Molecular Clocks – Deamidation

by Robinson & Robinson

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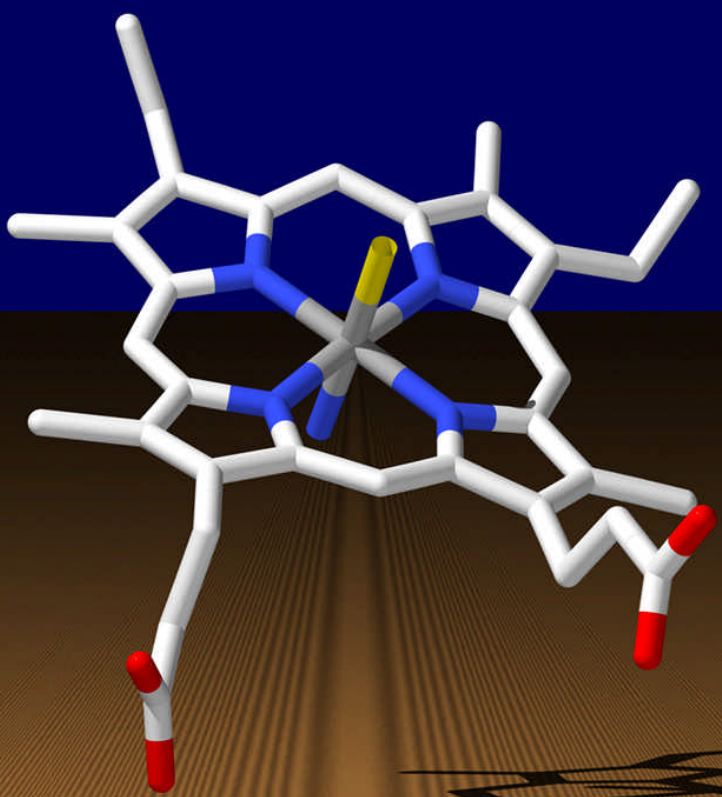
Asn & Gln Deamidation in Peptides & Proteins

Molecular Clocks

Deamidation of Asparaginyl and Glutaminyl Residues in Peptides and Proteins



Noah E. Robinson
Arthur B. Robinson

Molecular Clocks

Two of the twenty ordinary amino acid residues in peptides and proteins, Asn and Gln, especially Asn, are uniquely unstable under physiological conditions. The non-enzymatic deamidation half-times of these residues are genetically determined throughout the range from less than a day to more than a century with many within the biological lifetimes of the proteins, organelles, and organisms of which they are a part.

Deamidation of Asn or Gln introduces a negative charge at the deamidating residue and some isomerization as well. Consequently, the structure of the deamidating peptide or protein is profoundly altered.

Recent experimental and computational research has shown that deamidation is pervasive. Through miniature amide clocks imbedded in each protein, the protein pool in a living thing is a dynamic, time-dependent array of macromolecules.

Why do peptides and proteins include residues that are unstable and cause fundamental molecular changes during their biological lifetimes?

These timed changes of structure must be of substantial value to living things. Otherwise, they would be unnecessarily disruptive to the integrity of the molecules essential to life, and would not be present.

We suggest that they are present because amide residues can serve as ubiquitous molecular regulators for the timing and control of biological processes.

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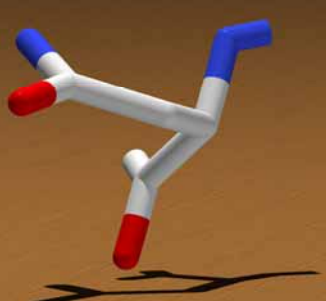

MOLECULAR CLOCKS

Deamidation of Asparaginyl and Glutaminyl Residues in Peptides and Proteins

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Deamidation of Asparaginyl and Glutaminyl Residues in Peptides and Proteins

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We suggest that they are present because amide residues can serve as ubiquitous molecular regulators for the timing and control of biological processes.[1]

N. E. Robinson
A. B. Robinson

1 N. E. Robinson and A, B, Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci.* **99**, 5283 (2002); N. E. Robinson and A. B. Robinson, *Mech. Ageing Dev.* **125**, 259 (2004).

Dedicated to

Laurelee Ruth Robinson

Preface

It is now about two centuries since the amino acid asparagine was discovered and 125 years since the discovery of glutamine. Only 70 years ago, the residues of asparagine and glutamine were shown to be ordinary constituents of proteins. During the next 35 years, the chemistry of the deamidation of asparaginyl and glutaminyl residues in peptides and proteins began to be elucidated.

Unfortunately, the analytical procedures of that period, especially acid hydrolysis followed by amino acid analysis, prevented deamidation from being easily seen by most investigators. Deamidated forms of proteins were frequently noticed during electrophoretic separations, but these were generally dismissed as biologically uninteresting impurities that were believed to arise from protein degradation during purification.

Between 35 and 30 years ago, three changes occurred. Deamidation was proved to occur *in vivo*; deamidation was shown to be under simple genetic control and to take place under physiological conditions over a wide range of biologically relevant time intervals; and it was suggested that deamidation is a biological molecular timing mechanism, with two instances of this being experimentally demonstrated.

These discoveries increased interest in peptide and protein deamidation, so that more investigators examined their preparations for deamidated products. Even though the available experimental techniques were still laborious, more work was initiated to understand the details of this process.

Up to the present, apparent *in vivo* or *in vitro* deamidation has been observed in more than 200 peptides and proteins. It has turned out that deamidation leads to several degradation products rather than simply to L-aspartyl and L-glutaminyl as was first supposed. Substantial progress has been made in understanding deamidation reaction mechanisms, and an enzyme system that removes some of the apparently deleterious isomeric products has been discovered and characterized.

Careful studies have been made of deamidation in a few individual proteins. Unfortunately, limitations in experimental techniques and the lack of needed quantitative theoretical and experimental information have caused these studies to be long, arduous efforts requiring many years of dedicated work.

Now, however, the advent of new experimental procedures, the availability of extensive three-dimensional protein structure information, and the development of new computational methods have combined to make possible reliable quantitative calculations and predictions of deamidation rates.

This work and the discovery of additional *in vivo* systems wherein deamidation plays biologically important roles is causing a rapid expansion of deamidation research. It is evident that this field of inquiry has grown too large to be adequately summarized in an ordinary review.

We are mindful of the tale of a small boy who asked his father several questions about penguins. So, his father presented him with the gift of a learned book on penguins. Perusing his new possession, the boy remarked, "This book tells me more about penguins than I wanted to know." Nevertheless, there is a need for a comprehensive book about deamidation. The companion book to this volume, Protein Deamidation by N. E. Robinson and A. B. Robinson (2004), condenses the subject for those who prefer a succinct account.

We hope that this book will prove useful and that the inadvertent but inevitable errors and omissions will be found by our readers and communicated to us, so that later editions may be improved.

We owe our ability to write this book to many teachers, coworkers, and colleagues and to the researchers whose work is referenced herein.

For their special personal and professional help, we are very grateful to Professor and Mrs. R. Bruce Merrifield, Professor Martin D. Kamen, Professor Harry Gray, Dr. Jane Orient, Dr. Zachary Robinson, Arynne Robinson, Bethany Robinson, Joshua Robinson, Matthew Robinson, and Jeremy Snavely.

Noah E. Robinson
Arthur B. Robinson
August 2004

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Introduction

OUR present knowledge about deamidation of glutaminyl and asparaginyl residues has accumulated gradually over the past two centuries, within which several specific milestones may be defined. Superimposed upon those milestones has been a gradual accumulation of information about the deamidation of proteins in general and of several proteins in particular, which have been of special interest.

The improvement of analytical techniques, the advent of chemical and biological means for synthesis of specific peptides and proteins, and the creation of the large databases of knowledge of one-dimensional and three-dimensional protein structure have now created an environment in which more rapid progress can be made.

The molecular clock hypothesis of the biological importance of deamidation has, without refutation and with some experimental support, been extant now for more than 30 years. Recent experiments and computations have markedly expanded this hypothesis and provided compelling evidence of its ubiquitous importance.

In order to provide perspective as an aid to understanding the detailed chapters that follow, this book begins with a general overview of the history and current state of knowledge about deamidation of glutaminyl and asparaginyl residues in peptides and proteins.

It then proceeds to an expanded review of current knowledge.

Our primary purpose is to provide a book that will aid in the advance of understanding of deamidation in biological systems. Therefore, this book is written primarily for scientists engaged in that work.

References to publications directly relevant to the text are redundantly footnoted so that each section stands alone, and inclusive reference lists are also provided for exploration of a particular aspect. All publications referenced in this book have been carefully considered by the authors, who have attempted to organize them in a useful way.

We hope that the reader will find this account interesting and helpful. We also hope that readers will tell us about any omissions, misinterpretations, or other errors that they may find herein, so that future editions may be improved.

An abbreviated chronology of the history of deamidation research is provided in Figure I-1.

A Chronology of Deamidation

1806 - 2004

Deamidation		Molecular Clock Hypothesis	
1806	Asparagine Discovered *Vauquelin & Robiquet*	1966	Amide Molecular Clock Hypothesis Originated *Robinson*
1851	Asparagine Shown Optically Active *Pasteur*	1970	Amide Molecular Clock Hypothesis Published *Robinson, McKerrow, & Cary*
1877	Glutamine Discovered *Schulze*	1970-04	Elucidated and Expanded *Robinson, Robinson, & coworkers*
1932	Asn and Gln in Proteins Verified *Domodaran, Jaaback, & Chibnall*	1968-74	Demonstrated for Turnover of Cytochrome c *Flatmark, Sletten, Robinson, McKerrow, & Legaz*
1952	First Purification of a Naturally Deamidated Peptide/Protein-Insulin *Harfenist & Craig*	1970-74	Demonstrated for Turnover of Aldolase *Lai, Chen, Horecker, Midelfort, Mehler, McKerrow, & Robinson*
1954	Imide Mechanism Demonstrated *Sondheimer & Holley*	1980-90	Demonstration as Counter of Triosephosphate Isomerase Catalytic Cycles *Gracy & coworkers*
1959	Enzymatic Deamidation of Gln *Waelsch & coworkers*	2002	Demonstration as Timer of Cell Apoptosis *Deverman, Weintraub, & coworkers*
1966-67	Demonstration of *In Vivo* Nonenzymatic Deamidation of Cytochrome c *Flatmark & Sletten*	2001-02	Development of Method for Computing Amide Clock Settings From Three-Dimensional Structure. *Robinson & Robinson*
1966-70	Amide Molecular Clock Hypothesis Formed *Robinson & coworkers*	2002-03	Computational Proof of Pervasive and Ubiquitous Genetically-Determined Amide Clocks in Living Systems. *Robinson*
1970-74	Primary, Secondary, and Tertiary Structure Dependence Demonstrated and its Range Shown to be More than 6 days to 10 years. *Robinson & coworkers*		
1982-84	Protein O-Methyltransferase Specificity for D-Asp and IsoAsp Demonstrated *Clark, McFadden, O'Conner & Murray*		
1985-89	Ubiquitous Character of Protein O-Methyltransferase Demonstrated *Clark, Aswad, & coworkers*		
1989-01	Imide Mechansim Elucidated in Detail *Capasso & coworkers*		
2001-04	Quantitative Structure Dependence of Deamidation Elucidated *Robinson & Robinson*		
2002-04	Deamidation Rates for All Asn in Three-Dimensional Structure Database Computed *Robinson*		

FIG. I-1 Chronology of Deamidation Research.

CHAPTER 1

Asparagine and Glutamine

1-1. DISCOVERY OF ASPARAGINE AND GLUTAMINE

Asparagine, the first amino acid to be isolated from natural sources, was found in asparagus in 1806.[1] It was named in 1826[2] and crystallized

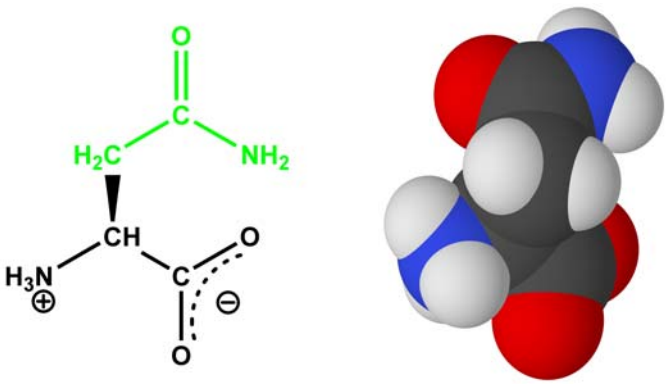

FIG. 1-1 Asparagine

as the L isomer and shown to be optically active in 1851.[3] Amide hydrolysis of asparagine to aspartic acid was first carried out in 1827[4] and elucidated in 1832,[5] and the empirical formulas for aspartic acid and asparagine were determined in 1833 and 1838.[6] In 1886, asparagine crystals were obtained that tasted sweet rather than bitter and were found to be the D isomer.[7] Pasteur then suggested that the ability of the taste nerves to distinguish between the L and D isomers might result

1 L. N. Vauquelin and P. J. Robiquet, *Ann. Chim. (Paris)* **57**, 88 (1806).

2 A. Dulong, *J. Pharm.* **12**, 278 (1826).

3 L. Pasteur, *Ann. Chim. Phys.* **31**, 67 (1851).

4 A. Plisson, *J. Pharm.* **13**, 477 (1827).

5 J. Liebig and J. L. Wohler, *Ann. Chem.* **3**, 268 (1832); J. Pelouze, *Justus Liebig's Annalen Der Chemie Und Pharmacie* **5**, 283 (1833).

6 J. Liebig, *J. L. Ann. Chem.* **7**, 146 (1833); J. Liebig, *J. L. Ann. Chem.* **27**, 125 (1838).

7 A. Piutti, *Compt. Rend.* **103**, 134 (1886).

from the proteins in these nerves being optically asymmetric. Aspartic acid was first isolated from a protein hydrolysate in 1868.[8] The first synthesis of Asn peptides, LeuAsn and GlyAsnLeu, was carried out in 1907.[9] Asparagine was first isolated from proteins by enzymatic digestion in 1932.[10]

Glutamic acid was isolated from a hydrolysate of gliadin in 1866.[11] The conversion of glutamic acid into pyrrolidonecarboxylic acid was

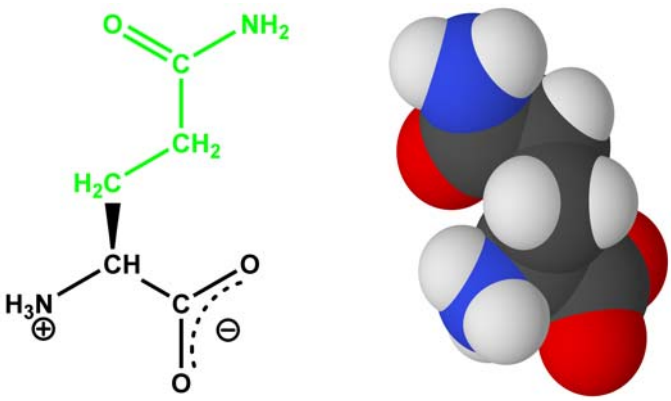

FIG. 1-2 Glutamine

reported in 1914.[12] Glutamine was first isolated from beet roots in 1877 and 1883.[13] The presence of residues of glutamine and asparagine in proteins was deduced from the presence of excess ammonia in protein hydrolysates in 1873,[14] and glutamine was isolated from proteins by enzymatic digestion in 1932.[15]

This early history of asparagine and glutamine is condensed and adapted from a detailed review in 1961.[16]

8 H. J. Ritthausen, *J. Prakt. Chem.* **103**, 233 (1868); H. J. Ritthausen, *J. Prakt. Chem.* **106**, 445 (1869); H. J. Ritthausen, *J. Prakt. Chem.* **107**, 218 (1869).

9 E. Fischer and E. Koenigs, *Deutsche Chem. Ges. Bur.* **4**, 2048 (1907).

10 M. Damodaran, *Biochemical Journal* **26**, 235 (1932).

11 H. Ritthausen, *J. Prakt. Chem.* **99**, 454 (1866).

12 F. W. Foreman, *Biochemical Journal* **8**, 463 (1914).

13 E. Schulze, *Ber.*, **10**, 85 (1877; E. Schulze and E. Bosshard, *Ber.* **16**, 312 (1883).

14 H. Hlasiwetz and J. Habermann, *Ann.* **169**, 150 (1873).

15 M. Damodaran, G. Jaaback, and A. C. Chibnall, *Biochemical Journal* **26**, 1704 (1932).

16 J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids* **3**, Wiley, New

1-2. DEAMIDATION OF ASPARAGINE AND GLUTAMINE

It was found that glutamine is much more rapidly deamidated than either asparagine or glutaminyl residues at internal or C-terminal positions in peptides.[17] Glutamine deamidation produces ammonia and pyrrolidonecarboxylic acid,[18] is accelerated by phosphate[19] and arsenate, and is strongly pH dependent.[20] See Figure 1-3. Glutamic acid also cyclizes to pyrrolidonecarboxylic acid, but under much more vigorous conditions than required for glutamine.[21] This reaction is 98%

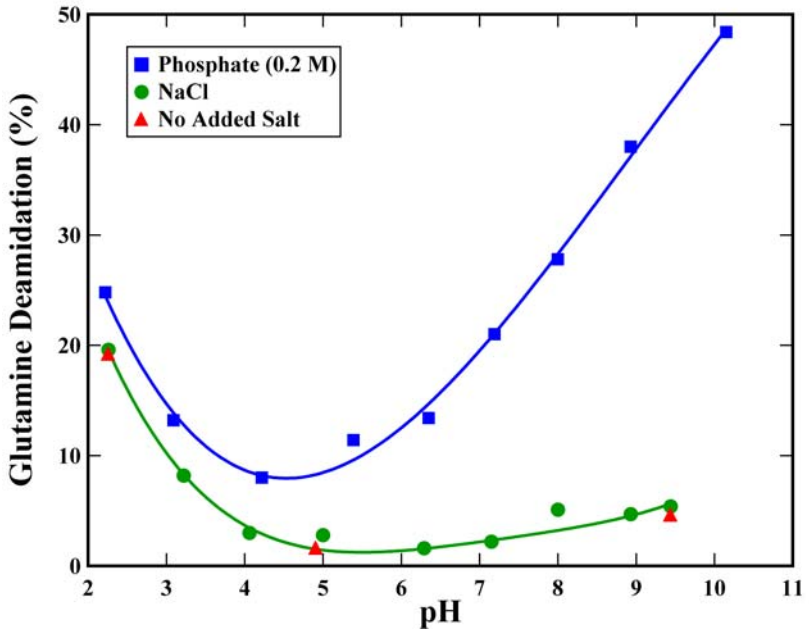

FIG. 1-3 Dependence of glutamine deamidation upon pH and phosphate. Percent deamidation in 12 hours at 37°C of 0.05 M L-glutamine in veronal buffer with 0.2 M phosphate, ■; NaCl at same ionic strength as that of phosphate, ●; and no added salt, ▲. Adapted from Gilbert, Price, and Greenstein.[20]

York, 1856 & 1929 (1961).

17 H. Thierfelder and E. von Cramm, *Z. Physiol. Chem.* **105**, 58 (1918).

18 A. C. Chibnall and R. G. Westall, *Biochemical Journal* **26**, 122 (1932); H. B. Vickery, G. W. Pucher, H. E. Clark, A. C. Chibnall, and R. G. Westall, *Biochemical Journal* **29**, 2710 (1935).

19 P. B. Hamilton, *J. Biological Chemistry* **158**, 375 (1945).

20 J. B. Gilbert, V. E. Price, and J. P. Greenstein, *J. Biological Chemistry* **180**, 209 (1949).

21 H. Wilson and R. K. Cannan, *J. Biological Chemistry* **119**, 309 (1937).

complete in 50 hours at 100°C at pH 4 or pH 10. Pyrrolidonecarboxylic acid can be quantitatively converted to glutamic acid by 2N HCl or 0.5N NaOH in 1 to 2 hours at 100°C.

It was discovered in 1939[22] that acid hydrolysates of proteins contain substantial quantities of D-glutamic acid. These hydrolysates can contain 5 to 10% D-glutamic acid[23] and small amounts of D-cystine. The other commonly occurring amino acids were not found to be appreciably racemized under these conditions.[24]

The first isolation and characterization of the amidated and deamidated forms of a naturally occurring peptide was accomplished by counter-current distribution of insulin in 1952.[25] Insulin and its singly deamidated product were separated.

These insulin experiments were performed in Flexner Hall at Rockefeller University in New York City. In this modest laboratory building, Lyman Craig developed the countercurrent distribution machine, Stanford Moore and William Stein developed the amino acid analyzer,[26] and Bruce Merrifield[27] invented solid phase peptide synthesis and accomplished the first chemical synthesis of a protein. Many other advances in biochemistry also originated in Flexner Hall.

It was recently decided that R. Bruce Merrifield's laboratory should be preserved as a National Monument. At his suggestion, the plan was changed so that Flexner Hall is now a National Monument.

Most of the existing information on the deamidation rates of peptides has also depended upon Flexner Hall. During a visit there in 1964 by A. B. Robinson, who was then a graduate student of Martin D. Kamen, Bruce Merrifield taught his new technique of solid phase synthesis to him. This later made possible deamidation rate determinations of 65 model peptides in the early 1970s,[28] which first demonstrated the extensive sequence dependence of deamidation. In 1999 and 2000, N. E. Robinson and A. B. Robinson synthesized 913 Asn and Gln peptides in Bruce Merrifield's lab-

22 F. Kögl and H. Erxleben, *Z. Physiol. Chem.* **258**, 57 (1939).

23 G. H. Wiltshire, *Biochemical Journal* **55**, 46 (1953).

24 J. A. Miller, *Cancer Research* **10**, 65 (1950); G. H. Wiltshire, *Brit. J. Cancer* **7**, 137 (1953).

25 E. J. Harfenist and L. Craig, *J. American Chemical Society* **74**, 3083 & 3087 (1952); E. J. Harfenist, *J. American Chemical Society* **75**, 5528 (1953).

26 S. Moore and W. H. Stein, *J. Biological Chemistry* **192**, 663 (1951); W. H. Stein and S. Moore, *Scientific American* March (1951).

27 R. B. Merrifield, *J. American Chemical Society* **85**, 2149 (1963); R. B. Merrifield, *Peptides: Synthesis, Structures, and Applications,* Academic Press, New York, 94 (1995).

28 This work by Robinson and coworkers is summarized and referenced in A. B. Robinson and C. J. Rudd, *Current Topics in Cellular Regulation* **8**, 247 (1974).

oratory in Flexner Hall with the help of Professor Merrifield and Mrs. Merrifield. The deamidation rates of 477 of these peptides have been reported.[29]

Early experiments on peptides emphasized glutaminyl residue deamidation. It was shown that the amide side chains of the dipeptide LeuGln and of L-asparagine were more stable than many of the amides in proteins.[30] The amides of GlnGlu, GlnGly, GlnGlyGly, and L-glutamine were found to be much more easily deamidated than LeuGln and L-asparagine at pH 4 and 100°C.[31] The deamidation half-times of GlnGlu, GlnGly, and GlnGlyGly at 37°C were found to be less than 1 day at pH 2 and approximately 3 days at pH 8. The primary products were the pyrrolidone derivatives.

Deamidation of L-glutamine at 100°C, pH 5 was determined to be approximately 40-fold faster than of L-asparagine because pyrrolidonecarboxylic acid was formed.[32] Experiments on the deamidation of L-glutamine in 11 different buffers at pH 8, 37°C showed that the L-glutamine deamidation rate is markedly increased by phosphite, arsenate, and bicarbonate, and moderately increased by phosphate and borate. The pH minimum of deamidation was between 4 and 6 and depended upon the type of buffer ion.[33]

These investigators also studied enzymatic deamidation of

Table 1-1 pK_a Values for Amino Acids

	α-COOH	β/γ-COOH	α-NH_2
Asparagine	2.02		8.80
Glutamine	2.17		9.13
Aspartic Acid	1.88	3.65	9.60
Glutamic Acid	2.19	4.25	9.67

29 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).; N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001); N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

30 A. C. Chibnall and R. G. Westall, *Biochemical Journal* **26**, 122 (1932).

31 J. Melville, *Biochemical Journal* **29**, 179 (1935).

32 H. B. Vickery, G. W. Pucher, H. E. Clark, A. C. Chibnall, and R. G. Westall, *Biochemical Journal* **29**, 2710 (1935).

33 J. B. Gilbert, V. E. Price, and J. P. Greenstein, *J. Biological Chemistry* **180**, 209 (1949).

L-glutamine in liver extracts as a function of buffer type. Phosphate, arsenate, and sulfate markedly increased the enzymatic deamidation rate of L-glutamine in liver extracts, which had a pH optimum of 8 regardless of buffer type.

The pK_a values[34] for asparagine, glutamine, aspartic acid, and glutamic acid are given in Table 1-1.

Deamidation of glutaminyl and asparaginyl residues in peptides and proteins has many characteristics that are different than for glutamine and asparagine. The charged N-terminal and C-terminal groups are moved away from the amide side chains; the amino acid residues on either side of the amide residues and their effects on deamidation vary according to sequence; and secondary, tertiary, and quaternary three-dimensional structure introduces deamidation impeding and, occasionally, deamidation accelerating effects. Nevertheless, many of these early observations of deamidation of glutamine, asparagine, and short glutaminyl peptides are directly relevant to the understanding of deamidation of peptides and proteins.

34 *CRC Handbook of Chemistry and Physics*, 75th Edition, CRC Press, 7-1 (1994).

CHAPTER 2

Asn and Gln in Peptides and Proteins

2-1. ASPARAGINYL RESIDUES

Bound together in peptides and proteins by peptide bonds, the amino acid "residues" remaining after elimination of H_2O during bonding are given three-letter designations such as Asn for the asparaginyl residue and Gln for the glutaminyl residue. The peptide bonds themselves are constrained in planar configurations by resonance stabilization as shown in Figures 2-1 and 2-2.[1] For the most part, rotation within the backbone is restricted to two bonds as shown in Figure 2-1

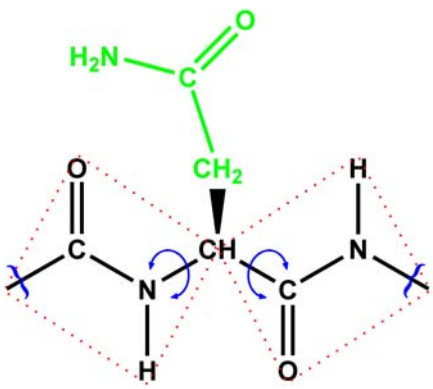

FIG. 2-1 Asparaginyl residue

Two of the 61 messenger RNA codons that code for amino acid residues represent Asn and two represent Gln. The mean and median percentages of Asn in a representative set of 4,835 proteins for which three-dimensional structures have been determined are 4.5% and 4.2%, respectively. These values for Gln are 3.9% and 3.7%. The probabilities of occurrence from random messenger RNA are 0.0328 for both Asn and Gln. Therefore, using median percentages, Asn occurs 28% more and Gln occurs 13% more than would be expected in proteins that are

1 L. Pauling, *The Nature of the Chemical Bond*, 3rd Edition, Cornell, Ithaca, 281 (1962).

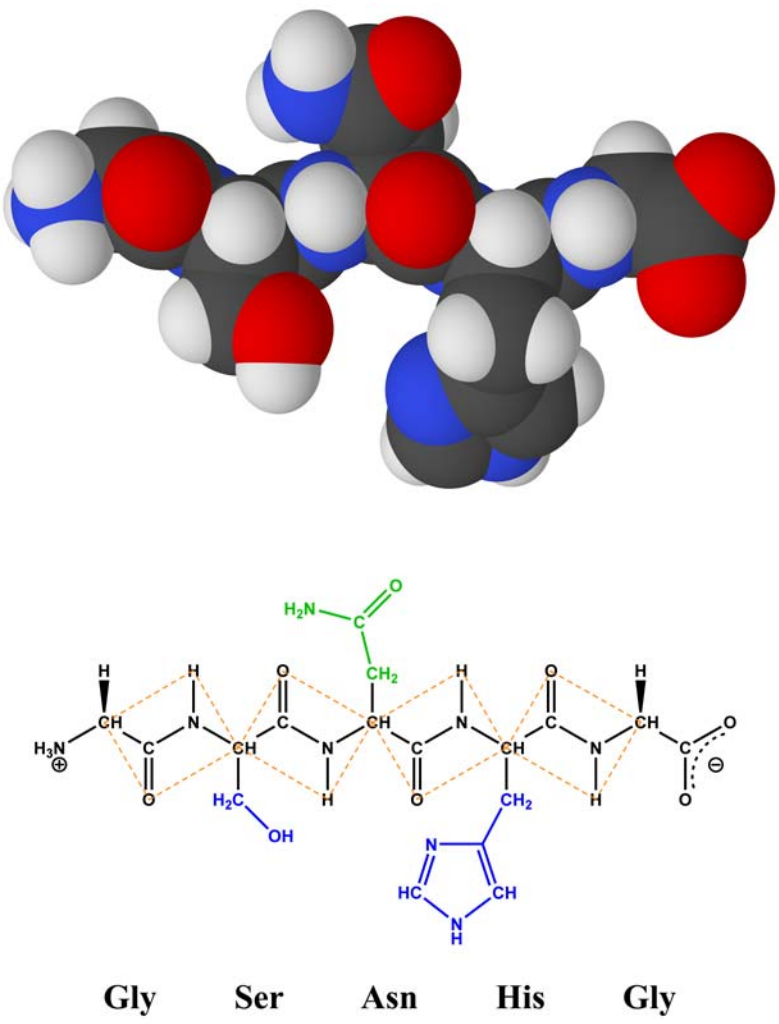

FIG. 2-2 One of the primary structures of GlySerAsnHisGly at neutral pH.

produced from randomly assembled messenger RNA. Figure 2-3 shows the distribution function of occurrence of Asn in proteins. About 72% of proteins have more Asn and 63% have more Gln than expected by chance.

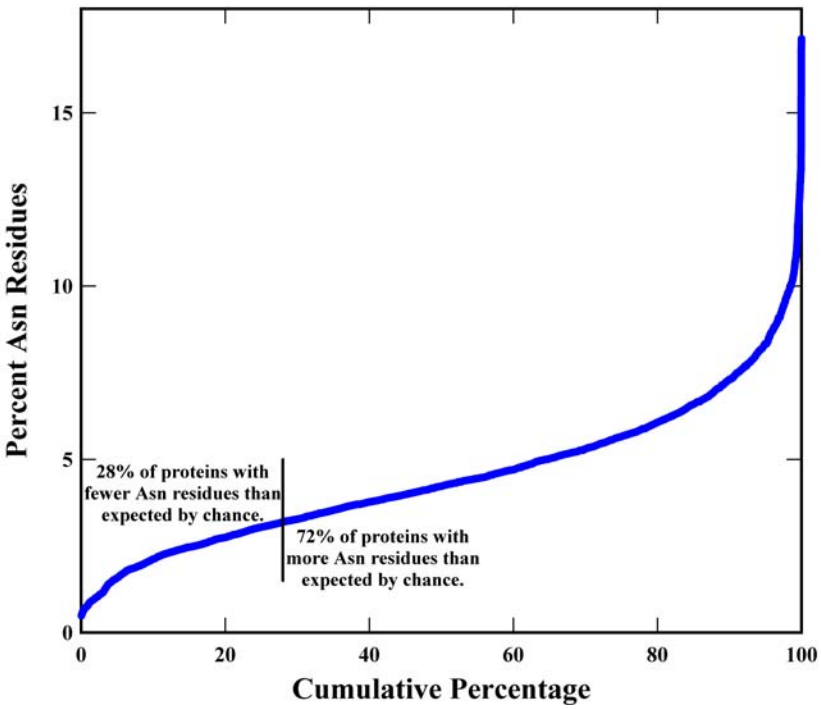

FIG. 2-3 Cumulative distribution function of occurrence of Asn in proteins. Percentages are calculated from the January 2003 PDB databank. Proteins with no Asn residues were first removed, and the remaining set was filtered to include only proteins which were less than 50% homologous in 4 residue segments and which were less than 1000 residues and greater than 50 residues in length. The remaining set contained 4835 representative proteins. The position of the division line shown was corrected for the slight skew introduced by removing proteins with no Asn residues (2003R).

2-2. GLUTAMINYL RESIDUES

Figure 2-5 shows the structure of the Gln residue, which differs from Asn by only a single CH_2 group elongation of its side chain. This small change, however, causes profound differences in the deamidation chemistry and biological uses of Asn and Gln.

It has been said of the great chemist R. B. Merrifield that he "is in love with each of the 20 amino acids."[2] It is necessary for a protein chemist to develop at least a very close friendship with each of these 20. In this regard, Gln is a more constant and less fickle friend than Asn.

2 Mrs. R. B. Merrifield, private conversation (1979).

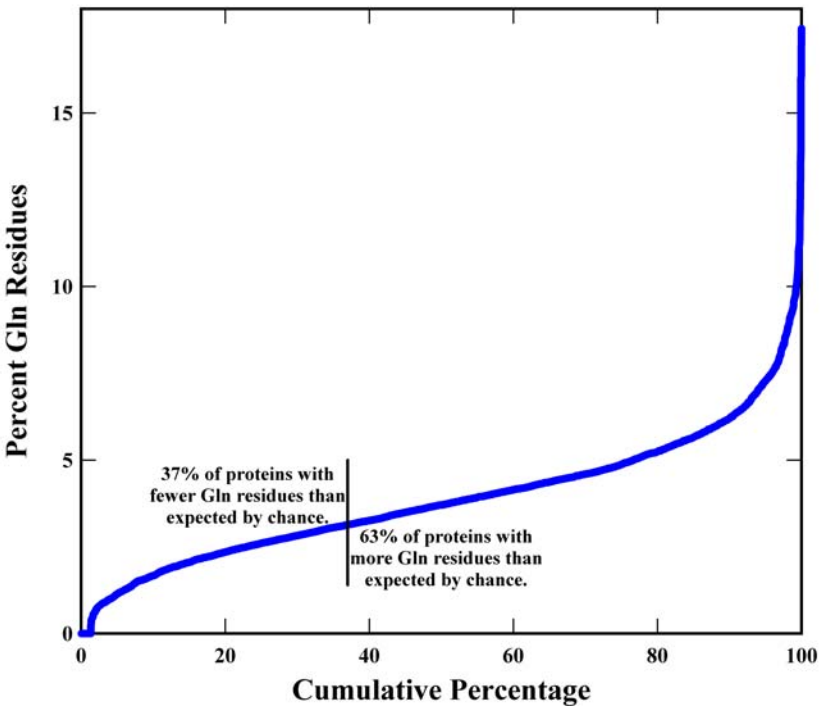

FIG. 2-4 Cumulative distribution function of occurrence of Gln in proteins. Percentages are calculated from the January 2003 PDB databank. Proteins with no Asn residues were first removed, and the remaining set was filtered to include only proteins for which 4 residue segments were less than 50% homologous and which were less than 1000 residues and greater than 50 residues in length. The remaining set contained 4835 representative proteins (2003R).

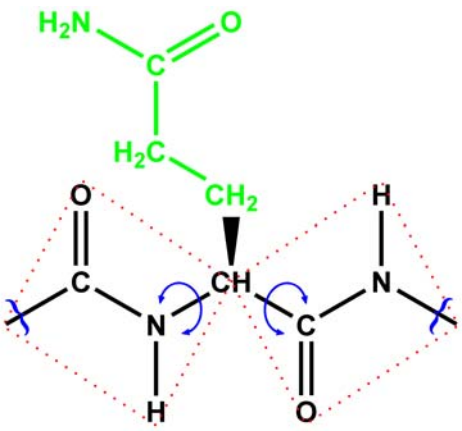

FIG. 2-5 Glutaminyl Residue

Gln is far more stable than Asn under physiological conditions and, when it does change, its peregrinations are less complex.

Figure 2-4 shows the distribution of occurrence of Gln in proteins.

2-3. C-TERMINAL AMIDES

In addition to Asn and Gln, proteins are sometimes found to be amidated on the C-terminal carboxyl group as shown in Figure 2-6. These C-terminal amides are relatively rare.

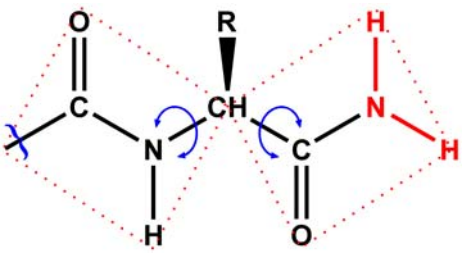

FIG. 2-6 C-Terminal Amide.

2-4. AMINO ACID RESIDUE IONIZATION STATES

In this book, we follow the usual convention of representing the sequences of peptides and proteins by means of three-letter abbreviations of the amino acid residues of which they are composed. Thus, "GlySerAsnHisGly" represents a pentapeptide of the indicated sequence – proceeding from the amino terminal end on the left to the carboxyl terminal end on the right. At pH 7.4, however, this peptide is actually a mixture of peptides in different states of ionization. Both the N-terminal α-NH_2 and the histidine side-chain imidazole are only partially ionized at pH 7.4. The pK_as of these two groups are 7.8 and 6.4, respectively.[3] Thus, in aqueous solution at pH 7.4, GlySerAsnHisGly is a mixture of four principal forms with different degrees of ionization. The properties of the peptide reflect the average of this mixture. Figure 2-7 shows a titration curve for GlySerAsnHisGly.

Since the pK_as of amino acid residue side chains are affected by protein structure, titrations of proteins give different side-chain pK_a values

3 N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483-493 (2001).

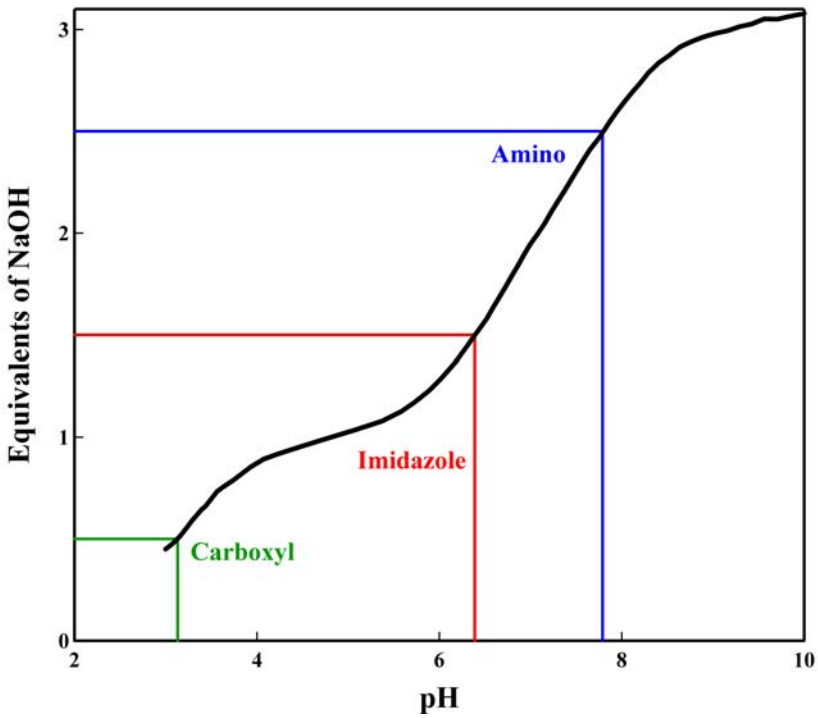

FIG. 2-7 Titration curve for GlySerAsnHisGly showing pK_as of the α-COOH, imidazole, and α-NH_2 of 3.1, 6.4, and 7.8 respectively. Solvent conditions were 1.0×10^{-3} M peptide, 37°C, 0.15 M Tris base titrated with 6 N HCl (2003R).

for similar residues. Table 2-1 lists values expected from model compounds[4] and the combined ranges of protein experimental values reported in three tabulations.[4,5]

4 C. Tanford, *Physical Chemistry of Macromolecules*, Wiley, New York, 556 (1961).

5 A. White, P. Handler, and E. L. Smith, *Principles of Biochemistry,* 4th Edition, McGraw-Hill, New York, 120 (1968); C. K. Mathews and K. E. van Holde, *Biochemistry,* 2nd Edition, Benjamin/Cummings, Menlo Park (1996).

Table 2-1 pK_a Values for Amino Acid Residues

	Model Compounds	Proteins
α-COOH	3.75	3.0 - 4.0
Side-chain-COOH	4.6	3.0 - 4.8
α-NH_2	7.8	7.6 - 9.0
Side-chain-NH_2	10.2	9.4 - 10.6
Imidazole	7.0	5.6 - 7.4
Phenolic	9.6	9.5 - 10.8
Guanidyl	>12	11.6 - 12.6
Sulfhydryl		8.0 - 9.0

CHAPTER 3

Enzymatic Deamidation

3-1. ENZYMATIC DEAMIDATION OF GLN

In 1950, Borsook and coworkers discovered a guinea pig liver transamidase that requires Ca^{++} and catalyzes reaction between the Gln side chain and lysine.[1]

Waelsch and coworkers[2] showed that this guinea pig liver transglutaminase-catalyzed reaction occurs with a wide variety of amines, wherein the amide nitrogen is replaced by the amine nitrogen with release of ammonia. The enzyme is specific for Gln and does not catalyze reaction with Asn. It can also exchange the amide group for H_2O, resulting in deamidation of Gln. This transglutaminase exhibits specificity for particular Gln primary sequences and is sensitive to steric hindrance. For example, it deamidates the B chain of unfolded insulin but does not react with folded insulin.

Transglutaminases are widely found in mammals as are low levels of Gln-Lys cross-linkages. After initial blood clotting, the clots are stabilized by transglutaminase-produced Gln-Lys linkages.[3]

Glutaminases are extensively distributed in the seeds of grain, beans, and other plants. These enzymes are especially active during seed germination. They specifically deamidate Gln residues, but do not have the crosslinking activity of transglutaminase, nor do they exhibit protease activity.[4]

1 Borsook, H., Deasy, E. L., Haagen-Smit, A.J., Keighley, G., and Lowy, P.H., *J. Biological Chemistry* **184**, 529 (1950); Schweet, R. and Borsook, H., *Federation Proceedings* **12**, 266 (1953).

2 N. K. Sarkar, D. D. Clarke, and H. Waelsch, *Biochimica et Biophysica Acta* **25**, 451 (1957); D. D. Clarke, M. J. Mycek, A. Neidle, and H. Waelsch, *Archives of Biochemistry and Biophysics* **79**, 338 (1959); M. J. Mycek, D. D. Clarke, A. Neidle, and H. Waelsch, *Archives of Biochemistry and Biophysics* **84**, 528 (1959); M. J. Mycek and H. Waelsch, *J. Biological Chemistry* **235**, 3513 (1960); L. Lorand, *Neurochemistry International* **40**, 7 (2002).

3 J. E. Folk, *Annual Review of Biochemistry* **49**, 517 (1980).

4 I. A. Vaintraub, N. K. Beltei, and A. D. Shutov, *Prikladnaya Biokhimiya I Mikrobiologiya* **17**, 166 (1981); I. A. Vaintraub, L. V. Kotova, and R. Shaha, *FEBS Letters* **302**, 169 (1992); I. Vaintraub, L. Kotova, and R. Shaha, *Physiologia Plantarum* **96**, 662 (1996); S. Yamaguchi and M. Yokoe, *Applied and Environmental Microbiology* **66**, 3337 (2000).

Substantial amounts of enzymatic deamidation of Gln have been observed in and during isolation of cat brain proteins, but no Asn deamidation has been observed. Enzymatic deamidation of Gln is thought to be a significant source of cerebral ammonia.[5]

Guinea pig liver transglutaminase requires that the Gln residue have at least one residue on its carboxyl-terminal side and two residues on its amino-terminal side. Bacterial peptidoglutaminases have also been found that are specific for carboxyl-terminal Gln.[6]

Gln deamidation enzymatic activity resides in transglutaminases, proteases, and glutaminases, and is widely distributed from microorganisms to mammals. Enzymatic deamidation of Gln is of special interest in the food industry as exemplified by 1988HM, 1988HS, 1991H1, 1991H2, 1994H, 1996CB1, 2001SL, and 2002JK1.

See also Chapter 12-11, Food Proteins, Chapter 15-2, Celiac Disease, and Chapter 15-3, Bacterial Toxins.

Additional studies of enzymatic Gln deamidation include 1949KJ, 1958N, 1962WM, 1980FP, 1983O, 1973KS, 1991SN, 1992LK1, 1993LC, 1995IY, 1995N, 1996CL1, 1996CL, 1996OS, 1998KL, 1998KS, 2000ND, 2001C, 2002KY, and 2002J.

3-2. ENZYMATIC DEAMIDATION OF ASN

While enzymatic deamidation of Gln is widely observed and is involved in several ordinary and pathological processes, there is no known example of a naturally occurring enzyme that deamidates XxxAsnYyy within a peptide or protein. If Asn does, in fact, serve as a ubiquitous molecular clock, this striking lack of natural asparaginases might be expected. Asparaginase scrambling of the genetically specified timed intervals would be undesirable.

Asparaginases that act on free asparagine are well known.[7] There is a substantial research literature concerning this, which is beyond the scope of this book.

5 D. B. Tower and J. R. Wherrett, *Acta Neurologica Scandinavica* **38**, 21 (1962); J. R. Wherrett and D. B. Tower, *Journal of Neurochemistry* **18**, 1027 (1971).

6 J. E. Folk and P. W. Cole, *J. Biological Chemistry* **240**, 2951 (1965); M. Kikuchi, H. Hayashida, E. Nakano, and K. Sakaguch, *Biochemistry* **10**, 1222 (1971); S. Yamaguchi, D. J. Jeenes, and D. B. Archer, *European Journal of Biochemistry* **268**, 1410 (2001).

7 O. Wagner, E. Irion, A. Arens, and K. Bauer, *Biochemical and Biophysical Research Communications* **37**, 383 (1969); J. W. Wriston Jr. and T. Yellin, *Advances in Enzymology and Related Areas of Molecular Biology* **39**, 185 (1973).

Enzymes have been found that deamidate carboxyl terminal Asn[8] and amino terminal Asn.[9] The latter are thought to play a part in the N-rule pathway for turnover of very short lived proteins. In this pathway, Asn and Gln are designated as "tertiary" destabilizing n-terminal residues, which require conversion into Asp or Glu and then coupling to Arg. Arg is designated a "primary" destabilizing residue.[10]

Enzymatic glycosylation of Asn in the peptide sequences $(Xxx)_nAsnYyyThr/Ser(Zzz)_n$ has been reported.[11] Synthetic "asparaginases" that deamidate AsnGly sequences have been made by producing antibodies to similar compounds. These antibodies accelerate AsnGly deamidation by 10 to 500-fold.[12]

Self-catalysis of Asn deamidation has been artificially produced. AspTrp in the active site of haloalkane dehalogenase was replaced by AsnTrp. With a deamidation half-time of only 1.5 days in 0.025 M Tris-H_2SO_4, pH 7.5, 4 °C, the enzyme was nonenzymatically reconverted to AspTrp. No isoAsp was detected. Apparently, the three-dimensional structure of the enzyme catalyzed the reconversion.[13]

A remarkable self-catalyzed transasparagination has been found in the construction of the 660 Å icosahedral head of bacteriophage HK97. After assembly, the head is knitted together with 420 Asn(356)-Lys(169) cross-linkages. These are formed through an internal catalysis from the 420 subunit proteins themselves that involves Glu(363).[14]

While enzymatic deamidation of internal Asn in peptides and proteins may yet be discovered, it is evidently at least a very rare occurrence. Conversely, enzymatic deamidation of internal Gln is frequently found and exhibits ordinary dependence upon steric effects. Moreover, enzymatically catalysed crosslinking reactions involving XxxGlnYyy

8 M. Kikuchi and K. Sakaguchi, *Archives of Biochemistry and Biophysics* **148**, 315 (1972).

9 S. A. Balogh, Y. T. Kwon, and V. H. Denenberg, *Learning and Memory,* **7**, 279 (2000).

10 A. Varshavsky, *Cold Spring Harbor Symposia on Quantitative Biology* **60**, 461 (1995).

11 R. S. Clark, S. Banerjee, and J. K. Coward, *J. Organic Chemistry* **55**, 6275 (1990).

12 R. A. Gibbs, S. Taylor, and S. J. Benkovic, *Science* **258**, 803 (1992); L. J. Liotta, R. A. Gibbs, S. D. Taylor, P. A. Benkovic, and S. J. Benkovic, *J. American Chemical Society* **117**, 4729 (1995).

13 F. Pries, J. Kingma, and D. B. Janssen, *FEBS Letters* **358**, 171 (1995).

14 W. R. Wikoff, L. Liljas, R. L. Duda, H. Tsuruta, R. W. Hendrix, and J. J. Johnson, *Science* **289**, 2129 (2000).

deamidation are also common, but are, as yet, unknown for XxxAsnYyy.

This difference between Asn and Gln may reflect the difference in their usefulness as biomolecular clocks.

CHAPTER 4

Nonenzymatic Deamidation

4-1. NONENZYMATIC DEAMIDATION OF ASN AND GLN

In 1937, Wilson and Cannan studied the rates of interconversion and equilibrium constants for glutamic acid and pyrrolidonecarboxylic acid as a function of pH and ionic strength in dilute aqueous solutions between 78 °C and 118 °C.[1] With a maximum at pH 6.6, the pyrrolidone is favored, but in extremes of pH, at or beyond 2 M HCl or 0.5 M NaOH, glutamic acid is favored. This allows quantitative measurement of Glu in peptides and proteins after hydrolysis in 6N HCl. They concluded that this is a slow proton transfer reaction. This is an early rigorous study of some reaction mechanisms relevant to deamidation.

Most peptide deamidation experiments have been carried out far from thermodynamic equilibrium with emphasis only on the forward components of the reactions. As has been shown with ribonuclease A, however, equilibrium processes are relevant to protein deamidation.[2]

In 1949, Dekker, Stone, and Fruton isolated PyrGlnGln from a marine alga and converted it to GluGluGlu with 1N NaOH by virtue of the equilibrium discovered by Wilson and Cannan and base-catalyzed deamidation.[3]

In 1952, it was found that asparagine, GlyAsn, AsnGly, glutamine, GlyGln, and GlnGly are 8, 25, 20, 92, 37, and 98% deamidated, respectively, in 10% trichloracetic acid for 1 hour at 70 °C. Thus, two Asn peptides were discovered that deamidate more rapidly than asparagine. It was also demonstrated that Gln deamidates more rapidly when n-terminal and free to form the pyrrolidone.[4]

In 1953, Clayton and Kenner reported the conversion of the Glu in toluene-*p*-sulphonylGlyPheGluGlycyclohexylamide to the glutarimide

1 H. Wilson and R. K. Cannan, *J. Biological Chemistry* **119**, 309 (1937).

2 S. Capasso and P. D. Cerbo, *J. Peptide Research* **56**, 382 (2000).

3 C. A. Dekker, D. Stone, and J. S. Fruton, *J. Biological Chemistry* **181**, 719 (1949).

4 H. K. Miller and H. Waelsch, *Nature* **169**, 30 (1952).

in pyridine, thionyl chloride at 20 °C for 2 hours and then hydrolysis of the product to isoGlu in 0.5N NaOH at 20 °C for 10 minutes.[5]

Leach and Lindley measured the deamidation of asparagine and AsnGly as functions of acid pH and temperature, finding deamidation half-times in pH 0 HCl, 80 °C of 0.063 and 0.043 days and ΔH° of 19.8 and 18.5 Kcal/mole, respectively.[6]

In 1954, Sondheimer and Holley synthesized carbobenzoxysuccinimide methyl ester from carbobenzoxyAsn methyl ester and also the analogous glutarimide. Succinimides and glutarimides are illustrated in Chapter 5. They hydrolyzed these in H_2O to the corresponding Asn, isoAsn, Gln, and isoGln compounds and suggested that mechanisms involving these imides might be found in biological systems. They found the glutarimide to be less stable than the succinimide in H_2O at basic pH.[7] Further studies of the succinimide and glutarimide intermediates and mechanisms were conducted in 1955 and 1956,[8] and Benzinger and Hems even measured the free energy of ATP hydrolysis by means of an ATP-dependent glutamine synthetase and the interconversion of glutamine and glutamic acid.[9]

In studies of terminus amide and carboxyl end-blocked |GluGly| and |GluPhe|, Battersby and Reynolds[10] were able to form the glutarimide for GluGly but not for GluPhe, which is in accord with recent peptide rate experiments,[11] and Liefländer obtained almost exclusively isoGlu forms during base hydrolysis of GluGlyGlu, presumably through a glutarimide mechanism.[12]

In 1963, Pisano, Freedman, and Cohen[13] observed isoAspHis in human urine even during 12 days of pure carbohydrate diet and isoAspGly and isoAspSer during a high collagen diet. IsoAspGly,

5 D. W. Clayton and G. W. Kenner, *Chemistry and Industry*, 1205 (1953).

6 S. J. Leach and H. Lindley, *Transactions of the Faraday Society* **49**, 915 (1953).

7 E. Sondheimer and R. W. Holley, *J. American Chemical Society* **76**, 2467 (1954).

8 A. R. Battersby and J. C. Robinson, *J. American Chemical Society* **259** (1955); D. W. Clayton, G. W. Kenner, and R. C. Sheppard, *IBID*, 371 (1956).

9 T. H. Benzinger and R. Hems, *Proc. Natl. Acad. Sci. USA* **42**, 896 (1956).

10 A. R. Battersby and J. J. Reynolds, *J. Chemical Society*, 524 (1961).

11 N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

12 M. Lieflander, *Z. Physiol. Chem.* **320**, 35 (1960).

13 J. J. Pisano, J. Freedman, and L. Cohen, *Federation Proceedings* **22**, 2688 (1963).

isoAspHis, isoAspVal, and isoGluLeu along with GlyAsp, GlyPro,and GluLeu were detected in dog urine.[14]

The human urine distribution functions of isoAsp peptides were found to be very narrow for single individuals, relatively narrow for groups of individuals on low protein diets, and wide and high for groups of individuals on high protein diets.[15]

In 1966, isoAsp peptides were found in enzymatic digests of hemoglobin, lysozyme, fibrin, and collagen.[16]

In 1967, Hideaki Fukawa[17] carried out a series of sequence-dependent decomposition studies of Gln peptides at 100 °C, 15 hours, 0.067 M phosphate, pH 4.5 to 9.2. These included GlnGly, GlnGlnGly, ProGlnGly, ProGln, LeuGlnGly, LeuGlnPro, GlyGln, AlaGln, ValGln, PheGln, and LeuGln with half-times of 0.12, 0.59, 1.4, 2.2, 3.1, 4.6, 4.6, 5.2, 6.0, 6.0, and 10.6 days, respectively, at pH 6.8. Deamidation half-times in H_2O without phosphate were substantially longer at pHs 4.5, 6.8, and 9.2 – the ratio of H_2O vs. phosphate at pH 6.8 ranging from 1.4 to 4.6 with an average of 2.6 longer half-time for peptides in H_2O as compared with 0.067 M phosphate. Therefore, decomposition of these peptides at high temperature through deamidation and, in some cases, chain cleavage showed a 2-fold apparently steric slowing by the amino-side residue and a 2 to 3-fold increase from phosphate catalysis.

Other interesting reports related to nonenzymatic deamidation of Gln include 1951ML, 1952QY, 1952RY, 1953LL, 1955BG, 1956TF, 1956BR, 1958SI, 1959TF, 1959L, 1960SK, 1961GW, 1961KV, 1961GW1, 1963W, 1964V, 1967DV, 1970MS1, 1971NT, 1989MP, 1991VK, 1993CP, and 1994BK.

Prior to 1970 there were no reported deamidation measurements for Asn or Gln peptides at neutral pH and ordinary temperatures. The only reports for Asn were for AsnGly and GlyAsn at pH < 1 and temperatures of 70 and 80 °C.

In 1966, Torgier Flatmark discussed, at a Kamen symposium at the La Valencia hotel in La Jolla, California, his work with Knut Sletten on the rate of deamidation of rat cytochrome c *in vivo*.[18] Stimulated by Flatmark's work, Arthur Robinson, at that same symposium, proposed

14 L. A. Wright and T. F. Nicholson, *Canadian Journal of Physiology and Pharmacology* **43**, 961 (1965).

15 F. E. Dorer, E. E. Haley, and D. L. Buchanan, *Biochemistry* **5**, 3236 (1966).

16 J. J. Pisano, E. Prado, and J. Freedman, *Archives of Biochemistry and Biophysics* **117**, 394 (1966); E. E. Haley, B. J. Corcoran, F. E. Dorer, and D. L. Buchanan, *Biochemistry* **5**, 3229 (1966).

17 H. Fukawa, *J. Chemical Society of Japan* **88**, 459 (1967).

18 T. Flatmark and K. Sletten, *J. Biological Chemistry* **243**, 1623 (1968).

the hypothesis that deamidation of Asn and Gln might serve as molecular clocks for the timing of biological processes. He further proposed that these clocks could be set to any desired time interval by genetic control of the primary, secondary, and tertiary structure surrounding the amide. At the time of this proposal, the only known rates of deamidation under physiological solvent conditions were those determined by Flatmark for cytochrome c in pH 6.8, 0.15 M phosphate at 37 °C of 13 and 5 days, respectively, for the first and second deamidations. The identities of the deamidating residues were unknown but were initially believed to be Gln residues[19] because the deamidation of glutamine was known to be faster than that of asparagine.

During 1966-1967, Robinson synthesized four peptides, GlyLysAsnLysGlyArg, TyrSerAspAlaAsnLysArg, AsnLeuGlu, and AlaSerAsnGlu[20] by Merrifield solid-phase peptide synthesis to explore this hypothesis. The LysAsnLys sequence was synthesized as part of a collaboration with Flatmark, who ultimately identified LysAsnLys as the second and ThrAsnGlu-C-terminal as the first deamidating sequences in cytochrome c. This LysAsnLys peptide was found to deamidate during synthesis, probably in the HF resin-release and deblocking reaction.

This study was then extended by Robinson, McKerrow, and Cary with synthesis and deamidation measurements in pH 7.0, 0.15 M sodium phosphate buffer at 37 °C of GlyAlaAsnAlaGly, GlyThrAsnThrGly, GlyLysAsnLysGly, GlyArgAsnArgGly, GlyGluAsnGluGly, and GlyGlyThrAsnGlu with resultant deamidation rates between 11 and 90 days. Also, a correlation was found between percentage of amide residues and *in vivo* turnover rate in 12 proteins, and non-randomness in the residues beside Asn and Gln in 43 proteins was detected.[21]

During the next 3 years, Robinson and co-workers examined the deamidation of peptides and proteins under physiological solvent conditions in substantial detail. Their experimental design was to directly measure the sequence-determined deamidation rates of model peptides and then to infer by difference the extent of protein secondary, tertiary, and quaternary structure effects on deamidation. They synthesized 65 peptides labeled with C^{14} and measured the deamidation rates in pH 7.4,

19 T. Flatmark, *Acta Chemica Scandinavica* **20**, 1487 (1966).

20 A. B. Robinson, *PhD Thesis, University of California at San Diego, Chemistry*, (1967).

21 A. B. Robinson, J. H. McKerrow, and P. Cary, *Proc. Natl. Acad. Sci. USA* **66**, 753 (1970).

0.15 I, phosphate buffer at 37 °C and in a variety of other solvent conditions. A summary of these rate determinations is included in Table 6-1. This work involved 65 variations in sequence, which included, among others, all of the Asn and Gln sequences in cytochrome c, lysozyme, and histone IV and the rapidly deamidating sequence of aldolase.[22] Most of these peptides were of the form GlyXxxAsn/GlnYyyGly.

Deamidation was measured by synthesis of C^{14} labeled peptides, incubation of the peptides in glass vials, paper electrophoresis, autoradiographic location of the peptides on the paper strips, soaking of appropriate diced sections of the paper strips in liquid scintillation fluid, and then C^{14} measurement.

Conclusions from this work included:

1. The sequence-dependent range of deamidation is at least from 6 days to 10 years. This has now been extended to from 1 day to 30 years.[23]

2. Deamidation rates for Asn are usually faster than for Gln.

3. Deamidation rates are increased by His and Ser neighboring to Asn with the effect larger for the carboxyl-side residue.

4. Deamidation rates are affected by steric hindrance from the side chains of the nearest neighboring residues to Asn and Gln.

5. Deamidation in proteins is generally and substantially slowed by secondary and tertiary structure.

6. Deamidation rates in proteins can be entirely sequence controlled when the amide is near a flexible end of the protein chain as in cytochrome c and aldolase.

7. Deamidation can be accelerated by a change in protein secondary and tertiary structure. This was demonstrated by means of peptide rates

22 J. H. McKerrow and A. B. Robinson, *Analytical Biochemistry* **42**, 565 (1971); A. B. Robinson and J. W. Scotchler, *J. Int. Research Communications* **1**, 8 (1973); A. B. Robinson, J. W. Scotchler, and J. H. McKerrow, *J. American Chemical Society* **95**, 8156 (1973); A. B. Robinson and S. Tedro, *Int. J. Peptide and Protein Research* **5**, 275 (1973); J. H. McKerrow, *PhD Thesis, University of California, San Diego, Chemistry* (1973); *J. W. Scotchler*, *PhD Thesis, University of California, San Diego, Chemistry* (1973); A. B. Robinson and J. W. Scotchler, *Int. J. Peptide and Protein Research* **6**, 279 (1974); A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **71**, 885 (1974); A. B. Robinson, J. H. McKerrow, and M. Legaz, *Int. J. Peptide and Protein Research* **6**, 31 (1974); J. H. McKerrow and A. B. Robinson, *Science* **183**, 85 (1974); A. B. Robinson and J. W. Scotchler, *Int. J. Peptide and Protein Research* **6**, 279 (1974); J. W. Scotchler and A. B. Robinson, *Analytical Biochemistry* **59**, 319 (1974); A. B. Robinson and C. J. Rudd, *Current Topics in Cellular Regulation* **8**, 247 (1974).

23 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001); N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001); N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

and the protein rates determined by Flatmark and Sletten for cytochrome c.

8. Sequence-determined deamidation rates for cytochrome c and aldolase are the same as their *in vivo* turnover rates.

9. Phosphate buffer apparently accelerates deamidation rates under physiological pH and temperature conditions as compared with Tris.

10. Peptide deamidation rates increase with ionic strength and temperature in the physiological pH and temperature range.

Considered together, the findings listed in 1 through 10 strengthened the biological molecular clock hypothesis.

Later refinement of the statistical correlations of residue sequences around Asn and Gln in a large protein set[24] also supported this hypothesis.

The peptides used in this work were all synthesized by Merrifield solid-phase peptide synthesis followed by resin cleavage and deblocking with anhydrous HF.[25] The HF treatment caused some deamidation. This problem has largely been eliminated by modern peptide synthesis procedures.[26] Deamidation has long been a problem during peptide synthesis. This was especially true of peptides and proteins synthesized by solid phase peptide synthesis and involving anhydrous HF.

This early work on nonenzymatic deamidation of Asn and Gln provided qualitative and semi-quantitative information about the reactions and their potential biological significance.

Some specific proteins were investigated as examples, including cytochrome c, aldolase, lysozyme, and histone IV. However, the systematic and comprehensive information required for a quantitative understanding of protein deamidation awaited new analytical techniques and the protein structure information that arose during the subsequent 25 years.

In the period between 1974 and 2000, the mechanisms of the deamidation reactions were thoroughly explored; additional biological

24 A. B. Robinson and L. R. Robinson, *Proc. Natl. Acad. Sci. USA* **88**, 8880 (1991).

25 J. Lenard and A. B. Robinson, *J. American Chemical Society* **89** (1967) 181; A. B. Robinson and M. D. Kamen, *Structure and Function of Cytochromes*, Ed. M. D. Kamen and I. Sekuzu, University of Tokyo and University of Park Press, 383 (1968); A. B. Robinson, *Solid Phase Peptide Synthesis*, Ed. J. M. Stewart and J. D. Young, 41 (1969); A. B. Robinson and C. J. Rudd, *Current Topics in Cellular Regulation* **8**, 247 (1974).

26 S. Mojsov, A. R. Mitchell, and R. B. Merrifield, *J. Organic Chemistry* **45**, 555 (1980); For an example see N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001).

information, including the presence of isoAsp and D-Asp methylating enzymes, was accumulated; deamidation was observed in another 150 protein species; and some additional peptide work was done.

Then, between 2000 and 2004 systematic and quantitative peptide work was finally carried out,[23] and this information was combined with protein structure data to provide a comprehensive understanding of protein deamidation.[27]

Current knowledge about nonenzymatic deamidation under biologically relevant conditions has, therefore, accumulated primarily between 1966 and 2004. This information forms most of the subject matter of the chapters of this book.

Interest in deamidation continues to increase as is indicated by the publication frequencies illustrated in Figure R-1. See reference section, page 317.

4-2. DEAMIDATION COEFFICIENT AND DEAMIDATION INDEX

It is useful, especially with molecules containing more than one amide, to have a distinct quantitative nomenclature for deamidation. For this reason, the terms deamidation coefficient, C_D, and deamidation index, I_D, have been introduced.[28] $C_D \equiv$ (deamidation half-time)/100 in days for a single amide. $I_D \equiv [\sum(C_{Dn})^{-1}]^{-1}$, where C_{Dn} is the nth amide residue. I_D is therefore the single-residue deamidation half-time/100 for the whole protein molecule with all amide residues considered.

If we assume, for example, that the three Asn in a particular protein have $C_{D1} = 1.0$, $C_{D2} = 6.0$, and $C_{D3} = 100$, then $I_D = 1/(1 + 1/6 + 1/100) = 0.85$. The fastest amide in the protein would then have a deamidation half-time of 100 days, while a net one-half deamidation of the protein with all three Asn considered would require 85 days.

Current values for C_D and I_D for Asn that have been experimentally measured or computed in more than 18,000 peptides and proteins are available at www.deamidation.org. Computation of values for any other Asn in proteins for which three-dimensional structures are available can be obtained by email at this Internet site.

27 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

28 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

CHAPTER 5

Mechanisms of Reactions Involving Asn and Gln

5-1. INTRODUCTION

In the early years of research on deamidation, it was generally believed that this reaction is simple general acid and general base catalyzed hydrolysis, with the reaction rate, therefore, at a minimum near neutral pH and rising as conditions become more acidic or more basic. Reaction products of the deamidation of L-Asn and L-Gln were expected to be entirely L-Asp and L-Glu with, a little racemization to D-Asp and D-Glu at basic pHs.

While the pH minimum of deamidation was actually observed to be about pH 5 for both peptides and proteins, this shift from neutral was ascribed to as-yet-unknown aspects of the reaction, which was not understood in any significant detail.

Early investigators were interested primarily in the occurrence and function of deamidation itself and therefore did not speculate about the intricacies of the deamidation mechanism, even though there were already clues in the organic chemistry literature as to the ultimate results.

It has turned out that, while deamidation does occur through hydrolysis, a special mechanism is predominantly important, especially in the physiological region near pH 7. It is the existence of this mechanism that shifts the minimum to pH 5. Moreover, the nature of this mechanism is such that it leads to a wide variety of reaction products.

Since reaction rates are non-equilibrium phenomena, are often dependent upon transient chemical species that are difficult to observe directly, and are subject to complex influences of solvent and other factors, there is often uncertainty concerning a proposed mechanism. This is the case with deamidation. A significant amount of experimental deamidation rate data has still not been reconciled with the currently accepted reaction mechanisms, especially with regard to participating residues in the peptide chain, catalysis by buffer ions, and other solvent properties.

Nevertheless, the current model is supported by many experimental observations and is probably largely correct. We will, therefore, describe this current model in detail and then review and summarize the experimental research with reference to it. This description should not, however, be interpreted by the reader as incontestable, established fact.

As Richard Feynman observed,[1] "Scientific knowledge is a body of statements of varying degrees of certainty – some most unsure, some nearly sure, none *absolutely* certain." Reaction mechanisms are in a less sure category.

5-2. CURRENT MECHANISTIC MODEL

A multitude of products results from the tendency of Asn and Gln to form rings. In the case of ordinary deamidation, these rings involve the backbone nitrogen of the carboxyl side residue, or, in the case of cleavage, involve the amide nitrogen. We will describe these reactions for Asn. Since Asn reaction rates are much faster than Gln, most experimental studies have been of Asn. The experimental evidence for these mechanisms is reviewed in Section 5-3.

As illustrated in Figure 5-1, the backbone nitrogen of the carboxyl-side residue participates in an ordinary acid-base equilibrium, in which the concentration of II is proportional to $[OH^-]$ and, therefore to $[H_3O^+]^{-1}$. The concentration of II increases with pH. The deprotonated back-bone nitrogen reacts with the Asn carbonyl carbon to form cyclic tetrahedral intermediate III. III is postulated, but has not been observed.

Since molecular motion is faster than hydrogen ion exchange at the backbone nitrogen, the properly aligned side chain probably reacts only if deprotonation has occurred prior to alignment. Yet, ring formation is much slower than hydrogen ion exchange, so there is plenty of time for the deprotonation of I to II to arrive at thermodynamic equilibrium before reaction takes place. Reaction of I to II is, therefore, simply an equilibrium process not a rate process. Its temperature dependence arises from the dependence of the equilibrium constant on temperature. Inductive effects, electrostatic effects, and other factors determine the acidity of the backbone nitrogen.

The reaction of II to III depends largely upon steric hindrance and conformational space because the Asn side chain must be correctly aligned in order to react. First, relatively temperature independent steric and conformational effects are introduced by the van der Waals radii of

1 R. P. Feynman, *Frontiers in Science*, Basic Books, (1958).

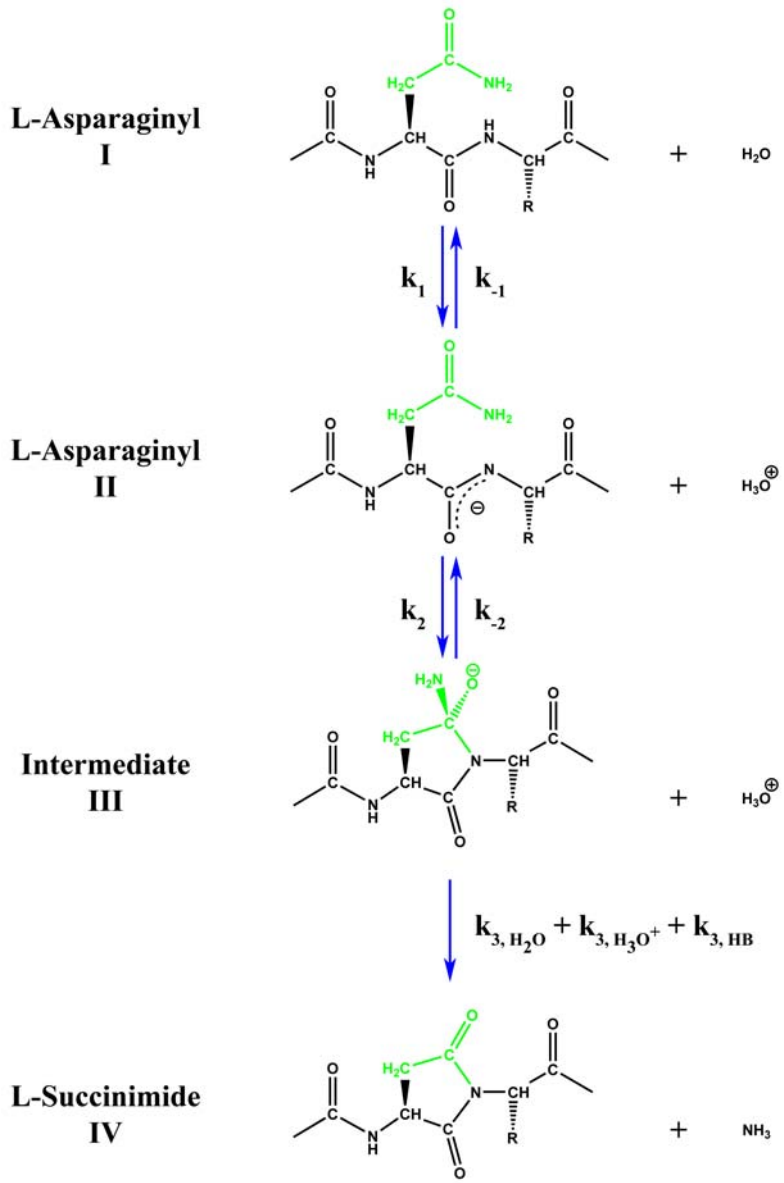

FIG. 5-1 Imide Formation

the side chain atoms of the carboxyl side residue and the other atomic constraints involved. Relative peptide deamidation rates for more than 400 different peptide sequences have been found to conform closely to these steric effects.[2] Second, especially in proteins, steric hindrance also results from hydrogen bonds and other weaker interactions that are more temperature dependent. So, this reaction involves a largely temperature dependent component and also a relatively temperature independent component.

Finally, the reaction of III to IV requires a proton source to facilitate removal of NH_3. This proton is provided by H_2O, H_3O^+, or any other general acid, HB, which is usually a buffer ion.

Therefore the first step of the conversion of Asn I to succinimide IV is proportional to $[OH^-]$, and the third step is proportional to $k_{3,H2O} + (k_{3,H3O+})[H_3O^+] + (k_{3,HB})[HB]$.

This has the result of providing an unusual pH profile for the deamidation reaction. As pH decreases below 5, the equilibrium is shifted away from II by decreasing $[OH^-]$, and acid-catalyzed hydrolysis of Asn becomes the predominant deamidation pathway. Between pH 5 and pH 6.5, $[H_3O^+]$ catalyzes conversion of III to IV so rapidly that catalysis by buffers HB is minimized. As pH 7 is approached, however, $[H_3O^+]$ decreases to such an extent that H_2O provides a significant part of the necessary protons as do buffers HB if they are present. Above pH 9, there is no significant contribution from $[H_3O^+]$. The reaction rate rises rapidly with pH between 5 and 6.5 and above pH 8, but rises more slowly between 6.5 and 8 while the transition between proton donors takes place.

Moreover, since the first step is proportional to $[OH^-]$ and the third step, if buffer catalyzed, is proportional to [HB], the overall reaction rate:

$$k_{observed} \propto [OH^-][HB]$$

It is therefore determined by the concentration of a specific base and a general acid. Yet, since $[B^-][H^+]/[HB] = K_B$ and $[H^+][OH^-] = K_{H_2O}$, then:

$$k_{observed} \propto [B^-]$$

So, reaction sequence I to IV is apparently general base catalyzed.

At physiological pH of 7.4, where, for biochemical purposes, most deamidation rates have been measured, the reaction sequence is in transition, so all of these processes except direct hydrolysis are significant.

2 N. E. Robinson and A. B Robinson, *J. Peptide Research* **63**, 437 (2004).

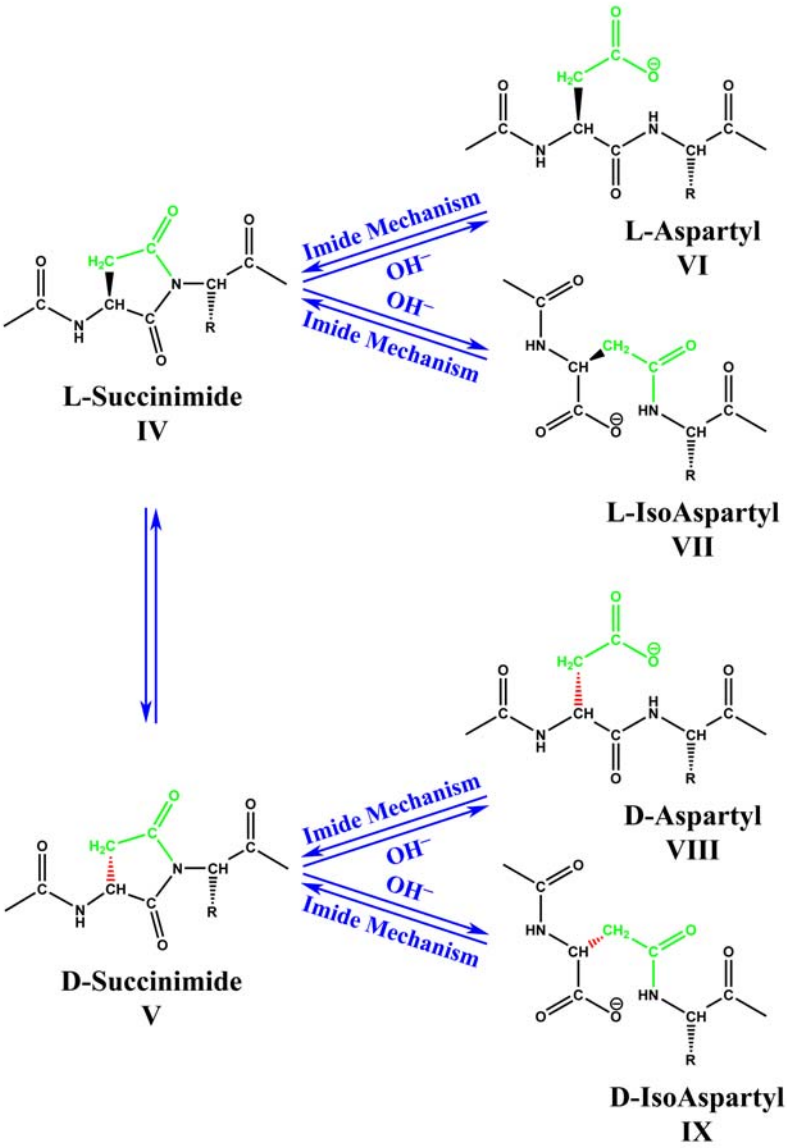

FIG. 5-2 Imide Isomerization

If the imide cannot form, as in AsnPro, or is less favorable, as with Gln, then direct hydrolysis is also important. Decomposition of succinimide IV is rapid, so conversion of I to IV controls the deamidation rate.

The reaction rate constant is, therefore written as:

$k_{observed} \propto (p1)(p2)(p3)$ or $d[I]/dt = (K_1)([I])([H^+]^{-1})(k_2)(k_{3,H2O} + [k_{3,H3O+}][H_3O^+] + [k_{3,HB}][HB])/(k_2 + k_{3,H2O} + [k_{3,H3O+}][H_3O^+]) + [k_{3,HB}][HB])$

The probabilities of the three reaction steps are p_1, p_2, and p_3. This equation is derived using the model in Figure 5-1 and the steady state assumption that:

$d[III]/dt = (k_2[II]) - (k_{-2}[III]) - (k_{-2} + k_{3,H2O} + [k_{3,H3O+}][H_3O^+]) \approx 0$

As has been demonstrated by Capasso and coworkers as discussed and referenced in Section 5-3, the experimental data from several peptides with AsnGly sequences and the Asn(67)Gly in ribonuclease A are entirely explainable by this model.

Once succinimide IV has been formed, however, the situation becomes even more complicated.

The α carbon hydrogen of IV is labile, so IV begins to racemize to V. More rapidly, however, IV is cleaved by hydrolysis on either side of the imide nitrogen to give the L-Asp VI and L-isoAsp VII isomers along with some D-Asp VIII and D-isoAsp IX from racemized IV as shown in Figure 5-2. The reaction to produce isoAsp is faster than that for Asp. This is pH dependent. The ratio isoAsp:Asp at pH 7.4 is about 3:1 in peptides.

Moreover, the Asp forms can slowly back-react through the succimide by a mechanism analogous to that for Asn as illustrated in Figure 5-1. They also equilibrate to a mixture of their carboxylic acid and carboxylate forms. Thus, the equilibrium illustrated in Figure 5-3 is established. Figure 5-3 shows this for the L forms. The similar D equilibrium is, also established, with further gradual L and D racemization through the respective imides.

Since the pK of isoAsp is lower than that for Asp, at neutral pH less isoAsp is in the carboxylic acid form, which is favored in the back-reaction because it lacks the inhibiting negative charge. Therefore, isoAsp is still the predominant species at equilibrium. As the pH approaches the Asp pKs, this difference is reduced and eventually eliminated. Moreover, since imide hydrolysis is base catalyzed, at pH of about 4 or less the imide becomes the predominant species. Imide-containing peptides and proteins are easily isolated at low pH. At neutral pH, there is usually very little imide present.

In proteins, the situation is different. Protein structures are designed for stability with ordinary L-amino acids. L-isoAsp has, in general, a structure that is not as suitable to a protein as is the original L-Asn or L-Asp. This can lead to a higher equilibrium free energy for the L-isoAsp protein form than for the L-Asp form. In this case, L-Asp is favored at equilibrium. In

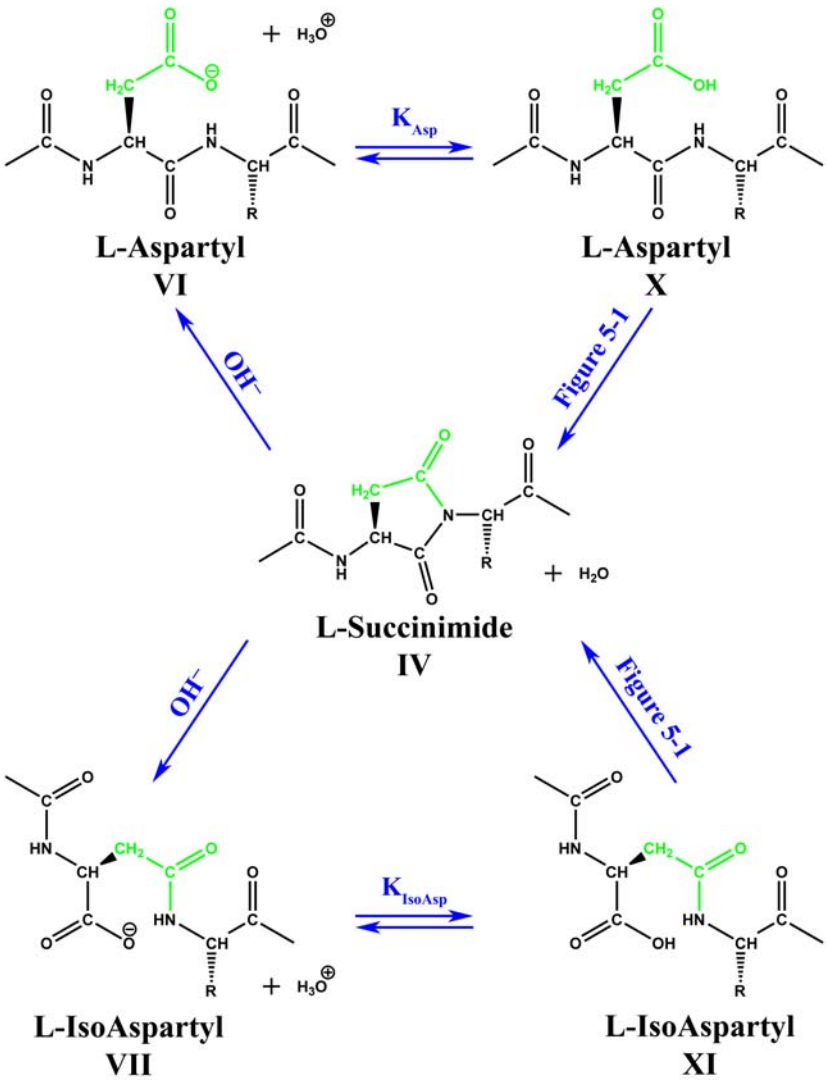

FIG. 5-3 Asp – IsoAsp Equilibration

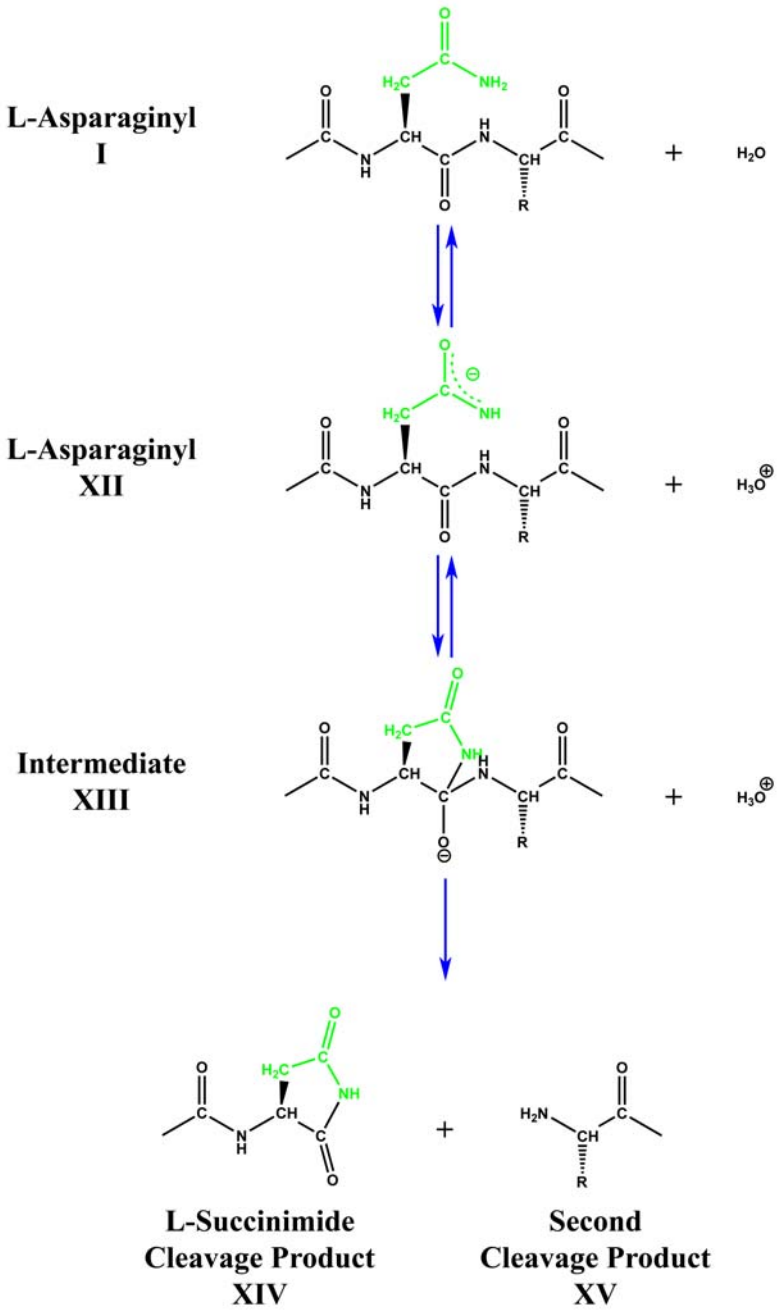

FIG. 5-4 Cleavage

ribonuclease A, for example, the equilibrium ratio L-isoAsp : L-Asp is 1:2.

Since protein structure can affect both the initial and equilibrium isoAsp:Asp ratios and also the racemization rate of the imide, these ratios cannot be easily used to study deamidation in proteins, nor can the quantities of L-isoAsp, D-Asp, and D-isoAsp be relied upon for quantitative measurement of protein deamidation. The presence of these isomers is, however, a good qualitative indicator that deamidation and/or imide-mediated Asp isomerization is taking place.

Alternatively, as illustrated in Figure 5-4, an imide XIV can be formed by reaction between the amide nitrogen and the backbone carboxylic carbon of Asn, which leads to backbone cleavage. This requires deprotonation of the amide nitrogen, which has a pK about 2 units higher than that for the backbone nitrogen, so cleavage is generally much slower than deamidation. In 0.15 M Tris, pH 7.4, 37 °C, where Asn peptide deamidation half-times range from about 1 to 400 days, Asn cleavage rates range from about 200 to more than 10,000 days.[3] Cleavage of AsnPro is the fastest sequence and, since its backbone nitrogen lacks a proton suitable for the reaction sequence in Figure 5-1, AsnPro deamidates by slow hydrolysis. Therefore, cleavage is the principal degradative pathway for AsnPro. As illustrated in Figure 5-5, hydrolysis and slow racemization of succinimides XIV and XV lead to L-Asn, L-isoAsn, D-Asn, and D-isoAsn amino-side cleavage products in addition to the carboxyl-side cleavage product produced during succinimide formation.

As pH decreases below 5, acid catalysis of deamidation takes place at an increasing rate and becomes quite fast below pH 2. This can take place, as illustrated in Figure 5-6, through ordinary acid catalysis or through an imide. That the former is most prevalent is indicated by the marked drop of the isoAsp:Asp ratio as pH decreases. Ordinary base catalysis also occurs at high pH as shown in Figure 5-7, but the rapidity of the imide mechanism at high pH usually obscures this. Also, as shown in Figure 5-7, Asp can undergo chain cleavage through formation of the anhydride by means of a mechanism similar to chain cleavage at Asn. By a similar mechanism, carboxyl-terminal Asn undergoes deamidation through anhydride formation, where the carboxyl-terminal carboxyl takes the place of Asp and the amide nitrogen that of the backbone nitrogen. This occurs for n-terminal Asn(21) in the A-chain

3 N. E. Robinson and A. B. Robinson, unpublished results.

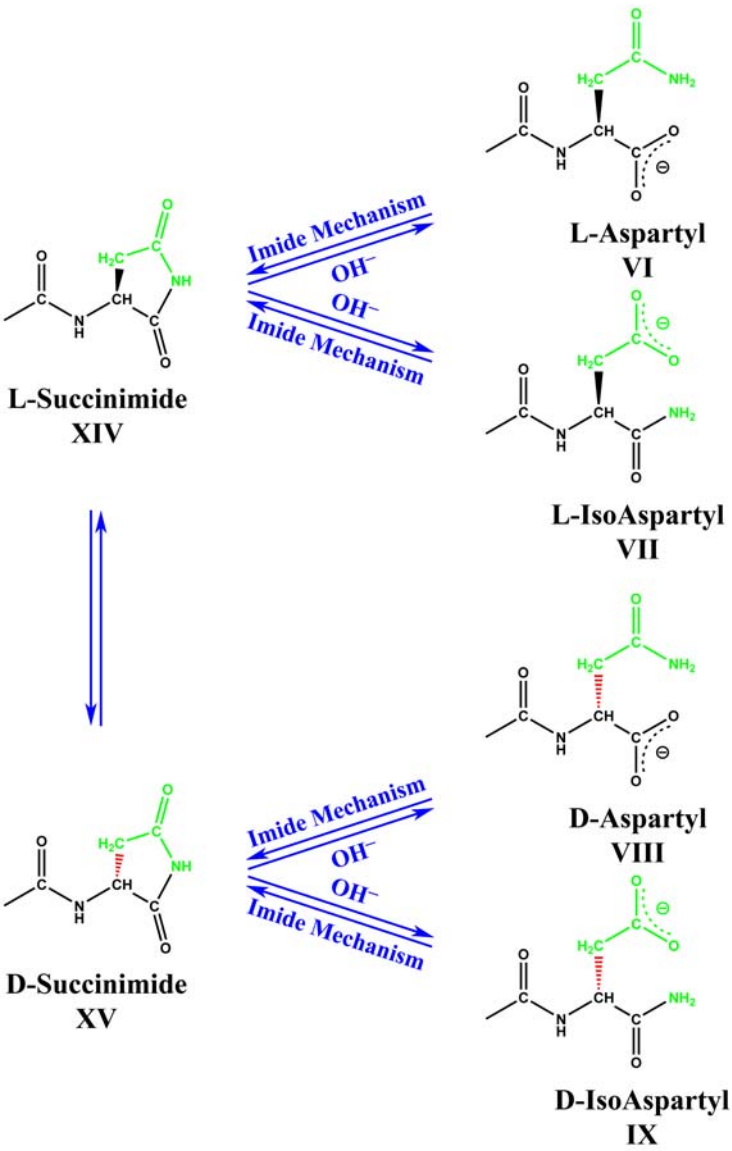

FIG. 5-5 Cleavage Product Isomerization

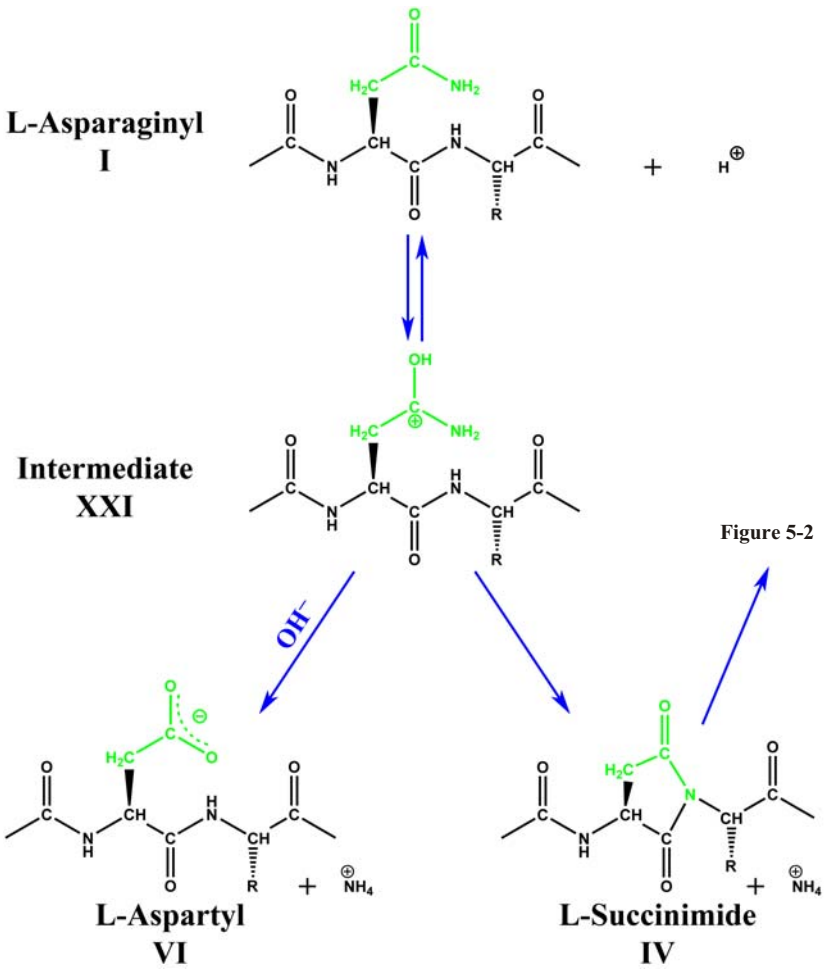

Figure 5-2

FIG. 5-6 Acid Hydrolysis of Asn

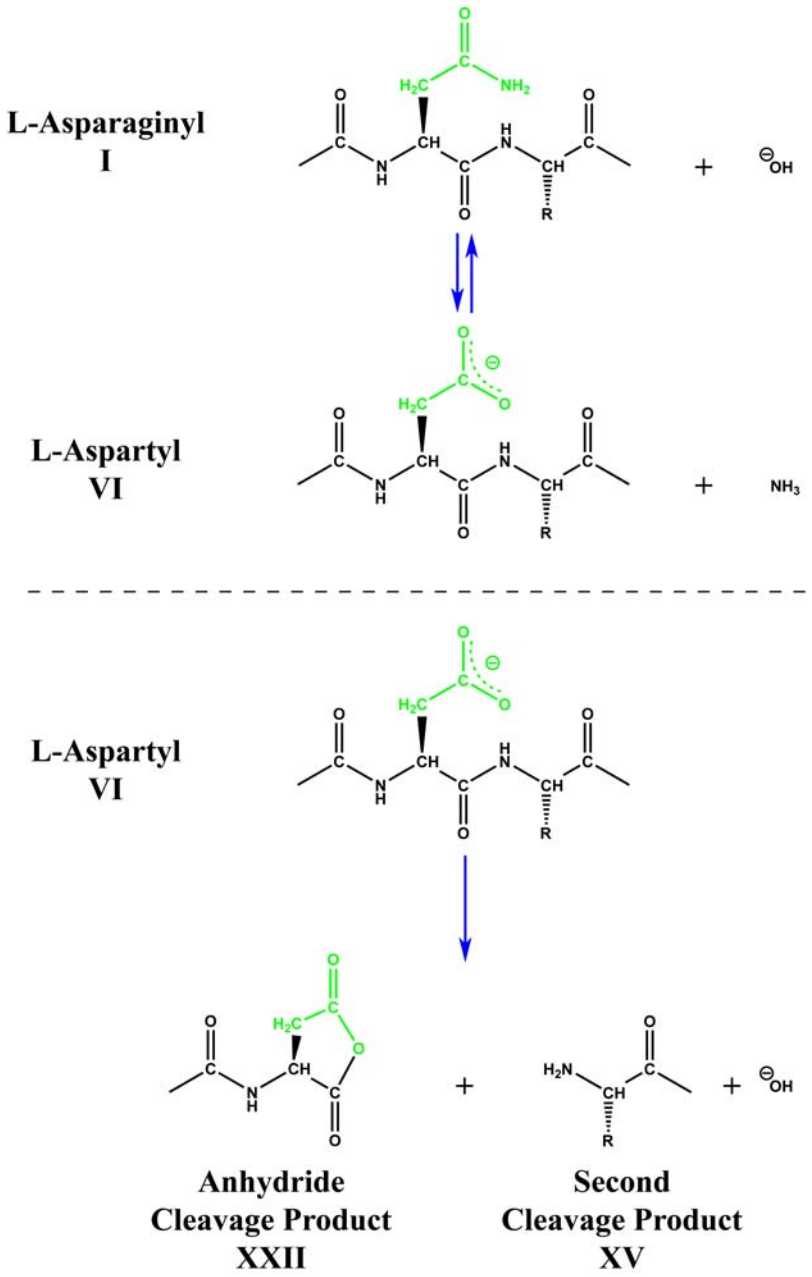

FIG. 5-7 Base Hydrolysis of Asn and Anhydride Cleavage at Asp

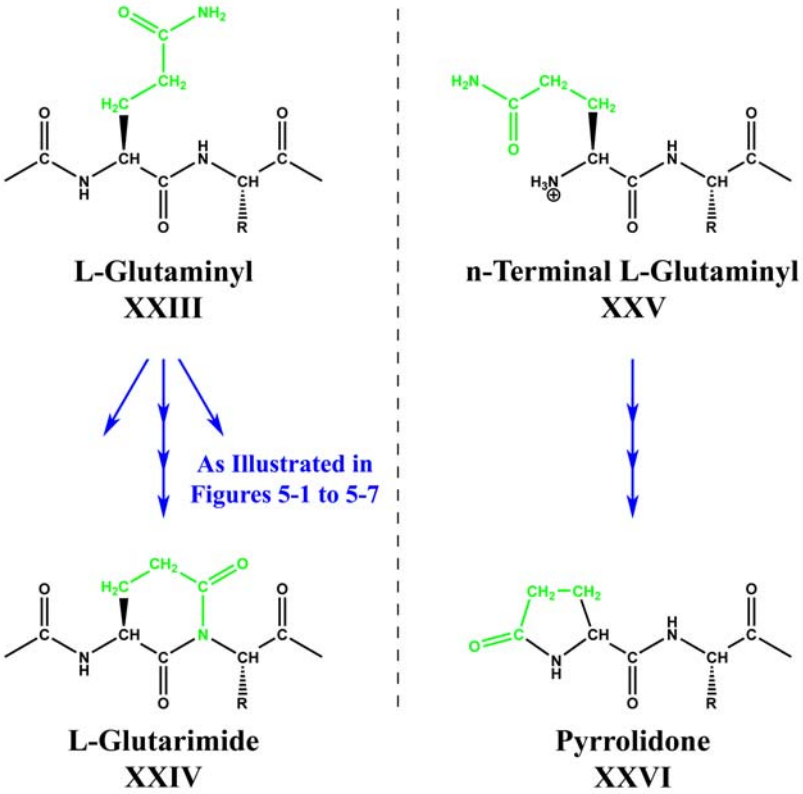

FIG. 5-8 Deamidation of Gln

of insulin at acidic pH, while Asn(3) in the B-chain deamidates above pH 6 by the usual succinimide mechanism.[4]

As illustrated in Figure 5-8, Gln can participate in the same series of reaction sequences as Asn, but it forms a six-membered glutarimide instead of a 5-membered succinimide. Formation of the glutarimide is less favorable, so imide deamidation of Gln is slower than Asn by about two orders of magnitude. An exception, however, is the stable 5-membered pyrrolidone that can form when Gln is the n-terminal residue in the peptide chain. This pyrrolidone forms for n-terminal Gln and for free glutamine. In the case of Asn or free asparagine, this ring is 4-membered and much less favorable, but it does form.[5] For this reason, deamidation of free glutamine is much faster than deamidation of free

4 J. Brange, *Acta Pharmaceutica Nordica* **4**, 209 (1992).

5 E. A. Talley, T. J. Fitzpatrick, and W. L. Porter, *J. American Chemical Society* **78**, 5836 (1956).

asparagine. Methylation of the side-chain amide of free asparagine leads to imide formation and a 10-fold increase in deamidation rate.[6]

The deamidation reaction rates of ordinary Asn residues through the imide mechanism in physiological solvent conditions are, therefore, largely affected by several factors:

These factors that control deamidation rate are unique, so the overall reaction does not lend itself to a simple activation energy explanation. Since each factor leads to an individual probability of reaction, we can write the reaction probability for the first three under physiological pH conditions as P = (p1)(p2)(p3), where p1, p2, and p3 are not entirely independent, but are largely so. This is the origin of the equation for $k_{observed}$ given above. The elements that most affect deamidation include:

1. The equilibrium deprotonation of the involved nitrogen, which depends upon pH, rises with increasing concentration of OH^-. The intrinsic acidity of this nitrogen depends upon inductive and electrostatic effects and other structure-dependent factors and, therefore, upon peptide sequence. It is also dependent upon solvent polarity and probably, although not yet demonstrated, upon the structure of water. Since this reaction, achieves equilibrium much more rapidly than the reactions that follow, it is governed by pH and its equilibrium constant.

2. Formation of the cyclic intermediate depends primarily upon steric hindrance and the amount of available conformational space of the different atoms in the peptide. In peptides at ordinary temperatures, this reduces to a simple problem in 3-dimensional geometry, wherein atom size and rotational freedom determine reaction rate.[2] This effect is expected to be relatively temperature independent.

In proteins under physiological conditions, peptide steric hindrance is joined by hindrance from the protein structure, which results from the complex combination of hydrogen bonds, van der Waals bonds, ionic bonds, solvent structure, and other factors that hold the protein in its unique conformation.

These impediments in proteins can be quantitatively predicted from 3-dimensional protein structure by observing the exact position of the amide side chain and the protein structure rearrangements necessary to allow that side chain to rotate into the position required for the cyclic intermediate.[7] These rearrangements sometimes involve the breaking of weak bonds, so this hindrance can be relatively temperature dependent.

6 A. V. Klotz and B. M. Higgins, *Archives of Biochemistry and Biophysics* **291**, 113 (1991).

7 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

3. Leaving group probability for the tetrahedral intermediate, while somewhat affected by electrostatic, inductive, and structural factors, depends primarily upon the availability of a donated proton and is therefore highly pH and buffer ion dependent. If not decomposed, the tetrahedral intermediate can revert to the open form, so timely abstraction of the leaving group accelerates deamidation.

At physiological pH, this leaving group removal is facilitated through general acid catalysis by H_2O, $[H_3O^+]$, or [HB]. When combined with the specific base equilibrium $[OH^-]$ dependence of the first reaction step, catalysis by HB renders the overall reaction kinetically equivalent to general base catalysis by $[B^-]$.

Dependence of the deamidation reaction rate in proteins upon [HB], while still important, appears to be less so than in peptides. This may be the result of reduced HB access to the deamidation site as a result of 3-dimensional protein structure.

4. Factors 1, 2, and 3 can be affected by chemically active substituents of the neighboring residues in the peptide chain, especially the carboxyl nearest neighbor. These effects are, as yet, not well understood. When steric hindrance is removed from the reaction rate, the effects of carboxyl-side side chains with hydroxyl, sulfur, basic, acidic, and amide groups can be easily discerned.[8] The specific mechanisms of the effects that each of these types of groups have upon reactions 1, 2, and 3 are still unsettled questions.

While protein structure usually inhibits deamidation, there are many instances in which protein structure near the amide is sufficiently benign that the amide deamidates at its primary sequence controlled rate. There are also relatively rare instances in which protein structure actually increases the deamidation rate either by holding the amide side chain in the position required for formation of the cyclic intermediate or by providing access to a catalytic group.

Substantial amounts of the L-Asp VI and L-iso-Asp VII forms and lesser, but detectable, amounts of D-Asp VII and D-isoAsp IX forms are found in proteins as a result of *in vitro* and *in vivo* deamidation. Housekeeping enzymes that specifically convert L-isoAsp and D-Asp into L-Asp have been found to be widely distributed and to be essential for good health in living things. These enzymes are discussed in Chapter 16. They do not reverse the charge change at the deamidation site of $0 \rightarrow -1$ at neutral pH, which occurs upon deamidation.

5. Since the imide intermediates are formed on the carboxyl side of Asn, sequence dependence of deamidation is 10- to 20-fold more pro-

8 N. E. Robinson and A. B. Robinson, *J. Peptide Research* **63**, 437 (2004).

nounced on the carboxyl side as compared with the amino side. There is, however, sequence dependence from the nearest neighboring residues on both sides of the amide residue. In peptides with substantial freedom of movement, this dependence can also be detected for other residues further along the peptide chain in both directions as shown in Chapter 7. These effects of more distant residues are probably largely suppressed in proteins.

6. Solvent properties such as pH, temperature, ionic strength, catalytic components, polarity, viscosity, water structure, and other elements affect deamidation more or less strongly in accordance with circumstances.

The overall result of these mechanisms is that the half-times of sequence-dependent Asn peptide deamidation at neutral pH and physiological temperature extend from about 0.5 days to 500 days, and Gln deamidation half-times extend from about 600 days to 20,000 days. Under these conditions, Asn deamidation proceeds >95% through the succinimide with the exception of AsnPro. Up to deamidation half-times of about 5,000 days, Gln deamidation proceeds >80% through the glutarimide. The Gln peptides with longer half-times and AsnPro peptides deamidate primarily through hydrolysis. This situation is summarized quantitatively in Figures 6-1, 6-2, and 6-3.[9]

This complex of mechanisms is supported by a substantial number of experiments as reviewed below. It explains the products and relative reaction rates observed and is consistent with quantitative work to date in both peptides and proteins. Overall, the reaction mechanisms summarized in Figures 5-1 through 5-8 are supported by a large body of experimental evidence.

The qualitative discovery of these mechanisms and initial quantitative evaluation in primarily non-aqueous solvents was carried out, for the most part, by chemists interested in peptide synthesis. Since then, many investigators have tested various aspects of these mechanisms both qualitatively and quantitatively in aqueous systems, as is reviewed in Section 5-3.

9 N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004); N. E. Robinson and A. B. Robinson, *Mechanisms of Aging and Development* **125**, 259 (2004).

5-3. MECHANISTIC EXPERIMENTS

In Chapters 1 to 4 we reviewed the early deamidation literature including mechanistic studies up to about 1970. This included the work of Sondheimer and Holley in 1954[10] in which imide intermediates were observed and the suggestion made that these might be relevant in physiological systems. After 1970, sufficient experimental data began to accumulate to test this hypothesis, and the hypothesis was applied, restated, and reemphasized by additional investigators.[11]

In 1971, Gráf, Bajusz, Patthy, Barát, and Cseh[12] corrected a sequence error in pig and human adrenocorticotropic hormone, ACTH, by reference to the imide mechanism. The assignments were TyrProAsp(25)GlyAla and GluAspGln(30)LeuAla. Assuming an imide mechanism and, therefore, reasoning that the lability of ACTH with respect to deamidation is more consistent with a sterically unhindered Asn rather than a slow Gln, they investigated and corrected the sequence to TyrProAsn(30)GlyAla and GluAspGlu(30)LeuAla.

Bodanszky and Kwei,[13] in 1978, found a carboxyl side sequence dependence for succinimide formation in Asp dipeptides that is qualitatively similar to that observed for Asn peptides.

Imide formation during peptide synthesis has been a continual problem, especially in the case of AsnGly and AspGly as discussed by Mojsov, Mitchell, and Merrifield in 1980 and Bodansky and Martinez in 1981.[14]

Possible mechanisms for both nonenzymatic and enzymatic deamidation of Asn and Gln were summarized by Wold in 1985.[15] Meinwald, Stimson, and Scheraga[16] confirmed in 1986 that AsnGly deamidation in an end-blocked dipeptide in aqueous solution at neutral

10 E. Sondheimer and R. W. Holley, *J. American Chemical Society* **76**, 2467 (1954).

11 P. Bornstein and G. Balian, *J. Biological Chemistry* **245**, 4854 (1970); Y. Meinwald, E. R. Stimson, and H. A. Scheraga, *Int. J. Peptide and Protein Research* **28**, 79 (1986); T. Geiger and S. Clarke, *J. Biological Chemistry* **262**, 785 (1987); J. P. Tam, M. W. Riemen, and R. B. Merrifield, *Peptide Research* **1**, 6 (1988).

12 L. Graf, S. Bajusz, A. Patthy, E. Barart, and G. Cseh, *Acta Biochim. Biophys. Acad. Sci. Hung.* **6**, 415 (1971).

13 M. Bodanszky and J. Z. Kwei, *Int. J. Peptide and Protein Research* **12**, 69 (1978).

14 S. Mojsov, A. R. Mitchell, and R. B. Merrifield, *J. Organic Chemistry* **45**, 555 (1980); M. Bodanszky and J. Martinez, *Synthesis* **5**, 333 (1981).

15 F. Wold, *Trends in Biochemical Sciences* 4 (1985).

16 Y. Meinwald, E. R. Stimson, and H. A. Scheraga, *Int. J. Peptide and Protein Research* **28**, 79 (1986).

to basic pH proceeds through a succinimide intermediate with production of the Asp and isoAsp forms in a ratio of 3:1.

Most of our knowledge of reaction mechanisms involving Asn and Gln and analogous processes with Asp and Glu was therefore first[17] developed and consolidated in the field of chemical peptide synthesis. Studies of succinimide formation during peptide synthesis showed that AspGly, AspSer, AspAsn, and AspHis sequences are especially susceptible and that steric hindrance in the carboxyl neighbor residues impedes the reaction. These findings were qualitatively and quantitatively similar to those for sequence dependence of Asn deamidation under physiological conditions as demonstrated with 65 Asn and Gln pentapeptides between 1973 and 1974[18] and were definitively examined with 477 Asn and Gln peptides between 3 and 13 residues in length between 2001 and 2004.[19]

By the mid 1980s, most aspects of the overall reaction mechanisms of deamidation and related processes had been demonstrated and observed both qualitatively and quantitatively, and it was known that deamidation reaction rates were genetically controlled and varied over a wide range under physiological conditions. In the late 1980s, three separate efforts to understand these mechanisms more completely were undertaken by Capasso, Clarke, Borchardt, and their coworkers. These investigations occurred, for the most part, concurrently.

In 1989, Capasso and coworkers verified imide formation during deamidation of three end-blocked tripeptides at high pH and the strong dependence on basic pH commencing at about pH 6. Carboxyl neighbor sequence dependence was, as expected, Gly>Ser>Ala. Also in 1991, they conducted a study of a blocked Gln dipeptide, demonstrating both the glutarimide and hydrolysis deamidation mechanisms. In 1991, using end-blocked AsnGlyGly, they demonstrated a marked decrease in deamidation rate with addition of organic solvents to the aqueous system, 50% acetonitrile reducing the rate 25-fold, and verified a salt dependence of rate with $K_2HPO_4/KH_2PO_4 > (NH_4)SO_4 >> MgSO_4 > NaCl$ and Na_2SO_4, with a 6-fold range at 0.5 M and a buffer depend-

17 M. Bodanszky and J. Z. Kwei, *Int. J. Peptide and Protein Research* **12**, 69 (1978); M. Bodanszky and J. Martinez, *Synthesis* **5**, 333 (1981); J. P. Tam, M. W. Riemen, and R. B. Merrifield, *Peptide Research* **1**, 6 (1988).

18 See review in A. B. Robinson and C. J. Rudd, *Current Topics in Cellular Regulation* **8**, 247 (1974).

19 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001); N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001); N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

ence of phosphate > tris and Im > ammonia > carbonate. The requirement for an aqueous environment had been reported by earlier investigators.[12]

In 1992, Capasso and coworkers reported the crystal structure of the imide intermediate in the ACTH sequence of end-blocked ProAsuGlyAla and studied the folding of end-blocked AsuGlyAla in 1995. In 1993, on the basis of studies of end-blocked GlyAsnGlyGly, they proposed an additional reaction tetrahedral intermediate II. They suggested that, at pH < 6.5, ring formation and specific base catalysis of the backbone deprotonation are rate limiting, but that, at pH > 6.5, general and specific base catalysed rearrangement of III to the succinimide is rate limiting. This finding was for an entirely carboxyl-side unhindered peptide with facile ring formation. In 1995, they also undertook solution studies of peptide structure in succinimide peptides.

In 1996, Capasso and coworkers studied peptide bond cleavage next to Asn, and also carried out a study of the post-reaction equilibrium of isoAsp, Asp, and succinimide. In 2000, Capasso reviewed the literature values of phosphate catalyzed peptide deamidation rates and combined them in a systematic way, although the resulting values are only semiquantiative because early measurement methods were more primitive, phosphate catalysis was present, and variations in reaction conditions needed to be accommodated.

Between 1999 and 2001, they carried out deamidation studies of Asn(67)Gly in ribonuclease A and appropriate model peptides, confirming identical mechanisms to those established for peptides.

These papers provide kinetic and thermodynamic constants for many of the reactions. The relevant references are 1989CM, 1989CM1, 1991CM, 1991CM1, 1992CM, 1993CM, 1995CM, 1996CM, 1996C, 1999CS, 2000CB, 2000C, 2000CC, 2001CC.

In 1987, Geiger and Clarke studied degradation of the L-Asn, D-Asn, and L-Asp forms of the ACTH sequence ValTyrProAsnGlyAla. The deamidation half-time for L-Asn at 37 °C, pH 7.4, 0.1 M phosphate was 1.4 days, while for D-Asn it was 2.1 days. Succimide formation was 34-fold slower for the L-Asp form. Succinimide concentration reached a maximum of about 5.5% of total peptide at about 0.4 days and the racemization half-time of the succinimide was 19.5 hours as compared with 2.3 hours for hydrolysis to L-Asp and L-isoAsp. As expected, at 70 °C, only cleavage without deamidation was observed with AsnPro, while AsnIle gave both deamidation and cleavage. In 1989, Stephenson and Clarke measured deamidation rates for five additional analogues of this hexapeptide, which gave values similar to those of the

35 Asn pentapeptides determined 16 years earlier by Robinson and coworkers. In 1995, Brennan and Clarke extended these studies to six more Asn peptides and six Asp peptides with the expected results.

In 1993, Clarke and coworkers showed that the deamidation rates of their first six hexapeptides were diminished by organic solvents with low dielectric constant as had been demonstrated by Capasso, Mazzarella, and Zagari in 1991.

In a 1995 paper, however, and a later one by Radkiewicz, Zipse, Clarke, and Houk in 2001, Clarke and coworkers suggested that steric hindrance is of little or no importance in determining the sequence dependence of peptide deamidation rates. They attributed sequence dependence instead to electrostatic and inductive effects on the acidity of the backbone nitrogen.

This hypothesis was supported primarily by a correlations between their deamidation rates on 8 peptides, measured rates of sequence dependence of exchange of the proton of the backbone nitrogen by other investigators,[20] and corrective calculations.

Correlation does not prove causality; the rates of hydrogen exchange are, themselves, subject to steric effects; and the measured exchange rates do not necessarily correspond to equilibrium acidity. Also, it has now been demonstrated that the steric effects of sequence on deamidation are quantitative and are theoretically predictable.[8]

While electrostatic and inductive effects are important in governing the acidity of the amide backbone hydrogen, the hypothesis that these effects transcend and, in fact, entirely eclipse steric effects on overall deamidation rate is probably not correct.

The relevant references for this work of Clarke and coworkers are 1987GC, 1989SC, 1993BC1, 1995BC, and 2001RZ.

In 1990, Borchardt and coworkers reported the sequence, buffer, and pH dependence of deamidation of four hexapeptide analogues of ACTH. In 1992 they measured the deamidation of a tetrapeptide by mass spectrometry. In 1993, they found that Asp hydrolysis of one of their deamidated ACTH sequences was about 50-fold slower than Asn deamidation, in agreement with the findings of Clarke and coworkers in 1987. In 1993, they studied the deamidation of four 32-residue growth hormone-releasing factor analogs as a function of methanol-water mixtures, attributing the slower rates with methanol to partial α-helix for-

20 Y. Bai, J. S. Milne, L. Mayne, and S. W. Englander, *Proteins: Structure, Function, and Genetics* **17**, 75 (1993); G. P. Connelly, Y. Bai, M. Jeng, and S. W. Englander, *Proteins: Structure, Function, and Genetics* **17**, 87 (1993).

mation. In 1994, this work was extended to include various drying procedures suitable for minimizing deamidation.

Between 1996 and 2001, Borchardt and coworkers demonstrated that solute impediments slowed the rate of deamidation of AsnGly peptides as did various organic solvents and polymer matrixes. In addition to quantitative data on various formulations, the primary finding was that dipole effects in the solvent affect the deamidation rate. They reported that viscosity has little effect on deamidation rate, but Li, Hageman, and Topp reported a substantial slowing of deamidation with increased viscosity.[21] This is in accord with the experiments of Capasso, Clarke, and coworkers.

In 2000, in a study of four peptides, Borchardt and coworkers reported sequence dependence on His at the AsnGlyHis position. Carboxyl-side His and amino-side Ser have been especially interesting to investigators since the finding in 1974 that GlySerAsnHisGly has a deamidation half-time in pH 7.4, I 0.2, phosphate buffer, at 37 °C of 6.4 days as compared with the *in vivo* rabbit muscle aldolase protein deamidation SerAsnHis rate of 8 days.[22] Deamidation rates for various peptide analogues of SerAsnHis were also reported in 1991.[23]

In 2003, Borchardt and coworkers reported increased deamidation of lymphotoxin upon denaturation, apparently as a result of disruption of 3-dimensional structure, and racemization of a pentapeptide during deamidation at pH 10, 70 °C.

This work is reported in 1989SM, 1990BP, 1990PB1, 1990PB2, 1990PB, 1992SW, 1993OB, 1993SF, 1994OB1, 1994OB, 1994OP, 1994OP1, 1996XV, 1999MH, 1999LH, 2000XA, 2000LS1, 2000LS3, 2000GS1, 2001SS, 2002XS, 2003LB, and 2004SB.

In 1974, White suggested that an occasional alternative pathway for deamidation could be through reaction with nitrous acid,[24] especially at low gastric pH.[25] In 1976, Landon discussed cleavage at AspPro[26] and invoked the anhydride mechanism shown in Figure 5-7. Anhydride formation requires the backbone nitrogen to be protonated, so this takes place at mildly acid pH. It was suggested[27] that the Pro backbone leav-

21 R. Li, M. J. Hagenim, and E. Topp, *J. Peptide Research* **59**, 211 (2002).

22 J. H. McKerrow and A. B. Robinson, *Science* **183**, 85 (1974).

23 R. Tyler-Cross and V. Schirch, *J. Biological Chemistry* **266**, 22549 (1991).

24 L. K. Keefer and P. P. Roller, *Science* **181**, 1245 (1973)

25 E. H. White, Letter to Arthur Robinson, (1974).

26 M. Landon, *Methods in Enzymology* **XLVII**, 145 (1976).

27 D. Piszkiewicz, M. Landon, and E. L. Smith, *Biochemical and Biophysical Research Communications* **40**, 1173 (1970).

ing-group nitrogen is more basic than that of other residues, which enhances the protonation of the leaving group. This renders this reaction so much faster in AspPro sequences that it has been used for selective cleavage of proteins during sequence determination.

Selective cleavage of proteins at AsnGly was also developed.[28] Protein 3-dimensional structure is disrupted with guanidine, and imide formation is promoted with mild base. The resulting succinimide is then cleaved with hydroxylamine. Selectivity results from the ease with which AsnGly sequences form the imide. Hydroxylamine cleavage has been used to distinguish between succinimide and AsnGly.[29] Selective cleavage at AsnGly by hydroxylamine has also been used to release the desired product from recombinant fusion proteins.[30] Selective cleavage of peptides at AsnPro has also been carried out in 25% ammonia at 45 °C.[31]

Cleavage products are formed in most peptide deamidation experiments, but the reaction is usually much slower than deamidation.[32] Some examples of peptide cleavage after Asn are found in references 1993BC, 1995BC, 1997NF, and 2002TK.

In 1988, Lura and Schirch[33] reported the presence of a seven-membered ring in the peptide ValAsnGlyAla formed by reaction of the carbonyl carbon of Asn with the amino-terminal amino nitrogen. Ordinary deamidation was not observed, while formation of the seven-membered ring occurred about twice as fast as did formation of succinimide in N-acetylValAsnGlyAla.

Patel[34] studied the deamidation of ACTH and four hexapeptide analogues under a wide variety of solvent conditions and reported confirmation of the imide mechanism at neutral to high pH and direct hydrolysis at low pH.

In 1993, Klotz and Thomas reported[35] that deamidation of IleAlaProGlyGlyAsnGlyTyr proceeds with the usual mechanism, but

28 P. Bornstein and G. Balian, *Methods in Enzymology* **47**, 132 (1976).

29 M. Y. Kwong and R. J. Harris, *Protein Science* **3**, 147 (1994).

30 M. Antorini, U. Breme, P. Caccia, C. Grassi, S. Lebrun, G. Orsini, G. Taylor, B. Valsasina, E. Marengo, R. Todeschini, C. Andersson, P. Gellerfors, and J. Gustafsson, *Protein Expression and Purification* **11**, 135 (1997).

31 E. Tarelli and P. H. Corran, *J. Peptide Research* **62**, 245 (2003).

32 N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

33 R. Lura and V. Schirch, *Biochemistry* **27**, 7671 (1988).

34 K. Patel, *Pharmaceutical Biotechnology* **5**, 201 (1993).

35 A. V. Klotz and B. A. Thomas, *J. Organic Chemistry* **58**, 6985 (1993); A. V. Klotz, *Bioorganic Chemistry* **21**, 83 (1993).

that methylation of the amide side-chain nitrogen of Asn leads to slower degradation through a combination of deamidation and chain cleavage.

The glycopeptide antibiotic vancomycin is easily deamidated at Asn through the imide mechanism.[36] The structure of vancomycin holds the Asn side chain in a position ideal for imide formation. This conformation changes with pH, thus influencing the reaction.

In 1999, Kosky, Razzaq, Treuheit, and Brems showed,[37] in a study of end-blocked 18-residue peptides including the sequence ArgAsnAla and with and without helix-breaking residues, that deamidation was essentially halted by helix formation. Their relative rates of deamidation were quantitatively consistent with zero detectable deamidation in the helix form. In this case, the imide is inhibited, but hydrolysis probably continues.

It has been suggested that the structure of water may affect these reaction mechanisms,[38] but no verifying experiments have been performed. In view of the fact that clathrate water structures are expected to be stabilized by peptide and protein side chains at physiological temperatures,[39] this effect should be explored.

Between 2001 and 2004, Robinson and Robinson and their coworkers reported the sequence-dependent deamidation rates of 360 Asn pentapeptides, 52 Gln pentapeptides, and 65 other Asn peptides of between 3 and 13 residues under physiological solvent conditions.[19]

Robinson and Robinson[40] then combined these peptide rates with protein structure data for 28 proteins in which 38 specific Asn had been reported to deamidate out of a total Asn of 268. Observations of the exact positions of the Asn side chains and the structural alterations necessary to achieve the imide configuration were parameterized and optimized to provide the best predictions of relative deamidations within the 28 proteins. The computation procedure was then verified by comparison with the literature values for the absolute deamidation rates

36 A. S. Antipas, D. V. Velde, and V. J. Stella, *Int. J. of Pharmaceutics* **109**, 261 (1994); A. S. Antipas, D. V. Velde, and V. J. Stella, *Pharmaceutical Research New York* , S-279 (1994); A. S. Antipas, D. G. Vander Velde, S. D. S. Jois, T. Siahaan, and V. J. Stella, *J. Pharmaceutical Sciences* **89**, 742 (2000).

37 A. A. Kosky, U. O. Razzaq, M. J. Treuheit, and D. N. Brems, *Protein Science* **8**, 2519 (1999).

38 N. E. Robinson and A. B. Robinson, *J. Peptide Research* **63**, 437 (2004).

39 L. Pauling, *Science* **134**, 15 (1961); S. L. Miller, *Proc. Natl. Acad. Sci. USA* **47**, 1515 (1961); A. B. Robinson, K. F. Manly, M. P. Anthony, J. F. Catchpool. and L. Pauling, *Science* **149**, 1255 (1965).

40 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001).

in 10 proteins. This comparison showed that the procedure is remarkably accurate in predicting Asn deamidation rates.

The protein computation procedure was then computerized and applied to the entire 17,935 protein 3-dimensional structure data base as of January 2003, thereby predicting deamidation rates for all 170,014 Asn in these proteins.[41] Most of these deamidation rates have not been measured. Agreement between the computations and those that have been measured before and since is excellent. The computerized procedure was found to be more than 96% reliable in predicting the most unstable amides within a single protein.

The sequence-dependent peptide rates were found to conform precisely to observed regularities and theoretical predictions corresponding to steric control of deamidation from the carboxyl-side residue and specific effects from the hydroxyl, sulfur, basic, and acidic side-chain substituents. Amino-side effects were also observed as were inhibitory interactions between acidic and basic components in both positions.[42]

While these peptide and protein calculations do not definitively prove any specific deamidation mechanism, they were modeled upon the mechanism summarized in Figure 5-1. The marked success of these computations in predicting primary, secondary, tertiary, and quaternary structure-determined deamidation rates in peptides and proteins provides, therefore, substantial support for this mechanism.

Additional studies of interest to the mechanism of Asn and Gln deamidation and related reactions include 1954KM, 1976TT, 1978KI, 1983SL, 1988HA2, 1988YG, 1991F, 1992PS, 1993PG, 1994SN, 1994AP, 1994CM, 1997MA, 1998NY, 1998DC, 1999XS, 2000IY, 2000SS, 2001SK, 2001KA, 2001BB, 2002KA, 2002HB, 2002CL, 2003BU, and 2004GB.

41 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002)

42 N. E. Robinson and A. B. Robinson, *J. Peptide Research* **63**, 437 (2004)

CHAPTER 6

Primary Structure Dependence of Nonenzymatic Deamidation of Asn and Gln

6-1. PEPTIDE EXPERIMENTS

As described in Chapter 4, before 1970 the deamidation of a few dipeptides and tripeptides had been carried out under extreme conditions of pH and temperature. Some steric effects had been observed, but even the question of the relative rates of deamidation of Asn peptides vs. Gln peptides was unsettled. A substantial part of these experiments included amino-terminal Gln, so Gln appeared to deamidate faster than Asn.

The hypothesis that deamidations of Asn and Gln can serve as biomolecular clocks initiated a series of studies designed to determine the available settings for those clocks under physiologically relevant solvent conditions.[1] The strategy adopted was to determine the effect of primary sequence on the deamidation rate by means of peptide models of the type GlyXxxAsn/GlnYyyGly and to infer the effects of secondary, tertiary, and quaternary structure by difference in comparisons with protein deamidation rates. Deamidation rates of 35 Asn peptides and 30 Gln peptides were measured in pH 7.4-7.5 phosphate buffer, 37 °C, I 0.15-0.2.

The sequence-dependent deamidation half-times of these peptides varied between 6 days and 10 years, with the half-times for Asn peptides clearly shorter than those for Gln. Deamidation was found to be slowed by steric hindrance and accelerated by both negatively and positively charged residues and hydroxyl groups. The fastest deamidating sequence was GlySerAsnHisGly with carboxyl-side His.

1 A. B. Robinson, J. W. Scotchler, and J. H. McKerrow, *J. American Chemical Society* **95**, 8156 (1973); A. B. Robinson and S. Tedro, *Int. J. of Peptide and Protein Research* **5**, 275 (1973); J. H. McKerrow and A. B. Robinson, *Science* **183**, 85 (1974); A. B. Robinson, J. H. McKerrow, and M. Legaz, *Int. J. of Peptide and Protein Research* **6**, 31 (1974); A. B. Robinson and J. W. Scotchler, *Int. J. of Peptide and Protein Research* **6**, 279 (1974); A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **71**, 885 (1974); A. B. Robinson and C. J. Rudd, *Current Topics in Cellular Regulation* **8**, 247 (1974).

It was further shown that the peptides GlyThrAsnGlu and GlySerAsnHisGly deamidate at rates essentially the same as the nearest-neighbor sequences AlaThrAsn(103)Glu in cytochrome c and IleSerAsnHisAlaTyr in aldolase. These deamidations apparently initiate *in vivo* turnover of these enzymes. While these two deamidations in flexible regions near the ends of the protein chains were largely unaffected by secondary, tertiary, or quaternary structure, it was generally evident that most Asn residues in proteins deamidate at rates slower than those found for the corresponding peptides. As a special case, it was also found that the second sequence to deamidate in cytochrome c, AsnLysAsn(54)LysGly, is accelerated by a structural change in the protein that occurs as a result of the first deamidation.

Thus, by 1974, a wide range of sequence-controlled deamidation rates along with the elements of this control provided by steric and functional residue side chains had been qualitatively demonstrated, and it had been shown that protein structure could both enhance or suppress these rates, with suppression being the most common.

Between 1986 and 2000, deamidation rates of an additional 32 Asn sequences and one Gln sequence were measured. This work established the applicability of the succinimide and glutarimide deamidation mechanisms at physiological pH and temperature and extended the lower end of the range of sequence-controlled rates from 6 days to 1 day. Further studies comparing peptide rates to those of similar sequences in proteins were also carried out. See Table 6-1.

While only a few three-dimensional protein structures were available in the early 1970s, this number had grown to more than 10,000 by 2000. This wealth of structural information could not, however, be productively applied to the understanding and prediction of protein deamidation rates until a suitable and complete library of experimental sequence-controlled deamidation rates under standardized conditions was available.

Therefore, between 2000 and 2003, high-precision measurements by direct-injection mass spectrometry of the deamidation rates of 425 Asn peptides and 52 Gln peptides in pH 7.4, 37.0 °C, 0.015 Tris-HCl were carried out. These included measurements of 412 pentapeptides with varying nearest neighbor residues on each side of the amide[2] of the type GlyXxxAsn/GlnYyyGly and also 65 peptides between 3 and 13 residues in length. The latter were designed to test the relevance and ap-

2 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001); N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

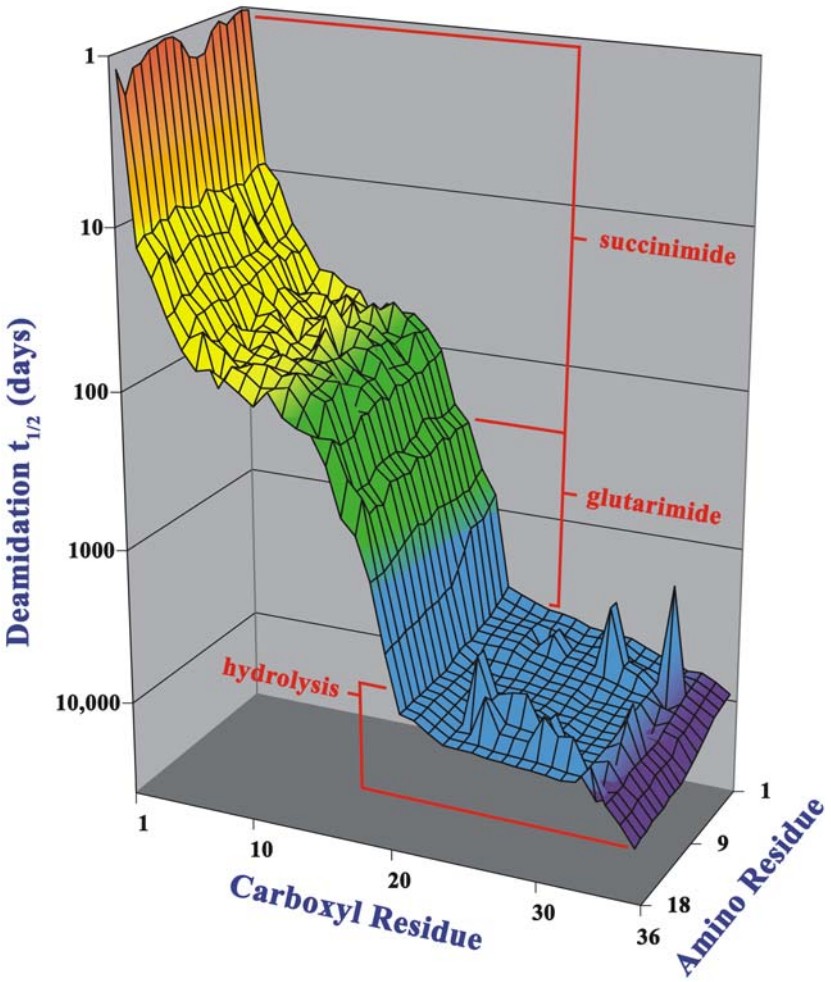

FIG. 6-1 Sequence dependence of Asn and Gln pentapeptide deamidation half-times. The amino-side and carboxyl-side residue sets have been ordered by increasing deamidation half-time. Each intersection of the black lines represents the deamidation half-time of a unique pentapeptide sequence of the type GlyXxxAsn/GlnYyyGly. Adapted from 2003R.

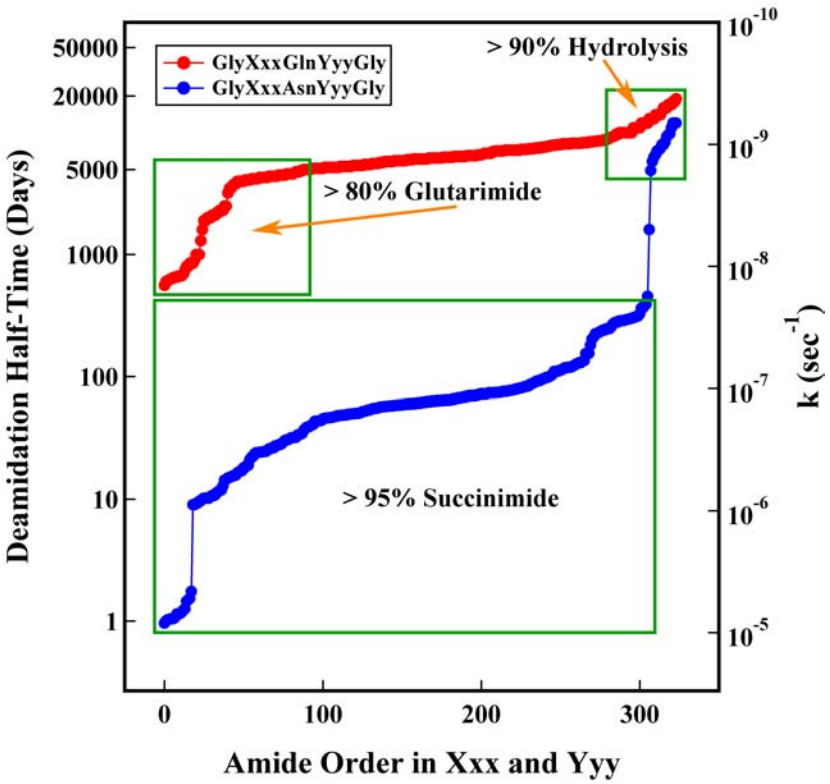

FIG. 6-2 Distribution of pentapeptide deamidation rates for peptides with sequences GlyXxxAsnYyyGly, ●; and GlyXxxGlnYyyGly, ●. Adapted from 2004RR1.

plicability of the pentapeptide models and to discover the effects of residues further removed from the amide.[3] In the longer of these peptides, secondary structure effects were also observed.

These 477 peptide deamidation experiments made possible the combination of experimental primary sequence dependent deamidation and three-dimensional protein structures in order to produce a computation method that can reliably predict the deamidation rates of Asn residues in proteins for which the three dimensional structures are known[4] and the computerization and application of this method to the 17,935 pro-

3 N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001).

4 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001).

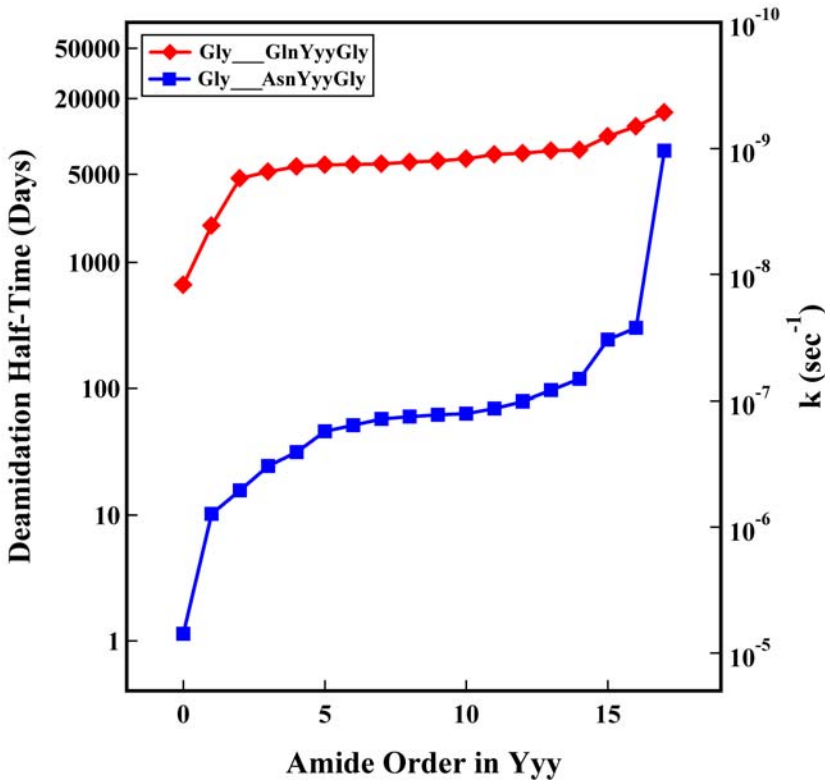

FIG. 6-3 Distribution of median pentapeptide deamidation rates for peptides with the sequences Gly___AsnYyyGly, ■; and Gly___GlnYyyGly, ◆. Adapted from 2004RR1.

teins for which three-dimensional structures had been reported as of January 2003,[5] as discussed in Chapter 9.

Figures 6-1, 6-2, and 6-3 summarize the sequence dependence of deamidation in peptides of the form GlyXxxAsn/GlnYyyGly in pH 7.4, 37 °C, 0.15 M Tris-HCl.

Table 6-1 lists the peptide deamidation rate experiments carried out between 1949 and 2004.

Tables 6-2 and 6-3 provide experimental and calculated values for 648 peptides of the form GlyXxxAsn/GlnYyyGly.[2] These values form the primary structure data set upon which current understanding and computation of structure-dependent deamidation depends. Further tabulations of related peptide deamidation rates are found in 2001RR,

5 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002); N. E. Robinson and A. B. Robinson, *Mechanisms of Ageing and Development* **125**, 259 (2004).

2001RR2, and 2004RR1. The latter of these three reports also provides the associated Arrhenius activation energies.

The double amide peptides are not included. Measurements of these half-times are in progress. These half-times are in the range of about 20, 40, 5,000, and 5,000 days for the third residues in GlyXxxAsnAsnGly, GlyXxxAsnGlnGly, GlyXxGlnAsnGly, and GlyXxxGlnGlnGly, respectively.[6]

These 648 values flow in a regular pattern across the range of naturally occurring sequences. Some special effects are evident. For example, the deamidation half-times for GlyTyrAsnGlyGly, GlyAspAsnGlyGly, and GlyGluAsnGlyGly are about 50% greater than for most other GlyXxxAsnGlyGly. A similar effect was also noticed by Bodanski in a series of blocked dipeptides and attributed to proton abstraction from NH.[7]

Also, peptides with paired nearest-neighboring basic and acidic residues, GluAsnLys, GluAsnArg, AspAsnLys, and AspAsnArg, clearly stand out as having deamidation half-times about 50% higher than the similar singular Glu, Asp, Lys, and Arg analogues. The same effect can be distinguished for LysAsnGlu, ArgAsnGlu, LysAsnAsp, and ArgAsnAsp, but it raises the deamidation half-times in these peptides by only about 10%. There are many other special sequence effects revealed by the sequence-dependent deamidation rates.

6 N. E. Robinson, B. R. Robinson, and M. L. Robinson unpublished results (2004).

7 M. Bodanszky and J. Z. Kwei, *Int. J. Peptide and Protein Research* **12**, 69 (1978).

Table 6-1 Nonenzymatic Peptide Deamidation Half-Times*

Sequence	Half-Time Days	Conditions	Reference
GlyAlaAsnAlaGly	22.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnAlaGly	24.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnAlaGly	24.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnAlaGly	26.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnAlaGly	25.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnAlaGly	21.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnAlaGly	24.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnAlaGly	25.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnAlaGly	25.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnAlaGly	23.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnAlaGly	22.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnAlaGly	24.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnAlaGly	31.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnAlaGly	24.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnAlaGly	24.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnAlaGly	30.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnAlaGly	24.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnAlaGly	27.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnArgGly	62.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnArgGly	67.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnArgGly	87.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

* The standardized set of pentapeptide deamidation half-times reported in 2001-2004 is listed in this table. This is followed by a complete listing of all other reported peptide deamidation half-times in chronological order. The standardized set is ordered alphabetically by carboxyl-side residue and then by amino-side residue.

Sequence	Half-Time Days	Conditions	Reference
GlyCysAsnArgGly	83.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnArgGly	80.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnArgGly	57.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnArgGly	48.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnArgGly	66.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnArgGly	62.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnArgGly	57.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnArgGly	58.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnArgGly	61.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnArgGly	72.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnArgGly	59.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnArgGly	51.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnArgGly	73.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnArgGly	56.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnArgGly	67.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnAspGly	31.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnAspGly	34.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnAspGly	29.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnAspGly	30.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnAspGly	32.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnAspGly	28.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnAspGly	31.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnAspGly	33.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnAspGly	32.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnAspGly	34.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnAspGly	26.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlyPheAsnAspGly	27.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnAspGly	48.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnAspGly	30.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnAspGly	27.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnAspGly	43.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnAspGly	28.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnAspGly	33.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnCysGly	63.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnCysGly	50.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnCysGly	54.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnCysGly	46.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnCysGly	44.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnCysGly	40.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnCysGly	43.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnCysGly	52.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnCysGly	53.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnCysGly	49.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnCysGly	49.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnCysGly	46.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnCysGly	60.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnCysGly	60.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnCysGly	55.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnCysGly	83.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnCysGly	48.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnCysGly	63.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnGluGly	74.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlyArgAsnGluGly	68.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnGluGly	46.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnGluGly	48.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnGluGly	60.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnGluGly	73.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnGluGly	69.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnGluGly	58.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnGluGly	56.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnGluGly	72.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnGluGly	72.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnGluGly	62.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnGluGly	92.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnGluGly	59.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnGluGly	60.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnGluGly	75.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnGluGly	41.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnGluGly	64.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnGlyGly	1.05	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnGlyGly	1.00	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnGlyGly	1.53	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnGlyGly	1.14	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnGlyGly	1.45	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnGlyGly	1.03	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnGlyGly	1.14	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnGlyGly	1.26	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnGlyGly	1.08	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlyLysAsnGlyGly	1.02	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnGlyGly	1.04	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnGlyGly	1.15	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnGlyGly	1.18	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnGlyGly	0.96	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnGlyGly	1.04	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnGlyGly	1.75	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnGlyGly	1.49	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnGlyGly	1.23	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnHisGly	9.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnHisGly	10.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnHisGly	9.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnHisGly	10.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnHisGly	9.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnHisGly	9.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnHisGly	10.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnHisGly	11.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnHisGly	10.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnHisGly	10.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnHisGly	10.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnHisGly	10.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnHisGly	12.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnHisGly	8.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnHisGly	9.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnHisGly	11.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnHisGly	10.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlyValAsnHisGly	10.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnIleGly	300	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnIleGly	311	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnIleGly	298	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnIleGly	304	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnIleGly	279	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnIleGly	287	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnIleGly	327	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnIleGly	384	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnIleGly	391	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnIleGly	313	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnIleGly	275	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnIleGly	287	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnIleGly	455	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnIleGly	285	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnIleGly	279	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnIleGly	286	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnIleGly	306	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnIleGly	366	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnLeuGly	124	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnLeuGly	128	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnLeuGly	111	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnLeuGly	119	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnLeuGly	130	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnLeuGly	104	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnLeuGly	116	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlyIleAsnLeuGly	154	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnLeuGly	155	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnLeuGly	119	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnLeuGly	113	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnLeuGly	118	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnLeuGly	181	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnLeuGly	110	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnLeuGly	110	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnLeuGly	133	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnLeuGly	118	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnLeuGly	154	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnLysGly	55.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnLysGly	49.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnLysGly	75.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnLysGly	46.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnLysGly	77.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnLysGly	48.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnLysGly	50.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnLysGly	64.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnLysGly	60.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnLysGly	53.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnLysGly	60.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnLysGly	58.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnLysGly	67.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnLysGly	55.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnLysGly	57.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlyTrpAsnLysGly	59.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnLysGly	55.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnLysGly	63.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnMetGly	59.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnMetGly	74.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnMetGly	57.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnMetGly	64.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnMetGly	59.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnMetGly	50.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnMetGly	63.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnMetGly	58.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnMetGly	62.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnMetGly	60.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnMetGly	56.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnMetGly	58.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnMetGly	78.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnMetGly	54.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnMetGly	47.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnMetGly	64.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnMetGly	64.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnMetGly	65.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnPheGly	65.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnPheGly	68.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnPheGly	70.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnPheGly	73.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnPheGly	70.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlyGlyAsnPheGly	64.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnPheGly	72.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnPheGly	61.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnPheGly	72.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnPheGly	70.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnPheGly	61.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnPheGly	69.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnPheGly	100	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnPheGly	52.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnPheGly	76.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnPheGly	71.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnPheGly	58.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnPheGly	66.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnProGly	7370	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnProGly	5790	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnProGly	11800	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnProGly	1550	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnProGly	8860	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnProGly	7170	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnProGly	8440	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnProGly	11600	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnProGly	10500	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnProGly	4940	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnProGly	9300	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnProGly	7990	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnProGly	6590	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlySerAsnProGly	7060	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnProGly	6290	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnProGly	—	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnProGly	9830	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnProGly	8030	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnSerGly	14.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnSerGly	14.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnSerGly	17.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnSerGly	19.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnSerGly	16.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnSerGly	11.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnSerGly	15.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnSerGly	14.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnSerGly	16.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnSerGly	15.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnSerGly	15.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnSerGly	18.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnSerGly	18.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnSerGly	15.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnSerGly	17.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnSerGly	15.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnSerGly	11.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnSerGly	18.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnThrGly	43.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnThrGly	50.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnThrGly	52.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlyCysAsnThrGly	48.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnThrGly	36.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnThrGly	39.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnThrGly	47.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnThrGly	46.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnThrGly	46.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnThrGly	58.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnThrGly	43.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnThrGly	39.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnThrGly	63.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnThrGly	45.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnThrGly	50.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnThrGly	38.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnThrGly	38.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnThrGly	49.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnTrpGly	130	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnTrpGly	127	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnTrpGly	80.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnTrpGly	111	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnTrpGly	98.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnTrpGly	77.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnTrpGly	95.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnTrpGly	86.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnTrpGly	74.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnTrpGly	98.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnTrpGly	92.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlyPheAsnTrpGly	102	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnTrpGly	122	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnTrpGly	76.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnTrpGly	72.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnTrpGly	135	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnTrpGly	120	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnTrpGly	88.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnTyrGly	73.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyArgAsnTyrGly	90.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnTyrGly	70.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnTyrGly	83.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnTyrGly	94.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnTyrGly	63.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnTyrGly	82.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnTyrGly	79.3	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnTyrGly	75.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnTyrGly	96.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnTyrGly	74.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnTyrGly	75.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnTyrGly	114	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnTyrGly	64.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnTyrGly	80.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnTyrGly	92.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnTyrGly	70.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnTyrGly	79.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAlaAsnValGly	254	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR

Sequence	Half-Time Days	Conditions	Reference
GlyArgAsnValGly	247	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyAspAsnValGly	241	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyCysAsnValGly	229	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGluAsnValGly	268	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyGlyAsnValGly	224	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyHisAsnValGly	247	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyIleAsnValGly	295	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLeuAsnValGly	294	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyLysAsnValGly	246	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyMetAsnValGly	211	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyPheAsnValGly	203	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyProAsnValGly	364	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlySerAsnValGly	233	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyThrAsnValGly	237	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTrpAsnValGly	226	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyTyrAsnValGly	241	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
GlyValAsnValGly	291	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR
AlaAlaAsnAlaAla	29.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaProAlaAsnAlaProAla	195	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaGluAlaAsnAlaGluAla	31.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaAlaAlaAsnAlaAlaAla	31.2	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaIleAlaAsnAlaIleAla	25.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAlaAsnAlaAlaAla	25.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAlaAsnAlaSerAla	21.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaAlaAlaAsnAlaHisAla	16.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAlaAsnAlaHisAla	16.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2

Sequence	Half-Time Days	Conditions	Reference
AlaLysAlaAsnAlaLysAla	14.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaTyrAlaAsnAlaTyrAla	14.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaHisAlaAsnAlaHisAla	12.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaArgAlaAsnAlaArgAla	10.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaIleAlaAlaAsnAlaAlaIleAla	45.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaGluAlaAlaAsnAlaAlaGluAla	43.4	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaAlaAlaAlaAsnAlaAlaAlaAla	37.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAlaAlaAsnAlaAlaSerAla	29.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAlaAlaAsnAlaAlaAlaAla	28.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaAlaAlaAlaAsnAlaAlaHisAla	25.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaHisAlaAlaAsnAlaAlaHisAla	23.7	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAlaAlaAsnAlaAlaHisAla	21.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaLysAlaAlaAsnAlaAlaLysAla	14.1	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAlaAlaAlaAsnAlaAlaAlaSerAla	1490	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAlaAlaAlaAsnAlaAlaAlaHisAla	630	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaIleAlaAlaAlaAsnAlaAlaAlaIleAla	278	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaHisAlaAlaAlaAsnAlaAlaAlaHisAla	59.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaGluAlaAlaAlaAsnAlaAlaAlaGluAla	55.9	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaLysAlaAlaAlaAsnAlaAlaAlaLysAla	15.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAlaAlaAlaAlaAsnAlaAlaAlaHisAla	1150	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAlaAlaAlaAlaAsnAlaAlaAlaSerAla	960	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaIleAlaAlaAlaAlaAsnAlaAlaAlaIleAla	150	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaGluAlaAlaAlaAlaAsnAlaAlaAlaGluAla	36.5	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaHisAlaAlaAlaAlaAsnAlaAlaAlaHisAla	32.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaLysAlaAlaAlaAlaAsnAlaAlaAlaLysAla	18.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaAsnAla	270	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2

Sequence	Half-Time Days	Conditions	Reference
AlaAlaSerAsnAlaAla	27.8	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlyAsnGly	98.0	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlyGlyAsnGlyGly	1.083	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlySerGlyGlyAsnGlyGlyGlyGly	0.935	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlyGlyGlyAsnGlyGlyGly	0.916	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlyGlyGlyGlyAsnGlyGlyGlyGlyGly	0.845	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlySerGlyGlyAsnGlyGlyHisGly	0.828	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlyGlyGlyGlyAsnGlyGlyHisGly	0.820	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlySerGlyAsnGlyGlyGly	0.752	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlySerGlyAsnGlyHisGly	0.648	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlyGlyGlyAsnGlyHisGly	0.646	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlyGlySerAsnGlyGly	1.003	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AcAsnHisAlaAla	8.10	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaAlaAsnHisAlaAla	8.44	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlyGlyAsnHisGlyGly	7.41	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
SerAsnHis	39.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AcSerAsnHisNH$_2$	9.01	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AcAlaAlaSerAsnHisAlaAlaNH$_2$	8.05	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaSerAsnHisAla	7.83	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlyGlySerAsnHisGlyGly	7.42	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AcAlaSerAsnHisAlaNH$_2$	7.35	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaAlaSerAsnHisAlaAla	7.27	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AcGlyGlySerAsnHisGlyGlyNH$_2$	7.05	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaAlaAlaSerAsnHisAlaAlaAla	6.78	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AcGlySerAsnHisGlyNH$_2$	6.63	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
GlyGlyGlySerAsnHisGlyGlyGly	6.47	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2

Sequence	Half-Time Days	Conditions	Reference
GlySerAsnHisGly	—	pH 1.0 to 9.0, 37 °C, 0.15M Tris-HCl	2001RR2
AsnHisAlaAla	5.75	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaAlaSerAsnNH$_2$	37.6	pH 7.4, 37 °C, 0.15M Tris-HCl	2001RR2
AlaAlaSerAsn	3440	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
AlaAlaAlaAlaNH$_2$	6590	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyAlaGlnAlaGly	5300	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyAspGlnAlaGly	7090	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyGluGlnAlaGly	7100	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyGlyGlnAlaGly	5880	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyLysGlnAlaGly	4320	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyMetGlnAlaGly	4400	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyPheGlnAlaGly	6270	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyProGlnAlaGly	4730	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlySerGlnAlaGly	6420	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyValGlnAlaGly	4460	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyAlaGlnArgGly	7190	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyHisGlnArgGly	4530	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyLysGlnArgGly	2300	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyProGlnArgGly	6370	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyThrGlnArgGly	5110	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyAlaGlnAspGly	7450	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyHisGlnAspGly	5630	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyTrpGlnAspGly	8780	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyAlaGlnCysGly	1910	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyArgGlnCysGly	1040	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyLysGlnGluGly	5350	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1

Sequence	Half-Time Days	Conditions	Reference
GlyAlaGlnGlyGly	605	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyArgGlnGlyGly	659	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyCysGlnGlyGly	559	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyGlyGlnGlyGly	649	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyHisGlnGlyGly	855	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyIleGlnGlyGly	621	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyLeuGlnGlyGly	668	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyThrGlnGlyGly	668	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyTyrGlnGlyGly	849	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyAspGlnHisGly	2540	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyLysGlnHisGly	5100	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlySerGlnHisGly	6520	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyAlaGlnIleGly	6170	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyGluGlnIleGly	4230	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlySerGlnIleGly	5870	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyValGlnIleGly	5570	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyAspGlnLysGly	4600	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyValGlnLysGly	5210	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyGlyGlnMetGly	4500	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyIleGlnMetGly	5140	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyGlyGlnPheGly	7610	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyTrpGlnPheGly	8580	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyGlyGlnProGly	10040	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyGlyGlnSerGly	5710	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyAlaGlnThrGly	5070	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyTrpGlnThrGly	5580	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1

Sequence	Half-Time Days	Conditions	Reference
GlyGlyGlnTrpGly	15300	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyLysGlnTrpGly	10400	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyTyrGlnTrpGly	17900	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyAlaGlnTyrGly	10000	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
GlyGlyGlnValGly	6470	pH 7.4, 37 °C, 0.15M Tris-HCl	2004RR1
PyrGlnGln	1	1M NaOH, 25 °C, 24 hours	1949DS
AsnGly	0.2	70 °C, 10% trichloroacetic acid, 1 hour	1952MW
GlnGly	0.98	70 °C, 10% trichloroacetic acid, 1 hour	1952MW
GlyAsn	0.25	70 °C, 10% trichloroacetic acid, 1 hour	1952MW
GlyGln	0.37	70 °C, 10% trichloroacetic acid, 1 hour	1952MW
Asparagine	0.08	70 °C, 10% trichloroacetic acid, 1 hour	1952MW
Glutamine	0.92	70 °C, 10% trichloroacetic acid, 1 hour	1952MW
AsnGly	0.043	pH 0, 70 - 90 °C, 0.2N to 1.0N HCl	1953LL
Asparagine	0.063	pH 0, 70 - 90 °C, 0.2N to 1.0N HCl	1953LL
GlnGlnGly	0.59	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
LeuGlnGly	3.1	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
ProGlnGly	1.4	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
GlnGly	0.12	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
LeuGlnPro	4.6	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
AlaGln	5.2	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
GlyGln	4.6	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
LeuGln	10.6	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
PheGln	6	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
ProGln	2.2	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
ValGln	6	pH 6.8, 100 °C, 0.067M Phosphate	1967F1
GlyAlaAsnAlaGly	40	pH 7, 37 °C, Phosphate	1970RM

Sequence	Half-Time Days	Conditions	Reference
GlyAlaAsnAlaGly	50	pH 10, 37 °C, Phosphate	1970RM
GlyArgAsnArgGly	11	pH 7, 37 °C, Phosphate	1970RM
GlyArgAsnArgGly	9	pH 10, 37 °C, Phosphate	1970RM
GlyGluAsnGluGly	22	pH 6.8, 37 °C, Phosphate	1970RM
GlyGluAsnGluGly	7	pH 10, 37 °C, Phosphate	1970RM
GlyGlyThrAsnGlu	20	pH 6.8, 37 °C, Phosphate	1970RM
GlyThrAsnGluGly	2	pH 10.4, 37 °C, Phosphate	1970RM
GlyGlyThrAsnGlu	2.2	pH 10.4, 37 °C, Phosphate	1970RM
GlyLysAsnLysGly	90	pH 7, 37 °C, Phosphate	1970RM
GlyLysAsnLysGly	10	pH 10, 37 °C, Phosphate	1970RM
GlyThrAsnThrGly	18	pH 10, 37 °C, Phosphate	1970RM
GlyArgAsnArgGly	115	pH 7.4, 37 °C, Tris-HCl	1971MR
GlyArgAsnArgGly	20	pH 7.4, 37 °C, Phosphate	1971MR
GlyArgAsnArgGly	—	pH 10, 37 °C, Carbonate - I of 0.2 to 1.35	1971MR
GlyThrAsnGluGly	1.42	pH 10, 37 °C, Borate	1971MR
GlyThrAsnGluGly	0.46	pH 10, 37 °C, Carbonate	1971MR
GlyThrAsnGluGly	0.21	pH 10, 37 °C, Phosphate	1971MR
GlyThrAsnGluGly	27	pH 7.4, 37 °C, Borate	1971MR
GlyThrAsnGluGly	12	pH 7.4, 37 °C, Phosphate	1971MR
GlyThrAsnThrAsn	—	pH 10, 5 to 37 °C, Carbonate	1971MR
GlyArgAsnArgGly	37	pH 7.4, 37 °C, 0.0766M Phosphate	1973M
GlyArgAsnAlaGly	18	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyAspAsnAlaGly	44	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyHisAsnAlaGly	45	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyPheAsnAlaGly	47	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyGluAsnAlaGly	49	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS

Sequence	Half-Time Days	Conditions	Reference
GlySerAsnAlaGly	52	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyLysAsnAlaGly	61	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyCysAsnAlaGly	68	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyThrAsnAlaGly	68	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyMetAsnAlaGly	77	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyTyrAsnAlaGly	85	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyGlyAsnAlaGly	87	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyTrpAsnAlaGly	87	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyAlaAsnAlaGly	95	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyProAsnAlaGly	100	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyValAsnAlaGly	111	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyLeuAsnAlaGly	217	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyIleAsnAlaGly	507	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyHisGlnAlaGly	96	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyMetGlnAlaGly	102	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyAspGlnAlaGly	209	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyGluGlnAlaGly	226	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyLysGlnAlaGly	280	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyArgGlnAlaGly	389	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyGlyGlnAlaGly	418	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyAlaGlnAlaGly	538	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyLeuGlnAlaGly	663	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyTyrGlnAlaGly	689	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyTrpGlnAlaGly	713	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlySerGlnAlaGly	889	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyPheGlnAlaGly	1060	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS

Sequence	Half-Time Days	Conditions	Reference
GlyIleGlnAlaGly	1087	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyProGlnAlaGly	1114	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyValGlnAlaGly	3278	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyThrGlnAlaGly	3409	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyAlaGlnArgGly	188	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyLysGlnArgGly	223	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyArgGlnArgGly	285	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyAlaGlnIleGly	1094	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyTyrGlnLeuGly	884	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyAlaGlnLysGly	157	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyLysGlnLysGly	251	pH 7.4, 37 °C, 0.0766M Phosphate	1973RS
GlyThrGlnAlaGly	431	pH 7.4, 37 °C, Joklik Medium - including 0.011M Phosphate	1973RS1
GlyIleGlnGlyGly	196	pH 7.4, 37 °C, Joklik Medium - including 0.011M Phosphate	1973RS1
GlyPheGlnGlyGly	248	pH 7.4, 37 °C, Joklik Medium - including 0.011M Phosphate	1973RS1
GlyTyrGlnLeuGly	174	pH 7.4, 37 °C, Joklik Medium - including 0.011M Phosphate	1973RS1
GlyMetAsnAlaGly	78	pH 7.5, 37 °C, 0.15M Phosphate	1973RT
GlyThrAsnArgGly	16	pH 7.5, 37 °C, 0.15M Phosphate	1973RT
GlyArgAsnArgGly	71	pH 7.5, 37 °C, 0.15M Phosphate	1973RT
GlyCysAsnAspGly	28	pH 7.5, 37 °C, 0.15M Phosphate	1973RT
GlyCysAsnIleGly	100	pH 7.5, 37 °C, 0.15M Phosphate	1973RT
GlyArgAsnLeuGly	113	pH 7.5, 37 °C, 0.15M Phosphate	1973RT
GlyIleAsnSerGly	18	pH 7.5, 37 °C, 0.15M Phosphate	1973RT
GlyArgAsnThrGly	28	pH 7.5, 37 °C, 0.15M Phosphate	1973RT
GlyPheAsnThrGly	123	pH 7.5, 37 °C, 0.15M Phosphate	1973RT
GlyThrGlnAlaGly	>3000	pH 7.5, 37 °C, 0.15M Phosphate	1973RT
GlyLeuGlnIleGly	>3000	pH 7.5, 37 °C, 0.15M Phosphate	1973RT

Sequence	Half-Time Days	Conditions	Reference
GlySerAsnHisGly	6.4	pH 7.4, 37 °C, 0.0766M Phosphate	1974MR
GlyThrAsnGlu	16	pH 7.4, 37 °C, 0.0766M Phosphate	1974RM
GlyProAsnLeuGly	277	pH 7.4, 37 °C, 0.0766M Phosphate	1974RM
GlyAlaAsnLysGly	54	pH 7.4, 37 °C, 0.0766M Phosphate	1974RM
GlyLysAsnLysGly	94	pH 7.4, 37 °C, 0.0766M Phosphate	1974RM
GlyGluAsnProGly	80	pH 7.4, 37 °C, 0.0766M Phosphate	1974RM
GlyAlaGlnCysGly	113	pH 7.4, 37 °C, 0.0766M Phosphate	1974RM
GlyValGlnLysGly	421	pH 7.4, 37 °C, 0.0766M Phosphate	1974RM
GlyAspAsnIleGly	75	pH 7.4, 37 °C, 0.0766M Phosphate	1974RS
GlyGluAsnValGly	145	pH 7.4, 37 °C, 0.0766M Phosphate	1974RS
GlyArgGlnAlaGly	—	pH 3.6 to 8.6, 40 to 60 °C, I of 0.2 to 1.6	1974RS
GlyLeuGlnAlaGly	—	pH 3.6 to 8.6, 40 to 60 °C, I of 0.2 to 1.6	1974RS
GlyArgGlnGlyGly	305	pH 7.4, 37 °C, 0.0766M Phosphate	1974RS
GlyIleGlnGlyGly	735	pH 7.4, 37 °C, 0.0766M Phosphate	1974RS
AcAsnGlyNHCH$_3$	1	pH 13, 37 °C, 0.1N NaOH, 1 hr	1986MS1
AcAsnGlyNHCH$_3$	1	pH 11, 37 °C, 0.1M Et_3N, 20 hr	1986MS1
AcAsnGlyNHCH$_3$	1	pH 9, 37 °C, 0.1M Tris-HCl, 48 hr	1986MS1
ValTyrProAsnGlyAla	1.4	pH 7.4, 37 °C, 0.1M Phosphate	1987GC
ValTyrProAsnGlyAla	0.0063	pH 7.4, 100 °C, 0.1M Phosphate	1987GC
ValTyrProAsnLeuAla	0.2	pH 7.4, 100 °C, 0.1M Phosphate	1987GC
ValTyrProAsnProAla	0.31	pH 7.4, 100 °C, 0.1M Phosphate	1987GC
AcValAsnGlyAla	3.33	pH 7.3, 37 °C, 0.02M Phosphate	1988LS
AcValAsnGlyAla	0.33	pH 7.3, 60 °C, 0.02M Phosphate	1988LS
ValAsnGlyAla	1.5	pH 7.3, 37 °C, 0.02M Phosphate	1988LS
ValAsnGlyAla	0.146	pH 7.3, 60 °C, 0.02M Phosphate	1988LS
BocAsnAlaGlyNH_2	6.25	pH 8.93, 25 °C	1989CM1

Sequence	Half-Time Days	Conditions	Reference
BocAsnGlyGlyNH_2	8.9	pH 7.39, 25 °C, Extrapolated in phosphate to zero buffer - measured as function of pH from 4.93 to 9.93	1989CM1
BocAsnGlyGlyNH_2	0.5	pH 8.93, 25 °C	1989CM1
BocAsnGlyAlaNH_2	0.54	pH 8.93, 25 °C	1989CM1
BocAsnSerGlyNH_2	3.54	pH 8.93, 25 °C	1989CM1
ValTyrProAsnAlaAla	20.2	pH 7.4, 37 °C, 0.1M Phosphate	1989SC
ValTyrProAsnSerAla	8	pH 7.4, 37 °C, 0.1M Phosphate	1989SC
GluLeuThrAlaAlaAsnAlaAlaAlaAlaAla AlaAlaThrAlaArg	—	—	1989SM
ValTyrProAsnGlyAla	—	pH from 5 to 12, 37 °C	1990PB1
ValTyrProAsnGlyAla	4.2	pH 7, 37 °C, 0.005M Phosphate	1990PB1
ValTyrGlyAsnGlyAla	0.51	pH 7.5, 37 °C, 0.1M Phosphate, NaCl	1990PB2
ValTyrProAsnGlyAla	0.83	pH 7.5, 37 °C, 0.1M Phosphate, NaCl	1990PB2
ValTyrProAsnSerAla	5.55	pH 7.5, 37 °C, 0.1M Phosphate, NaCl	1990PB2
ValTyrProAsnValAla	107	pH 7.5, 37 °C, 0.1M Phosphate, NaCl	1990PB2
AcGlnGlyNHCH_3	350	pH 7.4, 37 °C, 0.01M Phosphate	1991CM
BocAsnGlyGlyNH_2	—	pH , 25 °C, Buffer, ionic strength, organic solvent dependence	1991CM1
ValSerAsnAlaVal	2.75	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
GlySerAsnAlaGly	3	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValThrAsnAlaVal	4.13	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValSerAsnArgVal	3.17	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValSerAsnAspVal	2.71	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValSerAsnGluVal	—	pH 8 to 10, 60 °C, 4 Buffers including Phosphate	1991TS
ValSerAsnGlyVal	0.24	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValSerAsnHisVal	1.08	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValSerAsnIleVal	13.8	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValSerAsnLeuVal	10	pH 7.3, 60 °C, 0.02M Phosphate	1991TS

Sequence	Half-Time Days	Conditions	Reference
ValSerAsnLeuVal	1.38	pH 10, 60 °C, Including NH_4OH dependence	1991TS
ValAlaAsnSerVal	1.75	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
GlyAlaAsnSerGly	1.79	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValArgAsnSerVal	1.67	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValGlyAsnSerVal	1.42	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValHisAsnSerVal	1.88	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValLeuAsnSerVal	1.92	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValSerAsnSerVal	1.88	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValSerAsnSerVal	1.88	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValThrAsnSerVal	2.17	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValValAsnSerVal	1.88	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValAlaAsnThrVal	5	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValSerAsnThrVal	10	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValThrAsnThrVal	3.92	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
GlyAlaAsnValGly	13.8	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ValSerAsnValVal	17.5	pH 7.3, 60 °C, 0.02M Phosphate	1991TS
ThrAsnSerTyr	—	pH 10, 37 °C, 0.10M NH_4HCO_3	1992SW
ValTyrProAsnGlyAla	1.21	pH 7.4, 37 °C, 0.1M Phosphate	1993BC1
AcGlyAsnGlyGlyNHCH$_3$	4.8	pH 7.4, 37 °C, Includes dependence on 4 buffers	1993CM
ValTyrProAsnGlyAla	—	pH 1 to 12 , 37 °C, Wide variety of buffers	1993P
32 residue GRF analogue - Asn(8)	0.36	pH 10, 37 °C, 0.1M Carbonate - 40% CH_3OH	1993SF
ValTyrProAsnGlyAla	—	Various pH, T, % H_2O, and additives	1994OP
ValTyrHisAsnCysAla	8	pH 7.4, (37) °C, 0.1M Phosphate, Extrapolated from 70 °C	1995BC
ValTyrProAsnCysAla	9.8	pH 7.4, (37) °C, 0.1M Phosphate, Extrapolated from 70 °C	1995BC
ValTyrHisAsnGlyAla	1.25	pH 7.4, 37 °C, 0.1M Phosphate	1995BC
ValTyrProAsnHisAla	23	pH 7.4, (37) °C, 0.1M Phosphate, Extrapolated from 70 °C	1995BC

Sequence	Half-Time Days	Conditions	Reference
ValTyrProAsnLeuAla	67	pH 7.4, 70 °C, 0.1M Phosphate, Extrapolated from 70 °C	1995BC
ValTyrProAsnPheGlyAla	68.5	pH 7.4, (37) °C, 0.1M Phosphate, Extrapolated from 70 °C	1995BC
ValTyrHisAsnProAla	None	pH 7.4, 70 °C, 0.1M Phosphate	1995BC
ValTyrProAsnProAla	None	pH 7.4, 70 °C, 0.1M Phosphate	1995BC
ValTyrProAsnValAla	107	pH 7.4, (37) °C, 0.1M Phosphate, Extrapolated from 70 °C	1995BC
ValTyrProAsnGlyAla	1.03	pH 7.5, 37 °C, 0.1M Phosphate	1998LS2
AcCysLysAsnGlyGlnThrAsnCysNH$_2$	2.7	pH 7.4, 37 °C	1999CS
AcTyrGlyArgAlaAlaAlaAlaArgAsnAlaAlaAlaArgAlaAlaAlaAlaAlaArgNH$_2$	48	pH 7.4, 37 °C, 0.01M Borate, 1M NH_4Cl	1999KR
AcCysAcLysAsnGlyGlnThrAsnCysNH$_2$	—	Various pH's , 37 °C	2000CB
GlyGlnAsnGlyGly	1.55	pH 7, 37 °C, 0.1M Phosphate	2000GS1
GlyGlnAsnGlyHis	2.33	pH 7, 37 °C, 0.1M Phosphate	2000GS1
GlyGlnAsnHisHis	2.46	pH 7, 37 °C, 0.1M Phosphate	2000GS1
GlyGlnAsnValHis	19.7	pH 7, 37 °C, 0.1M Phosphate	2000GS1
ValTyrProAsnGlyAla	—	pH 10, 37 °C, 0.1M Bicarbonate, various soluion polarities and viscosities	2000LS3
AcAsnGly	3	pH 8.8, 37 °C, Carbonate	2000XA
AsnGly-cyclic	9	pH 8.8, 37 °C, Carbonate	2000XA
AsnGly-cyclic	0.3	pH 11.8, 37 °C, Carbonate	2000XA
GlyAsn-cylclic	—	pH 8.8, 37 °C, Carbonate	2000XA
GlyAsn-cylclic	9	pH 11.8, 37 °C, Carbonate	2000XA
ValTyrProAsnGlyAla	—	pH 10, 40 °C, Bicarbonate, various viscosities	2002LH

Table 6-2 First-Order Deamidation Half-Times of GlyXxxAsnYyyGly in days at pH 7.4, 37.0 ºC, 0.15 M Tris HCl*

Xxx\Yyy	Gly	His	Ser	Ala	Asp	AmCys	Thr	Cys	Lys	Met	Glu	Arg	Phe	Tyr	Trp	Leu	Val	Ile	Pro	Median†
Gly	**1.03**	**9.2**	**11.8**	**21.1**	**28.0**	**27.6**	**39.8**	**40.6**	**48.2**	**50.4**	**73.9**	**57.8**	**64.0**	**63.6**	**77.1**	**104**	**224**	**287**	**7170**	50.4
Ser	**0.96**	**9.0**	**15.1**	**24.1**	**30.3**	**41.3**	**45.7**	**60.2**	**55.5**	**54.9**	**59.7**	**59.7**	**52.2**	**64.7**	**76.8**	**110**	**233**	**285**	**7060**	55.5
Thr	**1.04**	**9.6**	**17.1**	**24.6**	**27.9**	**34.4**	**50.0**	**55.5**	**57.6**	**47.6**	**60.8**	**51.2**	**76.4**	**80.6**	**72.5**	**110**	**237**	**279**	**6290**	55.5
Cys	**1.14**	**10.8**	**19.0**	**26.4**	**30.6**	38.3	**48.7**	**46.0**	**46.6**	**64.5**	**48.3**	**83.1**	**73.9**	**83.9**	**111**	**119**	**229**	**304**	**1550**	48.7
AmCys	**1.14**	**10.9**	**15.4**	**21.5**	**32.9**	**39.3**	**41.7**	46.6	**48.9**	**56.5**	**45**	**58.8**	**63.3**	**78.8**	**81.3**	**100**	**215**	**250**	**3900**	48.9
Met	**1.04**	**10.2**	**15.2**	**22.1**	**26.4**	**33.8**	**43.6**	**49.6**	**60.4**	**56.9**	**72.4**	**58.8**	**61.9**	**74.0**	**92.7**	**113**	**211**	**275**	**9300**	57.9
Phe	**1.15**	**10.2**	**18.1**	**24.2**	**27.4**	**29.8**	**39.0**	**46.5**	**58.2**	**58.6**	**62.4**	**61.2**	**69.5**	**75.1**	**102**	**118**	**203**	**287**	**7990**	58.6
Tyr	**1.49**	**10.2**	**11.9**	**24.3**	**28.4**	**33.3**	**38.1**	**48.6**	**55.1**	**64.3**	**41.0**	**56.9**	**58.0**	**70.6**	**120**	**118**	**241**	**306**	**9830**	51.8
Asp	**1.53**	**9.7**	**17.0**	**24.0**	**29.4**	**45.8**	**52.4**	**54.1**	**75.9**	**57.3**	**46.8**	**87.2**	**70.1**	**70.4**	**80.3**	**111**	**241**	**298**	**11800**	55.7
Glu	**1.45**	**9.0**	**16.4**	**25.8**	**32.0**	**32.1**	**36.8**	**44.2**	**77.8**	**59.6**	**60.3**	**80.9**	**70.2**	**94.5**	**98.4**	**130**	**268**	**279**	**8860**	59.9
His	**1.14**	**10.7**	**15.7**	**24.6**	**31.2**	**33.8**	**47.2**	**43.9**	**50.2**	**63.1**	**69.4**	**48.9**	**72.1**	**82.3**	**95.4**	**116**	**247**	**327**	**8440**	50.2
Lys	**1.02**	**10.5**	**15.6**	**23.6**	**34.0**	**36.5**	**58.1**	**49.0**	**53.5**	**60.9**	**72.5**	**57.4**	**70.1**	**96.7**	**98.1**	**119**	**246**	**313**	**4940**	58.1
Arg	**1.00**	**10.0**	**14.3**	**24.4**	**34.7**	**42.3**	**50.7**	**50.5**	**49.6**	**74.4**	**68.3**	**67.4**	**68.3**	**90.0**	**127**	**128**	**247**	**311**	**5790**	67.4
Ala	**1.05**	**9.3**	**14.9**	**22.5**	**31.9**	**40.6**	**43.5**	**63.7**	**55.9**	**59.2**	**74.1**	**62.4**	**65.6**	**73.9**	**130**	**124**	**254**	**300**	**7370**	62.4
Leu	**1.08**	**10.7**	**16.7**	**25.1**	**32.1**	**33.6**	**46.1**	**53.5**	**60.1**	**62.6**	**56.7**	**62.1**	**72.4**	**75.7**	**74.5**	**155**	**294**	**391**	**10500**	60.1
Val	**1.23**	**10.2**	**18.2**	**27.5**	**33.5**	**34.7**	**49.9**	**63.2**	**63.8**	**65.7**	**64.8**	**67.4**	**66.6**	**79.2**	**88.9**	**154**	**291**	**366**	**8030**	64.8
Ile	**1.26**	**11.5**	**14.5**	**25.9**	**33.8**	**33.0**	**46.3**	**52.7**	**64.4**	**58.8**	**58.6**	**66.4**	**61.5**	**79.3**	**86.7**	**154**	**295**	**384**	**11600**	58.8
Trp	**1.75**	**11.3**	**15.5**	**30.7**	**43.6**	**42.9**	**38.9**	**83.1**	**59.4**	**64.2**	**75.7**	**73.9**	**71.1**	**92.6**	**135**	**133**	**226**	**286**	12000	67.6
Pro	**1.18**	**12.8**	**18.9**	**31.8**	**48.6**	**43.7**	**63.1**	**60.0**	**67.8**	**78.4**	**92.0**	**72.9**	**100**	**114**	**122**	**181**	**364**	**455**	**6590**	72.9
Mean	1.19	10.3	15.9	25.0	32.5	36.7	46.3	53.2	58.4	60.9	63.3	65.0	68.8	81.1	98	126	251	315	8300	60.9
St.Dev.	0.05	0.23	0.49	0.67	1.3	1.2	1.7	2.4	2.1	1.8	3.1	2.5	2.3	3.0	4.9	5.1	9.3	12.2	600	2.3
%St.Dev.	4.4	2.2	3.1	2.7	4.1	3.3	3.6	4.5	3.6	2.9	4.8	3.9	3.4	3.7	5.0	4.0	3.7	3.9	8.8	3.7
Median	1.14	10.2	15.6	24.4	31.9	34.7	46.1	50.5	57.6	59.6	62.4	62.1	69.5	79.2	95	119	241	300	8010	59.6

† Median does not include Yyy = AmCys. *The values listed in Tables 6-2 and 6-3 in bold type were experimentally determined. Those in ordinary type were estimated by interpolation and extrapolation from the experimental values. Adapted from 2004RR1.

Table 6-3 First-Order Deamidation Half-Times of GlyXxxGlnYyyGly in days at pH 7.4, 37.0 ºC, 0.15 M Tris HCl*

Xxx\Yyy	Gly	Cys	Met	Thr	Ser	Ala	His	Lys	Leu	Ile	Val	Arg	Glu	Asp	Phe	Pro	Tyr	Trp	Median
Cys	**560**	800	3200	3500	3800	4100	4200	4400	4800	4900	5000	5100	5600	6100	6500	7100	7900	9100	4800
Met	600	900	3500	3800	4100	**4400**	4400	4600	5000	5000	5000	5100	5800	6200	6600	7300	8200	9400	5000
Thr	**670**	1000	3700	4000	4200	4300	4500	4800	5200	5300	5100	**5100**	5900	6300	6800	7500	8400	9700	5100
Lys	650	1000	4000	4100	4200	**4300**	**6100**	4000	5300	5400	5700	**2300**	**5400**	5900	7000	7700	8800	**10000**	5300
Arg	**660**	**1000**	4100	4200	4300	4400	4900	4000	5400	5500	5800	2300	5400	5900	7100	8100	9200	11000	4900
Val	640	1300	4200	4300	4400	**4500**	5000	**5200**	5500	**5600**	5900	6100	6500	7000	7200	8500	9700	12000	5500
Pro	630	1600	4500	4600	4600	**4700**	5200	5500	5800	6000	6200	**6400**	6800	7200	7300	8900	10000	13000	5800
Ala	**610**	**1900**	4400	**5100**	5200	**5300**	5500	5700	6100	**6200**	6400	**7200**	7300	**7400**	7500	9300	**10000**	14000	6100
Gly	**650**	1900	**4500**	5200	**5700**	**5900**	5900	6000	6200	6300	**6500**	7200	7300	7600	**7600**	**10000**	12000	**15000**	6200
Leu	**670**	2000	4800	5300	5800	6000	6100	6100	6300	6500	6800	7200	7400	7800	8000	10000	12000	16000	6300
Ile	**620**	2000	**5100**	5300	5800	6200	6100	6100	6300	6500	7100	7200	7700	8100	8100	10000	12000	16000	6300
Phe	660	2000	5100	5300	5900	**6300**	6200	6200	6400	6400	7100	7200	8100	8200	8200	10000	12000	16000	6400
Ser	700	2100	5100	5400	6000	**6400**	**6500**	6300	6100	**5900**	6800	7200	8100	8200	8300	10000	13000	17000	6400
Glu	750	2100	5200	5400	6100	**7100**	2500	4600	4300	**4200**	6400	5200	8200	8300	8400	10000	13000	17000	5400
Asp	800	2100	5200	5400	6200	**7100**	**2500**	**4600**	6200	6400	6600	5200	8200	8400	8500	11000	13000	17000	6200
His	**850**	2200	5200	5500	6300	7200	7200	4000	6600	6700	6800	**4500**	5800	**5600**	8600	11000	14000	18000	6300
Tyr	**850**	2200	5300	5600	6400	7300	7400	7500	7800	7900	8000	8100	8300	8600	8700	11000	14000	**18000**	7800
Trp	850	2300	5300	**5600**	6500	7400	7500	7600	7900	8000	8200	8300	8500	**8800**	**8600**	11000	14000	19000	7900
Mean	690	1700	4600	4900	5300	5700	5400	5400	6000	6000	6400	5900	7000	7300	7700	9400	11200	14300	6000
St.Dev.	22	129	163	169	228	296	352	272	226	233	221	423	273	259	180	329	521	809	246
%St.Dev.	3.2	7.6	3.5	3.4	4.3	5.2	6.5	5.0	3.8	3.9	3.4	7.2	3.9	3.5	2.3	3.5	4.7	5.7	4
Median†	660	1950	4650	5250	5750	5950	6000	6050	6250	6400	6650	7200	7350	7700	7800	10000	12000	15500	6150

† Median without charged residues * See legend of Table 6-2.

6-2. PREDICTION OF PRIMARY SEQUENCE RATES

The measurement of 477 pentapeptide deamidation rates under standardized conditions increased fundamental understanding of the nature of sequence effects on deamidation by making possible a theoretical and semi-empirical treatment that allows the prediction of the carboxyl-residue-side sequence dependent rates without experimental measurement.[8,9]

It was discovered that the steric effect depends upon the size of the chemical group inhibiting reaction at the ring closure location, the variety of conformations available to that group, and the distribution function of occupation of those conformations. The aliphatic and aromatic side chains provide internally consistent and redundant means of determining the steric effects. These steric components can then be removed from the chemically active side chains to quantitatively reveal the catalytic effects of the hydroxyl, sulfur, carboxyl, amino, and other functional side chains.

Table 6-4 gives these substituent effects in units of (100)ln k, where k is the first order deamidation rate constant in sec^{-1}. The labeling convention is given in Figure 6-4. For convenience, these values include both the steric and catalytic components. Catalytic components can be determined separately by subtracting the appropriate hydrocarbon components given in the table. The values given in boldfaced type in the table are experimental, while the others are computed.

Figure 6-5 illustrates the way in which the values in Table 6-4 are determined. Figures 6-6 a and b show that steric hindrance diminishes, as expected, with the cube root of the distance of the substituent from the reactive center. Figure 6-7 illustrates the diminution of positive ion catalysis with distance from the reactive center. For a detailed description of this work, see 2004RR2.

The steric substituent effects have also been theoretically calculated.[8] The calculated values are closely similar to the experimentally observed values.

The effects of the carboxyl side residue on deamidation half-time can be computed by simply summing the carboxyl side substituent effects given in Table 6-4 and applying equations 6-1 or 6-2 and 6-3.

6-1. Asn peptides $\quad t_{1/2} = [(\ln(2))/86400][e^{[(\mathrm{Sum}/100) + 11.863]}]$

8 N. E. Robinson and A. B. Robinson, *J. Peptide Research* **63**, 437 (2004).

9 N. E. Robinson, *PhD Thesis, California Institute of Technology, Chemistry* (2003).

Table 6-4 Δ(100)ln(k) Coefficients for Calculating Deamidation Rates.*

	β	γ	δ	ε	ζ	η
– H	**0**	**102.3**	**36.1**	**7.2**	0.18	0
– CH_3	**306.8**	**214.5**	**59.2**	**8.1**	0.19	0
– CH_2 –	**204.5**	**178.4**	**52.0**	**7.9**	0.19	0
– CH – –	**102.3**	**142.2**	**44.7**	**7.7**	0.19	0
– – C – –	**0**	**106.1**	**37.5**	**7.5**	0.19	0
– C_5H_5	284.5	**207.4**	52.9	7.9	0	0
– C_5H_5OH	—	**220.6**	—	—	—	—
– C_8H_6N (Indole)	390.6	**239.4**	60.4	8.1	0	0
– $C_3H_3N_2^+$ (Imidazole)	—	**14.9**	—	—	—	—
– S –	—	**77.7**	**5.5**	—	—	—
– SH	—	**175.3**	—	—	—	—
– O –	—	19.5	11.5	-9.6	—	—
– OH	—	**55.6**	—	—	—	—
– CO_2-	—	**129.2**	**18.2**	—	—	—
– – NH^+	—	—	-136.0	—	-49.7	-42.1
– $N_3CH_5^+$ (Guanidino)	—	—	—	**-34.2**	—	—
– NH_3^+	—	—	—	—	**-49.7**	—

* Adapted from 2004RR2.

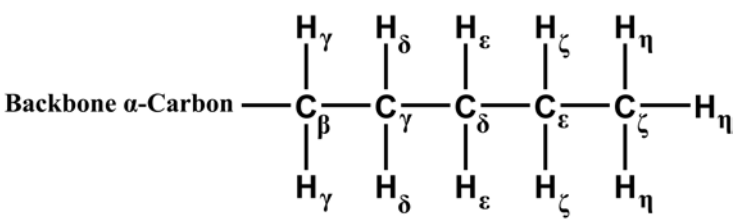

FIG. 6-4 Convention for designating positions along amino acid residue side chain as used in Table 6-4. This convention is used regardless of atom type and branching.

6-2. Gln peptides $t_{1/2} = [(\ln(2))/86400][e^{[(Sum/100) + 18.311]}]$

6-3. Hydrolysis correction $t_{1/2} = 1/(1/8000 + 1/t_{1/2\ calculated})$

These equations give values for $t_{1/2}$ of the Xxx median of the amino-side residue set of the naturally occurring residues in peptides of the form GlyXxxAsn/GlnYyyGly, where the substituent effects of Yyy are considered. The values 11.863 and 18.311 are the absolute values for AsnGly and GlnGly, respectively, in this system.

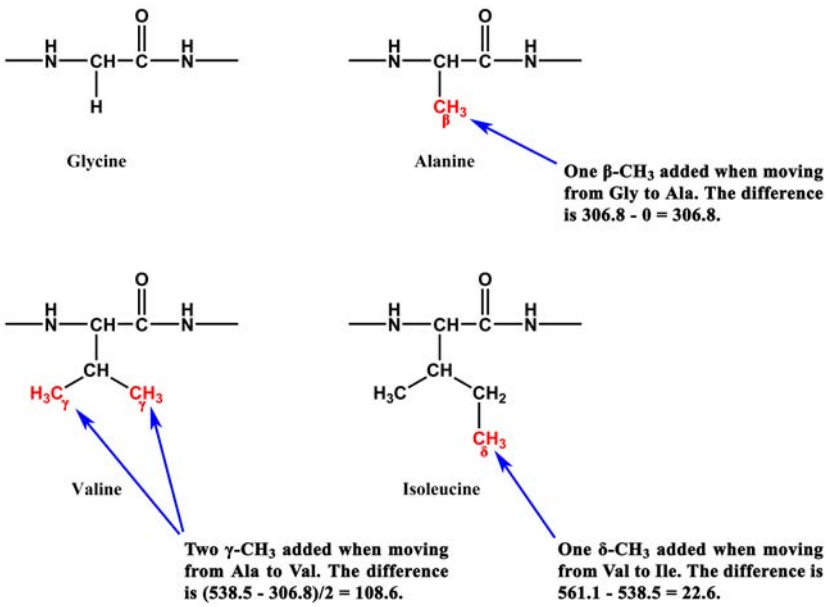

FIG. 6-5 Illustration of the derivation of the values in Table 6-4. For each residue to the left of Asn, (100)ln k is computed. These values are then normalized by subtraction from Gly. Thus Gly is 0 and Ala is 306.8, so the addition of a CH_3 adds 306.8. This is the value for a β-CH_3 in Table 6-4. The difference between Ala and Val is 108.6. This involves replacing two γ hydrogen atoms with γ-CH_3 groups. Proceeding to Ile, an additional 22.6 is added by the final replacement of a δ hydrogen atom with a δ-CH_3 group. Individual effects of each atom or group were derived from averages for Table 6-4. For example Table 6-4 gives a total of 102.3 + 214.5 + 214.5 = 531.3 for Ile which is close to the 538.5 shown here. For a detailed description see reference 2004RR2.

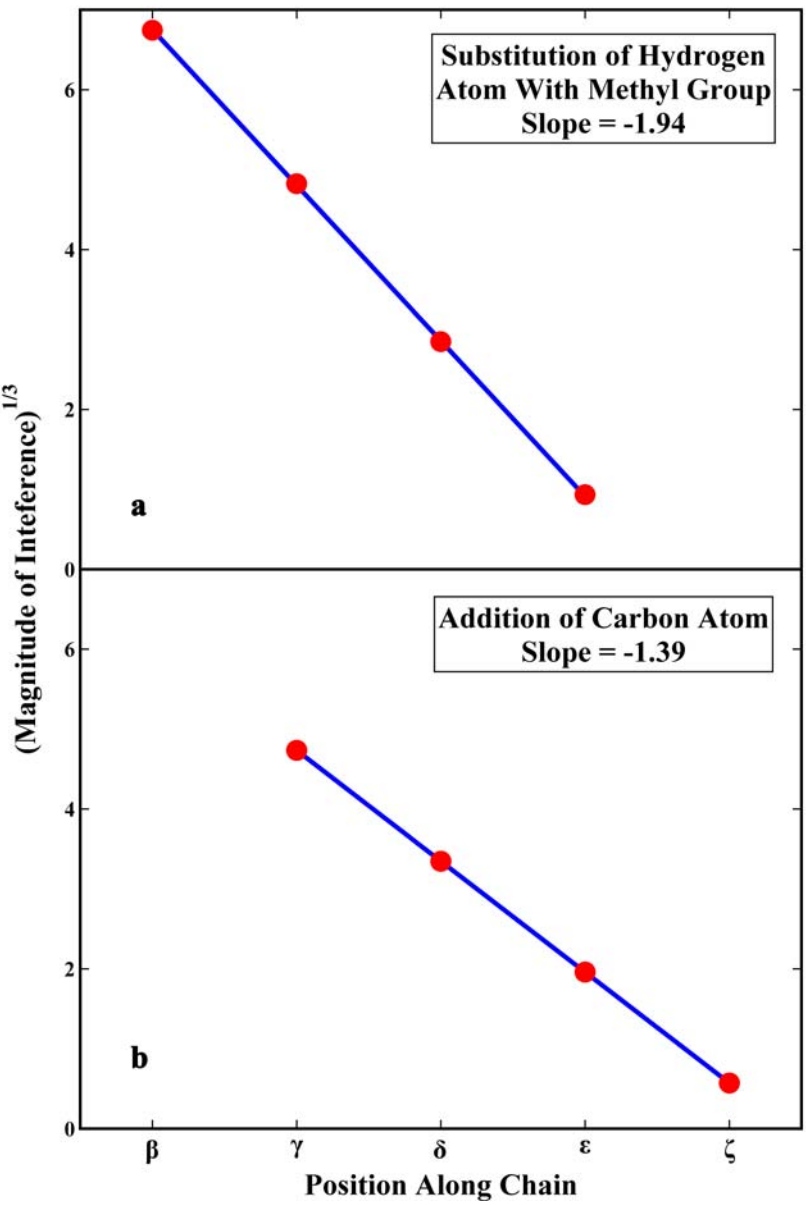

FIG. 6-6 Graph of the cube roots of the methyl substitution for hydrogen coefficients (a) and carbon atom coefficients alone (b) as a function of the position along the carboxyl residue side chain. The β, γ, and δ points for (a) were calculated directly from experimental data. The ε point is dependent on the individual atom calculations. The linearity of (b) was used to optimize the individual atom coefficients. As expected in this theoretical treatment, the fractional volume occupied by a side chain atom decreases with the cube root of the substituent number. Adapted from 2004RR2.

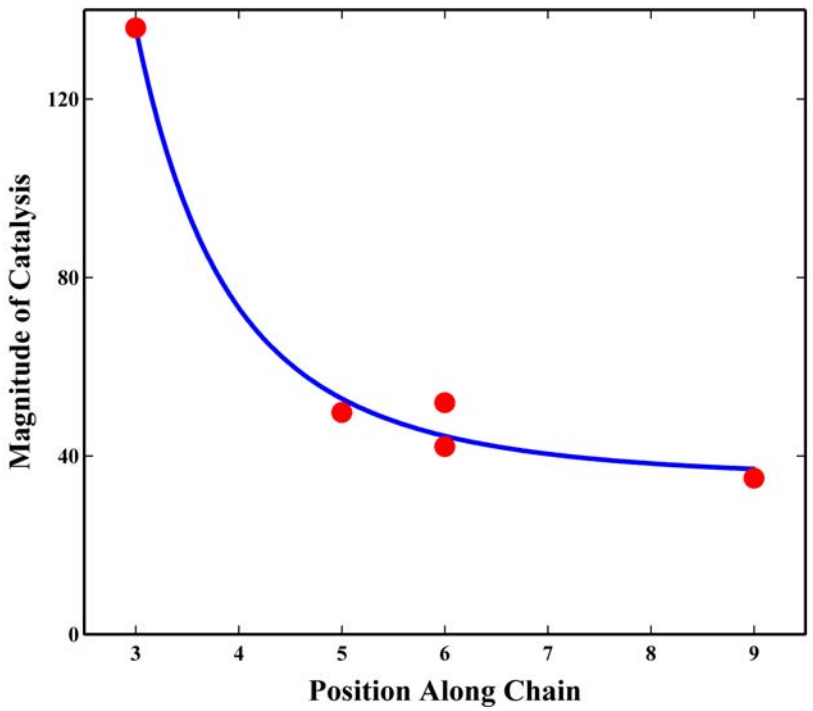

FIG. 6-7 Graph of the NH^+ catalysis coefficients for His, Lys, and Arg as a function of position along the carboxyl-side residue side chain and peptide backbone. Steric effect of NH is included. Adapted from 2004RR2.

For example,[10] the peptide GlyXxxAsnThrGly has the carboxyl-side substituent $-CH(CH_3)OH$. The substituent assignments are shown in Figure 6-8. Summing the β-CH, γ-CH3, and γ-OH:

AsnThr $t_{1/2} = [(\ln(2))/86400][e^{[((102.3+214.5+55.6)/100) + 11.863]}] = 47.2$

With hydrolysis $t_{1/2} = 1/(1/8000 + 1/47.2) = 46.9$

The experimental value for the Xxx median of GlyXxxAsnThrGly is 46.2.

At present, since the sequence dependence of hydrolysis of Gln peptides is unknown and measurements of Gln deamidation rates are subject to much greater experimental error because of the long deamidation half-times, a similarly detailed analysis of Gln peptides is not possible.

Figure 6-9 illustrates, however, that Asn and Gln peptides are amenable to the same sort of analysis. When a median hydrolysis correction

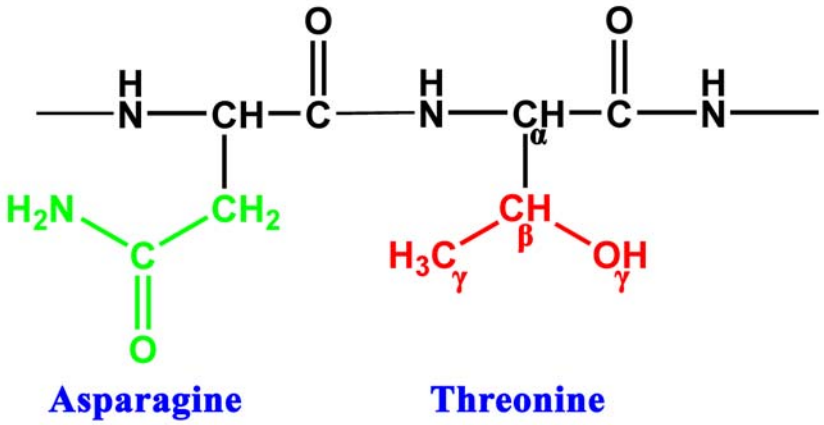

FIG. 6-8 Illustration of AsnThr Sequence (2004R).

is made and the fundamental difference between Asn and Gln is removed, the two-dimensional medians for Asn peptides and Gln peptides correspond within 9%. The greater scatter results from measurement uncertainty and hydrolysis dependence, which is a significant factor with slowly deamidating Gln peptides.

While the effects of steric factors alone have been quantitatively understood and modeled, the effects of functional groups are not as well understood. The measurement of deamidation rates for a variety of non-natural amino acids may elucidate this further. It appears, however, that the presence of positive or negative charges, or of nitrogen, oxygen or sulfur atoms, tends to accelerate the deamidation rate, as do many buffer ions. This is even true for peptides in which the catalytic group is several residues away.[8]

Much of this could be explained by the formation of special structures, many of which may be present only a small fraction of the time. Internal rings and other intramolecular structural types are possible. Another possibility, however, is the effect these groups have on the structure of water.

All of the apparently catalytic groups observed here have the property that they readily form hydrogen bonds. Water has a continuously flickering structure in which hydrogen bonds are being continually broken and reformed. The presence of peptide side-chains and also polarizable solutes have been calculated to have a large effect on water structure.[10]

10 L. Pauling, *Science* **134**, 15 (1961); S. L. Miller, *Proc. Natl. Acad. Sci. USA* **47**, 1515 (1961).

This effect could increase the structure of water over a wide localized area and therefore account for the fact that primary structure catalysis of deamidation is comparable in amount for several different substituents and extends over a long intramolecular distance.

Water structure and other types of structures have the potential to either speed or slow the deamidation reaction depending on the exact structure adopted. It might expected, however, that direct catalytic effects would be more pronounced.

A structure which hinders deamidation must be present a large fraction of the time to have an effect, even if it stops deamidation completely when it exists. This is observed in many protein amides which have relatively constant three-dimensional structures. It cannot, however, have a large effect in peptides or sections of proteins with labile three-dimensional structures. Catalytic effects, can operate over a very short fraction of the time, providing that they have a large effect when they are present. This is true of buffer ion effects as well as structural effects from water or other sources.

6-3. PREDICTION OF EFFECTS OF SEQUENCE CHANGES ON RATES

While absolute calculations by means of this method are restricted to pentapeptides of the type GlyXxxAsn/GlnYyyGly and similar molecules, relative half-time calculations are not. Therefore, if the rate of deamidation is known, changes of that rate as a result of changes in the carboxyl side residue can be estimated, even for non-natural substituents.[8]

For example, the sequence PheValAsn(B3)GlnHis of insulin deamidates with a half-time of 136 days in 37 °C, pH 7.4 sodium acetate, 0.1% methyl paraben, 0.7% NaCl.[11] Deamidation of insulin at Asn(B3) is a significant problem in pharmaceutical preparations. Numerous studies of storage conditions to minimize this reaction have been conducted. An alternative possibility would be to replace the carboxyl side Gln side chain $-CH_2CH_2CONH_2$ with the non-natural amide residue $-C(CH_3)_2CH_2CONH_2$.

To compute the effect of this substitution, one removes the AsnGly coefficient and calculates the coefficient for this insulin system.

$$- 1186.3 - (100)\ln((\ln(2)/136)(1/60)(1/60)(1/24)) = 478.3$$

11 J. Brange, L. Langkjaer, S. Havelund, and A. Volund, *Pharmaceutical Research* **9**, 715 (1992).

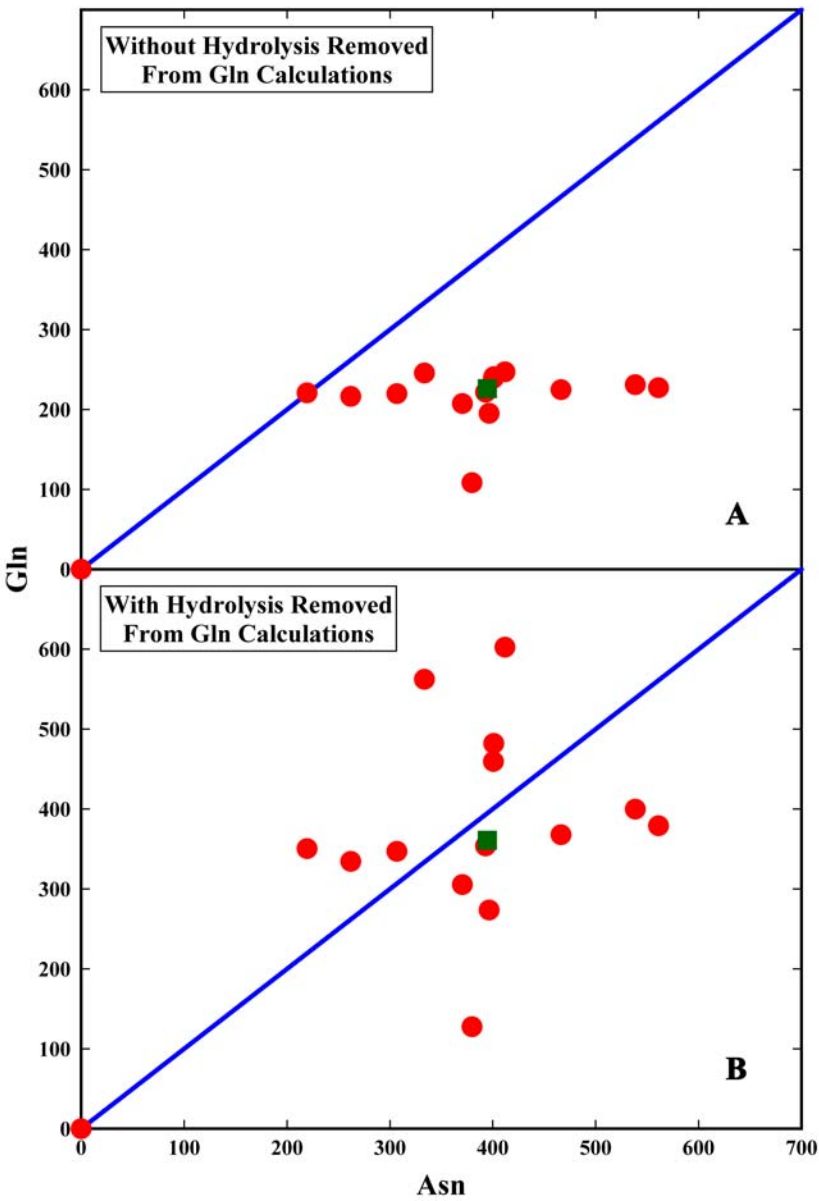

FIG. 6-9 (a) Normalized Gln (100)(ln(k)) without hydrolysis corrections vs. those for Asn (100)(ln(k)) with hydrolysis corrections as listed in Table 8-1. The fundamental difference in succinimide rate vs. glutarimide rate has been removed by normalization, which subtracts the Gln values from GlyXxxGlnGlyGly and the Asn values from GlyXxxAsnGlyGly. (b) As in (a) with both the Asn and Gln values corrected for hydrolysis. The two dimensional medians of the plotted points are indicated by the squares, ■. Adapted from 2004RR2.

Removing two hydrogen atoms and replacing them with two methyl groups in accordance with Table 6-2 allows calculation of the sum.

$$478.3 - 2\gamma H + 2\gamma CH_3 = 478.3 - (2)(102.3) + (2)(214.5) = 702.7$$

The deamidation half-time is then computed.

$$t_{1/2} = [(\ln(2))/86400][e^{[(702.7/100) + 11.863]}] = 1283 \text{ days}^{9}$$

Therefore, this modified insulin is expected to have a deamidation half-time about 10 times longer than the unmodified form.[8] Thus, the effects of carboxyl side Asn and Gln modifications can be estimated in a wide variety of situations. Such modifications could be of substantial value to the pharmaceutical industry.

This calculation method permits a better understanding of deamidation, provides a prediction procedure for protein engineering, and facilitates improved computation of peptide and protein primary, secondary, tertiary, and quaternary structure deamidation rates.

While the catalytic effects of chemically active side chains can be estimated from Table 6-4, the specific means by which these substituents exert their effects upon the reaction rate are still only partly understood. Inductive effects, hydrogen bonds, ionic bonds, effects on the structure of water, or other factors may be involved. Many of the specific effects of other solution components are also unexplained.

Amino side effects can be empirically estimated from the large number of rate measurements now available, but no theoretical explanation of these effects has, as yet, been reported. The amino side effect in peptides is usually 10- to 20-fold less than the carboxyl side effect. This makes separation of the various contributing factors more difficult. Since the amino side residue is farther removed from the reaction center, its effect in proteins may also be reduced by structural limitations.

6-4. PRIMARY CLOCKS

Every amide residue in a peptide or protein is a miniature molecular clock. The half-time of each of these clocks, whether it is a few hours or more than a century, is set by the molecular structure surrounding the amide. This structure is genetically determined.

In principle, this rate-determining structure can be considered as a concerted whole, without separating the essential elements of the struc-

ture into primary, secondary, tertiary, and quaternary components. This simplifying subdivision has, however, proved especially useful in deamidation as it has in many other aspects of peptide and protein chemistry. The strategy of measuring primary-structure-determined deamidation rates and then deducing by difference the effects of secondary, tertiary, and quaternary structure has proved robust.

Following this procedure, the primary sequence control of deamidation has been carefully measured. This has been done primarily in pentapeptides, since experiments with longer peptides and with proteins have shown the pentapeptide models to be quite suitable. Indeed, longer peptide models show complicating effects from secondary structure, which are preferably avoided in the primary measurements.

So far, these measurements have been carried out primarily in one set of solvent conditions. Qualitative extrapolations to other solvent conditions can be made, and experiments are in progress that will allow quantitative extrapolations. Ideally, the entire primary deamidation rate data set should eventually be reduced to zero buffer, pure water conditions, with coefficients available to adjust for buffer, ionic strength, temperature, and other factors. Recent advances in mass spectrometric deamidation rate determination make the completion of this task relatively easy.[12]

Figure 6-2 shows the cumulative distribution function of these primary structure deamidation half-times for pH 7.4, 0.15 M Tris-HCl, 37.0 °C at 0.001 M peptide. The actual half-time values for this figure are given in Tables 6-2 and 6-3. The plotted values in Figure 6-2 include 324 Asn peptides and 324 Gln peptides, of which 322 Asn and 52 Gln values are experimental with the remainder extrapolated and interpolated from the experimental.

The primary deamidation half-times under these conditions are distributed almost seamlessly between about 1 day and more than 10,000 days. The shorter deamidation half-times are mediated by the Asn succinimide mechanism, the longer by the Gln glutarimide mechanism, and the longest by the hydrolysis mechanism for long-lived Gln sequences and for AsnPro.

Figure 6-1 shows these primary rates in three-dimensional format, wherein the relative effects of the amino-side and carboxyl-side residues are illustrated. Each intersection of the solid lines in Figure 6-1 represents the deamidation of a unique GlyXxxAsn/GlnYyyGly peptide.

12 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

These measurements show that primary sequence alone provides a rich set of genetically determined molecular clocks that are found imbedded in most peptides and proteins. In cases where secondary, tertiary, and quaternary structure do not significantly intervene, these primary structure settings alone, adjusted for unique solvent conditions, determine the rates of these clocks in biological systems.

It turns out that most of the amide clock settings in proteins are determined by a combination of primary structure and of secondary, tertiary, and quaternary structure. See Chapter 9. All four of these aspects of structure are, of course, the result of genetically determined primary sequence. This separation into four structural types provides a mental model and a computational convenience.

Other related articles include 1962FM, 1963PK, 1967HT, 1972GP3, 1977B1, 1994DL1, 1995NF, and 2000ID.

6-5. AMIDE PROBES OF PROTEIN STRUCTURE

Demonstration of the marked suppression of deamidation in LeuAlaAsn(334)SerLeu in rabbit muscle aldolase by an α-helix gave rise to the suggestion[13] that amide residues can be used as molecular probes of secondary, tertiary, and quaternary structure in systems in which direct structure measurements are unavailable. Native Asn sequences or the insertion of appropriate sequences, especially AsnGly, and observation of the deamidation rate can give three-dimensional structure information about the Asn environment.

This method has been applied in a study of the modification of insulin to produce insulin amyloid fibrils.[14] In this case, the naturally occurring carboxyl terminus amide Asn(A21) was utilized. Since only half of this Asn deamidated in the fibrils, the investigators concluded that there were at least two different insulin-packing arrangements in the fibrils.

Also, PheAsnGly has been used as a probe of molecular motion in polyvinylpyrrolidone glasses.[15]

13 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

14 M. R. Nilsson and C. M. Dobson, *Protein Science* **12**, 2637 (2003).

15 T. Xiang and B. D. Anderson, *J. Pharmaceutical Sciences* **93**, 855 (2004).

6-6. AEROBIC ASCORBIC ACID

In the presence of aerobic ascorbic acid, peptide and protein solutions exhibit rapid and substantial release of ammonia.[16] Moreover, small peptides show an increase of negative charge upon subsequent electrophoresis. This was also observed in transferrin, where it was accompanied by extensive peptide bond cleavage. It has also been reported in encephalitogenic protein.[17]

These observations were first thought to be the result of ascorbic acid catalysis of deamidation.

Subsequent work showed, however, that free radicals generated by the oxidation of ascorbic acid were breaking peptide bonds and deaminating the resulting peptides.[18] A similar phenomenon was discovered earlier in the degradation of catalase by aerobic ascorbic acid.[19] Similar DNA breakage occurs in the presence of aerobic ascorbic acid.[20]

Peptide and protein damage by γ-irradiation may involve a similar mechanism.[21]

Ascorbic acid oxidation products might be found to cause some deamidation, but most of the observed ammonia release is apparently the result of peptide bond cleavage and deamination.

16 A. B. Robinson, K. Irving, and M. McCrea, *Proc. Natl. Acad. Sci. USA* **70**, 2122 (1973); A. B. Robinson and C. J. Rudd, *Current Topics in Cellular Regulation* **8**, 247 (1974).

17 F. C. Westall, M. Thompson, and A. B. Robinson, *Experientia* 32, 848 (1976).

18 A. B. Robinson and S. L. Richheimer, *Annals of the New York Academy of Sciences* **258**, 314 (1975); S. L. Richeimer and A. B. Robinson, *Orthomolecular Psychiatry* **6**, 290 (1977).

19 C. W. M. Orr, *Biochemistry* **6**, 3000 (1967).

20 A. Guidarelli, R. D. Sanctis, B. Cellini, M. Fiorani, M. Dacha, and O. Cantoni, *Biochemical Journal* **356**, 509 (2001).

21 H. Delincee and B. J. Radola, *Radiation Research* **58**, 9 (1974); M. J. Davies, *Archives of Biochemistry and Biophysics* **336**, 163 (1996); C. Houee-Levin and J. Berges, *The European Physical Journal D* **20**, 551 (2002).

CHAPTER 7

Secondary, Tertiary, and Quaternary Structure Dependence of Nonenzymatic Deamidation of Asn and Gln

7-1. INTRODUCTION

In the 1950s and 1960s, there were indications that deamidation would depend upon secondary, tertiary, and quaternary structure. Many instances of observed deamidation at that time occurred in protein sequencing work where higher order protein structure had been disrupted.

AsnGly sequences were found to be especially unstable, and Asx, denoting unknown amidation state, or Asp, where Asn was actually correct, commonly appeared in published protein sequences. As late as 1972, for example, the primary protein sequence reference[1] listed the sequence of one of the best characterized proteins of that time, hen egg white lysozyme, with two AspGly where AsnGly was, in fact, correct.

As primary-sequence-determined deamidation rates accumulated in the early 1970s, it became clear that most of the amide residues in proteins deamidate more slowly than their peptide counterparts. This has remained the case to the present as is illustrated by the compilation of all reports of deamidation of peptides and proteins from biological sources given in Table 11-1.

A recently reported example is ribonuclease A in which the folded protein was found to deamidate at CysLysAsn(67)GlyGln with deamidation half-time about 30-fold greater than for the corresponding free peptide.[2]

A review of the literature in 2001 found 38 instances in 28 proteins in which a deamidating Asn had been identified in a biologically produced protein and for which the three-dimensional structure had also

1 *Atlas of Protein Sequence and Structure*, 1972, Volume 5, M. O. Dayhoff, National Biomedical Research Foundation, Silver Spring, Maryland, USA.

2 S. Capasso and S. Salvadori, *J. Peptide Research* **54**, 377 (1999).

been reported.[3] Subsequently, several additional such instances have been found.[4] For 10 of these proteins, the *in vitro* or *in vivo* deamidation rates had also been determined.

In about half of those 38 instances, computations of the deamidation coefficients indicate that the *fastest* amide to deamidate is slowed by high-order structure.[3] The deamidation rates of most of the other Asn in these proteins are also reduced by higher-order structure. Among these proteins, in only one case has the deamidation rate been experimentally shown to be accelerated by higher-order structure at ordinary biologically relevant pH and temperature. A second case, interleukin II, was initially identified as accelerated, but this resulted from a misinterpretation of the reaction conditions.

Computations involving 13,335 proteins with 170,014 Asn indicate that only about 1% of Asn in proteins deamidate entirely under sequence control and without retardation by secondary, tertiary, or quaternary structure.[5] About half of these approximately 2,000 instances arise in sections of proteins that are unresolved in the structure determinations. Asn in these unresolved regions tend to be more unrestrained, but many may have restrictive structures that have not yet been seen.

So these estimates indicate that there are, among the 13,335 proteins for which three-dimensional structures had been reported by April 2001, at least 1,000 for which the first deamidation is expected to be primarily under sequence control. About 99% of the total individual Asn, however, have deamidation rates mediated by both primary and higher-order structure.

Computations have shown that, in the 28 proteins mentioned above, deamidation depends about 60% on primary structure and about 40% on higher order structure.[3] For 17,935 proteins for which three-dimensional structures were reported by January 2003, computations indicate that Asn deamidation depends, on average, about 50% on primary structure and 50% on secondary, tertiary, and quaternary structure.[6] As would be expected, the initial experimental observations of proteins have been biased (60:40) toward less structural suppression, while the entire set (50:50) lacks this bias.

3 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001).

4 www.deamidation.org; N. E. Robinson, *PhD Thesis, California Institute of Technology, Chemistry* (2003).

5 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

6 N. E. Robinson and A. B. Robinson, *Mechanisms of Ageing and Development* **125**, 259 (2004)

These computations are discussed in Chapter 9. They consider all aspects of secondary, tertiary, and quaternary structure simultaneously.

Experimental observations have been made of secondary structure dependence of deamidation in various longer peptides. These have included especially α-helix dependence.[7] Examples of other secondary structure studies include the effects of disulfide bridges and basic amino acid residues.[8]

Occasional rare instances have been found of structural acceleration of deamidation to shorter deamidation half-times than would be expected from primary structure. An interesting example is the Asp(105) to Asn(105) mutant of fluoroacetate dehalogenase from *Moraxella* sp. B, which spontaneously deamidates at GlyHisAsn(105)Arg with a half-time of about 10 days in pH 7.5, 4°C, 0.050 M phosphate to reconstitute the Asp(105) active form of the enzyme.[9]

Since the effects of higher order structure on deamidation and of deamidation on higher order structure are reviewed throughout this book in appropriate sections, the specific comments in sections 7-2 and 7-3 are abreviated.

7-2. SECONDARY STRUCTURE

Secondary structure effects on deamidation are easily demonstrated in longer peptides. Figure 7-1, for example, shows the deamidation rates of a series of peptides, $AlaXxx(Ala)_nAsn(Ala)_nYyyAla$. The markedly rising rates as a function of n reflect secondary structure. This may be α-helix formation. Some tertiary and quaternary structure may also be involved.

Table 7-1 shows an example of apparent secondary structure effect when n = 1. The hepta-peptide with Pro in positions 2 and 6 has a markedly slower deamidation rate.

Secondary structure effects demonstrated through synthesis of model peptides restricted, for example, by disulfide bridges and having sequences similar to those around specific amides found in proteins

7 A. A. Kosky, U. O. Razzaq, M. J. Treuheit, and D. N. Brems, *Protein Science* **8**, 2519 (1999); N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001); N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001).

8 S. Capasso and S. Salvadori, *J. Peptide Research* **54**, 377 (1999); S. Capasso, G. Balboni, and P. D. Cerbo, *Biopolymers* **53**, 213 (2000); C. Goolcharran, L. L. Stauffer, J. L. Cleland, and R. T. Borchardt, *J. Pharmaceutical Sciences* **89**, 818 (2000).

9 S. Ichiyama, T. Kurihara, Y. Kogure, S. Tsunasawa, H. Kawasaki, and N. Esaki, *Biochimica et Biophysica Acta* **1698**, 27 (2004).

have been used in several instances to detect secondary effects in proteins. These instances are referenced elsewhere in this book.

Rabbit muscle aldolase deamidates at the carboxyl end sequence IleSerAsnHisAlaTyr with a half-time of 9.4 days, in good agreement with the value for GlySerAsnHisGly of 9.0 days. The second Asn from the carboxyl end, AlaLeuAlaAsnSerLeuCysGlnGlyLys, however, has a half-time of more than 150 days, while GlyAlaAsnSerGly has a value of 11.4 days.[10] This Asn is in an α-helix in the protein, which markedly slows its deamidation. See Table 7-2.

Secondary structure effects on deamidation have been observed in other peptides[11] and can be expected to be demonstrable in most large peptides. There has been, however, no systematic experimental peptide work that allows quantitative or predictable secondary structure effects to be utilized in protein deamidation analysis. This is probably unnecessary.

Techniques for three-dimensional structure determination are advancing so rapidly that peptide and protein three-dimensional structures are becoming routinely available. As is shown in Chapter 9, experiments on proteins of known three-dimensional structure are sufficient to quantitatively determine structure effects on deamidation.

7-3. TERTIARY AND QUATERNARY STRUCTURE

The effects of tertiary structure on deamidation rates and, conversely, the effects of deamidation on tertiary structures play key roles in the biological function of deamidation. Understanding the former requires detailed understanding of the exact nature of the tertiary structure in the neighborhood of the amide, while the effect of the latter must, at present, be determined by empirical observation.

In general, deamidation tends to open the structure of proteins to greater susceptibility to proteolytic enzymes. Sometimes biological activity is increased, but more often it is decreased.

The effect of deamidation on an individual protein is, of course, specific to the exact changes that accompany the change of charge and conformation at the deamidating location in that protein.

The apparently ubiquitous distribution of methylating enzymes that reverse the isomerization that usually accompanies deamidation and the

10 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001); N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001).

11 M. Xie and R. L. Schowen, *J. Pharmaceutical Sciences* **88**, 8 (1999).

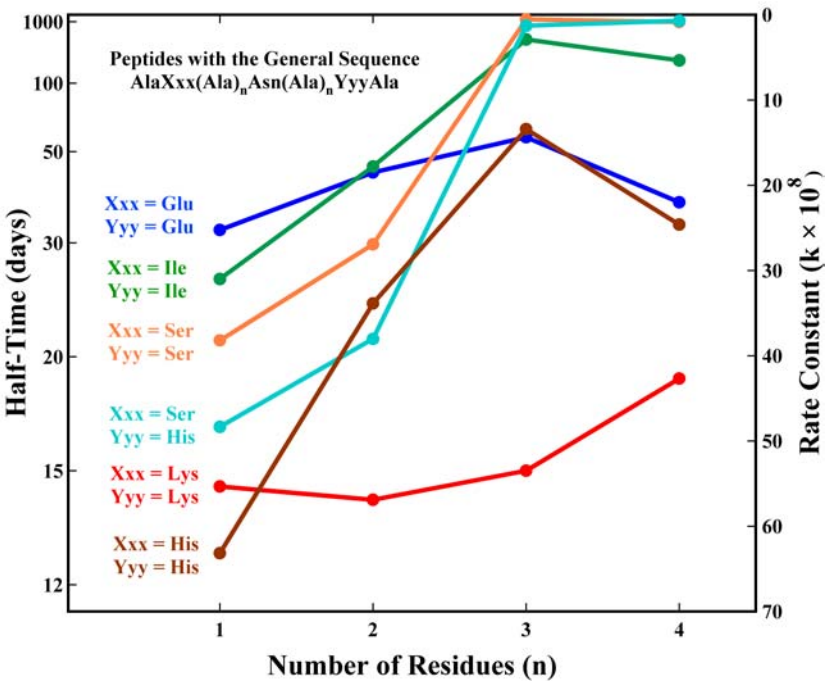

FIG. 7-1 Half-time as a function of sequence length for peptides of the type $AlaXxx(Ala)_nAsn(Ala)_nYyyAla$ (2003R).

diminished health of mutants deficient in these enzymes suggest that restoration of the usual backbone configuration may be a simple housekeeping function – as compared with the irreversible change in charge, which appears to be the primary protein-specific effect of deamidation. The relative biological importance of the charge change and of isomerization is, however, as yet undetermined.

Higher-order structures are usually inhibitory of protein deamidation rates. Since some instances of sequence dependence of deamidation were apparently the result of side chain catalysis, it was expected that some protein amides would also be catalyzed by secondary, tertiary, and quaternary structure through amino acid residue side chains that were not near those amides in primary sequence. So far, such examples are quite rare. If, as we discuss in Chapter 6, these apparent catalytic effects arise primarily from a general effect such as that on the structure of water, this rarity would be unsurprising.

Tertiary and quaternary structure effects on and of deamidation are central to many aspects of this subject and are, therefore, discussed

elsewhere in the various appropriate sections of this book, especially in Chapter 9.

Table 7-1 AlaXxxAlaAsnAlaYyyAla Deamidation Rates*

Peptide	t ½ Days	k x 10^6 Sec
AlaProAlaAsnAlaProAla	195	0.0411
AlaGluAlaAsnAlaGluAla	31.8	0.252
AlaAlaAlaAsnAlaAlaAla	31.2	0.257
AlaIleAlaAsnAlaIleAla	25.9	0.310
AlaSerAlaAsnAlaAlaAla	25.4	0.316
AlaSerAlaAsnAlaSerAla	21.0	0.382
AlaAlaAlaAsnAlaHisAla	16.9	0.475
AlaSerAlaAsnAlaHisAla	16.6	0.483
AlaLysAlaAsnAlaLysAla	14.5	0.553
AlaTyrAlaAsnAlaTyrAla	14.4	0.557
AlaHisAlaAsnAlaHisAla	12.7	0.632
AlaArgAlaAsnAlaArgAla	10.9	0.736

*Adapted from 2001RR2.

Table 7-2 Deamidation of Rabbit Muscle Aldolase and Model Peptides in 1.0 x 10^{-3} M Peptide or Protein, 37.00 ºC, pH 7.4, 0.15 M Tris-HCl in the Same Solution.

Peptide	t ½ Days	k x 10^{-6} Sec
Aldolase - IleuSerAsnHisAlaTyr	9.4	0.85
GlySerAsnHisGly	8.3	0.97
Aldolase - AlaLeuAlaAsnSerLeuCysGlnGlyLys	More than 150 days	
GlyAlaAsnSerGly	11.4	0.70

*Adapted from 2001RR.

CHAPTER 8

Dependence of Nonenzymatic Deamidation of Asn and Gln on Buffer Type, pH, Temperature, and Ionic Strength

8-1. BUFFER DEPENDENCE OF DEAMIDATION

As reviewed in Chapter 1, the buffer dependence of deamidation was initially discovered in experiments with the amino acid glutamine. It gradually became evident that deamidation is apparently subject to both specific and general acid and base catalysis. The relative contributions of these catalytic processes depend upon pH and upon the individual catalytic properties of the acids and bases.

During the work that established the broad sequence dependence of deamidation in model peptides, which has been summarized in review,[1] buffer dependence of deamidation was extended to include Asn peptides[2] and Gln peptides.[3] These experiments also established that the deamidation rates of Asn and Gln peptides at pH 7.4 increase with ionic strength and temperature.

The deamidation half-times for GlyThrAsnGluGly at pH 10, 37 °C, I = 1.0 were found to be 34, 11, and 5 hours in borate, carbonate, and phosphate buffers, respectively and, at pH 7.4, 37 °C, I = 0.15, were 27 and 12 days for borate and phosphate. The half-time for GlyArgAsnArgGly at pH 7.4, 37 °C, I = 0.15 was 115 days in Tris-HCl and 20 days in phosphate.[2] Figure 8-1 shows this dependence for two Gln peptides.[3]

In designing their experiments in the 1970s, the investigators did not know which buffer system would be most biologically relevant. Phosphate was chosen for the experiments.

1 A. B. Robinson and C. J. Rudd, *Current Topics in Cellular Regulation* **8**, 247 (1974).

2 J. H. McKerrow and A. B. Robinson, *Analytical Biochemistry* **42**, 565 (1971).

3 J. W. Scotchler and A. B. Robinson, *Analytical Biochemistry* **59**, 319 (1974).

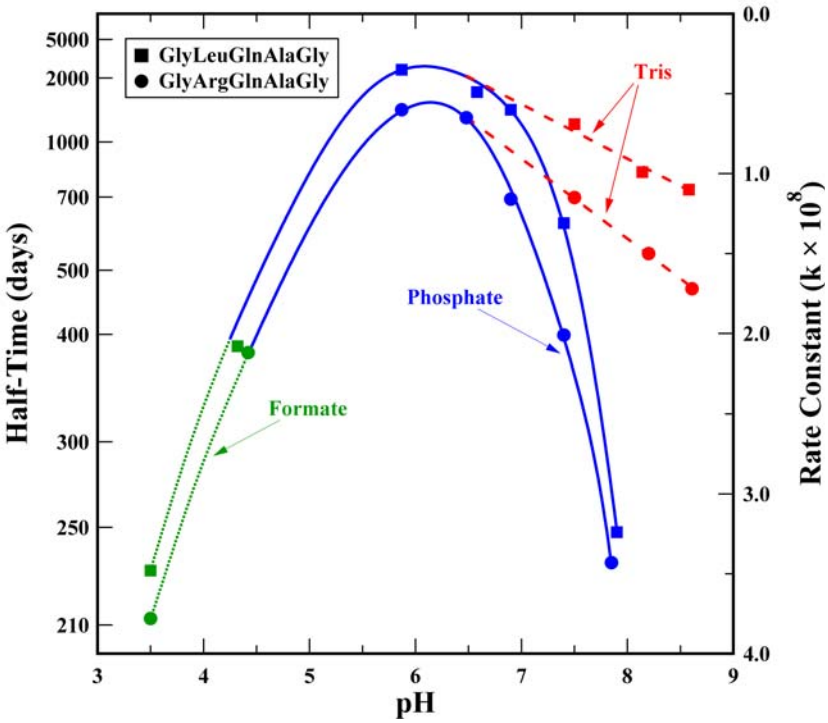

FIG. 8-1 The half-lives and first-order rate constants of deamidation vs. pH for GlyLeuGlnAlaGly, ■; and GlyArgGlnAlaGly, ●. Formic acid, sodium formate buffer was used at pH 3.6 and 4.4, ■, ●. KH_2PO_4, Na_2HPO_4 buffer was used at pH 5.8, 6.6, 7.0, 7.4, and 7.8, ■, ●. Tris, Tris-HCl buffer was used at pH 7.6, 8.2, and 8.6, ■, ●. The solutions were 37°C, ionic strength 0.2, and 0.001 M in peptide. The curved lines were visually interpolated between the measurements shown. Adapted from 1974SR.

When it was discovered that pentapeptide rates in phosphate closely duplicated the *in vitro* and *in vivo* deamidation rates of identical peptide sequences in aldolase and cytochrome c, it was concluded that phosphate buffers of the composition used were probably suitable. As it turned out, the catalytic effects of carboxyl-side Ser and His, the marked decrease of Gln rates as compared with Asn, the general dependence of deamidation upon steric hindrance and upon catalytic neighboring residues, and the broad range of genetically available sequence-controlled rates were successfully demonstrated in these experiments with 65 peptides in phosphate.[1]

Comparison, however, with recent quantitative and much more extensive measurements[4] demonstrates that, except for peptides with rela-

4 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001); N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483

tively short deamidation half-times, this early work was only semi-quantitative. While some of this is no doubt owing to better analytical techniques, it may well be that the choice of buffer was unfortunate. For quantitative work, Tris buffer or extrapolation to zero buffer concentration now appears to be more suitable. More research on deamidation as a function of peptide sequence and buffer type must be carried out before this matter is resolved.

The buffer dependence of deamidation in four AsnGly model peptides and in ribonuclease A has been investigated[5] and interpreted in the context of the mechanistic model as summarized in Chapter 5. This mechanism of imide formation involves three steps – equilibrium deprotonation of the backbone nitrogen, formation of the five-membered ring, and protonation of this ring to produce the imide and ammonia. The equilibrium step depends upon the concentration of hydroxyl ion, and the final step depends upon donation of a proton from an acid. Pre-equilibrium specific base catalysis followed by another step of general acid catalysis is kinetically indistinguishable from single-step general base catalysis. The relevant kinetic parameters have been measured for AcGlyAsnGlyGluNHMe at 37 °C.[6] Using these measurments, Figure 8-2 illustrates buffer dependence by means of the customary Brønsted plot of log k_b, the rate constant for catalysis by the base B^-, vs. pK_a, where K_a is the acid equilibrium constant of the base. This plot involves some details that are explained in appropriate texts.[7]

The Brønsted treatment depends upon the logical premise that a stronger acid is a better proton donor, while a stronger base is a better proton acceptor. Bases H_2O and OH^- corresponding to acids H_3O^+ and H_2O are included in Figure 8-2. Proton donors for step three include these acids, buffer components, and other solution acids in accordance with the rate constant equation.[6]

$$k_{observed} = k_{H2O} + k_{OH-}[OH^-] + \Sigma(k_{Bi}[B_i])$$

(2001); N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

5 S. Capasso, L. Mazzarella, and A. Zagari, *Peptide Research* **4**, 234 (1991); S. Capasso, L. Mazzarella, F. Sica, A. Zagari, and S. Salvadori, *J. Chemical Society-Perkin Transactions* **2**, 679 (1993); S. Capasso and S. Salvadori, *J. Peptide Research* **54**, 377 (1999).

6 S. Capasso, L. Mazzarella, F. Sica, A. Zagari, and S. Salvadori, *J. Chemical Society-Perkin Transactions* **2**, 679 (1993).

7 W. P. Jencks, Catalysis in Chemistry and Enzymology, McGraw-Hill , (1969); S. W. Benson, *The Foundations of Chemical Kinetics, McGraw-Hill*, (1960); March, J., *Advanced Organic Chemistry*, John Wiley & Sons, 1992.

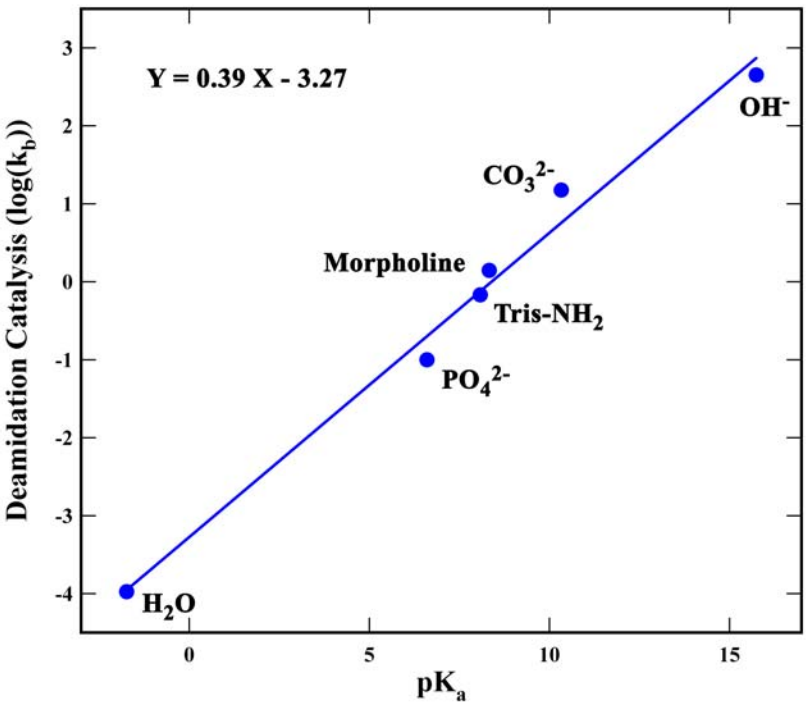

Figure 8-2 General base catalysis Brønsted plot for AcGlyAsnGlyGluNHMe deamidation. Base catalysis $\log(k_b)$ is plotted vs. pK_a, where k_b is the rate constant for catalysis and K_a is the acid equilibrium constant. Plotted from data in 1991CM.

Thus, the steps leading to imide formation are said to involve two steps mechanistically, yet are kinetically equivalent to a single step of apparently opposite catalytic nature.

Figure 8-3 shows a Brønsted plot of the general acid-catalysed part of this process with the data taken from a study of deamidation of $BocAsnGlyGlyNH_2$ in which specific base catalysis was removed by normalization,[8] leaving only the general acid-catalysed step. In the case of general acids, the Brønsted treatment requires use of $-pK_a$.

These treatments are for AsnGly peptides. Catalysis can also be reasonably expected to be dependent upon steric and other effects that are sequence dependent.

8 S. Capasso, L. Mazzarella, and A. Zagari, *Peptide Research* **4**, 234 (1991).

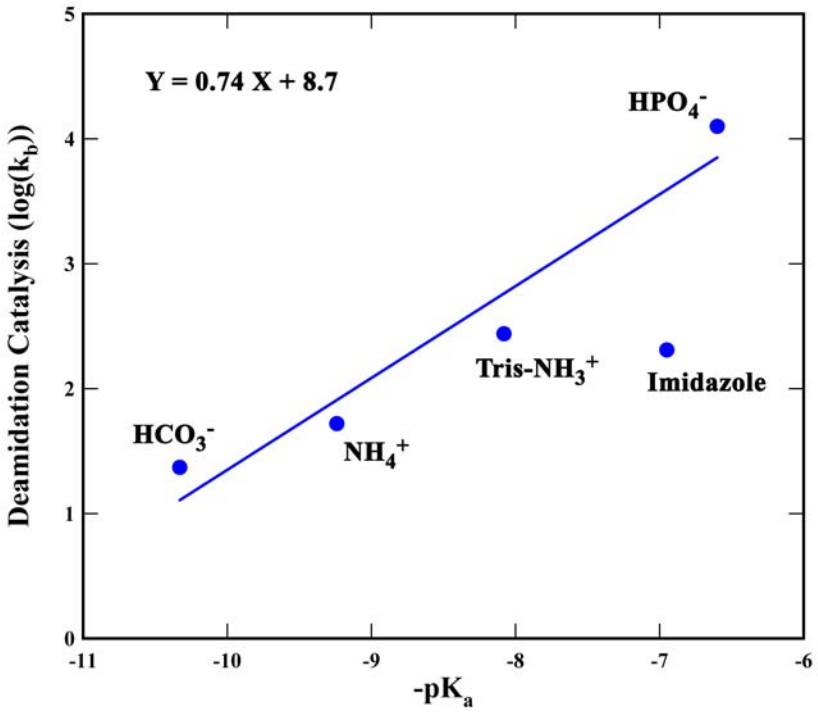

Figure 8-3 General acid Brønsted plot for BocAsnGlyGlyNH_2. Plotted from data in 1991CM.

The 35 Asn peptide deamidation rates measured in the 1970s[9] have been combined with 32 peptide rates measured subsequently[10] and the subsequent measurements have been corrected to the earlier conditions of pH 7.4, 37 °C, 0.0146 M $H_2PO_4^-$, and 0.062 M $HPO_4^=$.[11] The coefficients in this compilation permit the computation of median deamidation half-times for 12 Asn carboxyl-side residue sequences, in-

9 A. B. Robinson, J. W. Scotchler, and J. H. McKerrow, *J. American Chemical Society* **95**, 8156 (1973); A. B. Robinson and S. Tedro, *Int. J. Peptide and Protein Research* **5**, 275 (1973); J. H. McKerrow and A. B. Robinson, *Science* **183**, 85 (1974); A. B. Robinson, J. H. McKerrow, and M. Legaz, *Int. J. Peptide and Protein Research* **6**, 31 (1974); A. B. Robinson and J. W. Scotchler, *Int. J. Peptide and Protein Research* **6**, 279 (1974).

10 T. Geiger and S. Clarke, *J. Biological Chemistry* **262**, 785 (1987); R. Lura and V. Schirch, *Biochemistry* **27**, 7671 (1988); K. Patel and R. T. Borchardt, *Pharmaceutical Research* **7**, 703 (1990); K. Patel and R. T. Borchardt, *Pharmaceutical Research* **7**, 787 (1990); R. Tyler-Cross and V. Schirch, *J. Biological Chemistry* **266**, 22549 (1991); S. Capasso, L. Mazzarella, F. Sica, A. Zagari, and S. Salvadori, *J. Chemical Society-Perkin Transactions* **2**, 679 (1993); S. Capasso and S. Salvadori, *J. Peptide Research* **54**, 377 (1999).

11 S. Capasso, *J. Peptide Research* **55**, 224 (2000).

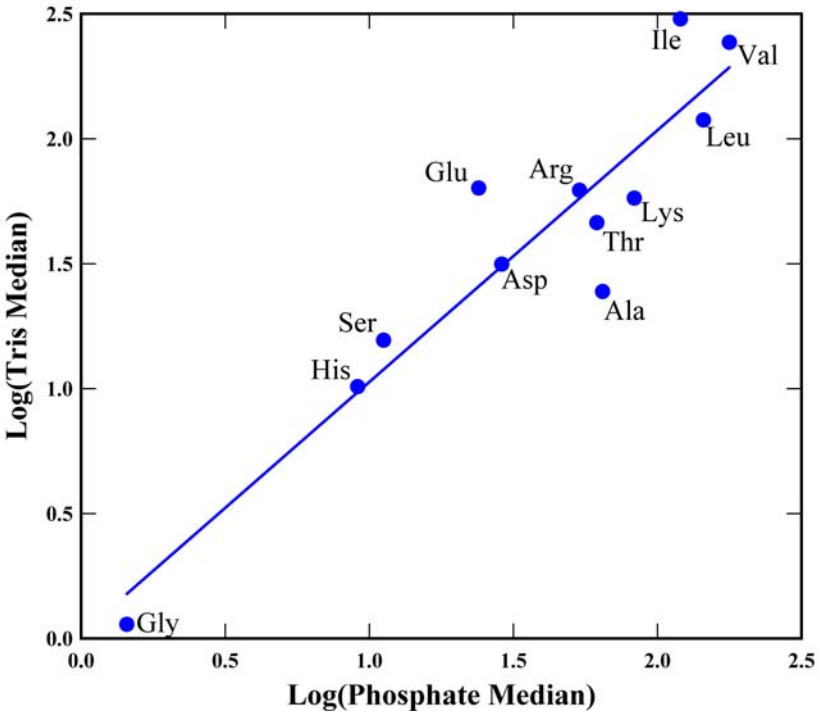

Figure 8-4 Comparison between Tris and phosphate deamidation half-times in 37 °C, pH 7.4, 0.125M Tris-HCl and 0.026 M Tris Base vs. 0.0146 M $H_2PO_4^-$ and 0.0162 M $HPO_4^=$.

cluding Gly, His, Ser, Asp, Glu, Arg, Thr, Ala, Lys, Ile, Val, Leu, with the medians computed from all 20 ordinary amino-side residues except for Gln and Asn. Log deamidation half-times for these peptides are compared with the medians for the same sequences measured in pH 7.4, 37 °C, 0.124 M Tris-HCl, and 0.026 M Tris base[12] in Figure 8-4.

Figure 8-4 includes experimental errors, especially in the phosphate values which are derived from fewer experimental values measured in several laboratories over a period of 30 years under varied conditions. The illustrated variations also apparently include sequence dependence of catalysis. For example, Gly, the least hindered, lies below the line, while Ile and Val, the most hindered, lie above the line, with Tris appearing to be more susceptible to steric hindrance than phosphate. These relative effects can, of course, be caused by any of the many other ways in which a catalyst may interact with the peptide in addition to steric factors.

12 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

The least-squares fitted line passes through 0,0 and 2,2, so the overall rates, all sequences considered, are essentially identical. Capasso estimates that $k_{phosphate}/k_{water}$ = 13.4. Correcting for phosphate concentration, this is 2.03, so phosphate accounts for about half of the deamidation in Figure 8-4 as, therefore, does Tris. The buffer acid concentrations are, however, 0.124 and 0.0146 for Tris and phosphate, respectively.

Computing 0.124/0.0146 = 8.5, phosphate is seen to be, therefore, an 8.5-fold more effective general acid catalyst of the third step than is Tris. For AsnGly, however, phosphate is only 2-fold more catalytic, while, for sterically hindered Ile, phosphate is 20-fold more catalytic than is Tris, without correction for water.

There are also indications that, as would be expected, structural factors are important to catalysis of protein deamidation. In a buffer-dependence study of deamidation of triosephosphate isomerase at 37 °C, pH 10, Yüksel and Gracy reported[13] that the observed deamidation rates fit a Brønsted plot for general base catalysis, but that the Tris rate was much lower than expected.

In ribonuclease A,[14] the catalytic constant for Tris base above pH 7 is 23-fold lower for deamidation of CysLysAsn(67)GlyGln compared with the peptide AcCys(Me)LysAsnGlyGlnThrAsnCys(Me)NH_2. For the peptide with the disulfide bridge formed, it was 2-fold lower. Below pH 7, no buffer catalysis was detected for either the peptides or the protein.

Deamidation of Asn(67)Gly in ribonuclease A is 60-fold slower than for AsnGly in unhindered peptides, primarily as a result of extensive secondary and tertiary structure interference with imide ring formation,[15] so it is not surprising that buffer catalysis is also hindered.

It has been found that Asn(45)GlyLys in horse α-lactalbumin has a deamidation half-time in 37 °C, pH 7.4, 0.15 M Tris-HCl of 3.6 days, which is 2.2-fold longer than that for 37 °C, pH 7.4, 0.15 M phosphate of 1.6 days. However, in 37 °C, pH 7.4, 0.020 M Tris-HCl, the half-time is 1.6 days as in 0.15 M phosphate.[16] This is apparently the result of some intricacy of structure dependence of α-lactalbumin on buffer ions.

13 K. Ü. Yüksel and R. W. Gracy, *Archives of Biochemistry and Biophysics* **248**, 452 (1986).

14 S. Capasso and S. Salvadori, *J. Peptide Research* **54**, 377 (1999).

15 N. E. Robinson, *PhD Thesis, California Institute of Technology, Chemistry* (2003).

16 J. -M. Girardet, M. -A. N'negue, A. S. Egito, S. Campagna, A. Lagrange, and J. -L. Gaillard, *International Dairy Journal* **14**, 207 (2004).

It is likely that, as more buffer-dependence data becomes available, it will be found that the genetically determined deamidation rates of proteins will be affected less by solution ions than are peptides, especially in regions of proteins where deamidation is substantially dependent on secondary, tertiary, and quaternary structure. Moreover, significant departures from the relative catalytic effects of different solutes expected from acid-base theory are likely to be found.

Most studies of deamidation rates have been conducted with reference to its physiological importance, so pH 7.4 has been of special interest. Studies of peptide and protein stability during chemical synthesis, extraction from biological mixtures, purification, storage, and investigations of reaction mechanism have led, in addition, to studies over a wide range of pH.

8-2. PH DEPENDENCE OF DEAMIDATION

Deamidation rates exhibit minima in the region around pH 4 to 6, with marked increases at lower and higher pH. Figure 8-5 illustrates this for the the first two deamidations of cytochrome c – first at AlaThrAsn(103)GluCOOH and second at AsnLysAsn(54)LysGly.[17] Deamidation of Asn(103) is entirely under sequence control, while Asn(54) deamidation is accelerated by a structural change caused by the deamidation of Asn(103).[18] Note that the second deamidation is faster than the first, so it would, of course, be first were it not dependent upon the first.

Figure 8-6 shows the deamidation rates of GlySerAsnHisGly vs. pH. Deamidation at low pH is, in this case, probably due primarily to acid hydrolysis.

The pH dependence in the physiological region shows a slowly rising plateau between pH 7 and pH 8 as illustrated for AcGlyAsnGlyGlyNHMe in Figure 8-7.[19] Similarly shaped curves have been found for deamidation of CysLysAsn(67)GlyGln in ribonuclease A and for model peptides of this region of the protein.[20] Deamidation rate rises inexorably with pH, but this rise slows between pH 7 and 8. While this has been studied only for AsnGly sequences, it may be found

17 T. Flatmark, *Acta Chemica Scandinavica* **20**, 1487 (1966).

18 A. B. Robinson, J. H. McKerrow, and M. Legaz, *Int. J. Peptide and Protein Research* **6**, 31 (1974).

19 S. Capasso, L. Mazzarella, F. Sica, A. Zagari, and S. Salvadori, *J. Chemical Society-Perkin Transactions* **2**, 679 (1993).

20 S. Capasso and S. Salvadori, *J. Peptide Research* **54**, 377 (1999).

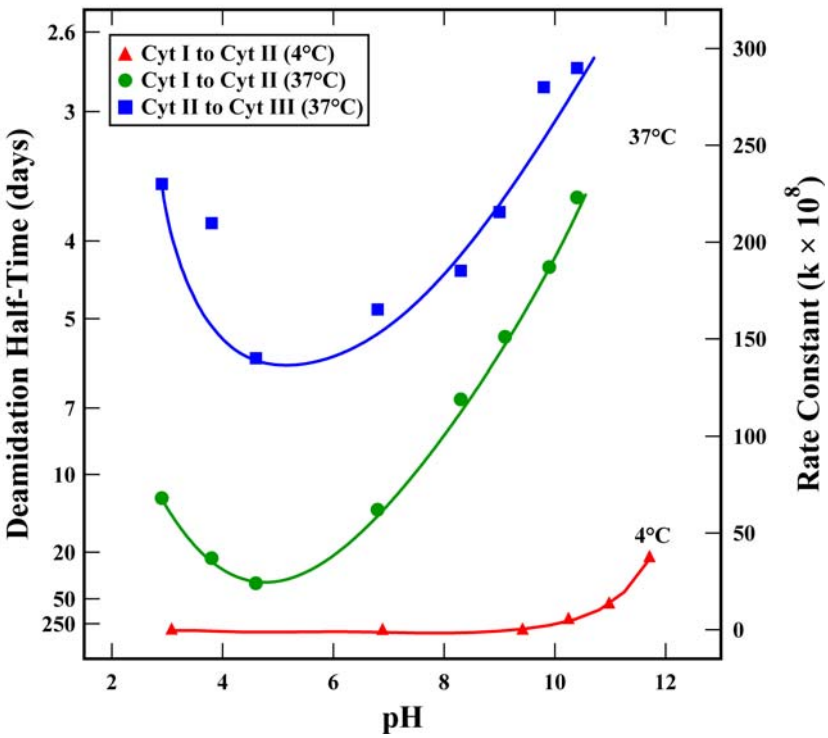

FIG. 8-5 Effect of pH and temperature on the first-order rate constant for the conversion of Cy I into Cy II, ▲, ●; and of Cy II into Cy III, ■. Formate and borate buffers of ionic strength 0.1 were used. Adapted from 1966F.

to be general. The curve shape for ribonuclease A is similar to that for the corresponding peptide, even though ribonuclease A deamidation is greatly inhibited by several aspects of structure that are qualitatively similar in effect to those responsible for sequence dependent deamidation in peptides.

The rising trend with increasing pH reflects, assuming the currently accepted mechanism, increased deprotonation of the backbone nitrogen in the first step of the imide reaction, and also increased base catalysis. In the case of Asn at neutral pHs and except for AsnPro, this is primarily the case, while Gln is a mixture of imide and hydrolysis.

Between pH 7 and 8 a transition takes place. This apparently reflects a change in catalysis of step three of the reaction. Since the physiological pH of 7.4 lies in this region, the reaction mechanism is more complicated than for other regions of pH.

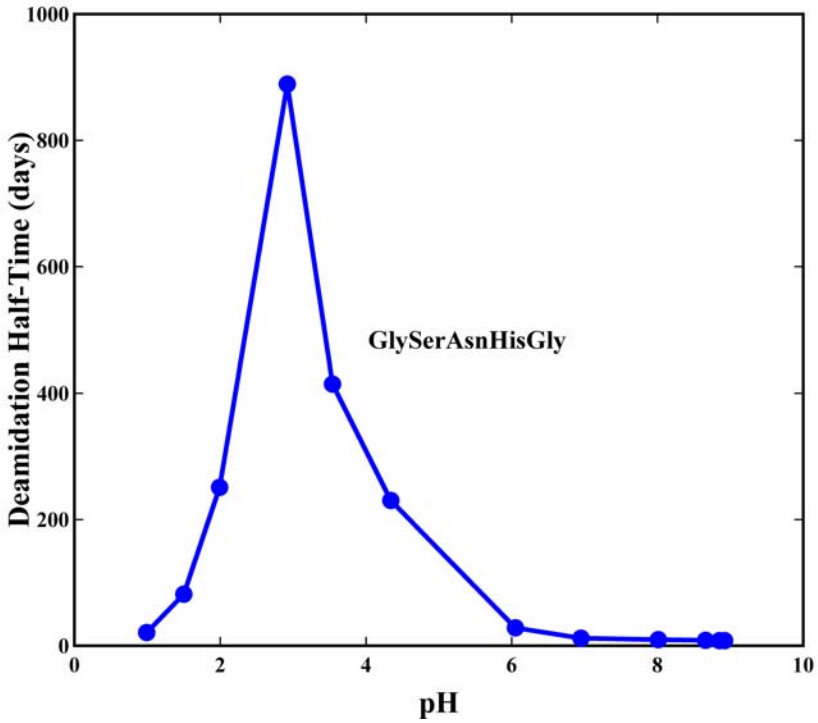

FIG. 8-6 Deamidation half-times of a 1.0×10^{-3} M solution of GlySerAsnHisGly as a function of pH in 37°C, 0.15 M Tris base adjusted to pH with 6 N HCl. The pH values shown are measured averages of the 1st, 9th and 17th point in deamidation solutions after the deamidation reactions had taken place. Adapted from 2001RR2.

In the various pH regions, deamidation of Asn and Gln is subject to specific and general acid and base catalysis, steric hindrance, and numerous other substituent interactions.

It is evident that deamidation rates depend strongly upon pH and upon catalysis by water and solution components such as buffer ions that are, themselves, pH-dependent. Moreover, the relative effects on deamidation of these components are dependent upon primary, secondary, tertiary, and quaternary peptide and protein structure. At present, there is too little data about these phenomena to permit reliable quantitative generalizations.

Therefore, deamidation studies should be conducted with very carefully measured pH, and, where quantitative pH-dependence data is needed, it must be gathered empirically for the system of interest.

It is also important to recognize that the deamidation of a completely pure peptide or protein does not always reflect the deamidation of a sin-

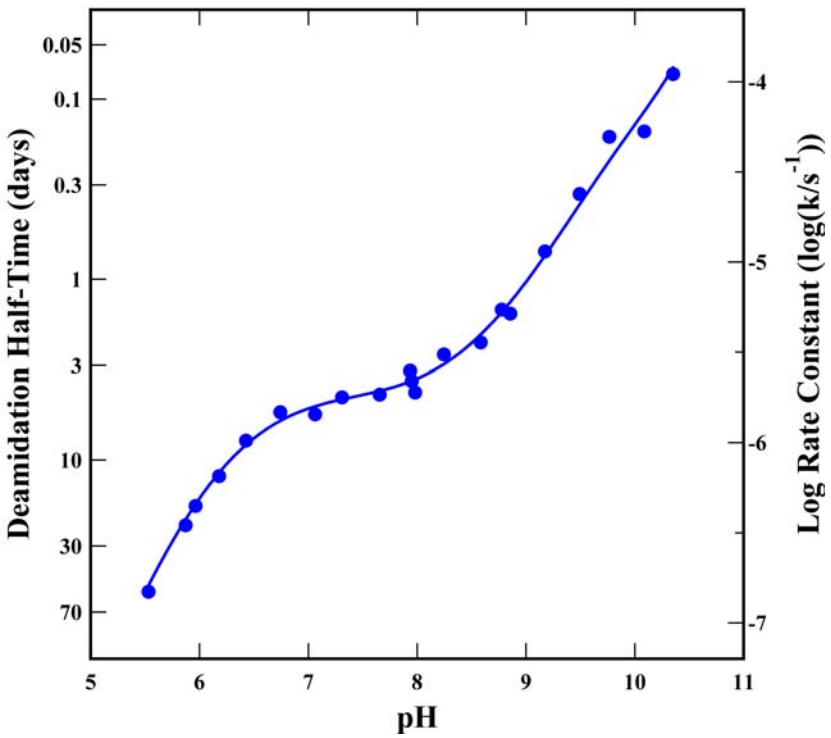

FIG. 8-7 pH dependence of AcGlyAsnGlyGlyNHMe. Adapted from 1999CS.

gle molecular species, even if only one deamidating residue is involved. The pKs of the amino acid residue side chains introduce heterogeneity.

Figure 2-7 shows a titration curve for GlySerAsnHisGly.[21] The measured pKs are 3.1, 6.4, and 7.8, as shown. Note that both the amino group and the imidazole group contribute heterogeneity, so there are several molecular species in the solution. The measured first-order deamidation rate of the peptide is a weighted average of the rates for those different species. Similar situations arise in proteins.

8-3. TEMPERATURE DEPENDENCE OF DEAMIDATION

Deamidation rate increases with temperature. Early investigators reported rate increases of 1.7 and 2.0-fold per 10 °C for GlyLeuGlnAlaGly and GlyArgGlnAlaGly, respectively in pH 7.4, I =

21 N. E. Robinson, *PhD Thesis, California Institute of Technology, Chemistry* (2003).

0.2, phosphate buffer, and 3.6-fold per 10 °C for GlyThrAsnThrGly in, pH 10, I = 1.0, carbonate buffer.[22] Similarly, the deamidation rate of ValTyrProAsnGlyAla increases 2.4-fold per 10 °C in pH 7.4, 0.1 M phosphate buffer.[23]

The deamidation rate of triosephosphate isomerase, TPI, however, is reported to increase only 1.2-fold per 10 °C in pH 7.0, 0.050 M triethanolamine or phosphate buffer.[24] The deamidation half-time of the unstable AsnGly sequences of TPI in these experiments averages 30 days, whereas AsnGly peptide deamidation half-times at pH 7 are on the order of about 1 day. Deamidation of TPI is substantially hindered by protein structure. The minimal temperature dependence of TPI probably reflects stability, in the range of temperatures of these measurements, of the three-dimensional structure components that have slowed its deamidation rate by a factor of 30-fold.

It has been shown that reduction of deamidation rates by steric hindrance of Asn carboxyl-side residues in pentapeptides can be quantitatively explained by a statistical model for which temperature dependence would be expected to be diminished because it depends largely upon covalent bonds and size of the sterically hindering groups. At ordinary temperatures, these bonds are not compromised.[25] See Chapter 6.

Similarly, some of the deamidation impediments in proteins are essentially stable at ordinary temperatures. Unless a specific structural impediment to deamidation is substantially disrupted as a function of temperature, the temperature dependence of that impediment would be expected to be diminished.

Unfortunately, a part of the published deamidation rate data has been collected at high pH and temperature, presumably because investigators lacked the patience to wait for slower reactions under moderate conditions. Often this data is qualitatively and even quantitatively extrapolated to lower pH or temperature in order to draw physiologically relevant conclusions.

This practice may be especially unwise in the case of temperature dependence, which is likely to be far more dependent upon peptide and protein-specific structure than is now generally recognized. This is cer-

22 J. H. McKerrow and A. B. Robinson, *Analytical Biochemistry* **42**, 565 (1971); J. W. Scotchler and A. B. Robinson, *Analytical Biochemistry* **59**, 319 (1974).

23 T. Geiger and S. Clarke, *J. Biological Chemistry* **262**, 785 (1987).

24 K. Ü. Yüksel and R. W. Gracy, *Archives of Biochemistry and Biophysics* **248**, 452 (1986).

25 N. E. Robinson and A. B. Robinson, *J. Peptide Research* **63**, 437 (2004).

tainly the case in proteins as reviewed, for example, for collagen in Chapter 12.

8-4. IONIC STRENGTH DEPENDENCE OF DEAMIDATION

In 1966, Flatmark reported[26] that the rate of deamidation of cytochrome c at AlaThrAsn(103)GluCOOH increased by a factor of 2 with an increase of ionic strength of about $\Delta I = 0.4$ at pH 11, 4 °C. He also found that deamidation of cytochrome c was very rapid in the presence of 80% saturated ammonium sulfate, conditions commonly used for protein purification. Asn(103) deamidation proceeds at the peptide rate without substantial interference from protein structure.[27]

Subsequently, it was reported[22] that the rate of deamidation of GlyArgAsnArgGly at pH 10, 37 °C increased by 2-fold with $\Delta I = 0.8$, while the deamidation rates of GlyLeuGlnAlaGly and GlyArgGlnAlaGly at pH 7.4, 37 °C increased 2-fold with $\Delta I = 0.8$ and 1.1, respectively. In these experiments, ionic strength was varied with NaCl. This is illustrated in Figure 8-8.

In 1991, a study[28] of deamidation of BocAsnGlyGlyNH_2 found a substantial accelerating effect by K_2HPO_4/KH_2PO_4 and $(NH_4)_2SO_4$, a moderate effect from $MgSO_4$, and essentially no effect by NaCl and Na_2SO_4 in concentrations up to 2 M. So, little ionic strength dependence was observed.

It is reasonable to expect ionic strength dependence of deamidation. This could arise in several different ways, but the importance of this effect is, as yet, unknown. There is indication, in the results reported to date, that ionic strength dependence might be significantly structure dependent.

26 T. Flatmark, *Acta Chemica Scandinavica* **20**, 1487 (1966).

27 A. B. Robinson, J. H. McKerrow, and M. Legaz, *Int. J. Peptide and Protein Research* **6**, 31 (1974).

28 S. Capasso, L. Mazzarella, and A. Zagari, *Peptide Research* **4**, 234 (1991).

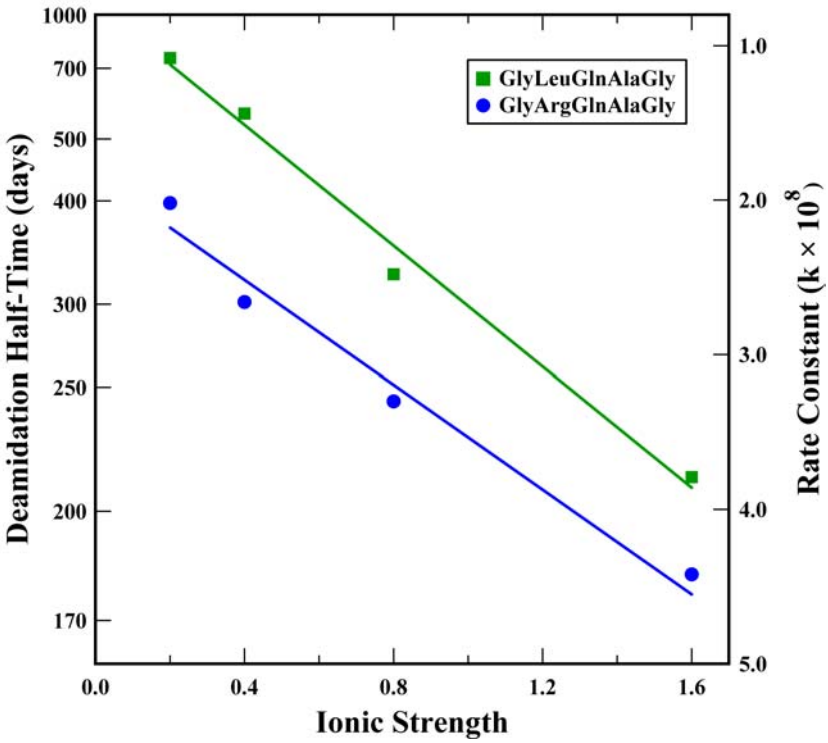

FIG. 8-8 Half-times and first-order rate constants of deamidation vs. ionic strength for GlyLeuGlnAlaGly, ■; and GlyArgGlnAlaGly, ●. The solutions were KH_2PO_4, Na_2HPO_4 buffer at pH 7.4, ionic strength 0.2, 37.0°C, and were 0.001 M in peptide. Higher ionic strengths were obtained by addition of NaCl according to the equation $I = 1/2\Sigma\, mz^2$, where m is the molality and *z* is the charge of each ion present. The lines are least-square straight lines for the measurements shown. Adapted from 1974SR.

CHAPTER 9

Nonenzymatic Deamidation Rates of Proteins

9-1. DEAMIDATION PREDICTION CALCULATIONS

Most deamidation rates of Asn in proteins are approximately equal to the sequence-controlled rates modulated through slowing by higher order structure. This modulation involves diminution of the ease with which the Asn side chain is able to align itself in an accommodating way with the reaction center in order to facilitate ring formation. In those cases where higher order structure does not slow the rate of deamidation, that rate is usually determined by primary structure. Both the primary structure component and the modulation component are under precise genetic control. This control renders every Asn a miniature molecular clock with a specific genetically determined deamidation rate.

Deamidation of Gln is similar to Asn, but, as a result of the longer deamidation half-times of Gln residues in peptides and proteins, little quantitative information about deamidation of Gln in proteins is, as yet, available.

A computation procedure for quantitatively combining the primary-structure determined deamidation rate and the higher order structure modulation of that rate has been developed for Asn in proteins.[1] This method has been computerized and applied to all proteins in the entire three-dimensional protein structure data base.[2]

In this computation, the exact three-dimensional position of each amide side chain is observed along with the side chain movement necessary to allow positioning of the side chain for optimum formation of the cyclic intermediate. The energy required for this repositioning is derived from a parameterized function that sums the necessary molecular rearrangements. The adjustable constants in this function are optimized with reference to experimentally observed protein deamidations. This

1 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001).

2 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

calculation provides computed deamidation coefficients, C_D, for each Asn and deamidation indexes, I_D, for each protein.

The calculation does not take into account rare specialized interactions in which proteins may increase or decrease deamidation rates in unusual ways because too little quantitative data about such interactions is currently available.

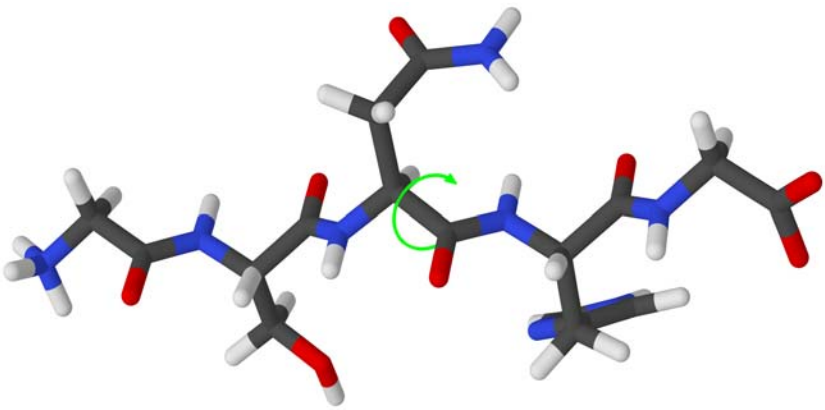

FIG. 9-1 Rotation required to form succinimide intermediate (2004R).

To a significant extent, the calculation evaluates impediments to free rotation of the peptide backbone to facilitate the necessary alignment of the Asn side chain as illustrated in Figure 9-1 and the presence of interfering chemical bonds with atoms that are essential to deamidation.

The optimization procedure makes use of 38 Asn residues that have been specifically identified as deamidated in 28 proteins and for which three-dimensional protein structures either by x-ray diffraction or nuclear magnetic resonance have been determined. It was assumed that the reported Asn are the fastest deamidating amides in each protein. While the conditions of deamidation vary widely for these 38 cases, they were combined without regard for these differences.

No absolute deamidation rates were used to calibrate this computation procedure. Only 13 such absolute rates are known for Asn in proteins with measured three-dimensional structures. These were used solely to test the computation procedure after it had been developed and optimized.

A set of observations of the three-dimensional environment of each Asn was selected. These observations were made and tabulated before

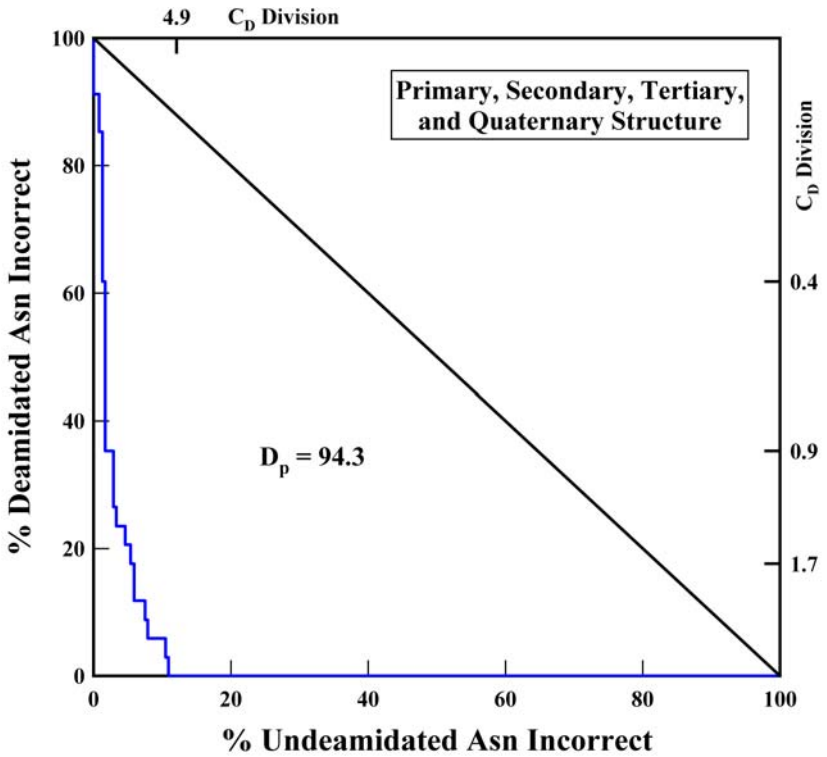

FIG. 9-2 Classification accuracies for the Asn residues in Tables 9-1 and 9-2 with all possible C_D division values used for the classification, excluding three Asn marked* in Table 9-1 and ‡ and ‡‡ in Table 9-2, and calculated deamidation resolving power D_p. Adapted from 2001RR3.

any calculations were carried out. The tabulated observations were not changed after calculations began.

$$C_D \text{ was defined: } C_D \equiv (0.01)\,(t_{1/2})(e^{f(Cm,\,CSn,\,Sn)})$$

where $t_{1/2}$ is the pentapeptide primary structure half-life, C_m is a structure proportionality factor, C_{Sn} is the three-dimensional structure coefficient for the nth structure observation, S_n is that observation, and $f(C_m, C_{Sn}, S_n) = C_m[(C_{S1})(S_1) + (C_{S2})(S_2) + (C_{S3})(S_3) - (C_{S4,5})(S_4)/(S_5) + (C_{S6})(S_6) + (C_{S7})(S_7) + (C_{S8})(S_8) + (C_{S9})(S_9) + (C_{S10})(1 - S_{10}) + (C_{S11})(5 - S_{11}) + (C_{S12})(5 - S_{12})]$. The structure observations, S_n, were selected as those most likely to impede deamidation, including hydrogen bonds, α helices, β sheets, and other factors. The functional form of C_D assumes that each of these structural factors is added to the reaction activation energy.

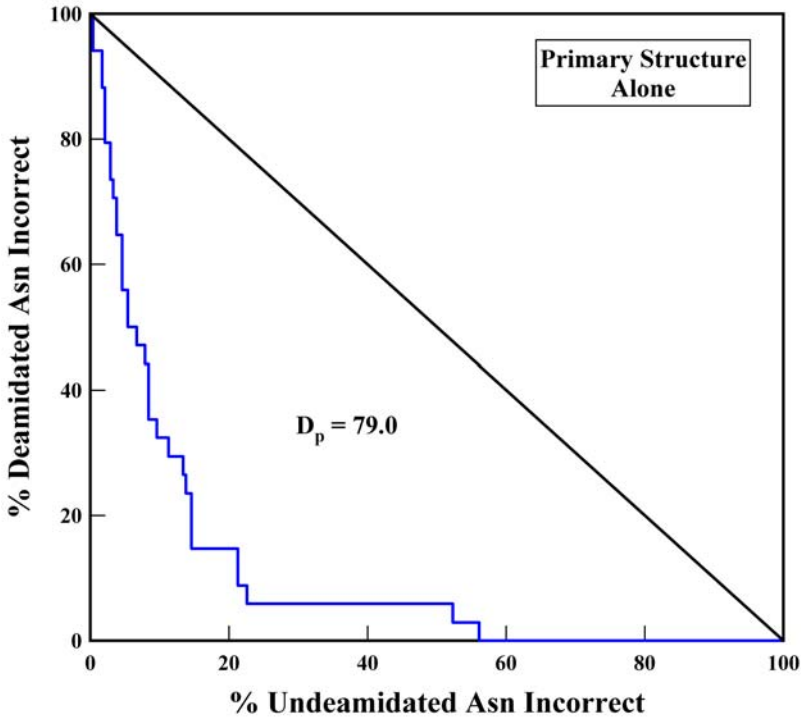

FIG. 9-3 Tabulation and calculation as in Fig. 9-2, but with use of only the primary structure part of the coefficients C_D. C_m=0. Adapted from 2001RR3.

The observed Sn were:

For Asn in an α-helical region:

S_1 = distance in residues inside the α-helix from the NH_2 end, where S_1 = 1 designates the end residue in the helix, 2 is the second residue, and 3 is the third. If the position is 4 or greater, S_1 = 0.

S_2 = distance in residues inside the α helix from the COOH end, where S_1 = 1 designates the end residue in the helix, 2 is the second residue, and 3 is the third. If the position is 4 or greater or $S_1 \neq 0$, then S_2 = 0.

S_3 = 1 if Asn is designated as completely inside the α helix, because it is 4 or more residues from both ends. If the Asn is completely inside, S_3 = 1, S_1 = 0, and S_2 = 0. If $S_1 \neq 0$ or $S_2 \neq 0$, then S_3 = 0.

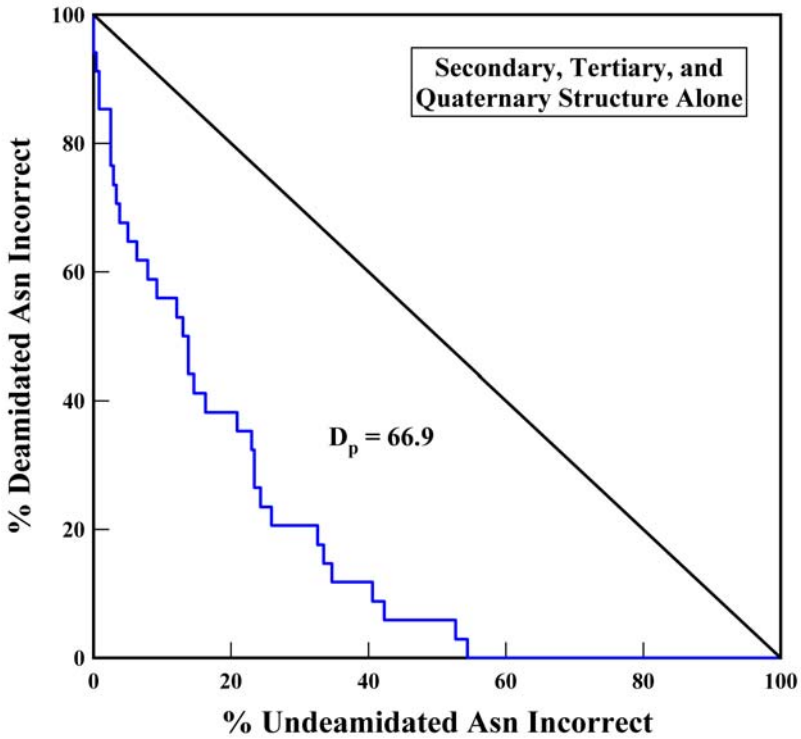

FIG. 9-4 Tabulation and calculation as in Fig. 9-2, but with use of only the secondary, tertiary, and quaternary structure part of the coefficients C_D. All $t_{1/2}$=1. Adapted from 2001RR3.

For flexibility of a loop including Asn between two adjacent antiparallel ß sheets:

S_4 = number of residues in the loop.
S_5 = number of hydrogen bonds in the loop. $S_5 \geq 1$ by definition.

For hydrogen bonds:
S_6 = the number of hydrogen bonds to the Asn side chain CO group. Acceptable values are 0, 1, and 2.
S_7 = the number of hydrogen bonds to the Asn side chain NH_2 group. Acceptable values are 0, 1, and 2.
S_8 = the number of hydrogen bonds to the backbone N in the peptide bond on the COOH side of Asn. Hydrogen bonds counted in S_6 or S_7 are not included. Acceptable values are 0 and 1. This nitrogen is used in the five-membered succinimide ring.

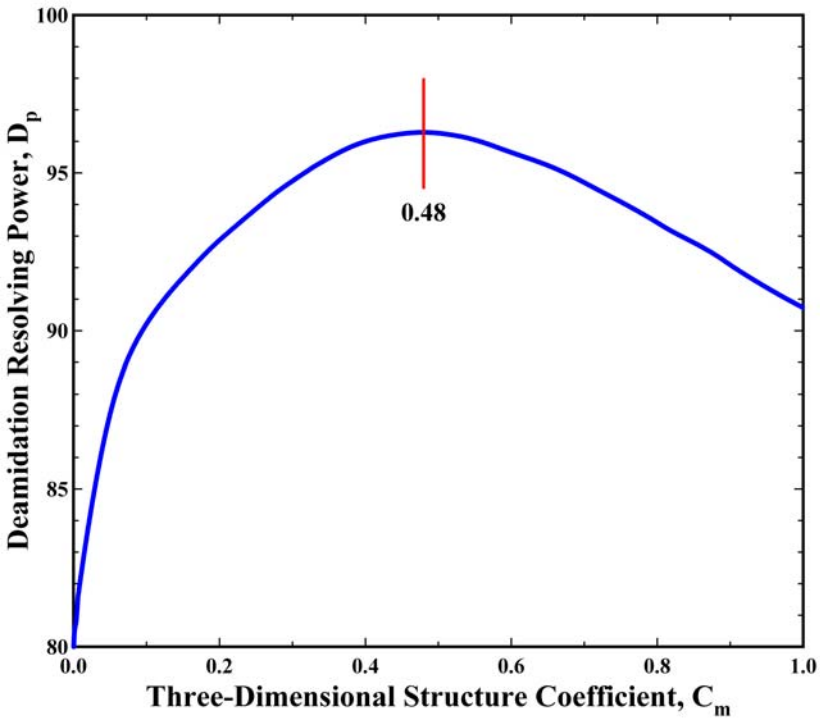

FIG. 9-5 Deamidation resolving power as a function of C_m. Adapted from 2002R.

S_9 = additional hydrogen bonds, not included in S_6, S_7, or S_8, that would need to be broken to form the succinimide ring.

For Asn situated so that no α-helix, ß-sheet, or disulfide bridge structure is between the Asn and the end of the peptide chain:

S_{10} = 1 if the number of residues between the Asn and the nearest such structure is 3 or more. If the number of intervening residues is 2, 1, or 0, or Asn is not between structure and chain end, then S_{10} = 0.

If the Asn lies near to any α-helix, β-sheet, or disulfide bridge structures:

S_{11} = the number of residues between the Asn and the structure on the NH_2 side, up to a maximum of 5. Values of 0, 1, 2, 3, 4, and 5 are acceptable.

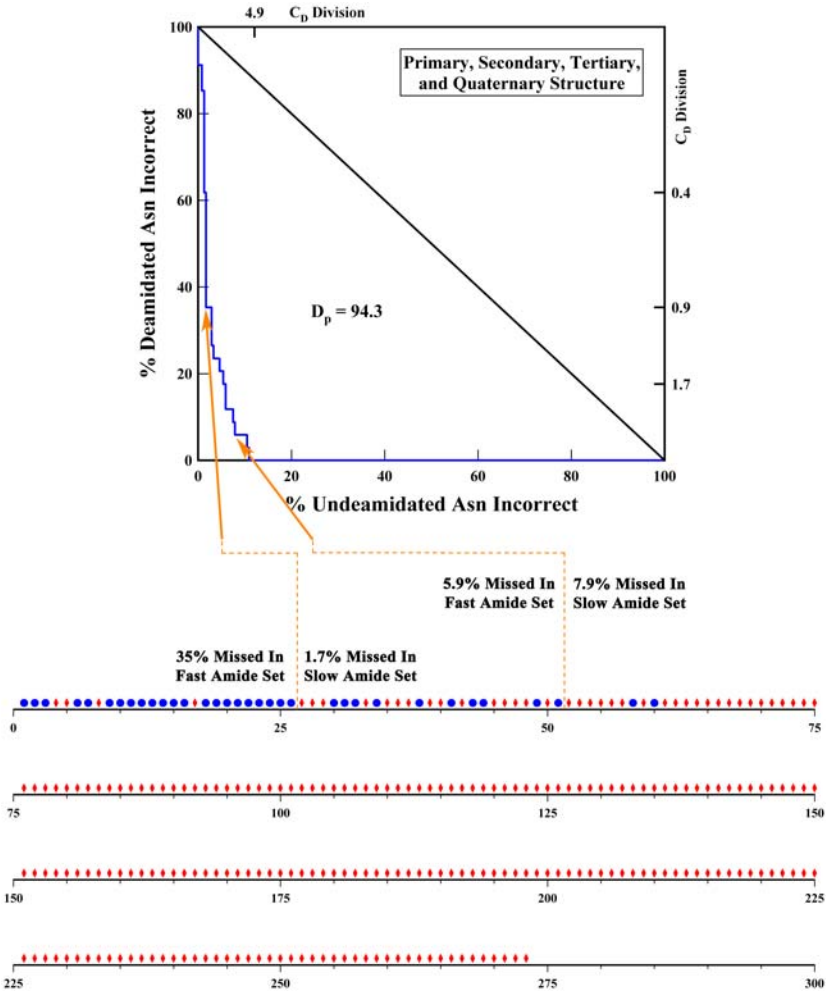

FIG. 9-6 Percent errors in classification made by cutting the (100)(C_D) axis at each possible position. The normalized area between the diagonal line and the plot (Dp) would be 100 for a perfect division of fast and slow amides and 0 for a completely random distribution. Two example cuts are shown. The percentages calculated are based on the number not in the set compared to the total. In the left slice, 12 of the 34 fast amides were missed, giving 35% on the y axis. Only 4 of the 238 slow amides were not put in the slow set, so this is 1.7% on the x axis. This is repeated at all 272 possible positions to produce the plot shown. The experimental observations were made over a wide variety of pH, temperature and buffer types and probably do not include all deamidating residues. Therefore, a Dp score of 100 may be impossible. The figure is from 2003R.

S_{12} = the number of residues between the Asn and the structure on the COOH side, up to a maximum of 5. Values of 0, 1, 2, 3, 4, and 5 are acceptable.

Hydrogen bonds selected by the Swiss Protein Data Bank (PDB) viewer were accepted if the bond length was 3.3 Å or less, and there was room in the structure to accommodate the van der Waals radius of the hydrogen atom. The Swiss PDB viewer, according to the customary criteria, selected α helices and ß sheets.

C_D values were optimized as a function of values for C_m and C_{Sn} to maximize the value of the deamidation resolving power, D_P. The optimized values were $C_m = 0.48$, $C_{S1} = 1.0$, $C_{S2} = 2.5$, $C_{S3} = 10.0$, $C_{S4,5} = 0.5$, $C_{S6} = 1.0$, $C_{S7} = 1.0$, $C_{S8} = 3.0$, $C_{S9} = 2.0$, $C_{S10} = 2.0$, $C_{S11} = 0.2$, and $C_{S12} = 0.7$.

For example, the ß-Lys-Asn(145)His sequence of hemoglobin is not in an α-helix or in a loop between two ß sheets, so S_1 through $S_4 = 0$, $S_5 = 1$. There is one hydrogen bond to the amide side chain nitrogen and one other to be broken to form the imide, but there are none to the amide carboxyl or the backbone nitrogen, so $S_6 = 0$, $S_7 = 1$, $S_8 = 0$, and $S_9 = 1$. This Asn is near the carboxyl end of the chain and one residue from an α-helix on the amino side, so $S_{10} = 0$, $S_{11} = 1$, and $S_{12} = 5$. The GlyLysAsnHisGly half-time is 10.5 days. Therefore, $C_D = (0.01)(10.5)e^{(0.48)[(1)(1)+(2)(1)+(2)(1-0)+(0.2)(4)]} = (0.105)e^{(0.48)(5.8)} = (0.105)(16.184) = 1.70$.

The D_P calculation method as developed previously for the evaluation of quantitative procedures in diagnostic medicine[3] was used as illustrated in Figures 9-2 to 9-4. A total of 264 Asn residues listed in Tables 9-1 and 9-2 were arranged in order of calculated C_D values and then divided into all possible two group sets arising from division at all possible C_D values. The errors at these division points for the optimized parameters are graphed in Figure 9-2. Figures 9-3 and 9-4 show graphs for primary structure and higher order structure alone. If the classification of Asn stabilities were perfect, then the graphs in Figures 9-2 to 9-4 would be straight lines along the axes, appearing as points in the origin. If there were no correlation between the calculations and the experimental data, the graphs would be along the diagonal lines. D_P is defined as the percentage of the area between the diagonal and the origin that has been successfully removed by the deamidation estimation procedure.

3 A. B. Robinson and F. C. Westall, *J. Orthomolecular Psychiatry* **3**, 70 (1974); A. B. Robinson and L. Pauling, *Clinical Chemistry* **20**, 961 (1974).

Each parameter in this computation procedure has been optimized with respect to the computed D_P. Figure 9-5 illustrates this procedure in the optimization of the three-dimensional structure coefficient C_m.

The method of calculation of D_P is further illustrated by Figure 9-6.[4] The graph in Figure 9-6 shows the percentage errors in classification made by cutting the axis at each possible position. The normalized area between the diagonal line and the plot of D_P would be 100 for a perfect division of fast and slow amides and 0 for a completely random distribution.

Two example cuts are shown. The percentages calculated are based on the number excluded from the set by the point of division as compared with the total. In the left slice, 12 of the 34 amides were missed, giving 35% on the y axis. Only 4 of the 229 slow amides were excluded from the slow set, so this is 1.7% on the x axis. This procedure is repeated at all 272 possible points of division to produce the plot shown.

The experimental observations were made over wide ranges of pH, temperature, and buffer types and probably do not include all deamidating residues. Therefore, a Dp score of 100 may be impossible with this data.

Two of the hemoglobin Asn mutations involve large undetermined structural changes in the protein, one by a frame-shift and the other causing the loss of the heme group, so suitable higher order criteria could not be tabulated. Higher order effects apparently markedly accelerate deamidation of Asn(54) in cytochrome C. These three Asn were not used in calculating D_P.

In addition to D_P, the Asn ranks within each protein as shown in Table 9-1 are especially interesting because these ranks avoid the complication that the different proteins were subjected to a wide variety of differing deamidating conditions. All 70 Asn in the hemoglobin set shown in Table 9-2 were incubated *in vivo* at 37°C for an average of 60 days in human blood.

In Tables 9-1 and 9-2, the amides are arranged in increasing order of C_D and the amides known to deamidate are enclosed in boxes. This illustrates the remarkable precision of this procedure.

Although the Asn residues designated as deamidating have been reported from experiments, those designated as un-deamidating depend on negative results. In many cases, ammonia evolution or protein separation experiments have shown that additional unstable amides may be present in these proteins. This is reflected in the asymmetry seen in Fig-

4 N. E. Robinson, *PhD Thesis, California Institute of Technology, Chemistry* (2003).

Table 9-1 Ordered deamidation coefficients and experimentally determined deamidating Asn residues in 23 proteins.

Aldolase	
Ser-Asn360-His	0.22
Thr-Asn119-Gly	0.93
Gln-Asn180-Gly	4.89
Pro-Asn231-Met	19.5
Ala-Asn334-Ser	76.4
Leu-Asn284-Ala	129
Glu-Asn166-Ala	346
Glu-Asn50-Thr	724
Ile-Asn282-Leu	790
Glu-Asn319-Leu	979
Ile-Asn287-Lys	1394
Val-Asn70-Pro	1587
Ala-Asn168-Val	2105
Glu-Asn54-Arg	2830
	0.17
Angiogenin	
Lys-Asn61-Gly	0.29
Glu-Asn109-Gly	0.38
Asp-Asn3-Ser	1.7
Gly-Asn49-Lys	18.5
Glu-Asn59-Lys	20.3
Ile-Asn43-Thr	21.5
Gly-Asn63-Pro	55.1
Glu-Asn68-Leu	71.5
Arg-Asn102-Val	1610
	0.15
Calbindin	
Lys-Asn56-Gly	0.03
Pro-Asn21-Gln	8.01
	0.03
cAMP-Dependent Protein Kinase	
Gly-Asn2-Ala	0.21
Ile-Asn340-Glu	1.53
Gly-Asn67-His	4.28
Gly-Asn283-Leu	11.5
Val-Asn99-Phe	11.9
Gln-Asn36-Thr	12.7
Tyr-Asn216-Lys	23.3
Ser-Asn326-Phe	39.2
Val-Asn289-Asp	40.7
Lys-Asn293-His	42.4
Asp-Asn113-Ser	53.9
Glu-Asn32-Pro	89.1
Leu-Asn90-Glu	180
Arg-Asn271-Leu	251
Ser-Asn115-Leu	275
Glu-Asn171-Leu	413
	0.16
Cytochrome c	
Thr-Asn103-Glu	0.68 ‡
Pro-Asn31-Leu	12.3
Lys-Asn54-Lys	51.1 *
Ala-Asn52-Lys	749
Glu-Asn70-Pro	1310
	0.64
Epidermal Growth Factor	
Leu-Asn16-Gly	0.08
Asn1-Ser	0.26 ‡‡
Cys-Asn32-Cys	8.19
	0.06
Fatty Acid Binding Protein	
Phe-Asn105-Gly	0.42
Asp-Asn89-Lys	1.98
His-Asn61-Glu	4.73
Met-Asn2-Phe	30.2
Thr-Asn111-Thr	159
Glu-Asn14-Phe	202
	0.32
Fibroblast Growth Factor	
Ser-Asn18-Gly	0.21
Gly-Asn7-Tyr	0.64
Lys-Asn114-Gly	1.38
Phe-Asn2-Leu	3.08
Glu-Asn92-His	6.3
Pro-Asn80-Glu	9.21
Lys-Asn106-Trp	51.5
Tyr-Asn95-Thr	74.8
	0.13
Glucoamylase	
Val-Asn181-Gly	0.35
Arg-Asn69-Gly	0.96
Tyr-Asn313-Gly	1.18
Asp-Asn145-Gly	2.48
Ser-Asn395-Gly	13.1
Trp-Asn171-Gln	13.1
Ala-Asn236-Phe	18.9
Phe-Asn110-Val	22.4
Arg-Asn430-Ser	35.7
Leu-Asn292-Asp	42.9
Gly-Asn315-Pro	55.1
Asp-Asn45-Pro	68.6
Ala-Asn277-His	77.1
Thr-Asn247-Thr	98.2
Leu-Asn20-Asn	99.9
Ala-Asn426-Asn	127
Ser-Asn9-Glu	189
Asn-Asn427-Arg	190
Ser-Asn93-Pro	192
Asn-Asn21-Ile	241
Arg-Asn161-Asp	465
	0.18
Growth Hormone	
His-Asn152-Asp	0.81
Thr-Asn149-Ser	1.17
Ala-Asn99-Ser	1.64
Ser-Asn63-Arg	4.07
Gln-Asn12-Ala	128
Ser-Asn72-Leu	170
Lys-Asn159-Tyr	496
	0.34
Hypoxanthine Guanine Phosphoribosyl-transferase	
Cys-Asn106-Asp	3.06
Leu-Asn202-His	7.31
Pro-Asn25-His	8.33
Lys-Asn128-Val	16.8
Arg-Asn87-Ser	45.4
Tyr-Asn195-Glu	80.5
Tyr-Asn153-Pro	89.1
Leu-Asn85-Arg	1344
	1.45
Insulin	
B-Val-Asn3-Gln	1.17
A-Cys-Asn21	6.06 ‡‡
A-Glu-Asn18-Tyr	115
	0.97
Interleukin 1β	
Leu-Asn32-Gly	0.04
Asn-Asn137-Ser	0.93
Gly-Asn136-Asn	1.69
Ile-Asn37-Gln	13
Ser-Asn53-Asp	22.8
Lys-Asn66-Leu	55.4
Pro-Asn119-Trp	433
Gln-Asn35-Ile	550
Phe-Asn102-Lys	780
	0.04
Lysozyme	
Gly-Asn103-Gly	0.06
Met-Asn106-Ala	0.58
Ile-Asn59-Ser	4.6
Arg-Asn113-Arg	7.43
Arg-Asn46-Thr	11.5
Asp-Asn19-Tyr	15.2
Arg-Asn74-Leu	22.8
Thr-Asn44-Arg	23.8
Phe-Asn39-Thr	47.4
Cys-Asn65-Asp	60.1
Val-Asn93-Cys	201
Gly-Asn27-Trp	245
Cys-Asn77-Ile	277
Ser-Asn37-Phe	807
	0.05
Ribonuclease-A	
Lys-Asn67-Gly	0.85
Ser-Asn24-Tyr	11.5
Val-Asn44-Thr	14.4
Ala-Asn103-Lys	16.1
Thr-Asn71-Cys	19.5
Pro-Asn94-Cys	29.8
Lys-Asn62-Val	70.8
Gly-Asn113-Pro	131
Arg-Asn34-Leu	141
Cys-Asn27-Gln	308
	0.66
Ribonuclease Seminal	
Lys-Asn67-Gly	0.31
Gly-Asn17-Ser	2.1
Thr-Asn71-Cys	4.37
Val-Asn44-Thr	60.6
Pro-Asn94-Cys	77.7
Cys-Asn27-Leu	145
Ser-Asn24-Tyr	787
	0.25
Ribonuclease-U2	
Tyr-Asn68-Gly	0.14
Ala-Asn32-Gly	1.62
Asp-Asn77-Tyr	12.5
Ser-Asn16-Asp	36.8
Thr-Asn8-Cys	85.7
Thr-Asn91-Thr	131
Gly-Asn12-Val	132
Asp-Asn38-Tyr	145
Ile-Asn20-Thr	147
	0.13
T-Cell Surface Glycoprotein CD4	
Leu-Asn52-Asp	3.99
Lys-Asn30-Ser	5.71
Ala-Asn103-Ser	12.3
Lys-Asn137-Ile	21.3
Gly-Asn66-Phe	29.8
Gln-Asn164-Gln	66.2
Ser-Asn32-Gln	69.5
Lys-Asn73-Leu	89.5
Gly-Asn39-Gln	92.7
	1.56
Thioltransferase	
Thr-Asn51-His	4.92
Thr-Asn55-Glu	119
Val-Asn7-Cys	1076
	4.70
Triose Phosphate Isomerase	
Thr-Asn71-Gly	0.78
Met-Asn15-Gly	1.77
Gln-Asn65-Cys	4.55
Ile-Asn245-Ala	31.5
Gly-Asn11-Trp	35.9
Asp-Asn153-Val	165
Leu-Asn29-Ala	208
Ser-Asn195-Val	360
	0.47
Trypsin	
Leu-Asn115-Ser	1.14
Tyr-Asn95-Ser	1.28
Ile-Asn48-Ser	4.82
Leu-Asn34-Ser	6.11
Ser-Asn97-Thr	10.6
Lys-Asn223-Lys	12.1
Ser-Asn245	21.1
Ala-Asn25-Thr	32.7
Leu-Asn100-Asn	40.9
Gly-Asn143-Thr	44.4
Gly-Asn79-Glu	50.5
Asp-Asn72-Ile	75.5
Ile-Asn74-Val	99.7
Ser-Asn179-Met	108
Cys-Asn233-Tyr	355
Asn-Asn101-Asp	537
	0.42
Phosphocarrier Protein - Hpr	
Pro-Asn12-Gly	0.21
Ser-Asn38-Gly	0.57
	0.15

Squares designate Asn reported as deamidated. *This unshaded square designates an unusual protein structure that accelerates deamidation. ‡Uses primary $t_{1/2}$ from Ref. 6. ‡‡Uses primary $t_{1/2}$ from Ref. 12. ___ designates deamidation index, I_D. Adapted from 2001RR3. As originally published, Interleukin 2 was also included, but its reaction conditions were not appropriate for this listing.

Table 9-2 Ordered deamidation coefficients for 70 Asn residues in wildtype and mutant human hemoglobins and experimentally determined deamidating Asn residues.

Hemoglobin - 7.78			
α-Ser-Asn50-Gly	0.18	β-Val-Asn61-Ala	141
β-Leu-Asn82-Gly	0.19	α-Val-Asn11-Ala	141
α-Pro-Asn78-Gly	0.67	β-Gly-Asn108-Met	160
β-Lys-Asn145-His	1.7	β-Ala-Asn139-Asp	164
β-Asp-Asn80-His	1.73	β-Gly-Asn17-Val	177
β-Val-Asn19-Gly	2.53	β-Ala-Asn139-Thr	223
β-Ser-Asn73-Gly	4.92	β-Asp-Asn80-Arg	240
β-Ala-Asn63-Gly	5.39	β-Gly-Asn65-Lys	247
α-Val-Asn56-Gly	6.31	α-Gly-Asn60-Lys	247
α-Pro-Asn78-Ala	11.1	α-Asp-Asn7-Thr	269
α-Leu-Asn87-Ala	11.7	β-Pro-Asn59-Val	274
α-Asp-Asn75-Met	12.4	β-Leu-Asn92-Cys	(274) ‡‡
α-Phe-Asn47-Leu	13	β-Leu-Asn89-Glu	291
β-Gly-Asn120-Glu	14.5	β-Asp-Asn80-Leu	305
β-Val-Asn21-Glu	18.7	β-Gly-Asn108-Leu	330
α-Val-Asn74-Asp	20.8	β-His-Asn117-Phe	370
α-Pro-Asn78-Thr	22	α-Val-Asn133-Thr	414
β-Asp-Asn80-His	26.7	α-Leu-Asn126-Lys	498
β-Ala-Asn143-Lys	33.1	α-Ser-Asn85-Leu	564
β-Leu-Asn79-Asn	33.1	α-Asp-Asn127-Phe	581
β-Cys-Asn94-Lys	35	β-Glu-Asn102-Phe	582
β-Ser-Asn52-Ala	47.3	β-Val-Asn19-Val	600
α-His-Asn90-Leu	49	β-Gly-Asn108-Val	711
β-Gly-Asn47-Leu	58.6	α-Ala-Asn6-Lys	749
β-Pro-Asn52-Ala	62.5	α-Val-Asn94-Pro	892
β-Trp-Asn38-Gln	72.9	β-Glu-Asn102-Leu	1077
α-Thr-Asn68-Ala	78.1	β-Val-Asn99-Pro	1081
α-Ser-Asn139-Thr	(81.5) ‡	α-Thr-Asn9-Val	1215
β-His-Asn144-Tyr	100	α-Lys-Asn61-Val	1262
β-Asp-Asn95-Leu	106	β-Ala-Asn139-Val	1303
α-Ala-Asn64-Ala	115	β-Gly-Asn57-Arg	1313
β-Ala-Asn139-Ala	115	β-Gln-Asn132-Val	2155
α-Val-Asn97-Phe	131	β-Glu-Asn102-Ile	2312
β-Val-Asn19-Glu	134	α-Gly-Asn16-Val	2360
β-Val-Asn19-Met	135	β-Gly-Asn57-Pro	2690

‡Frame shift mutation and ‡‡heme loss mutation, so three-dimensional structures are unknown and C_D derived from wild-type hemoglobin is not applicable. Squares designate Asn reported as deamidated. ___ designates wildtype deamidation index, I_D. Adapted from 2001RR3.

ure 9-2, wherein some of the "% deamidated Asn incorrect" at low C_D values are probably correctly assigned but not yet reported. Some of the Asn residues listed in Tables 9-1 and 9-2 with low C_D values will probably be eventually found to easily deamidate.

The values of C_D depend on 17 x-ray diffraction and 6 NMR structures. Although the deamidation of aldolase Asn (360) is known to be entirely sequence controlled *in vivo* and *in vitro* with no higher order

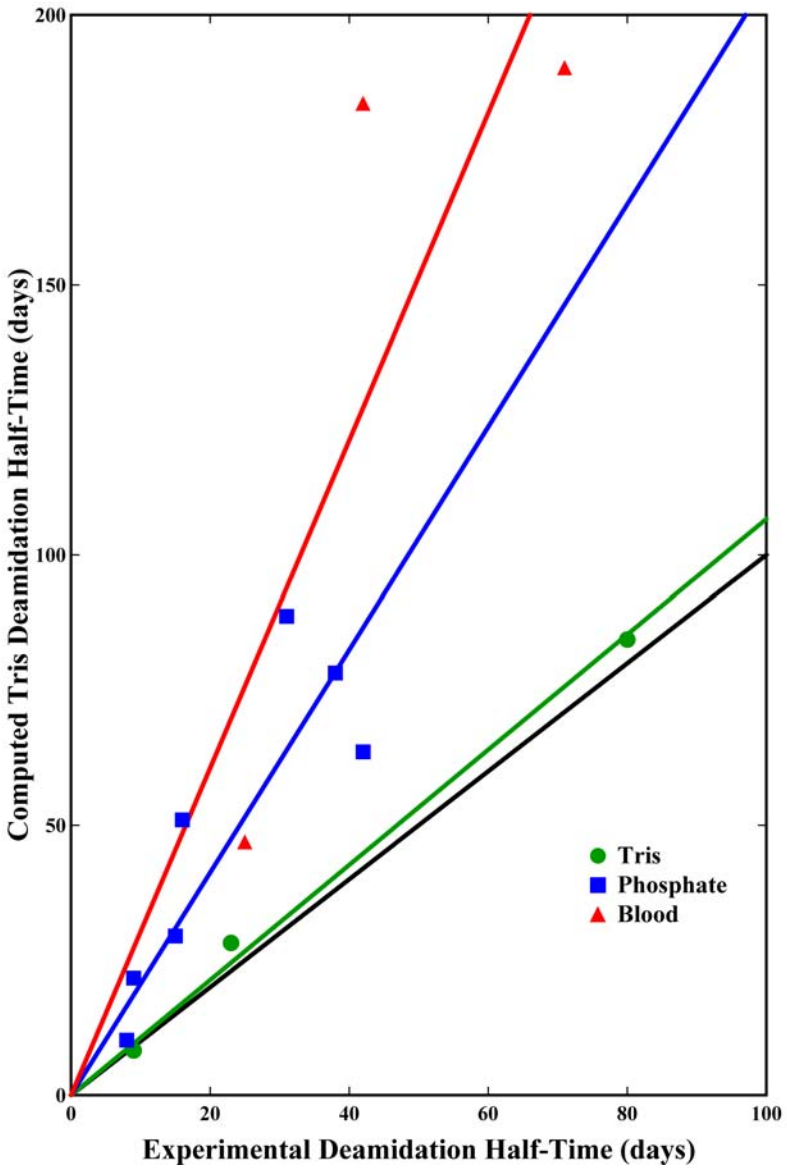

FIG. 9-7 Calculated single deamidation half-times for 10 individual Asn and 3 combinations of Asn residues in 10 different protein types vs. the corresponding experimentally observed deamidation half-times. Experiments were *in vitro* in Tris, ●, and phosphate, ■, buffers and *in vivo* in human blood, ▲. Buffer conditions in Tris and phosphate varied among these investigations but were comparable to pH 7.4, 37°C, 0.15 M. Some of the scatter in the figure is probably the result of these variations. If the calculated values and experimental values were identical, the points would lie on the solid black line, as do the values determined in Tris buffers. Catalysis of deamidation is higher by phosphate than by Tris and may be even higher in erythrocytes. Adapted from 2001RR1.

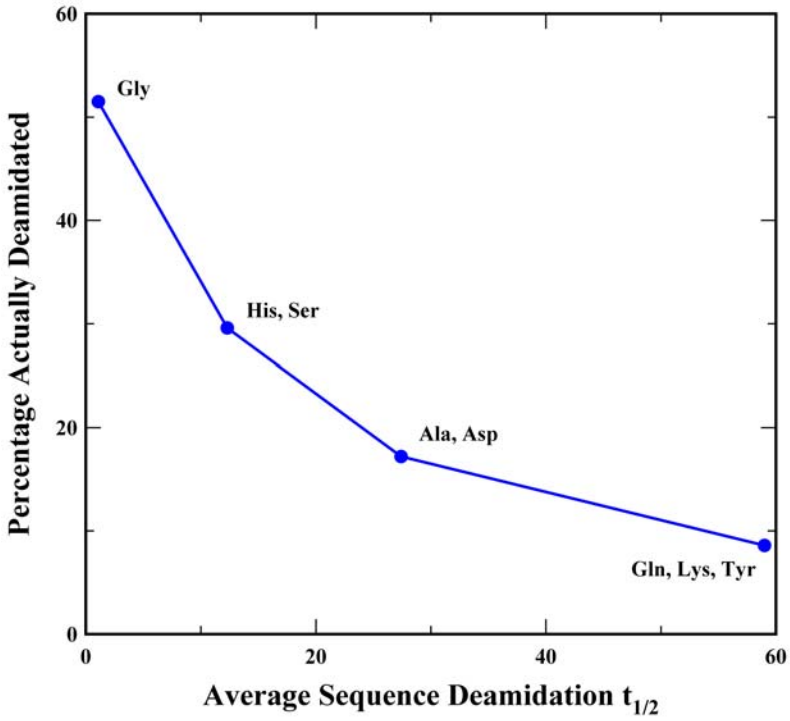

FIG. 9-8 Percentages of rapidly deamidating Asn residues listed in Table 9-1 and 9-2 that would be correctly guessed by simply assuming that Asn residues with COOH-side Gly, His, Ser, Ala, Asp, Gln, Lys, or Tyr deamidate vs. average pentapeptide deamidation half-times (2001RR) for those specific Asn sequences. Adapted from 2001RR3.

suppression,[5] the x-ray crystal structure shows one suppressing hydrogen bond. This aldolase computed C_D is, therefore, 0.22. It should be 0.08. Solution structures are best used when available.

Multiplication of the coefficient of deamidation, C_D, by 100 provides a semi-quantitative prediction of Asn deamidation half-times in 37°C, pH 7.4, 0.15 M Tris-HCl buffer. Figure 9-7 shows a comparison between the computed values of $(100)C_D$ for pH 7.4, 37 °C, 0.15 M Tris-HCl buffer and experimentally observed values for 13 Asn in 10 proteins.

The computed values compare favorably with the experimental values in Tris buffer. In phosphate buffer, the experimental deamidation rates are, on average, 2-fold higher than calculated, and the 3 *in vivo* hu-

5 C. Y. Lai, C. Chen, and B. L. Horecker, *Biochemical and Biophysical Research Communications* **40**, 461 (1970); C. F. Midelfort and A. H. Mehler, *Proc. Nat. Acad. Sci. USA* **69**, 1816 (1972); J. H. McKerrow and A. B. Robinson, *Science* **183**, 85 (1974).

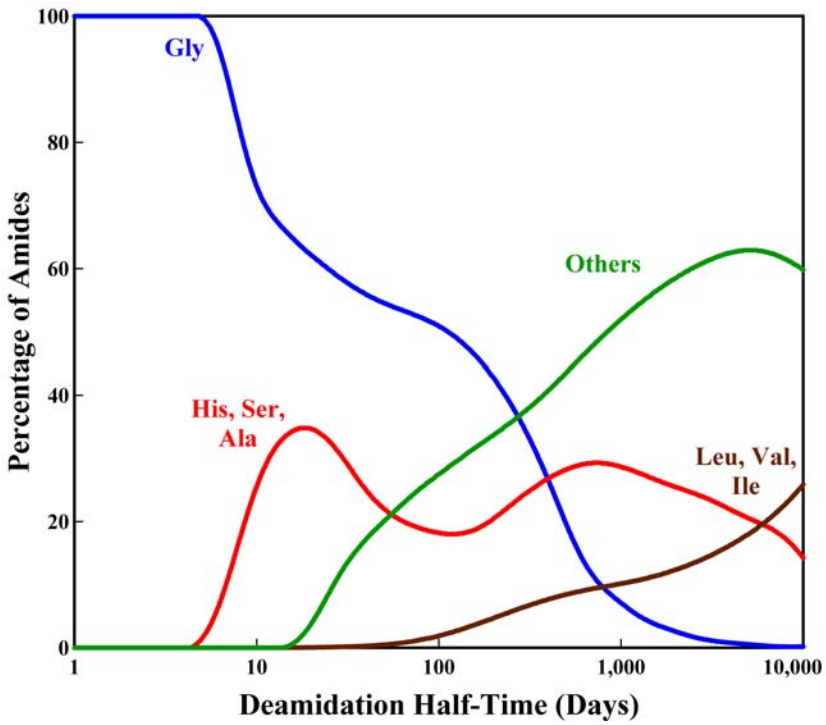

FIG. 9-9 Cumulative distribution plots for 131,809 amides in PDB databank for predicted half-times in 37.00 °C, pH 7.4, 0.15 M Tris with various carboxyl-side residues. Adapted from 2002R.

man blood values average 3-fold higher. This result is as expected because deamidation at neutral pH is subject to catalysis by solution ions. Phosphate is a stronger catalyst of deamidation in peptides and proteins than Tris. Tissue culture medium is even more catalytic of deamidation than phosphate.[6] Least-squares lines as shown in Figure 9-7 give experimental deamidation rates relative to the computed values in Tris, phosphate, and *in vivo* blood erythrocytes of 1.06, 2.07, and 3.01, respectively.

The agreement between the calculated values and Tris experimental values in Figure 9-7 does not arise from computational forcing. The computational method uses experimental sequence-determined pentapeptide deamidation rates in Tris buffer and a parametric higher order structure function with adjustable constants. The optimization

6 A. B. Robinson and J. W. Scotchler, *J. Int. Research Communications* **26**, 1 (1973).

method for these constants used only the ordered Asn residue instabilities in a wide variety of proteins and buffer types. No experimental absolute deamidation rates were used. The agreement arises because the computational method correctly estimates the relative primary and higher order contributions to the deamidation rate of each Asn, and the primary rates were experimentally determined in Tris.

It was once customary to guess which Asn residues might most easily deamidate on the basis of the carboxyl-side residue. Using the deamidating Asn residues in this data set, the sequence assumptions that these types of Asn residues most easily deamidate are 49% in error even in the very unstable AsnGly sequences, 70% in the AsnSer and AsnHis sequences, 83% in AsnAla and AsnAsp, and 91% in AsnGln, AsnLys, and AsnTyr. The converse non-deamidation assumptions are 51%, 30%, 17%, and 9% in error, respectively. See Figure 9-8. In comparison, Figure 9-2 shows that a division criterion of $C_D = 3$ leads to less than 6% errors in classification of all easily deamidating and all relatively stable Asn residues, simultaneously. A criterion of $C_D = 5$ includes 100% of easily deamidating Asn residues, except for Asn 54 in cytochrome C.

Figure 9-9 shows the normalized distribution functions of various carboxyl side residues as a function of Asn deamidation computed half-times in the 13,335 proteins of the PDB database as of April 2001.

Table 9-3 displays these distributions numerically.

Carboxyl-side Asn has a deamidation half-time comparable to that for Ala, so it would be expected to have values comparable to Ala in Figure 9-9 and Table 9-3. Carboxyl-side Gln is comparable to Thr.[7]

This calculation method, based on the sequence-controlled deamidation rates of Asn model peptides and the Asn three-dimensional environment in proteins, permits a useful estimation of the instability with respect to deamidation of Asn in proteins.

For a diverse group of protein types, this method is at least 94% reliable in determining relative deamidation rates within single proteins, as illustrated in Tables 9-1 and 9-2. A refined and computerized version of this method has been shown to be 96% reliable by the same criteria.[2] This reliability is underestimated, because the evaluation considers all of these protein amides simultaneously even though their deamidations were observed under a wide variety of experimental conditions. Moreover, some experimentally known Asn instabilities in these proteins

7 N. E. Robinson, B. R. Robinson, and M. R. Robinson, *Unpublished work in progress*.

Table 9-3 Percentages of carboxyl side residues in asparagine deamidation ranges.

Residue	%, < 5 days	%, < 10 days	%, < 25 days	%, < 100 days
Tris ½ time				
Gly	4.2	6.0	11	38
His		0.46	2.4	7.7
Ser		0.19	2.3	9.8
Ala			1.3	5.6
Cys			0.23	1.1
Thr			0.1	2.0
Asp			0.060	4.8
Lys			0.014	1.8
Glu				3.0
Gln				1.7
Arg				1.6
Asn				1.5
Phe				1.1
Met				0.93
Tyr				0.89
Trp				0.37
Leu				0.33
2xTris 1/10 time				
Gly	14	27	53	87.3
His	3.6	5.9	12	33.2
Ser	2.9	8.1	14	34.6
Ala	1.9	4.2	7.2	17.9
Cys	0.23	0.84	2.6	7.3
Thr	0.14	1.5	4.2	12.3
Asp	0.93	1.9	6.8	19
Lys	0.071	1.2	4.4	10.4
Glu	0.057	1.6	5.3	13.1
Gln	0.041	1.4	3.9	10.1
Arg	0.035	1.0	3.4	9.4
Asn	0.15	1.4	5.0	13.5
Phe		0.46	2.3	7.3
Met	0.036	0.75	2.4	5.4
Tyr	0.038	0.36	1.2	5.4
Trp		0.16	0.74	5.0
Leu		0.26	1.3	5.4
Val			0.01	2.8
Ile			0.013	1.3

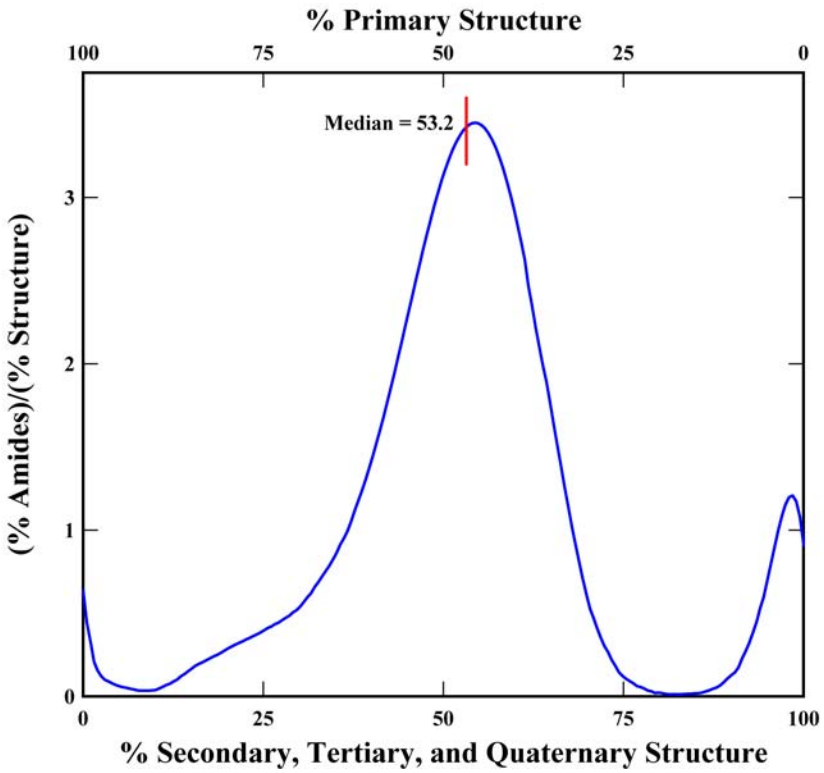

FIG. 9-10 Plot of contributions of primary vs. secondary, tertiary, and quaternary structure to overall deamidation C_D for 17,935 proteins (2003R).

have not yet been characterized, so the data probably incorrectly classified some Asn as relatively stable that are actually easily deamidated.

When used to determine the reportedly most unstable Asn residues within a single protein as illustrated in Tables 9-1 and 9-2, this method correctly identifies the most unstable Asn residues for 31 of 35 residues in 27 proteins and, in the remaining 4 cases, is in error by only one residue.

This method does not allow for special three-dimensional structures that change deamidation rates in unusual ways. There are still too few reported instances of these to permit their theoretical estimation. In LysAsn(54)Lys in cytochrome C, the reported experimentally determined protein rate is faster than the sequence determined rate. Also, in two instances, MetAsn(15)Gly in triosephosphate isomerase and LysAsn(54)Lys in cytochrome C, deamidation is reported to take place after a prior deamidation of the protein changes the structure in an ac-

commodating way. The changed structures have not yet been experimentally determined. Although this calculation method cannot yet predict these special effects, it aids in their recognition.

Figures 9-2 to 9-4 serve as a reasonable basis for estimating that Asn deamidation in these proteins is, on average, determined approximately 60% by primary structure and 40% by higher order structure. These percentages apply to higher order effects that diminish deamidation rates below those of primary structure alone. In 1 case out of 35 – about 3% of deamidating Asn and 0.4% of all Asn examined here – higher order structure is reported to actually accelerate deamidation. Calculations of C_D in 17,935 proteins involving more than 200,000 Asn indicate that the primary structure component is about 50% and the higher order component 50% in proteins in general as illustrated in Figure 9-10.

9-2. PROTEIN DEAMIDATION VALUES

The computation method described above was developed manually, tested, and then applied to 126 human proteins.[8] In order to make it generally applicable and more reliable, the entire procedure was computerized.[9] These automatic calculations determine the exact position of the Asn side chain, the molecular rearrangements necessary for it to assume the cyclic configuration for reaction, the optimized calculation constants, and C_D and I_D for the Asn and the protein.

This computerized method was first applied to the 13,335 proteins and 170,014 Asn with suitable three-dimensional structures recorded in the Protein Data Bank[10] as of April 2001.[9] This calculation has now been updated to include the 17,935 proteins in the Protein Data Bank as of January 2003. These values and additional future updates and related experimental measurements are available at www.deamidation.org.

Figures 9-11a and 9-11b summarize the I_D and protein single-deamidation half-times from these calculations.

These protein single-deamidation half-times vary between about 4 hours and more than a century. They provide molecular clocks suitable

8 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001).

9 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

10 H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, and P. E. Bourne, *Nucleic Acids Research*, 235 (2003), http://www.rcsb.org/pdb.

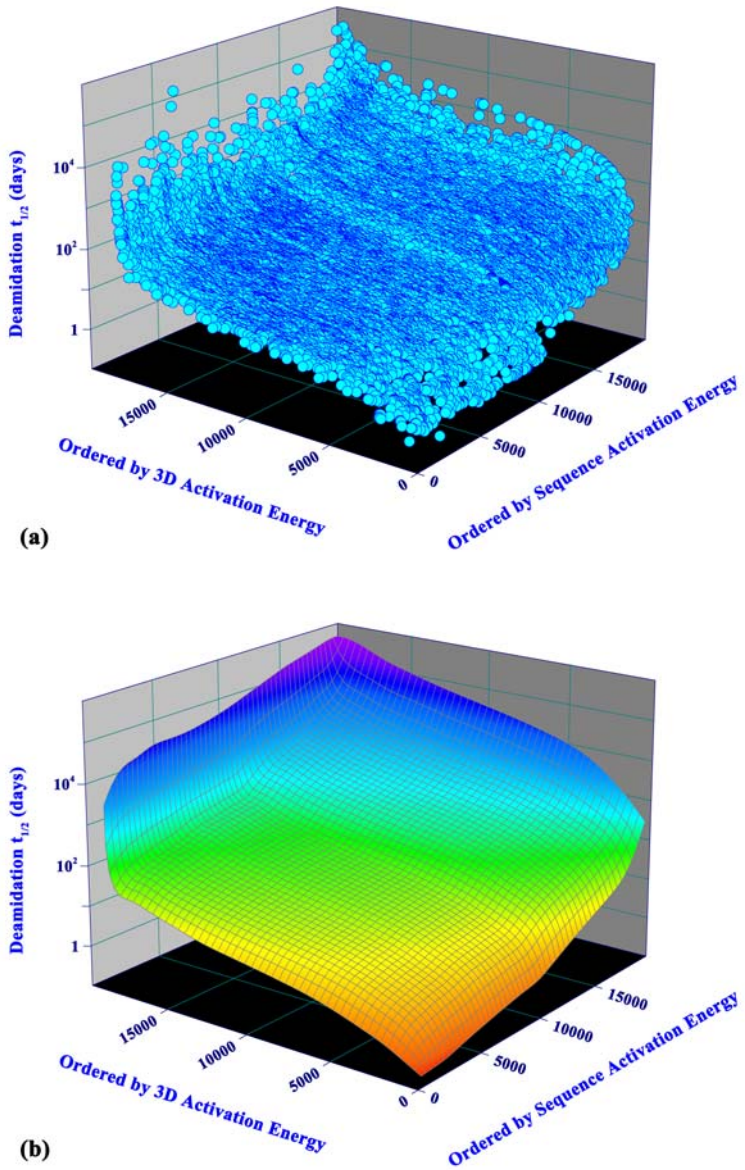

FIG. 9-11 (a) Computed single deamidation half-times in 0.15M Tris-HCl, pH 7.4, at 37.0°C for all of the 17,935 amide-containing peptides and proteins with suitable three-dimensional structures listed in the Protein Data Bank as of January 2003. The values have been ordered by primary structure and three-dimensional structure deamidation activation energies on the y and x-axes, respectively. Deamidation half-time in days computed as $t_{1/2} = 100 \times I_D$ is plotted on the z-axis. (b) Surface fitted to the values in (a). The yellow and orange parts of this surface include peptides and proteins with single deamidation half-times in 0.15 M Tris-HCl, pH 7.4, at 37.0 °C of about 10 days or less. Adapted from 2003R.

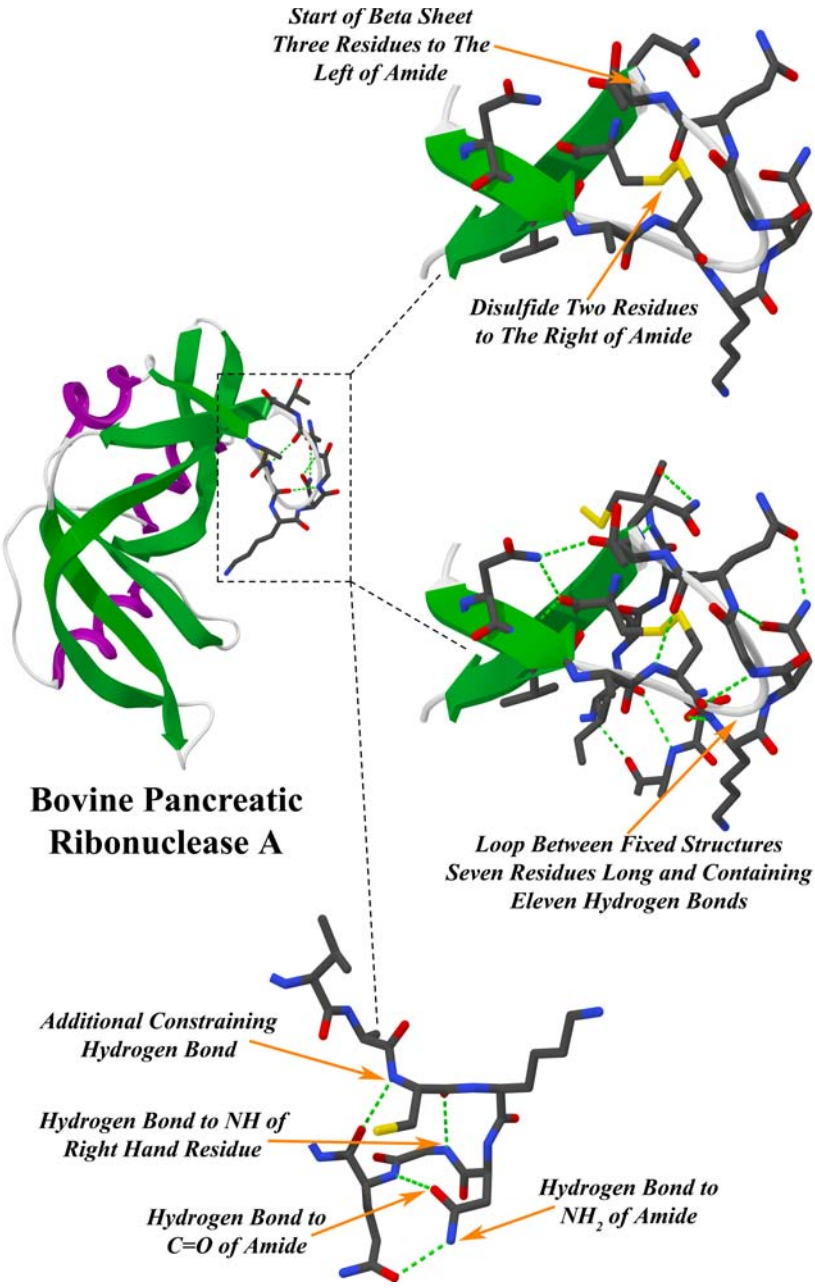

FIG. 9-12 The deamidation of LysAsn(67)Gly in ribonuclease-A (2003R).

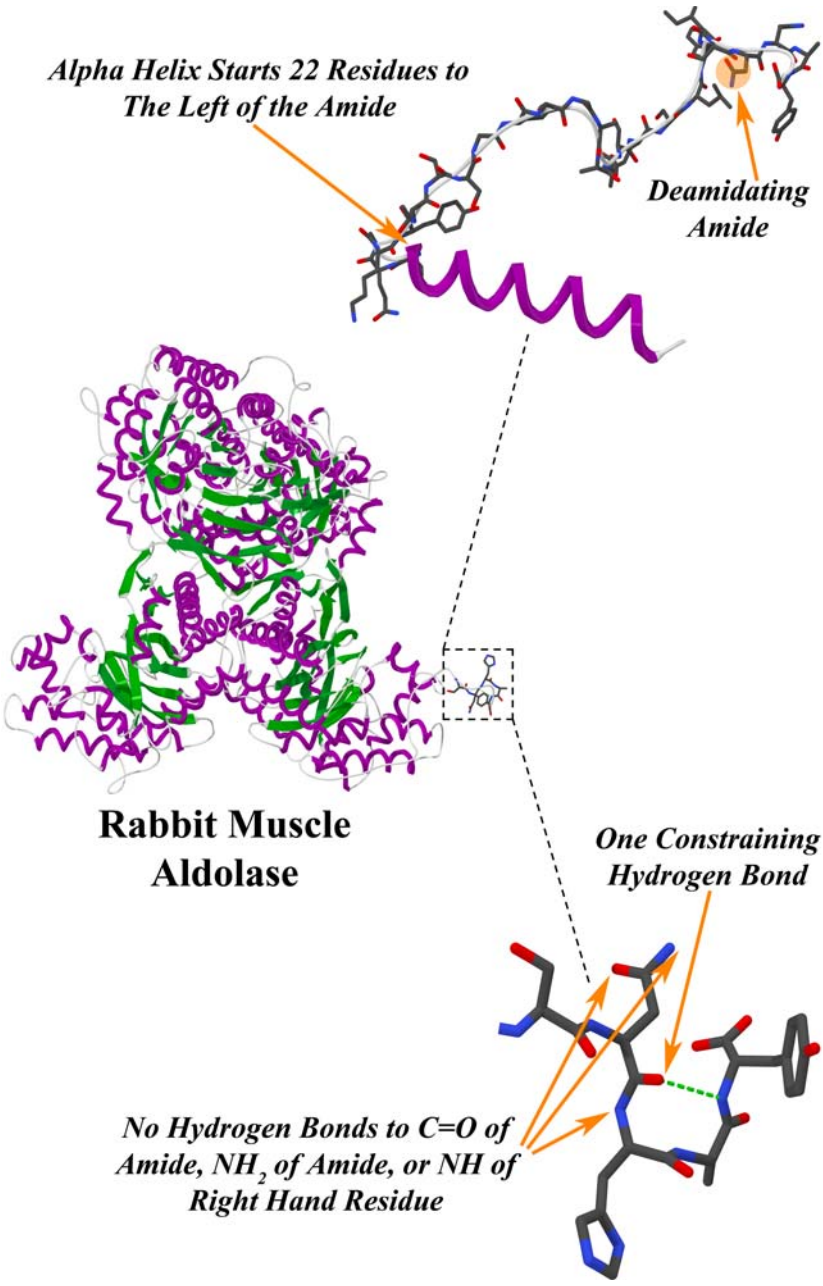

FIG. 9-13 The deamidation of SerAsn(360)His in rabbit muscle aldolase (2003R).

for timing most of the biological processes in the organelles, cells, tissues and organisms in which these proteins are imbedded.

The accuracy of this calculation method is presently limited by the availability of precise experimental protein deamidation observations with which to calibrate it. As additional such measurements become available, these computations will be refined.

At present, it is estimated[11] that the individual computed values of C_D are reliable within a factor of about 2 or better. In general, these calculated Tris values are lower than those found in phosphate buffer systems and *in vivo*. In the absence of other information and in physiologically reasonable solvents, the actual protein deamidation rate can be expected usually to lie between the computed value and about twice that value. Chapter 12 reviews several specific examples of the accuracy of these computations as compared with experimental measurements.

This accuracy is suitable for most current biological studies. Moreover, the relative values of C_D within single proteins are quite reliable. Therefore, the computations provide the deamidation rate of each Asn and each protein and a good estimate of the relative Asn deamidations contributing to that rate.

The primary structure vs. tertiary structure distribution function for the 17,935 proteins is shown in Figure 9-11. Figures 9-12 and 9-13 show the structural impediments to deamidation for Asn(67) in bovine ribonuclease A and for Asn(360) in rabbit muscle aldolase.

Ribonuclease A imposes several constraints. The computerized calculation method, based on an x-ray diffraction crystal structure, predicts the deamidation half-time for this Asn(67) residue to be 70 days.[12] An earlier similar calculation using manual structure viewer data gave 85 days. Direct experimental measurement in Tris-HCl, 37 °C, pH 7.4, extrapolated to zero buffer concentration, gives 67 days. The zero buffer experimental value and the Tris computed value of 70 days are directly comparable as explained in Chapter 12-22. The reason for this is that the computed Tris value is 40 days for the NMR solution structure of ribonuclease A. Removal of Tris catalysis raises this value about 2-fold. The computational change from 70 to 40 is the result of one hydrogen bond that is present in the crystal structure but not present in solution. Figure 9-12 illustrates the structural constraints used in this completely automatic calculation.

11 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

12 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

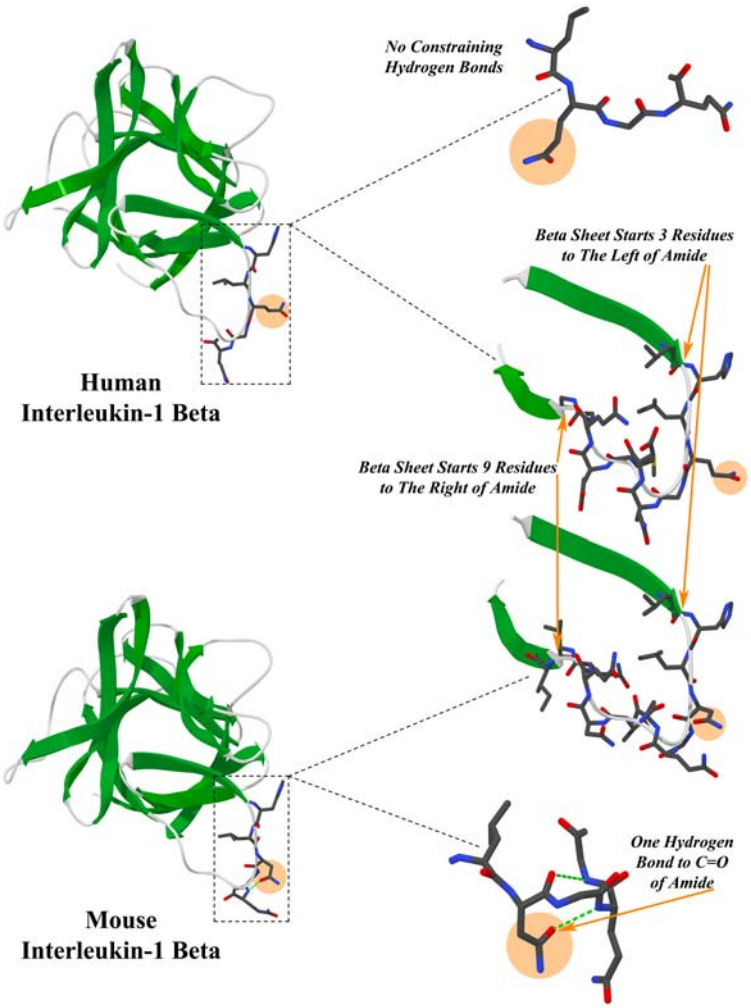

FIG. 9-14 Comparison between human (5I1B) and mouse (8I1B) interleukin-1β. Adapted from 2003R.

Rabbit muscle aldolase has an experimental deamidation half-time of 9.4 days, and rabbit muscle aldolase-A-fructose 1-6-bisphosphate complex PDB 6ALD has a computed half-time of 9.0 days, both in 0.015 M Tris-HCl, 37 °C, pH 7.4 buffer. No impediments to imide formation are present in the structure. On the other hand, uncomplexed

rabbit muscle aldolase PDB 1ADO has a computed half-time of 23 days. 1ADO is the structure illustrated in Figure 9-13. The hydrogen bond shown leads to this doubled value, although this bond may not be present in solution. PDB 6ALD does not have this bond, so the computed value is identical to the experimental value for GlySerAsnHisGly and identical to the measured experimental value for the protein.

Figure 9-14 shows an additional example. Mouse interleukin-1β deamidates at HisLeuAsn(32)GlyGln with a half-time in pH 8.5, 0.1 M Tris at 37 °C of 1.5 days and a 3 to 4-fold reduction of biological activity.[13] Human interleukin-1β, however, has a deamidation half-time in pH 7.0, 0.01 M Tris at 30 °C of between 160 and 330 days.[14] The human sequence is HisLeuGln(32)GlyGln.

The peptides GlyLeuAsnGlyGly and GlyLeuGlnGlyGly have deamidation half-times in 37 °C, pH 7.4, 0.15 M Tris of 1.1 days and 670 days, respectively.[15]

Computation using two PDB 3-dimensional structures, 2MIB and 8I1B, gives 4.8 days and 3.8 days for the deamidation half-time of Asn(32) in mouse interleukin-1β at pH 7.4, 0.15 M Tris at 37 °C.[16] Allowing for the difference in pH, this is in good agreement with the experimental value.

In the human protein, the computed half-time is the same as the sequence half-time because there are no constraints. However, the Gln side chain in the protein is found in an ideal orientation to form the glutarimide ring. This and the temperature and pH differences, apparently mildly accelerate its rate, so that the computed half-time is about 2-fold higher than the experimental value. The rate, therefore, is 2-fold lower. There are occasional other instances of ideal side chain alignments in proteins that are expected to increase deamidation rates.[17]

Interleukin-1β is therefore an interesting case in which substitution of Gln for Asn at the primary deamidation site increases the I_D and deamidation half-time by about two orders of magnitude.

13 E. M. Cassidy, B. T. Wakim, A. G. Ferguson, and A. M. Samarel, *J. Molecular and Cellular Cardiology* **23**, 589 (1991).

14 L. C. Gu, E. A. Erdös, H. Chiang, T. Calderwood, K. Tsai, G. V. Visor, J. Duffy, W. -C. Hsu, and L. C. Foster, *Pharmaceutical Research* **8**, 485 (1991).

15 N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

16 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

17 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002); N. E. Robinson, *Caltech Thesis*, *Chemistry* (2003).

9-3. PROTEIN DEAMIDATION DISTRIBUTION FUNCTIONS

The protein computation method permits calculation of the distribution functions of deamidation half-times for groups of proteins. Figures 9-15a and 9-15b show the cumulative deamidation distribution function for 1,371 Asn in 126 human proteins and the differentiated function. Figures 9-16a and 9-16b show these function for 131,809 Asn in 10,369 proteins from a wide variety of organisms. These functions have also been calculated for mouse, rat, chicken, *Bacillus subtilis*, and *Escherichia coli* proteins. Although not identical, these distributions are quite similar, with mouse, *B. subtilis*, and *E. coli* having somewhat higher numbers of unstable amides in their proteins as compared with human, rat, and chicken[2]. *Drosophila* proteins have also been separately tabulated with similar results.[18]

Since deamidation rates are determined by a large number of similarly sized partially independent variables, the differentiated functions in Figures 9-15b and 9-16b would be expected to be gaussian. It is seen that they are gaussian at high deamidation half-times, but are substantially distorted toward faster deamidation rates in the low deamidation half-time region. This distortion arises, in part, from the dissimilarly sized AsnGly primary sequence deamidation rates, but it is primarily a result of apparent genetic preference for Asn with low deamidation half-times.

Since deamidation of Asn is disruptive of protein structure, it would be expected that the Asn with low deamidation half-times would be genetically suppressed rather than accentuated – unless deamidation is being used for beneficial biological purposes. There is definitely no such suppression. These distribution functions, therefore, strongly support the hypothesis that Asn deamidation functions as a beneficial biomolecular clock. These functions are represented in tabular form in Tables 9-4 and 9-5.

Since these computations utilize the primary structure determined deamidation rates measured in 0.15 M Tris-HCl, pH 7.4, at 37 °C and a function that estimates the ratio between the primary rates and the higher order rates, it is expected that these estimates are for proteins in 0.15 M Tris-HCl, pH 7.4, at 37 °C. It may be, however, that inhibitions of buffer catalysis or other effects imposed by secondary, tertiary, and quaternary protein structure reduce the influence on these rates from Tris catalysis. In that case, the real protein rates would lie somewhere

18 N. E. Robinson and A. B. Robinson, *Mechanisms of Ageing and Development* **125**, 259 (2004).

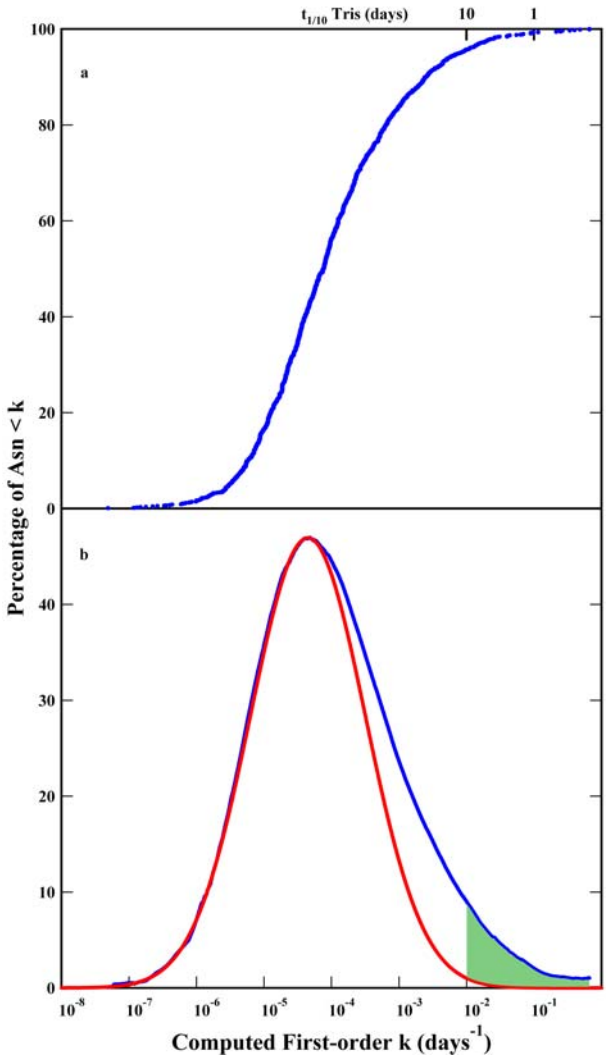

FIG. 9-15 *(a)* Cumulative distribution function of the calculated first-order rate constants for deamidation of 1,371 Asn residues in 126 human proteins. As indicated, the Asn residues involved in the initial deamidation of these proteins comprise a relatively small part of the complete set. Computed percentages of the Asn residues that are 1/10 deamidated at 1 and 10 days in Tris are 1% and 4%, respectively, as shown. If this deamidation were not of positive biological value, more slowly deamidating sequences and structures could easily have been used. *(b)* Differentiated values of the distribution function showing the special class of unstable Asn residues present in human proteins. Also shown is a Gaussian function that fits the distribution function except for that part arising from the especially unstable Asn residues. The shaded area contains those Asn residues computed to be one-tenth or more deamidated in 10 days in pH 7.4, 37°C, 0.15 M Tris-HCl. This shaded area for phosphate, physiological fluids, or longer time intervals would be a larger part of the illustrated deviation from Gaussian. Adapted from 2001RR1.

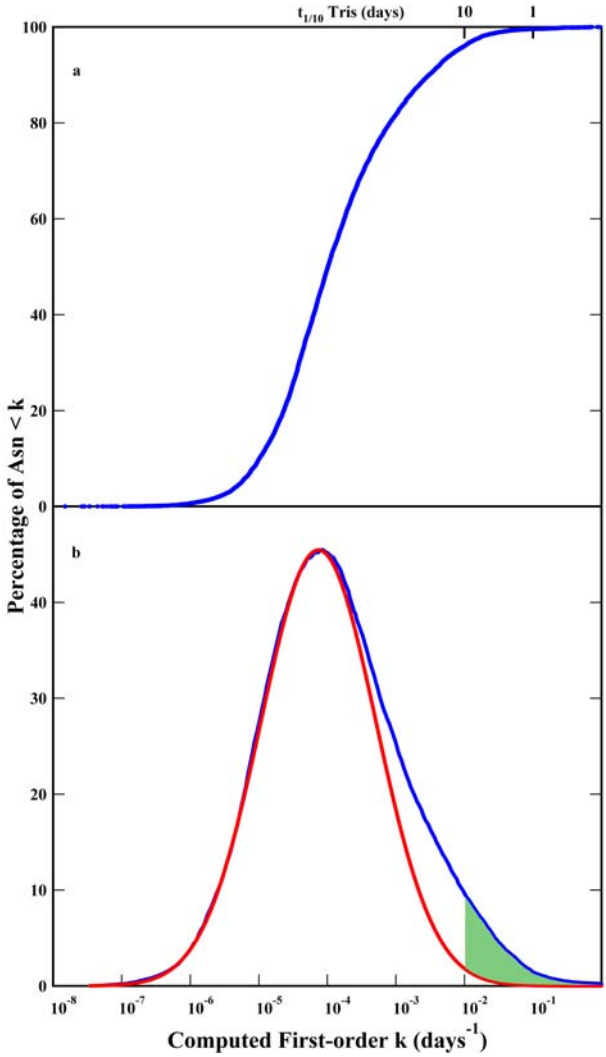

FIG. 9-16 *(a)* Cumulative distribution function of the calculated first-order rate constants for deamidation of 170,014 Asn residues in 17,935 proteins. Asn residues involved in the initial deamidation of these proteins comprise a relatively small part of the complete set. Computed percentages of the Asn residues that are 1/10 deamidated after 10 days in pH 7.4, 37°C, 0.15 M Tris-HCl is 4% as shown. *(b)* Differentiated values of the distribution function showing the special class of unstable Asn residues present in these proteins. Also shown with a red line is a Gaussian function that fits the distribution function, except for that part arising from the especially unstable Asn residues. The shaded area contains those Asn residues computed to be 1/10 or more deamidated in 10 days in pH 7.4, 37°C, 0.15 M Tris-HCl. Adapted from 2002R.

between those determined for Tris and those that would be determined using primary structure rates determined by extrapolation to zero buffer concentrations. Estimates of this effect and any necessary corrections await measurement of sufficient peptide and protein rates at zero buffer concentrations. These measurements are in progress.

It is evident that a substantial percentage of proteins deamidate during the lifetimes of the organelles, cells, and organisms of which they

Table 9-4 Percentages of human proteins computed to be more than 1/10 or 1/2 singly deamidated in Tris buffer after 1, 5, 10, and 50 days.

Days at 37°C pH 7.4	Deamidated by > 1/10		Deamidated by > 1/2	
	Tris	2 × Tris	Tris	2 × Tris
1	10%	13%	1.6%	4%
5	31%	43%	8%	13%
10	43%	56%	13%	20%
50	71%	82%	37%	49%

Table 9-5 Deamidation percentages for 17,935 peptides and proteins

Deamidation in 0.15M Tris-HCl, pH 7.4, at 37°C	Days	%
Deamidated by ≥ one-half of an amide		
	1	0.3
	5	3.7
	10	7.5
Deamidated by ≥ one-tenth of an amide		
	1	4.7
	5	26.6
	10	42.8
Deamidated by ≥ one-half of an amide at 2x rate		
	1	1.6
	5	7.5
	10	17.2
Deamidated by ≥ one-tenth of an amide at 2x rate		
	1	10.5
	5	42.8
	10	57.3

are a part. This is caused by a relatively small percentage of Asn and could be prevented by simple genetic specification.

Table 9-3 illustrates the small percentage of involved Asn even among those with the more rapidly deamidating primary sequences.

While more precise experimental data will eventually permit even more accurate computations of deamidation rates in proteins, the averaging involved in Figures 9-15 and 9-16 and Tables 9-3 to 9-5 is such that the overall quantitative information in these figures and tables is already quite reliable.

These computed distributions show for the first time, the quantitatively ubiquitous nature of deamidation in peptides and proteins.

It is apparent that a substantial percentage of the protein pool in living things is in a continuously changing state as a result of the miniature amide clocks embedded in each protein.[19]

19 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

CHAPTER 10

Structural Distributions of Asn and Gln in Proteins

10-1. PRIMARY STRUCTURE CORRELATIONS

The question of sequence preferences among the amino acid residues neighboring to Asn and Gln arose immediately in consideration of the hypothesis of biological usefulness of deamidation itself. Sequence preferences in a set of just 43 proteins containing 290 Asn and 22 Gln were published along with the hypothesis.[1] While interesting, these findings were very tentative in view of the small data set available.

These computations were of interest because, if amide residues served as molecular clocks, it would be expected that some time settings and therefore some amide environments would be more prevalent than others. Also, if deamidation resulted in protein turnover or had other biological consequences, it would be expected that consensus sequences that facilitate those consequences might be found among the residues surrounding the involved amides.

In 1973, a much more careful statistical analysis with a somewhat larger number of proteins was completed by Laurelee Robinson, Arthur Robinson, and Fred Westall.[2] These calculations showed that amino acid residue pairing certainly occurred non-randomly in proteins and that some of these non-random pairings involved the amide residues. The number of available protein sequences was still small, but increased rapidly thereafter.

In 1988, Laurelee Robinson undertook a definitive calculation of these probabilities with a data set including 1,465 peptides and proteins with 450,431 amino acid residues. Laurelee completed her work, but publication was delayed by her death in November 1988. An abbreviated account was eventually published.[3] After her death, another inves-

1 A. B. Robinson, J. H. McKerrow, and P. Cary, *Proc. Natl. Acad. Sci. USA* **66**, 753 (1970).

2 L. R. Robinson, A. B. Robinson, and F. C. Westall, *Unpublished Results* (1973).

3 A. B. Robinson and L. R. Robinson, *Proc. Natl. Acad. Sci. USA* **88**, 8880 (1991).

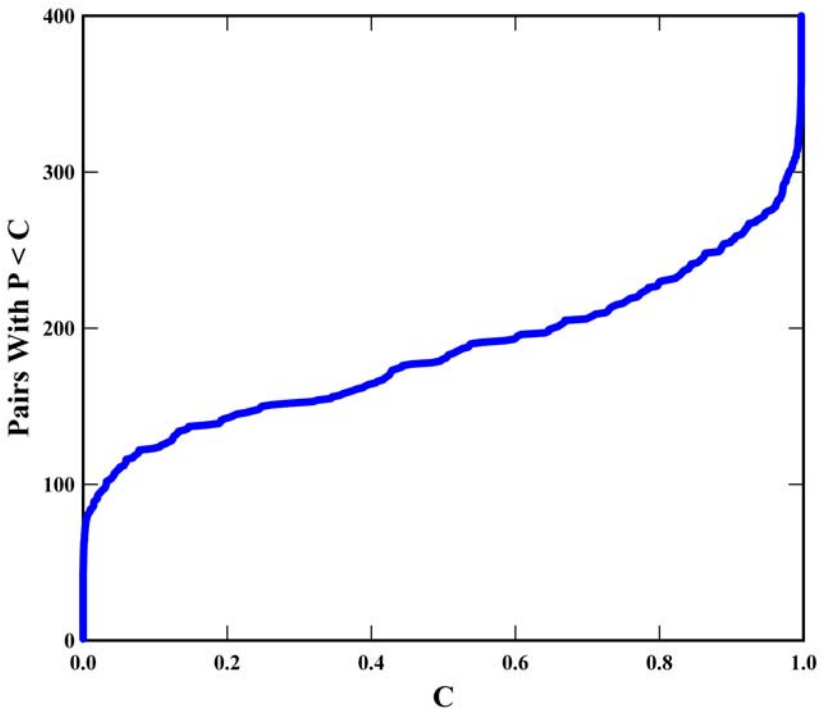

Fig. 10-1 Cumulative distribution function of probabilities, *P*, for the observed frequencies of side-by-side residue pairings in a set of 450,431 residues in 1465 unique peptides and proteins. The *P* values were calculated by reference to normal error functions centered on the frequencies expected from amino acid residue abundances in the set. *C* is the maximum *P* for each plotted group of pairs. Adapted from 1991RR.

tigator, who had been shown Laurelee's work and given detailed copies of her results before her death, published a rework and analysis of Laurelee's calculations under his own name.

Figures 10-1 to 10-4 summarize the calculations by Laurelee Robinson. Figure 10-1 shows the cumulative distribution function of residue pairings involving side-by-side pairing of all 20 naturally occurring amino acid residues in Laurelee's data set. About 130 preferred pairings and about 150 rejected pairings are present and detectable.

With a cutoff of $p < 0.001$ and, therefore, only about 0.4 pairings expected by chance, observed pairings fall off with distance between the pairs on the protein chain as illustrated in Figure 10-2.

After removal of pairing of identical residues, which probably accumulate for other reasons, preferred pairs are 42 at the side-by-side position and gradually diminish to 5 when 8 residues intervene. The

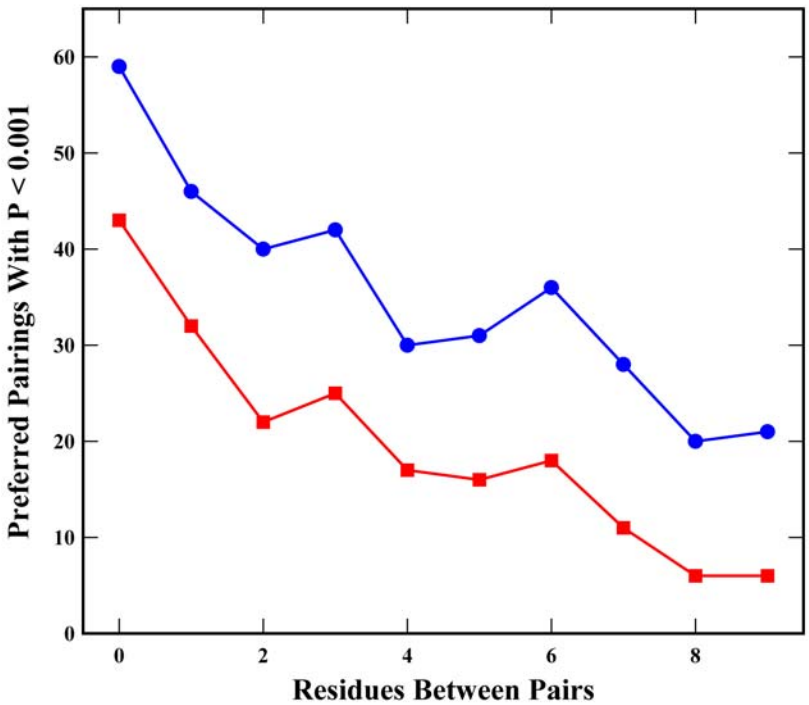

Fig. 10-2 Number of residue pairings with P < 0.001 as compared with random expectation in a set of 1465 unique peptides and proteins comprising 450,431 residues vs. the number of residues between the pairs under consideration. The 400 possible pairings for the 20 common amino acid residues were calculated for each point, comprising 4000 calculations for this graph. Only 0.4 pairings is expected by chance at each plotted point. Solid circles, ●, and line show the complete result of the calculations. Solid squares, ■, and line show this result with pairings of identical residues subtracted. Adapted from 1991RR.

increases near 3 and 6 intervening residues may be indicative of nearer neighbors as a result of α-helix formation. Of the 42 preferred pairs demonstrated in Figure 10-2, 8 involve Asn and none involve Gln. The number involving Asn as preferred is larger than for any of the other 19 naturally occurring amino acid residues.

The sequence AsnGly has p = 0.0036 in this 1,465 peptide and protein set. It is therefore a preferred sequence. Recent protein computations have now shown that this sequence is, in fact, markedly preferred in proteins.[4] While most AsnGly sequences in proteins are slowed through modulation by higher order structure, many of these sequences are among the fastest to deamidate and should be genetically rejected

4 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

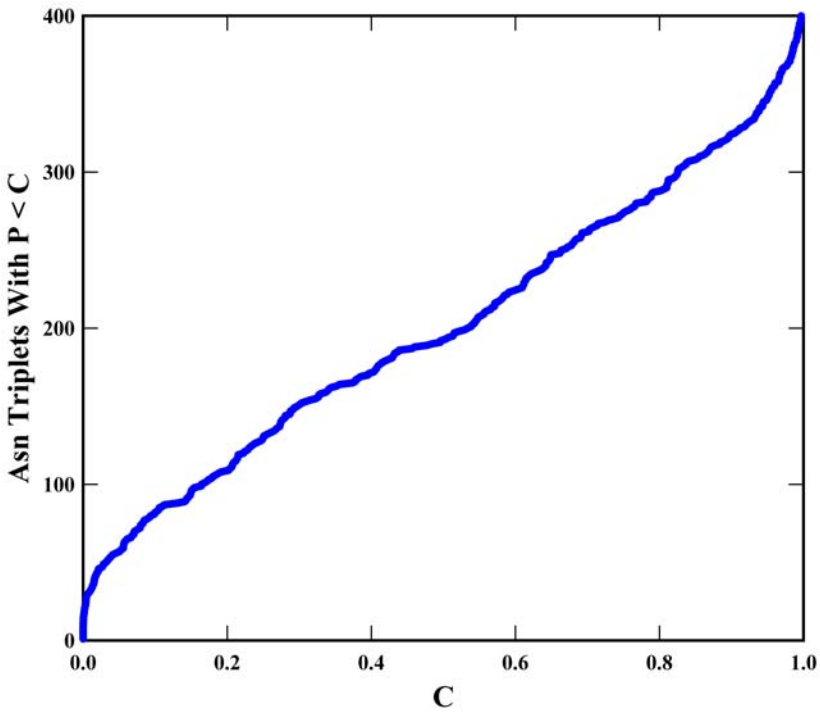

Fig. 10-3 Cumulative distribution function of probabilities, *P*, for the observed frequencies of 400 Gln triplet combinations in a set of 450,431 residues in 1465 unique peptides and proteins. The *P* values were calculated by reference to normal error functions centered on the frequencies expected from amino acid residue abundances in the set. *C* is the maximum *P* for each plotted group of triplets. Adapted from 1991RR.

rather than preferred, unless their deamidations are of positive biological value.

Figures 10-3 and 10-4 show these cumulative distribution functions for triplet sequences of the types XxxGlnYyy and XxxAsnYyy, respectively. About 70 Gln triplets and 50 Asn triplets are preferred, while 120 Gln and 50 Asn are rejected and detectable in this data set.

Among the 50 preferred Asn triplets, 6 involve Gly as an Asn nearest neighbor, with 5 of these sequences having Gly on the carboxyl side. These sequences are GlyAsnPro, AsnAsnGly, GluAsnGly, LysAsnGly, ArgAsnGly, and SerAsnGly, involving primarily chemically functional residues on the amino side.

Laurelee's calculations[3] also contain tabulated details for each of the preferred and rejected sequences, but we will defer review of these de-

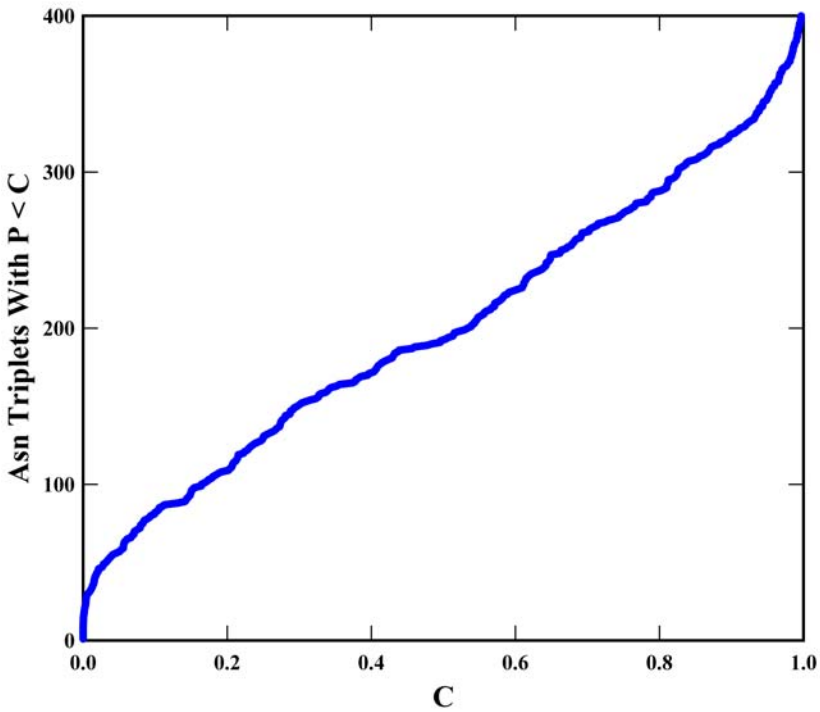

Fig. 10-4 Cumulative distribution function of probabilities, *P*, for the observed frequencies of 400 Asn triplet combinations in a set of 450,431 residues in 1465 unique peptides and proteins. The *P* values were calculated by reference to normal error functions centered on the frequencies expected from amino acid residue abundances in the set. *C* is the maximum *P* for each plotted group of triplets. Adapted from 1991RR.

tails, since the much larger data set that has accumulated since 1991 allows an updated calculation and a more detailed analysis.

These updated calculations are being analysed. When complete, they will be available at www.deamidation.org.[5]

5 N. E. Robinson and A. B. Robinson, *work in progress*.

10-2. SECONDARY STRUCTURE CORRELATIONS

As protein structure data became available, it was noticed that the distributions of amino acid residues in proteins show substantial amounts of order.[6] The most well-known example of this is the work of Chou and Fasman on the correlations between composition, sequence, and the presence of helix, β-sheet, and "random coil" structures.[7]

Correlations with DNA structure, protein folding, neighboring residues, chain flexibility, and β-turn properties were subsequently explored. See 1980TS, 1981HH, 1985ST, 1986VM, 1989RF, 1990BA, 1995MS.

In a study of 29 proteins, Chou and Fasman found that 50% of Asn occurred in β-turns.[8] Asn and Asp have been found to be especially prevalent at protein chain reverse turns with about half of all Asn in re-

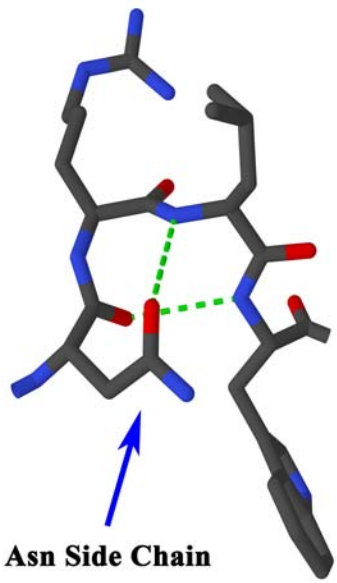

Fig. 10-5 Example of a type I β-turn. These are residues 123-126 of carboxypeptidase A. Protein Databank structure number 1ARM.

6 J. M. Zimmerman, N. Eliezer, and R. Simha, *J. Theoretical Biology* **21**, 170 (1968).

7 P. Y. Chou and G. D. Fasman, *Biochemistry* **13**, 211 (1974); P. Y. Chou and G. D. Fasman, *Trends in Biochemical Sciences*, 127 (1977).

8 P. Y. Chou and G. D. Fasman, *J. Molecular Biology* **115**, 135 (1977).

verse β-turns.[9] This is thought to be caused by their ability to stabilize these turns with hydrogen bonds.[10] These structures are unique. It was found that 40% of Asn were in positions 1 or 3 in the turns. Pro is in position 2 in 1/3 turns and the backbone amide hydrogen in position 4 is hydrogen bonded to the carboxyl group of position 1.[11]

Asn is also found unusually frequently as the amino-side end residue in α-helices. See 1988RR, 1993HR, 1994SS, 1997DM, 1997PS, 1998GN, and 1999WM.

Figures 10-5 and 10-6 show examples of Asn in a reverse turn and in an α-helix amide-side cap, respectively.

In occurring more frequently in turns and at amino-side helix caps, Asn is joined by other amino acids with similar hydrogen bonding geometries and capabilities, so it is not entirely unique. Nevertheless, these strategic locations may be found to correlate with its biological functions.

Other publications concerning secondary structure and Asn include 1980TL, 1995CK, 1997RB, and 1999WL.

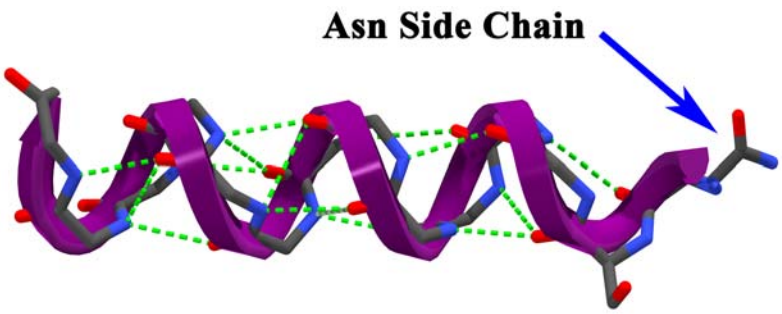

Fig. 10-6 Example of Asn in an α-Helix end cap. This is Asn(233) from thermolysin, Protein Data Bank structure number 8TLN.

9 D. C. Rees, M. Lewis, and W. N. Lipscomb, *J. Molecular Biology* **168**, 367 (1983); M. Fujinaga, L. T. J. Delbaere, G. D. Brayer, and M. N. G. James, *J. Molecular Biology* **183**, 479 (1985).

10 E. G. Hutchinson and J. M. Thornton, *Protein Science* **3**, 2207 (1994).

11 A. M. Sophocleous, personal communication (2002).

10-3. PROTEIN AMIDE COMPOSITIONS

In 1970 and 1974,[12] a correlation with $p \leq 0.0020$ between the percentage of residues that are Asn plus Gln in a protein and its *in vivo* life-time was reported for a set of 15 proteins. This correlation is shown in Figure 10-7. It was proposed that this correlation resulted from a tendency for short-lived proteins to have extra amide residues to aid in their rapid catabolic turnover and for long-lived proteins to reject short-lived amide sequences that would confer undesirable short-term instability.

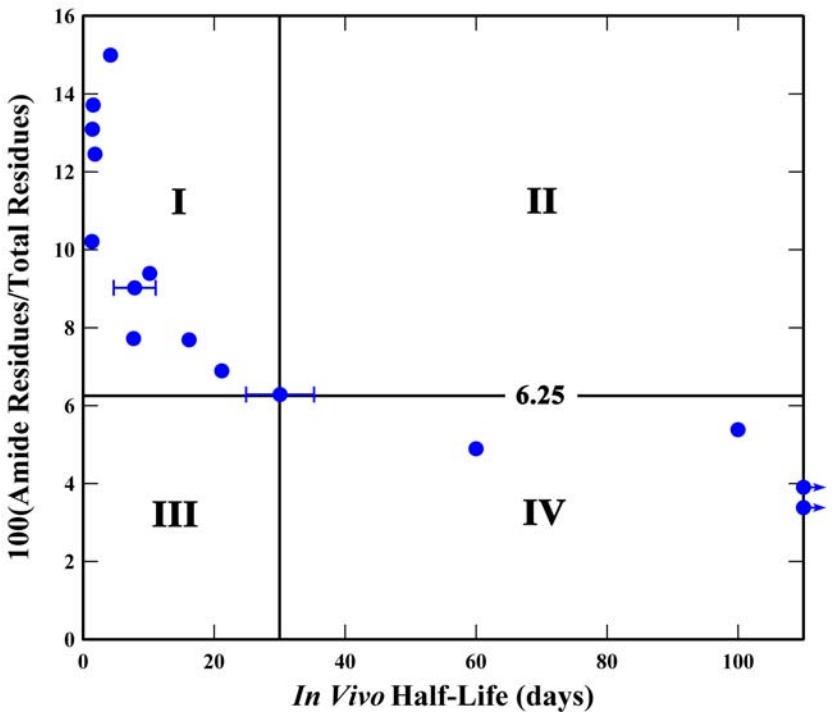

Fig. 10-7 Values for the relation between the *in vivo* lifetime of proteins vs the percentage of amide residues in the proteins. A horizontal line has been drawn at 6.25%, the percentage of triplets in the E. coli genetic code that designate glutaminyl or asparaginyl residues. A vertical line has been drawn at 30 days, the approximate value where the downward trend of the values intersects the 6.25% line. A χ^2 test of the distribution of values in Panels I, II, III, and IV gives a one-tailed $p = 0.003$ and a Wilcoxon test of the values in I and II vs those in III and IV gives a one-tailed $p = 0.0020$. The null hypotheses that this distribution is random is, therefore, rejected with 99.97% and 99.80% reliability for the two tests, respectively. Adapted from 1974R.

12 A. B. Robinson, J. H. McKerrow, and P. Cary, *Proc. Natl. Acad. Sci. USA* **66**, 753 (1970); A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **71**, 885 (1974).

During a comparison of the error-catastrophe hypothesis of aging[13] and the deamidation hypothesis of aging[12] as they relate to eye lens crystallins, Harding found[14] in 1976 that the correlation in Figure 10-7 was retained when the crystallins were added to the protein set, but only if Asn was plotted rather than both Asn and Gln. When Gln was added, crystallins did not fit this trend.

The correlation in Figure 10-7 was reported before the sequence dependence of deamidation was well known, especially the great differences between the deamidation rates of Asn peptides and Gln peptides. In light of that knowledge, it would be expected that this trend would be more applicable to Asn than to Gln.

In 1988 Rogers and Rechsteiner reported[15] a similar correlation of percentage of Asn plus Gln vs. the turnover rates of 35 proteins injected into HeLa cells. This correlation had $p \leq 0.01$.

In 1995, Vieille and coworkers reported[16] that the number of Asn plus Gln per molecule of xylose isomerase in various types of thermophilic bacteria diminishes as the growth temperature increases. Thermal inactivation of xylose isomerase was found to correlate closely with deamidation.[17] This appears to be a phenomenon similar to that illustrated in Figure 10-7, wherein increased temperature markedly increases the deamidation rate of proteins and is moderated by decreasing their amide content.

It is evident that long-lived proteins should not include short-lived amides, unless deamidation effects a biologically useful change. Therefore, their overall amide-content might be lower than the protein average.

The accumulation of amides in short-lived proteins, if the correlation proves generally applicable, might well play a role in protein turnover, but this remains to be experimentally demonstrated.

13 L. E. Orgel, *Biochemistry* **49**, 517 (1963).

14 J. J. Harding, *Biology of the Epithelial Lens Cells, INSERM* **60**, 215 (1976).

15 S. W. Rogers and M. Rechsteiner, *J. Biological Chemistry* **263**, 19850 (1988).

16 C. Vieille, J. M. Hess, R. M. Kelly, and J. G. Zeikus, *Applied and Environmental Microbiology* **61**, 1867 (1995).

17 J. M. Hess and R. M. Kelly, *Biotechnology and Bioengineering* **62**, 509 (1999); C. Chang, B. C. Park, D. Lee, and S. W. Suh, *J. Molecular Biology* **288**, 623 (1999).

CHAPTER 11

Deamidation of Peptides and Proteins in Biological Systems

11-1. UBIQUITOUS BIOLOGICAL OCCURRENCE

Until 2002, the extent of deamidation in biological systems was unknown.

Although a thorough review of the research literature shows about 200 protein types in which deamidation is believed to occur,[1] and about half that number in which it is proved to occur, this small group could be natural curiosities or, alternatively, the first evidence of a ubiquitous phenomenon. There has been no basis upon which to distinguish between these two possibilities or to test the hypothesis that the truth lies somewhere in between.

In 2002, this question was resolved.[2] The computerized calculation of the individual deamidation rates of 170,000 specific Asn in more than 18,000 peptides and proteins provided deamidation rates for a statistically significant sample of the biological protein pool. This discovery is reviewed in Chapter 9.

It is now established, therefore, that a large percentage of proteins deamidate to a substantial extent during their biological lifetimes. The protein pool is a dynamic, time-dependent array of protein molecules, the structures of which are continuously changing under the control of miniature amide clocks imbedded in each protein.

This time-dependent paradigm in which proteins are now known to function is fundamentally different from that previously assumed. Its existence should play a very significant role in the understanding of the functions of peptides and proteins in living systems.

1 See Chapter 11-2.

2 N. E. Robinson, *Proc. Natl. Acad. Sci. USA*, **99**, 5283 (2002).

11-2. EXPERIMENTAL OBSERVATIONS OF DEAMIDATION

At the present time, deamidation has been reported for about 200 distinct types of biological peptides and proteins. Heterogeneity that is probably the result of deamidation has been observed in many more. The number of observations is primarily a function of the protein purification and analysis methods in use.

We now know from experiment and from the computations reviewed in Chapter 9 that biologically significant deamidation rates are genetically specified for a large percentage of protein types. As analytical techniques improve, many thousands of proteins will be found to deamidate *in vivo*. In addition to limitations in analytical capabilities, protein turnover may also obscure some deamidation, since deamidated proteins tend to have increased susceptibility to catabolism. This reduces the steady-state concentration of deamidated forms.

Table 11-1 summarizes reports of deamidation in the research literature to date. In some cases, the deamidating amides have been identified and their rates of deamidation determined. In a greater number, identifications have been made, but rate experiments have not been performed. In many cases, however, deamidation has been found, but the specific deamidating amides have not been identified.

In some cases, charge heterogeneity is the primary evidence of deamidation. These have been included in Table 11-1 if the authors report that they think that deamidation is taking place.

Table 11-1 Experimentally Observed Deamidation in Biological Peptides and Proteins

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Acetylcholinesterase	Snake cobra venom			Purification + in vivo	1982RA, 1982RA1
Acid Phosphatase	Human prostate			Purification + in vivo	1989MW
Acidic Fibroblast Growth Factor	Human - polyanion stabilized	ProGlyAsn(8)TyrLys	7 at pH 8, 40°C; 28 at pH 7, 30°C	0.006M Phosphate, 0.001M EDTA, 0.12M NaCl	1995VV
Acid-Soluble Spore Protein	Bacillus subtilis PS 832			Heat killing at 80 to 90°C	1998SS
Acid-Soluble Spore Protein	Bacillus subtilis	Asn(48)Gly	0.04	0.025 M Phosphate, 0.005 M dithiothreitol, pH 7.5, 70°C	1997HS
Actin	Sea urchin egg			Purification + in vivo	1982DC
Actinoxanthin				Purification + in vivo	1981RZ
Adenylate Kinase	Rat liver			Purification + in vivo	1985WK
Adrenocorticotropic Hormone					See Ch. 12
A-layer Protein	Aeromonas salmonicida			Purification + in vivo	1992BE
Albumin	Wheat			Purification + in vivo	1973SP
Alcohol Dehydrogenase	Drosophila melanogaster			Purification + in vivo	1983WT
Alcohol Dehydrogenase	Rabbit liver			Purification + in vivo	1995KK
Alcohol Dehydrogenase	Sulfolobus solfataricus	LeuAsnAsn(249)SerGlu		0.10 Tris, pH 9, 96°C	1999GC
Alcohol Dehydrogenase	Yeast		0.012	0.05 M Phosphate, pH 7.8, 50°C	2001MN
Aldolase					See Ch. 12
Alkaline Phosphatase	Escherichia coli			Purification + in vivo	1977NA

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Alkaline Phosphatase	Human placenta			Purification + in vivo	1977WG
Alkaline Proteinase	Thermophilic Streptomyces			Purification + in vivo	1972MY
Amylase					See Ch. 12
Amylin(20-29)	Human pancreas - amyloid deposits in Type 2 diabetes	SerAsnAsnPhePro		pH 8, 60°C	2002ND
Amylin Proline Analog - Pramlintide	Human	LysCysAsn(3)ThrAla, LeuAlaAsn(14)PheLeu, GlySerAsn(35)ThrTyr, SerAsnAsn(22)PheGly, SerAsn(21)AsnPheGly		pH 4.0. 40 °C, 45 days	1998HD, 1999HD
Amyloid-Related Serum Protein	Human serum - Rheumatoid Arthritis	TyrSerAsn(23)MetArg [Ile/Leu]SerAsn(60)AlaArg AlaGluAsn(75)SerLeu		Digestion + purification + in vivo	1983SM
Angiogenin	Human	LysAsn(61)Gly GluAsn(109)Gly	17	0.05M Tris, pH 8.0, 37 °C	1992HS
Angiotensin	Frog plasma	Asn(1)ArgVal		In vivo - enzymatic - frog plasma asparaginase	1995CY
Antiflammin		AspMetAsn(4)LysVal	92	Phosphate, NaCl, pH 6.16, I 0.6, 37°C	1994WL
Antithrombin III	Human			Purification + in vivo	1977NN
Antithrombin III	Cow			Purification + in vivo	1977NN
Apolipoprotein A-I					See Ch. 12
Apolipoprotein A-II	Human serum			Purification + in vivo	1983SI
α-Arachin	Arachis hypogaea seeds			Purification + in vivo, NH_3	1969DN

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Asparaginase	Escherichia coli			Purification + in vivo	1971LL
Aspartase	Escherichia coli			pH 5-6, 55°C, 0.001M 2-mercaptoethanol or 0.05M acetate, pH 3.5, 0.01M dithiothreitol	1978TE
Aspartate Aminotransferase	Sheep heart			Purification + in vivo	1974JJ
Aspartate Aminotransferase	Pig heart			Purification + in vivo	1974JJ
Aspartate Aminotransferase			1st NH_3 = 9 2nd NH_3 = 22	0.01M Phosphate, pH 7.4, 25°C	1979WJ
Aspartate Aminotransferase	Human liver cytoplasm			Purification + in vivo	1981R
Aspartate Aminotransferase	Human erythrocytes			Purification + in vivo	1981R
Aspartate Aminotransferase	Human heart cytoplasm			Purification + in vivo	1982LH
Atrial Natriuretic Peptide	Human	GlyCysAsn(24)SerPhe			1996P
Bcl-x_L					See Ch. 12
Bence-Jones Protein TI		ThrGluGln(165)AspSer		Digestion + purification + in vivo	1972BW
Botulinal Toxin - Type A	Clostridium botulinum			Storage freeze dried	1994GJ
Calbindin D_{28k}	Human			pH 8	1999TL
Calbindin D_{9k}	Cow	LysAsn(56)GlyAsp		Purification + in vivo	1989CK

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Calcitonin	Salmon	LeuSerGln(14)GluLeu LysLeuGln(20)ThrTyr	Gln(14), pH 3, 92 days; Gln (20), pH 3, 65 days; Either Gln(14)and Gln(20), pH 3, 3,500 days each; None observed at pH 5	0.010M phosphate, NaCl, pH 3, I 0.10, 60°C and 0.010M acetate, NaCl, pH 4, 5, and 6, 60°C	1997WD
Calmodulin - without Ca++	Cow brain	GlyAsn(97)GlyThr GlyAsn(60)GlyThr	Asn(97) = 12 Asn(60) = 116	0.05M HEPES, 0.05% sodium azide, 0.001M EGTA, pH 7.4, 37°C	1987JL, 1989JS, 1993PH
Calmodulin - Ca++ bound	Cow brain			Purification + in vivo	1989OC
Calmodulin - without Ca++	Human erythrocytes			Purification + in vivo	1990OC1, 1990MW
cAMP-Binding Protein	Aplysia californica			Purification + in vivo	1982EP
cAMP-Dependent Protein Kinase	Cow, pig, rabbit, and rat muscle	Myr-GlyAsn(2)AlaAla		Purification + in vivo	1984LW, 1998JG, 2000KK, 2000PH, 2001TP
Carbonic Anhydrase	Toad erythrocytes			Purification + in vivo	1979SS
Carbonic Anhydrase	Cow erythrocytes			Purification + in vivo	1978GF, 1979BG
Carbonic Anhydrase	Sheep erythrocytes			Purification + in vivo	1979BG
Carbonic Anhydrase	Human erythrocytes	LysHisAsn(11)GlyPro LeuAsnAsn(63)GlyHis AsnPheAsn(232)GlyGlu		Purification + in vivo	1969FD, 1971FD, 1976HH, 1979BG
Casein					See Ch. 12
CD4	Human	LeuAsn(52)AspArg	1,200	0.13M phosphate, 0.1M NaCl, pH 7.2, 25°C	1991TP
Cell Surface Protein G	Streptococcus G148	IleAsn(7)Gly AlaAsn(34)Asp AspAsn(36)Gly		0.1 M NaOH, 2 hours	2002GL

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Cellobiohydrolase	Trichoderma reesei		0.06	0.1M NH_4OAc, 4M urea, pH 4.8, 70°C	1995JD
Chloroperoxidase	Caldariomyces fumago	AspAsnAsn(13)ThrLeu LeuProAsn(198)AsnAsp AspValGln(183)SerGly		Digestion + Purification + in vivo	1987KF
Cholera Enterotoxin	Vibrio cholerae - B subunit	PheLysAsn(44)GlyAla MetLysAsn(70)ThrLeu LeuAsnAsn(22)LysIleu ThrPheGln(49)ValGlu	1st form to 2nd, 4.0 days; 2nd form to 3rd, 3.0 days	Digestion + Purification + in vivo, for rates - 0.2M Phosphate, pH 8.2, 37°C	1976NN, 1977KM, 1977L, 1977RT, 1985TW, 1991SW1
Chorionic Somatomammotropin	Human		Protein 9.3 days; with 8M urea, 1.4 days; with 1M urea $(NH_4)_2SO_4$, 8.9 days	Phosphate, NaCl, pH 7.5, I 0.1, 37°C	1979TB, 1980M1
Chymotrypsin				Purification + in vivo	1964EC
CIF Protein	Soybean			pH 10.5, 60°C	1972SK
Class II Histocompatibility Antigen DRα-chain	Human	LeuArgAsn(127)GlyLys		Digestion + purification + in vivo	1984KK
Collagen					See Ch. 12
Colocin E3	Escherichia coli			Purification + in vivo	1972GK
β-Conglycin	Soybean seeds			In vivo - germination	1986WR
Corticotropin-Releasing Factor	Pig hypothalmus	AlaGluGln(26)LeuAla		Purification + in vivo	1986PS
Creatine Kinase	Human serum, muscle, and brain			Purification + in vivo	1988P, 1995WC1
Creatine Kinase	Rabbit muscle			Purification + in vivo	1995WC
Crystallin (Lens)					See Ch. 12
Cytochrome b5	Cow	HisAsnAsn(15)SerLys		Purification + in vivo	2000HM

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Cytochrome c					See Ch. 12
Cytochrome c-551	Pseudomonas	IleLysAsn(50)GlySer		Digestion + purification + in vivo	1963A
Cytochrome f	Charlock, Sinapis arvensis			Storage at minus 50°C	1978G
Dehalogenase	Mutant with Asn(145) in active site rather than wild-type Asp(145)	GlyAsnAsn(145)ThrAla	12 days or, with substrate, 29 seconds	0.05M HEPES, pH 7.5, 22 °C	1999XD
Deoxyribonuclease I	Human	GlyArgAsn(74)SerTyr	28 with Ca++ 5 without Ca++	Purification + in vivo - 64% deamidated. For rate experiment, 0.005M Phosphate, 0.001M $CaCl_2$, pH 7.3, 40°C or, without Ca++, substitute 0.001M EGTA for $CaCl_2$	1993CQ, 1994FS1, 1996S
Deoxyribonuclease	Human	GlyArgAsn(74)SerTyr	23 days	0.005M Tris, pH 7, 0.15M NaCl, 0.001M $CaCl_2$	1994CG
Dihydroorotase	Escherichia coli			Purification + in vivo	1984WC
DPN-specific Isocitrate Dehydrogenase	Pig heart			Purification + in vivo	1980RC
Elastase	Pig pancreas	GlnAsnAsn(77)GlyThr ArgThrAsn(148)GlyGln GlyGlyAsn(186)GlyVal LeuValAsn(204)GlyGln		Digestion + purification + in vivo	1970SH

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Endoglucanase I	Fungus Trichoderma reesei QM9414			0.1M Ammonium acetate, 8M urea, 0.0002M $CuCl_2$, pH 4.8, 70°C	1992DA
Enolase				Purification + in vivo	1976B, 1981B1
Enterotoxin C_2	Staphylococcus aureus			Purification + in vivo	1972DY
Envelope Glycoprotein E1	Hepatitis C Virus	GlyAsn(234)AlaSer		In vivo	1999SE
Epidermal Growth Factor	Mouse submaxillary gland	Asn(1)SerTyr	0.86	Purification + in vivo. Rate at 0.15M ammonium bicarbonate, pH 9.0, 37°C	1984KF, 1987DG, 1989GI, See Ch. 12
Epidermal Growth Factor	Human urine				See Ch. 12
Epidermal Growth Factor	Rat prostate	Asn(1)SerAsnThrGly		Purification + in vivo	1991NS, See Ch. 12
Extracellular Proteinase	Pseudomonas fluorescens 22F		0.035	0.2M Tris-maleate, 0.02M $CaCl_2$, pH 7.5, 90°C	1999SB
F1-ATPase - β chain	Cow heart mitochondria	Gln(1)AlaSerPro		Purification + in vivo	1985WF
Fatty Acid Binding Protein	Cow liver	GluPheAsn(105)GlyAsp ValGlnAsn(44)GlyLys		Digestion + purification + in vivo	1993DB
Fatty Acid Binding Protein	Rat liver	GluPheAsn(105)GlyAsp		Digestion + purification + in vivo	1994OO
Fibroblast Growth Factor	Human	Asn(7)			1995VV
Food Proteins					See Ch. 12
Formate Dehydrogenase	Potato tuber	MetProAsn(329)GlnAla ProAsnGln(330)AlaMet		Purification + in vivo	2003BS
Fumarase	Pig heart			Purification + in vivo	1971PC
Fusion Protein	Pig			Purification + in vivo	1990W

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Major G Protein α_o	Cow brain	IleAlaAsn(346)AsnLeu AlaAsnAsn(347)LeuArg		In vivo - found 30 to 40% deamidated in steady state in bovine brain.	1998MS, 1998MD, 1999MD, 1999EJ
β-Galactosidase	Aspergillus oryzae			0.2M Phosphate, pH 6.2, 55°C	1994YA
Glucagon		HisSerGln(3)GlyThr PheValGln(24)TrpLeu ArgAlaGln(20)AspPhe LeuMetAsn(28)Thr	Gln(24) = 9.4 Asn(28) = 23.7	0.14M Phosphate, NaCl, pH 2.4, I 0.15, 60°C	1987OD, 2000JR, 2002JK
Glucagon	Cow and sheep pancreas	LeuMetAsn(28)Thr		Purification + in vivo	1987OD
Glucoamylase	Aspergillus awamori	ValAsn(182)Gly		0.05M Sodium acetate, pH 4.5, 60°C	1983SL, 1994CB, 1994CF
Glucose Isomerase (Xylose Isomerase)	Streptomyces olivochromogenes		0.007 at pH 8 0.021 at pH 8 + 2M xylitol 0.14 at pH 7 + 2M xylitol	0.1M Tris, 0.010M $MgSO_4$, 90°C, immobilized on glass beads	1989VK, 1999CP
Glucose-6-Phosphate Dehydrogenase	Human and mouse erythrocytes, leukocytes, and eye lenses				1969FL, 1972HT, 1974HP, 1975KC, 1975GM, 1975WC, 1976KB, 1976KB1, 1976KB2, 1976SV, 1977KG, 1977KB, 1978L, 1981DK, 2002IC

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Glucose-6-Phosphate Isomerase	Human, cow, and other species			Purification + in vivo	1976BK, 1982G, 1984NT, 1986CG, 1988CC, 1988C, 1989FP, 1990SY1, 1993RY
Glutathione S-Transferase	Human liver			Purification + in vivo	1975KH
Glutathione S-Transferase	Human erythrocytes			Purification + in vivo	1978J
Glyceraldehyde-3-Phosphate Dehydrogenase	Methothermus fervidus		0.069	0.01M Phosphate, 0.15 mercaptoethanol, pH 7.3, 85°C	1994HJ
Glyceraldehyde-3-Phosphate Dehydrogenase	Pyrococcus woesei		0.053	0.01M Phosphate, 0.15 mercaptoethanol, pH 7.3, 100°C	1994HJ
Glycinin	Soybean seeds			In vivo - germination	1986WR
Granulocyte-Colony Stimulating Factor	Human	LeuGluGln(21)ValArg		0.001M HCl, pH 3.0, air-jet nebulization	1994NI, 1996P
Growth Hormone					See Ch. 12
Growth Hormone Releasing Factor					See Ch. 12
Gycerol-3-Phosphate Dehydrogenase	Rat liver and muscle			Purification + in vivo	1971RC, 1971FS
Hexokinase	Human erythrocyte			Purification + in vivo	1982SM
Hexon Protein	Human pancrease		~50	0.1 M Tris, 20% glycerol, pH 8.4, 20°C	2001BC1
High Mass Protein HMAP	Human, rat, and cow			Purification + in vivo	1998DO
High Molecular Weight Glycoprotein	Human skin fibroblasts			Purification + in vivo	1986VJ

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Hirudin	Leech Hirudo medicinalis	GlySerAsn(33)GlyLys HisAsnAsn(53)GlyAsp TyrLeuGln(65) - c-terminal		0.17M Phosphate, pH 9, 37°C	1992TM, 1993BL1, 1995DW, 1999GA, 2002DB
Hirulog 3-Thrombin Complex	Human	GlyAsn(53)Gly		Crystal structure disordered at this residue.	1992QP
Histone					See Ch. 12
HIV-1 Rev	HIV immunodeficiency virus	ProLeuGln(74)LeuPro		Digestion + purification + in vivo	1993LH
Homoserine Dehydrogenase	Maize shoots and roots			Purification + in vivo	1975DB
HPr Phosphocarrier Protein	Escherichia coli	AlaProAsn(12)GlyLeu ThrSerAsn(38)GlyLys	Asn(12) = 28 Asn(38) = 13	0.10M Phosphate, pH 7.4, 37°C	1971AW, 1984MK, 1993SH, 1994BA
HPr Phosphocarrier Protein	Escherichia coli	LeuThr(Gln57)GlyThr AspGluGln(71)LysAla		Boiling	1985WE, 1988KB
Hydrophobin HFBI	Fungus Trichoderma reesei	SerAsn(2)GlyAsn(4)GlyAsn ValCys, Asn(2) and Asn(4) deamidated		Purification + in vivo	2001AN
Hyperglycaemic Neurohormones	Crayfish Procambarus bouvieri sinus gland			Purification + in vivo	1988HA, 1988HA1
Hypoxanthine-Guanine Phosphoribosyltransferase	Human lymphoblasts and erythrocytes	TyrCysAsn(106)AspGln		Purification + in vivo	1974TF, 1977GM, 1982JR, 1982WL
Immunoglobulin					See Ch. 12

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Inhibitor Protein of Cyclic AMP-Dependent Protein Kinase	Cow and rabbit skeletal muscle			Purification + in vivo	1980WM
Inorganic Pyrophosphatase	Human and 26 other mammal erythrocytes			Purification + in vivo	1974FP
Insulin					See Ch. 12
Interferon	Engineered average sequence			Purification	1996PN, 2001PC
Interferon γ		AlaAspAsn(26)GlyThr PhePheAsn(84)SerAsn			1992PN, 1996P
Interleukin					See Ch. 12
Interleukin 1	Human	ArgAlaAsn(148)AspGln		Purification + in vivo	1986CL
Interleukin 1α	Human	ArgAlaAsn(36)AspGln	0.07	Purification + in vivo, rate at 0.05M tetraborate, pH 8.3, 30°C during capillary electrophoresis	1987WP, 1987WM1, 1990CB1, 1991GA, 1993MR, See Ch. 12
Interleukin 1β	Human	HisLeuGln(32)GlyGln	160 to 330	0.01M Tris, pH 7.0, 30°C	1991GE, See Ch. 12
Interleukin 1β	Mouse	HisLeuAsn(32)GlyGln	1.5	0.1M Tris, pH 8.5, 37°C	1991DW, See Ch. 12
Interleukin 2	Human	IleSerAsn(88)IleAsn	500	0.005M acetate, pH 5.0, 40°C	1992SH, See Ch. 12
Interleukin 3	Human	AspPheAsn(38)AsnIle		Purification + in vivo	1998BK, See Ch. 12
Interleukin 11	Human	AspHisAsn(49)LeuAsp	275	0.01M phosphate, 0.3M glycine, pH 7.0, 30 °C	2002ZC, See Ch. 12
Interleukin-1 Receptor Antagonist	Human	LeuThrAsn(136)MetPro	1,500	Storage at 30°C after freeze drying from 0.01M citrate, 2% glycine, pH 6.5	1996CR, 1996CB, 1996P
Invertase	Yeast			Loss of activity after 4 minutes in 0.1M acetate, pH 5.0, 100 °C	1980YO

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Isolectin	Wheat germ	CysGlnAsn(37)GlyAla		Digestion + purification + in vivo	1989WR
Kallikrein	Human and hog			Purification + in vivo	1982FA, 1983IK
lac Repressor	Escherichia coli	ValValAsn(25)GlnAla (25%), ValAlaGln(54)Gln(55)Leu Ala (35%), AspAspGln(131)AspAla & SerHisGln(153)AspGly (25%)		Digestion + purification + in vivo	1975BA
β-Lactamase	Bacillus licheniformis	AsnMetAsn(265)GlyLys GluLysAsn(273)GluLys		Purification + in vivo	1979A, 1991MJ
Lactate Dehydrogenase	Shrimp Palaemon serratus			Purification + in vivo	1984T
Lactate Dehydrogenase	Human serum, erythrocyte, cytosol			Purification + in vivo	1988RS
Lactoferrin	Human milk		11% of amides in 28 days	0.025M Phosphate, 0.85% NaCl, pH 7.0, 37°C	2001BN
Lactogen	Sheep placenta			Purification + in vivo	1978RW
Lactoperoxidase	Cow milk			Purification + in vivo	1966C, 1967CV
L-Alanine:2-Oxoglutarate Aminotransferase	Human liver			Purification + in vivo	1990KK
L-Amino Acid Oxidase	Rattle snake venom			Purification + in vivo	1969HW
Lectin	Fungus Arthrobotrys oligospora			Purification + in vivo	1996RK
Leghaemoglobin	Lupin and Serradella root nodules			Purification + in vivo	1972BD

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Leukaemia Protein p30	Mouse virus			Purification + in vivo	1980B
Lymphotoxin	Human	LysGlnAsn(19)SerLeu SerAsnAsn(41)SerLeu	Asn(19) 13.8 Asn(41) 80 or in 4M guanidinium chloride Asn(19) 0.45 Asn(41) 0.92	0.05M phosphate, pH 11, 40°C	2002XS
Lysozyme					See Ch. 12
Macrophage Migration Inhibitory Factor	Human liver and breast			Purification + in vivo	1998MB1
Major Cell-Envelope Membrane Protein	Escherichia coli			Purification + in vivo	1976SH
Major Internal Protein	Human virus SKA 21-3			Purification + in vivo	1981BD
Major Intrinsic Protein MP22	Human eye lens	ValSerAsn(246)GlyGln GluLeuAsn(259)ThrGln		In vivo - both Asn completely deamidated by 7 years of age.	2000SL1
Major Intrinsic Protein MP26	Cow lens membrane	GluSerAsn(246)GlyGln		Purification + in vivo	1991TE, 1997SF
Major Intrinsic Protein MP26	Rat lens membrane	GluSerAsn(244)GlyGln		Purification + in vivo	1995DC
Malate Dehydrogenase	Yeast Saccharomyces cerevisiae cytoplasm			Purification + in vivo	1978HN
Membrane Protein 4.1	Human erythrocytes	AsnIleAsn(478)GlyGln AsnAlaAsn(502)AlaVal	Asn(502) 41 Asn(478) much faster	In vivo - in erythrocytes	1992IG, 1992IM
Membrane Protein 4.1	Dog erythrocytes	ThrAlaAsn(502)SerVal		In vivo - in erythrocytes	1988IM, 1992IM
Membrane Protein 4.1	Human, dog, sheep, goat, cow, horse, pig, mouse, rabbit, cat			In vivo - in erythrocytes + purification	1988IM

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Methyl-Accepting Taxis Protein	Myxococcus xanthus			Purification + in vivo	1992MK1
β2-Microglobulin	Guinea pig urine			Purification + in vivo	1978CZ
β2-Microglobulin	Human serum	AlaGluAsn(17)GlyLys Asn(42)GlyGlu ArgValAsnHisVal		Digestion + purification + in vivo	1990OO, 1996MI, 2001KT, 2000SL2
MurA Enolpyruvyl Transferase	Enterobacter cloacae	ArgAsn(67)Gly		Purification (3 days, 4°C, pH 8), crystal growth (1 to 2 days, 19°C, pH 6.5), and in vivo	2000ES
Myelin Basic Protein					See Ch. 12
Myoglobin	Sperm whale, horse, cow		Cow - pH 7, phosphate, 37 °C, 35 days; pH 7, 7 °C, 635 days; 4M urea 155 days; 8M urea 64 days		1961EH, 1965E, 1967V, 1973Q, 1981GK
Myosin	Pig arterial smooth muscle			Purification + in vivo	1981DA
Myosin	Rat ventriculum	IleLeuAsn(99)AlaPhe GluLeuAsn(108)SerLys		0.2M Acetate or carbonate, pH 2 to 11, 37°C, 4 hours	1990CF, 1991CW
Myosin	Rabbit heart	GlyAlaAsn(13)SerAsn		In vivo - 10% deamidated in newborn rabbits and 30% in adult rabbits	2003WC
N-Acetyl-β-D-glucosaminidase	Human pancreas			Purification + in vivo	1991BO
NAD(P)H Dehydrogenase	Human blood			Purification + in vivo	1979UK
Neocarzinostatin	Streptomyces carzinostaticus			pH 3.2, 4 C	1977MK, 1986KT
Nerve Growth Factor	Human	AsnIleAsn(45)AsnSer		0.01M Acetate, pH 5.8, 37°C	1997EL

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
neu Differentiation Factor	Human	PheLysAsn(61)GlyAsn CysValAsn(180)GlyGly		Purification + in vivo	1996HL
Neurotrophic Factor	Human glial cell	GluArgAsn(16)ArgGln ProGluAsn(26)SerArg GlyLysAsn(39)ArgGly		Deamidate easily in vitro - conditions not given	2001MH
Neurotrophic Factor	Human ciliary	IleAlaAsn(195)AsnLys			1996P
Neutrophil Activating Peptide	Human monocyte			Purification + in vivo	1988SM
Non-histone Chromosomal Protein	Rat liver			Purification + in vivo	1983WP
Nucleoside Phosphorylase	Human eye lens, erythrocyte, liver, kidney, fibroblast			Purification + in vivo	1971EH, 1976SV
Ornithine Transcarbamylase	Cow liver			Purification + in vivo	1972MC
Outer Surface Protein	Borrelia burgdorferi			Purification + in vivo	1997BK
Ovalbumin	Chicken egg		92	Egg white at 37 °C	1985MS1, 1986KT1, 2001MT
Parathymosin	Rat liver	ThrGluAsn(101)GlyAla		Digestion + purification + in vivo	1988KP, 1988FG, 1987H
Parathyroid Hormone	Human parathyroid gland	AspValAsn(76)ValLeu (At pH 2) HisLeuAsn(16)SerMet (At pH 5 to 10)		0.02M Borate-citrate-phosphate, pH 2 to 10, 40 to 60 °C	1978KS, 1981HK, 1987GH, 1991ZS, 1995NK, 1997NF
Parotid Basic Protein Pb-1a, Pb-1b, and Pb-2	Human parotid saliva	PheHisGln(16)LysHis		Purification + in vivo	1977PA
Phenylalanine Hydroxylase					See Ch. 12
Phenylalanine(Histidine): Pyruvate Aminotransferase	Mouse liver			Purification + in vivo	1977MN

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Phosphodeoxyribomutase	Escherichia coli		Form I to Form II = 210	0.05M Tris-succinate, 0.001 EDTA, pH 6.9, 4°C. Also in vivo.	1975LH
Phosphoglucomutase	Human erythrocytes white blood cells			Purification + in vivo	1974TF, 1969M
Phosphoglucomutase	Rat erythrocytes			Purification + in vivo	1969M
6-Phospho-D-Gluconate Dehydrogenase	Human skin fibroblasts - Proeria			Purification + in vivo	1975GM
6-Phospho-D-Gluconate Dehydrogenase	Pseudomonas fluorescens			0.05M Phosphate, pH 7.2, 4°C	1978SK
3-Phosphoglycerate Kinase	Methothermus fervidus			Purification + in vivo	1994HJ
Phospholipase A2	Cobra naja naja naja venom			Purification + in vivo	1975DD
Phospholipase A2	Cow adrenal medulla lysosomes			Purification + in vivo	1990BF
p-Hydroxyphenylpyruvate Hydroxylase	Chicken liver			Purification + in vivo	1975WF
Phytohemagglutinin	Red kidney bean isolectin			Purification + in vivo	1981FL
Plasma Albumin	Human, cow, and rat			Purification + in vivo	1973WS, 1984AT, 1985LK, 1988P1
Plasminogen Activator	Human tissue	CysPheAsn(58)GlyGly GlyAsn(177)SerAsp CysAsn(37)SerGly	Asn (58) = 11	0.2M Arginine-phosphate, pH 7.3, 37°C	1993AP, 1994PG, 1995A
Potato Proteins	Potato tuber			Purification + in vivo	1973SF, 1980SP

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Protease PfpI	Archaeon Pyrococcus furiosus Intracellular			Purification + in vivo	1997HB
Prion PrP	Mouse neuronal glycoprotein	ThrAsn(107)LeuLys		H_2O, -20°C	2000QY
Prion PrP	Mouse	LysThrAsn(108)MetLysHis	25	0.10M Phosphate, pH 7.4, 37°C	1999SH
Prolactin	Sheep pituitary		32	0.05 Phosphate, pH 7, 37°C	1965LC, 1969CL, 1970LC1, 1980L, 1981CW, 1985HT, 1985HT1
Prolactin	Mouse pituitary		12	0.1M Carbonate, 0.02% NaN_3, 0.001M PMSF, pH 8.0, 37°C	1969CL, 1985HT, 1985HT1
Prolactin	Bovine pituitary	CysCMProAsn(6)GlyPro		Purification + in vivo	1969CL, 1970GC, 1971GB
Prolactin	Rat pituitary			Purification + in vivo	1969CL, 1982NR, 1995MD
Prolactin	Human pituitary		Human serum, 37°C = 3.2, phosphate = 13.4	Purification + in vivo, rates in human serum at 37°C and in 0.01M Phosphate, 0.9% NaCl, pH 7, 37°C	1972HG, 1975L, 1980NR, 1981NR, 1985NB, 1990PR
Protein G Albumin Binding Domain	Streptococcus G148			0.5M NaOH 5 hours	2000GL
Protein VP6	Rotavirus	GlnArgAsn(107)GlyIle		Digestion + purification + in vivo	2000EM
Protein A	Staphylococcus aureus			0.5 M NaOH, 30 minutes	2004LG

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Prothymosin α	Rat thymus	AlaGluAsn(28)GlyArg ProAlaAsn(35)GlnAsn		Digestion + purification + in vivo	1985HB, 1988FG
Prothymosin α	Human	AlaGluAsn(28)GlyArg ProAlaAsn(35)GlnAsn		Digestion + purification + in vivo	1986PH1
Purine-Nucleoside Phosphorylase	Human erythrocytes			Purification + in vivo	1974TF
Retinol-Binding Protein	Human serum and urine		15.3	Purification + in vivo, rate in 0.020M phosphate, 0.15M NaCl, 0.010M Na-cacodylate, pH 7.4, 37°C	1971RV, 1997MH
Rhinovirus-14 3C Protease	Human	GlyGlyAsn(164)GlyArg		Purification + in vivo	1999CJ
Ribonuclease					See Ch. 12
Ribonuclease U2	Ustilago sphaerogena	ValAlaAsn(32)GlyAsp			1986KU
Ribulose 1,5-biphosophate carboxylase/oxygenase	Tobacco Nicotiana Tabacum chloroplast and nucleus			Purification + in vivo	1979KR
Ricin D	Ricinus communis seeds			Purification + in vivo	1978WK
Serine Hydroxymethyltransferase	Rabbit liver cytosol	AlaValAsn(5)GlyAla AspGluAsn(220)GlyAla	Asn(5) 2.5 Asn(220) >8	Purification + in vivo, rates in 0.020M phosphate, 10% sorbitol, 0.000050M pyridoxal-5-phosphate, 0.001M dithiothreitol, 10 g/ml protease inhibitor, pH 7.3, 37°C	1990AB, 1993AF, 1995S, 1998SF, 1999SF
Serine Proteinase	Bacillus Licheniformis			Purification + in vivo	1982AS

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Serine Proteinase	Thermomonospora fusca YX			Purification + in vivo	1987GK
Sex-Dependent Protein α_{2u}	Male rat urine			Purification + in vivo	1971LI, 1972LN, 1972LN1
Somatomedin A (or Insulin-Like Growth Factor)	Human plasma	Gln(40)		Digestion + purification + in vivo	1984EC
Somatotropin	Cow and Pig	PheThrAsn(99)SerLeu			1987HH, 1990VS
Spermatozoal Protein AP1	Guinea Pig testes			Purification + in vivo	1975JH
Stem Cell Factor	Human		56	0.040M Tris, pH 7.2, 37°C	1998HC2
Superoxide Dismutase	Cow erythrocyte	ValHisGln(47)PheGly		Digestion + Purification + in vivo	1974SN
Superoxide Dismutase	Rat liver			Purification + in vivo	1976RG
Thioltransferase (Glutaredoxin)	Human red blood cells	ThrAsn(51)HisThr		Purification + in vivo	1994PG1
Thrombopoietin		TyrLeuAsn(227)ArgIle			1996P
Thylakoid Proteins	Oenothera chloroplast membrane			Purification + in vivo	1989HL
Transmembrane Secretory Component	Human	ThrGluAsn(87)AlaGln ValIleAsn(137)GlnLeu AlaGlnAsn(321)GlyArg TrpAsnAsn(429)ThrGly		Digestion + purification + in vivo	1989KS
Triosephosphate Isomerase				Purification + in vivo	See Ch. 12
Troponin T	Rabbit fast skeletal muscle			Purification + in vivo	1987BL1
Trypsin					See Ch. 12

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Trypsinogen	Cow pancreatic stone			Purification + in vivo	1982CM
Tryptophan Synthetase	Escherichia coli	Gln(1)ArgTyr		Purification + in vivo	1972LY
Tubulin	Cow, calf, rat, lamb brain and other tissues			Purification + in vivo	1981GM, 1988FL, 1996NO
Tubulin	Chicken embryo brain			Purification + in vivo	1979NB
Tumor Necrosis Factor	Human	LeuAlaAsn(39)GlyVal		Purification + in vivo	1989SN, 1990CB1, 1991CH
Tyrosinase	Mouse melanocytes normal and melanoma			Purification + in vivo	1978HN1
Urease	Jackbean meal			Purification + in vivo	1976FN
Uropepsinogen and Uropepsin	Human urine			Purification + in vivo	1984MI
Vascular Endothelial Growth Factor	Human	GlyGlnAsn(10)HisHis	pH 5.0, 51 days; pentapeptide model, 48 days; pH 8.0 - 6.9 pentapeptide model, 5.9	0.1M Phosphate, pH 5.0 and 8.0, 37°C	2000GS, 2000GS1
Vasopressin		AlaGlyCysTyrPheGln AsnCysProArgGly-NH_2		Purification	1997S

Protein	Source	Deamidating Sequence	Half-Time (Days)	Conditions	Reference
Viscumin	Mistletoe Viscum album	A chain - ProPheAsn(12)GlySer B chain - Asn(87)GlyMet B chain - Asn(230)GlyLeu B chain - TrpGlyAsn(91)GlyThr		Digestion + purification + in vivo	2001LM
Xylose Isomerase (Glucose Isomerase)	Streptomyces olivochromogenes		0.007 at pH 8 0.021 at pH 8 + 2M xylitol 0.14 at pH 7 + 2M xylitol	0.1M Tris, 0.010M $MgSO_4$, 90°C, immobilized on glass beads	1989VK
Xylose Isomerase	Thermotoga Neapolitana		pH 7.0 = 0.019 pH 7.9 = 0.013 pH 7.0 + 0.01M Phosphate = 0.037	0.100M MOPS, 0.01M MgSO4, 0.001M $CoCl_2$	1995VH, 1999HK
Zein	Maize prolamine			Purification + in vivo	1976GI

CHAPTER 12

Particular Peptides and Proteins

12-1. INTRODUCTION

In some peptides and proteins, deamidation has been studied more thoroughly than for most of those listed in Table 11-1. These include molecules of special historical interest in the development of knowledge about deamidation, molecules that have served as models for the study of the chemistry of deamidation, and molecules where deamidation is thought to be of special significance. Brief reviews of research on these particular peptides and proteins are presented in this chapter.

12-2. ADRENOCORTICOTROPIN

Adrenocorticotropin, ACTH, is released from the posterior pituitary gland upon stimulation by corticotropin-releasing hormone. ACTH then stimulates the synthesis of mineralocorticoids and glucocorticoids in the adrenal cortex.[1]

Deamidation of corticotropin was first reported in 1955 in preparations from sheep pituitary glands showing ACTH activity.[2] Purification by chromatography and countercurrent distribution resulted in a pure component designated as α-corticotropin. Another fraction was, however, also found that was entirely converted into α-corticotropin after 16 hours in 0.1 M $NaCO_3$. This reaction was accelerated by higher pH and temperature. The investigators labeled this an "alkali-labile active precursor" of α-corticotropin. They determined that the precursor had four amides per molecule, while α-corticotropin had two.

Soon thereafter, deamidation of pig β-corticotropin with $t_{1/2} = 1.03$ days in pH 9, 25 °C, sodium bicarbonate buffer was measured.[3]

1 C. K. Mathews and K. E. van Holde, *2nd Edition, Benjamin/Cummings, Menlo Park* , 684 (1996).

2 C. H. Li, I. I. Geschwind, J. S. Dixon, A. L. Levy, and J. I. Harris, *J. Biological Chemistry* **213**, 171 (1955).

3 R. G. Shepherd, K. S. Howard, P. H. Bell, A. R. Cacciola, R. G. Child, M. C. Davies, J. P. English, B. M. Finn, J. H. Meisenhelder, A. W. Moyer, and J. van der Scheer, *J. American Chemical Society* **78**, 5051 (1956).

β-corticotropin was determined to be a 39-residue peptide. Deamidation did not diminish its ACTH activity. The same peptide was later reported in sheep.[4]

The deamidating residue was initially thought to be Gln, but was correctly reassigned in 1971 to TyrProAsn(25)GlyAla.[5] This deamidation reduces biological activity by 2-fold.[6] The sequence was confirmed, and the $t_{1/2}$ of deamidation in 1N NH_4OH, 25 °C found to be about 1 hour.[7]

A deamidation $t_{1/2}$ of 15 days was found in pH 7.0, 0.005 M phosphate, 37 °C for ACTH, while that for the peptide ValTyrProAsnGlyAla was 4.2 days. At pH 9.6 in glycine buffer, these investigators found that $t_{1/2}$ decreased from 0.44 days at 0.01 M to 0.13 days at 0.10 M buffer.[8] The buffer and pH dependence of ACTH and the model peptide have been extensively investigated[9] with $t_{1/2}$ in pH 7.5, 0.005 phosphate, I 0.5, 37 °C found to be 1.9 days. In pH 7.5, 0.10 M phosphate, I 0.5, 37 °C, both the peptide and ACTH have $t_{1/2}$ = 0.83 days.

The crystal structure of the succinimide peptide BocProAsuGlyAlaOMethyl has been studied in an effort to understand the geometric aspects of ACTH deamidation.[10]

The deamidation of TyrProAsn(25)GlyAla in ACTH has been found, therefore, to be primary-structure controlled, with some indication of slowing by secondary peptide structure under mild deamidating conditions.

12-3. ALDOLASE

Aldolase is a tetrameric protein that catalyzes the cleavage of D-fructose-1,6-bisphosphate to produce dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. Reports of heterogeneity in rabbit

4 B. T. Pikering, R. N. Andersen, P. Lohmar, Y. Birk, and C. H. Li, *Biochimica Et Biophysica Acta* **74**, 763 (1963).

5 L. Graf, S. Bajusz, A. Patthy, E. Barart, and G. Cseh, *Acta Biochim. Biophys. Acad. Sci. Hung.* **6**, 415 (1971).

6 L. Graf, G. Hajos, A. Patthy, and G. Cseh, *Hormone and Metabolic Research* **5**, 142 (1973).

7 B. Riniker, P. Sieber, W. Rittel, and H. Zuber, *Nature New Biology* **235**, 114 (1972).

8 N. P. Bhatt, K. Patel, and R. T. Borchardt, *Pharmaceutical Research* **7**, 593 (1990).

9 K. Patel, *Pharmaceutical Biotechnology* **5**, 201 (1993).

10 S. Capasso, L. Mazzarella, F. Sica, A. Zagari, G. Cascarano, and C. Giacovazzo, *Acta Crystallographica Section B-Structural Science* **48**, 285 (1992).

muscle aldolase began in 1959, and demonstration that this was the result of deamidation at IleSerAsn(360)HisAlaTyr-COOH near the carboxyl-terminal ends of the monomers was accomplished in 1970.[11] It was also found that the deamidated components increase with age and are more easily digested by proteolytic enzymes.[12] Similar heterogeneity was observed in *Drosophila* aldolase.[13]

In 1972, Midelfort and Mehler demonstrated that Asn(360) is deamidated *in vivo* with $t_{1/2}$ = 6 to 7 days.[14] They found an apparent *in vivo* lifetime for the protein of 38 days, but revised this to 8 days by correction for reuse of the ^{14}C isoleucine tracer.[15] This correction, however, assumed that the *in vivo* degradation rates of the amidated and deamidated forms were the same. Some of this difference may arise from selective *in vivo* digestion of the deamidated form.

In 1974, McKerrow and Robinson found the peptide GlySerAsnHisGly to have $t_{1/2}$ = 6.4 days in pH 7.4, I 0.2, phosphate at 37 °C and suggested, based on the close agreement with the protein, that *in vivo* deamidation and turnover of rabbit muscle aldolase is controlled by primary structure-determined deamidation.[16]

GlySerAsnHisGly has $t_{1/2}$ = 9.0 days in pH 7.4, 37 °C, 0.15 M Tris-HCl, while Asn(360) in rabbit muscle aldolase has $t_{1/2}$ = 9.4 days under these conditions.[17] In the same solution with the protein, the peptide was $t_{1/2}$ = 8.3 days, the difference from 9.0 probably being an artifact of the simultaneous peptide and protein analysis.

Aldolase specific activity in 31-month-old mouse liver is about one-half that of 3-month-old mice, while the total amount of aldolase is unchanged.[18] This same change is also observed in 50-day-old nematodes as compared with 0-day-old.[19] Similarly, deamidated[20] human

11 C. Y. Lai, C. Chen, and B. L. Horecker, *Biochemical and Biophysical Research Communications* **40**, 461 (1970).

12 M. Koida, C. Y. Lai, and B. L. Horecker, *Archives of Biochemistry and Biophysics* **134**, 623 (1969).

13 O. Brenner-Hozach and F. Leuthardt, *Helvetica Chimica Acta* **54**, 302 (1971).

14 C. F. Midelfort and A. H. Mehler, *Federation Proceedings* **31**, A887 (1972).

15 C. F. Midelfort and A. H. Mehler, *Proc. Nat. Acad. Sci. USA* **69**, 1816 (1972).

16 J. H. McKerrow and A. B. Robinson, *Science* **183**, 85 (1974).

17 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001); N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001).

18 H. Gershon and D. Gershon, *Proc. Natl. Acad. Sci* **70**, 909 (1973).

19 P. Zeelon, H. Gershon, and D. Gershon, *Biochemistry* **12**, 1743 (1973).

20 F. Mennecier and J. -C. Dreyfus, *Biochimica et Biophysica Acta* **364**, 320 (1974).

aldolase A accumulates in human striated muscle,[21] and age-dependent accumulation of deamidated aldolase has been demonstrated in rabbit eye lenses.[22] These changes are all consistent with reduced protein turnover with age. A further account of rabbit muscle aldolase deamidation is in chapter 9.

Additional studies with relevance to aldolase deamidation include 1959DB, 1960KB, 1967M, 1968KP, 1969DH, 1969SK, 1972MM2, 1975DG, 1980BB, 1980OB, and 1988HK.

12-4. AMYLASE

Amylase, the first enzyme discovered, was found in malt in 1814 and proved to convert starch into sugar in 1833. Originally called "diastase", it constitutes about 10% of the protein in human saliva. Humans produce more than one gram of α-amylase per day with about 40% in saliva and most of the remainder in the pancreas.[23]

α-amylase is a calcium-requiring endoglycosidase that catalyzes the internal hydrolysis of α-1,4 glycosidic bonds in starch, glycogen, and other glucose polymers. It is named "α" because it leaves its products in the α configuration.[24]

Heterogeneity of crystalline human salivary amylase, which has 511 residues, was noticed in 1953[25] and 1964[26] and suggested in 1971[27] and 1972[28] to result from deamidation. Ten deamidated forms of human amylase were found with 6 in one genetic phenotype of the enzyme and 4 in another,[29] while chicken pancreas amylase had 3 deamidated forms.[30]

Amylase variants include separate gene products in the salivary glands and pancreas, enzymatic Asn-linked glycosylation and

21 E. Steinhagen-Thiessen and H. Hilz, *Mechanisms of Aging and Development* **5**, 447 (1976).

22 J. Banroques, C. Gregori, and F. Shapira, *FEBS Letters* **65**, 204 (1976).

23 C. Arglebe, *Advances in Oto-Rhino-Laryngology* **26**, 97 (1981).

24 J. J. Zakowski and D. E. Bruns, *CRC Critical Reviews in Clinical Laboratory Sciences* **21**, 283 (1984).

25 J. Muus, *C. R. Lab. Carlsberg, Ser. Chim.* **28**, 317 (1953).

26 J. Muus and J. M. Vnenchak, *Biochemistry* **204**, 283 (1964).

27 P. J. Keller, D. L. Kauffman, B. J. Allan, and B. L. Williams, *Biochemistry* **10**, 4867 (1971).

28 N. Jacobsen, K. L. Melvaer, and A. Hensten-Pettersen, *J. Dental Research Supplement to No. 2* , 381 (1972).

29 R. C. Karn, B. B. Rosenblum, J. C. Ward, and A. D. Merritt, *Isozymes, (Ed. C. L. Market), Academic Press, New York* **4**, 745 (1975).

30 L. M. Lehrner and G. M. Malacinski, *Biochemical Genetics* **13**, 145 (1975).

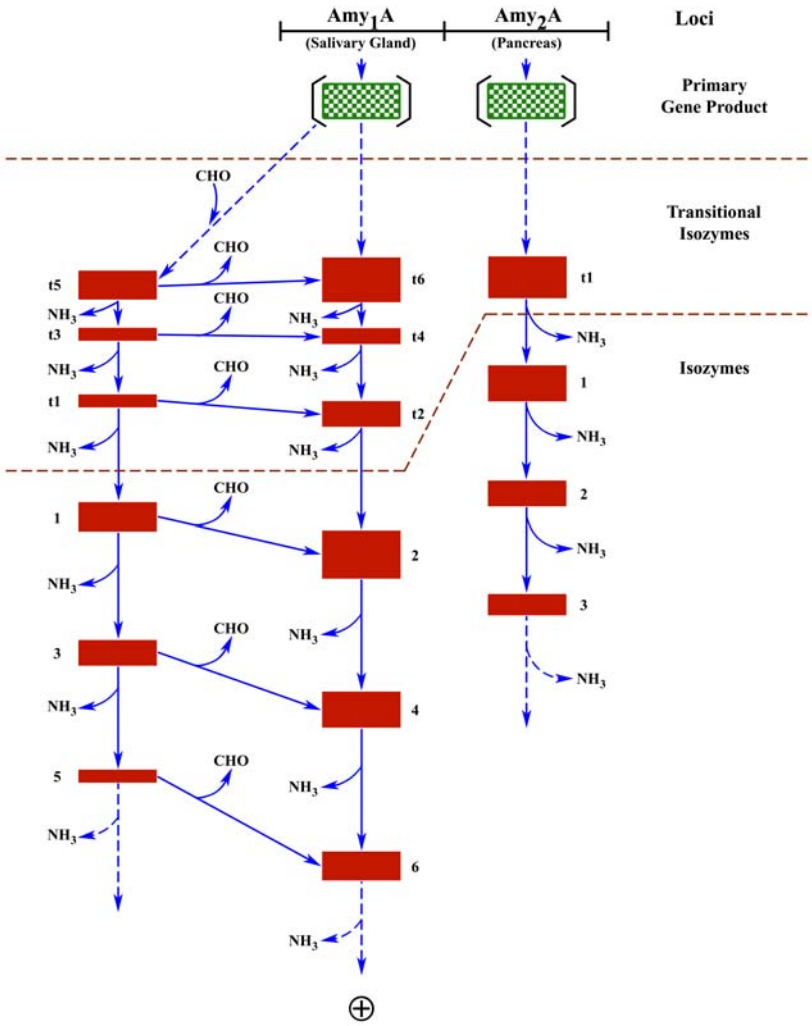

Figure 12-1 Model for the multiple isozymes expressed by the amylase Amy_1 and Amy_2 phenotypes. The position of *t* isozymes, not usually seen in standard electrophoretic systems, is enclosed by horizontal dashed lines. Adapted from 1975KR.

deglycosylation, and a series of deamidations. This situation has been modeled as shown in Figure 12-1.[29] A modified version of this model has been proposed more recently.[31]

A progressive increase in deamidated amylases with age between 0 and 89 years was found in humans.[32] Markedly increased prevalence of deamidated forms was also seen in the saliva of children with cystic fibrosis and their clinically healthy heterozygous parents.[33]

Deamidated forms of pancreatic amylase were found to be increased in victims of acute pancreatitis, and have been attributed to enzymatic deamidation by peptidoglutaminase.[34]

Deamidation $t_{1/2} = 3$, 15, and 25 days in pH 7.4, 25 °C Tris for α-amylase from pancreatic serum, normal pancreatic juice, and normal saliva, respectively, while *in vivo* deamidation was observed for serum and pancreatic juice, but not for saliva.[35]

Many efforts are being made to stabilize bacterial amylases for use in industrial applications at temperatures of 90 °C and higher. The conformational stability varies as a function of bacterial origin with deamidation being the principal limiting parameter.[36] In one study of 175 mutants, only three mutants wherein Asn was replaced at LeuAsn(172)Arg, SerAsn(188)Glu, and GluAsn(190)Gly had enhanced stability at 80 °C, pH 5.6, 0.1 M $CaCl_2$ with substitution of Asn(190) being the most effective. A study of 500 mutants of Baccillus licheniformis α-amylase resulted in a 50 °C range of temperature stability with some of the stabilizing mutations involving Asn.[37] Amylase deamidation can be reduced by sorbitol and other sugars.[38]

31 R. A. Bank, E. H. Hettema, F. Arwert, A. V. Nieuw Amerongen, and J. C. Pronk, *Electrophoresis* **12**, 74 (1991).

32 C. Arglebe, R. Chilla, and M. Opaitz, *Clinical Otolaryngology* **1**, 249 (1976).

33 R. Chilla, C. Arglebe, H. Lubahn, and K. Doering, *Clinical Otolaryngology* **1**, 309 (1976); K. M. Doering, C. Arglebe, H. Lubahn, and R. Chilla, *European Journal of Pediatrics* **126**, 185 (1977); R. Chilla, K. -M. Doering, H. Lubahn, and C. Arglebe, *Archives of Oto-Rhino-Laryngology* **214**, 367 (1977).

34 M. Ogawa, G. Kosaki, K. Matsuura, K. Fujimoto, N. Minamiura, T. Yamamoto, and M. Kikuchi, *Clinica Chimica Acta* **87**, 17 (1978); D. W. Weaver, D. L. Bouwman, A. J. Walt, D. Clink, S. Sessions, and J. Stephany, *Archives of Surgery* **117**, 707 (1982).

35 K. Lorentz and B. Flatter, *Enzyme* **24**, 163 (1979).

36 S. J. Tomazic and A. M. Klibanov, *J. Biological Chemistry* **263**, 3086 (1988); S. J. Tomazic and A. M. Klibanov, *J. Biological Chemistry* **263**, 3092 (1988).

37 N. Declerck, M. Machius, P. Joyet, G. Wiegand, R. Huber, and C. Gaillardin, *Biologia, Bratislava* **57**(Suppl. 11), 203 (2002).

38 K. Khajeh and M. Nemat-Gorgani, *Applied Biochemistry and Biotechnology* **90**, 47 (2001).

Additional studies with relevance to amylase deamidation include 1973J, 1976LM, 1979PF, 1979BM, 1979L, 1980R, 1977MK1, 1982L, 1984ZB, 1989B, 1984ZG, 1983MO, 2001KN.

12-5. APOLIPOPROTEIN

The deamidation of high-density lipoprotein was reported in 1971.[39] Heterogeneity of apolipoprotein A-I, a major high-density lipoprotein responsible for blood lipid transport and activation of lecithin:cholesterol acyltransferase,[40] was observed[41] and then characterized as involving six variants of differing charge with variants also found in apolipoproteins A-II, A-IV, B, C-II, C-III, E, and H.[42]

It has been shown[43] that conversion of apolipoprotein A-I proceeds stepwise from A-I_1 through A-I_5 with A-I_3 being the principal species. Deamidation half-times for A-I_3 and A-I_4 at pH 7.52, 0.1 M phosphate, 37 °C were 23 days and 28 days, respectively. In buffered plasma, A-I_3 and A-I_4 deamidation half-times were 26 and 25 days. *In vivo* residence times for A-I_3 and A-I_4 were 3.5 and 3.0 days, while their *in vivo* degradation rates were 8.8 and 1.7 days.

Recent analysis with high resolution immobilized gradient isoelectric focusing and time-of-flight mass spectrometry identified 12 isoforms of apolipoprotein A-I, substantially more than the original 5. It is evident that this protein undergoes deamidation at rates similar in magnitude to its *in vivo* degradation rate, although understanding of its many isoforms and their metabolic purposes and fates awaits further experimentation.

Additional studies with relevance to apolipoprotein deamidation include 1986MW, 1990MF, and 1992GW.

39 J. J. Albers, L. V. Albers, and F. Aladjem, *Biochemical Medicine* **5**, 48 (1971).

40 C. K. Mathews and K. E. van Holde, *2nd Edition, Benjamin/Cummings, Menlo Park* , 624 (1996).

41 A. C. Nestruck, G. Suzue, and Y. L. Marcel, *Biochimica et Biophysica Acta* **617**, 110 (1980); G. Ghiselli, E. J. Schaefer, J. A. Light, and H. B. Brewer Jr., *J. Lipid Research* **24**, 731 (1983).

42 D. L. Sprecher, L. Taam, and H. B. Brewer, Jr., *Clinical Chemistry* **30**, 2084 (1984).

43 G. Ghiselli, M. F. Rohde, S. Tanenbaum, S. Krishnan, and A. M. Gotto, Jr., *J. Biological Chemistry* **260**, 15662 (1985).

12-6. Bcl-x_L

Bcl-x_L is a member of the Bcl-2 family of proteins. It is found primarily on the outer membrane of mitochondria and inhibits a common pathway of cell death, or apoptosis.[44] It may play a special role in development and regulation within the human brain.[45]

Preparations of rat Bcl-x_L were found to contain both an amidated form and one that had been deamidated at AlaIleAsn(52)GlyAsn and AlaValAsn(66)GlyAla. Crystal structures of these forms showed identical backbone configurations in the deamidating regions.[46] Upon denaturation, a third sequence, GlnGluAsn(185)GlyGly, was also deamidated. Deamidation calculations showed that Asn(52) and Asn(66) have deamidation coefficients C_D = 0.01, while C_D for Asn(185) is 5.4.[47] Denaturation evidently disrupted the higher order structure suppression of deamidation. These deamidated forms have also been observed *in vivo* in normal human liver.[48]

Bcl-x_L functions as a molecular clock in the cellular response to DNA damage in human fibroblasts.[49] The deamidation rates of Asn(52) and Asn(66) introduce an approximately 24 hour delay into this system.[50] If this deamidation is not stopped through timely DNA repair, Bcl-x_L loses its ability to prevent apoptosis and cell death occurs.[50]

This pathway is of special interest because it may explain the unusual susceptibility of cancer cells to DNA-damaging agents. Cancer cells lack Rb proteins and p53, which is also involved in control of apoptosis. On the basis of experiments with liver tumors, it has been

44 T. Takehara and H. Takahashi, *Cancer Research* **63**, 3054 (2002).

45 O. Sohma, M. Mizuguchi, S. Takashima, M. Yamada, K. Ikeda, and S. Ohta, *J. Neuroscience Research* **43**, 175 (1996); M. Mizuguchi, O. Sohma, S. Takashima, K. Ikeda, M. Yamada, N. Shiraiwa, and S. Ohta, *Brain Research* **712**, 281 (1996).

46 M. Aritomi, N. Kunishima, N. Inohara, Y. Ishibashi, S. Ohta, and K. Morikawa, *J. Biological Chemistry* **272**, 27886 (1997).

47 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

48 T. Takehara and H. Takahashi, *Gastroenterology* **118**, 2433 (2000).

49 B. E. Deverman, B. L. Cook, S. R. Manson, R. A. Niderhoff, E. M. Langer, I. rosova, L. A. Kulans, X. Fu, J. S. Weinberg, J. W. Heinecke, K. A. Roth, and S. J. Weintraub, *Cell* **111**, 51 (2002); B. E. Deverman, B. L. Cook, S. R. Manson, R. A. Niederhoff, E. M. Langer, I. Rosova, L. A. Kulans, X. Fu, J. S. Weinberg, J. W. Heinecke, K. A. Roth, and S. J. Weintraub, *Cell* **115**, 503 (2003); S. J. Weintraub and S. R. Manson, *Mechanisms of Ageing and Development* **125**, 255 (2004); D. R. Alexander, *Cell Cycle* **3**, e19 (2004); T. Yoshikawa, K. Nariai, H. Uga, Y. Yumoto, M. Date, and H. Takahashi, *Hepatology* **38**, 761A (2003); R. Zhao, F. T. Yang, and D. R. Alexander, *Cancer Cell* **5**, 37 (2004); S. J. Weintraub, S. R. Manson, and B. E. Deverman, *Cancer Cell*, 3 (2004).

50 S. J. Weintraub and S. R. Manson, *Mechanisms of Ageing and Development* **125**, 255 (2004).

further proposed that malignant tumors acquire resistance to apoptosis by suppression of Bcl-x_L deamidation.

The presence of these two time-delay amide molecular clocks in Bcl-x_L has been described as a "chronometric buffer".[50]

The relevant biochemistry of Bcl-x_L and its importance to the development of anti-cancer drugs has been reviewed.[51]

12-7. COLLAGEN

Collagen, the primary component of connective tissue, comprises about one-third of the protein in the human body.[52] The amides of cow collagen are labile in 0.2 N HCl between 75 and 100 °C with a deamidation half-time at 75 °C of 0.15 days.[53]

The half-times for release of a single amide from kangaroo tail tendon in 0.1 M phosphate at pH 7.35 were found to be 8.1, 40, 180, and 290 minutes at temperatures of 100, 77, 65.6, and 60 °C, respectively.[54] This suggested that deamidation at lower temperatures would be facile.

Collagen, however, undergoes thermal shrinkage within a few minutes at lower temperatures. At 45 °C, kangaroo tail tendon release of ammonia was reduced to a level indistinguishable from background. If the tendon were first exposed to 65 °C for 5 minutes, then deamidation at 45 °C proceeded with a single-amide half-time of 1750 minutes. Similar results were obtained for rat tail tendon[55].

Evidently, the deamidation of this collagen is markedly suppressed by higher order structure, which must be disrupted before deamidation proceeds at the fast rate observed at higher temperatures.

During the tanning of leather by liming with 3% $Ca(OH)_2$ at 25 °C, it was found that the initial deamidation was not accompanied by formation of D-Asp. D-Asp, however, accompanied deamidation after 24 hours. It was concluded that hydrolysis was the primary mechanism of deamidation until the helical structure of collagen was sufficiently disrupted to allow succinimide formation.

51 C. Li and C. B. Thompson, *Science* **298**, 1346 (2002).

52 J. Gross, *Scientific American* **204**, 121 (1961).

53 J. M. Cassel and E. McKenna, *American Leather Chemists Association Journal* **48**, 142 (1953).

54 F. M. Sinex, *J. Gerontology* , 15 (1960).

55 O. Menderes, A. D. Covington, E. R. Walte, and M. J. Collins, *J. Society of Leather Technologists and Chemists* **83**, 107 (1999).

Therefore, it is to be expected that collagen deamidation probably proceeds primarily by hydrolysis in the human body and in other organisms that live at ordinary temperatures.

Hydrolysis of Asn and Gln proceeds with half-times in the range of about 10,000 days at 37 °C,[56] so studies of collagen fractions with very slow turnovers should show results similar to those for crystallins. As yet, no such studies have been reported.

Substantial heterogeneity in collagen preparations has been observed and sometimes attributed to deamidation, but this has not been experimentally verified. Additional studies of interest include 1965V, 1969B, 1969B1, 1970B, 1971B, 1972GP, 1972GP1, 1975GP, 1976BM, 1981B, 1981TH, 1982K, 1987BL, 1997RS, and 1998SB.

12-8. CRYSTALLIN AND MAJOR INTRINSIC PROTEIN

Crystallins, which comprise 90% of the soluble proteins in vertebrate eye lenses,[57] have been of special interest because human eye lenses undergo degenerative changes with age and because these proteins are thought to have *in vivo* turnover times of many years. The crystallins, therefore, provide a living laboratory in which to study very slowly deamidating proteins.

In 1969, calf eye lens α-crystallin, which is 30% of the water-soluble eye lens protein of mammals,[58] was found in two forms designated αA_1 and αA_2, differing with respect to amidation, changing in proportion with age and development, and thought to be the products of different genes.[59] It was shown, however, that this difference is not of genetic origin.[60] In 1972 evidence was presented that αA_1 crystallin results from postsynthetic deamidation of αA_2 crystallin at Gln(9).[61] This find-

56 N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

57 H. Bloemendal, *Science* **197**, 127 (1977).

58 G. J. van Kamp and H. J. Hoenders, *Experimental Eye Research* **17**, 417 (1973).

59 J. G. G. Schenmakers, J. J. G. Gerding, and H. Blemendal, *European Journal of Biochemistry* **11**, 472 (1969).

60 W. G. Palmer and J. Papconstantinou, *Proc. Natl. Acad. Sci. USA* **64**, 404 (1969); J. Delcour and J. Papaconstantinou, *Biochemical and Biophysical Research Comminications* **41**, 401 (1970).

61 H. Bloemendal, A. J. M. Berns, F. V. Der Ouderaa, and W. W. W. De Jong, *Experimental Eye Research* **14**, 80 (1972).

ing was extended to show that the conversion of αB_2 into αB_1 also involves deamidation and is age correlated.[62]

Van Kleef, de Jong, and Hoenders[63] measured the degradation of deamidated αA_2 and αB_2 in 22-week old calf lenses as a function of position in the lens between the cortex and nucleus, where the inner layers near the cortex were considered the older tissues. Degradation increased with nearness to the nucleus, with most of the αB_2 and about one-third of the αA_2 degradation attributable to deamidation. The remainder in αA_2 was chain cleavage at AsnGlu, AspAla, SerSer, and SerAla.

Deamidation in the innermost layers of the lens was about 20% for both proteins, corresponding, during the 22 weeks, to a deamidation half-time of about 500 days. The deamidating sequence in αA_2 was reported to be ProSerAsn(123)ValAsp. In human lenses, this deamidation occurred primarily in crystallin synthesized before birth and during infancy, but did not continue during adulthood.[64]

By 1979, the number of deamidated forms observed in human αA_2 and αB_2 had increased to 2 and 3, respectively, and it had been suggested that deamidated forms were involved in cataract formation.[65] Deamidation of β and γ crystallins was also observed.[66] However, in 1987, it was found that the heterogeneity originally attributed to deamidation of Asn(123) in αA_2 is actually due to phosphorylation of Ser.[67]

Gradually improving analytical methods were then brought to bear on this system in which long-term *in vivo* postsynthetic modifications of proteins can be observed. The result has been a steadily increasing number of reports of crystallin modification through acetylation, chain cleavage, deamidation, methylation, oxidation, phosphorylation, and other reactions. Of these, deamidation is the most prevalent. Moreover, as a result of the long incubation periods in living lenses, extensive Gln

62 H. J. Hoenders, G. J. van Kamp, L. Leim-The, and F. S. M. van Kleef, *Experimental Eye Research* **15**, 193 (1973); F. v. Kleef and H. J. Hoenders, *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie* **355**, 1216 (1974).

63 F. S. M. van Kleef, W. W. de Jong, and H. J. Hoenders, *Nature* **258**, 264 (1975).

64 J. J. Harding, *Biology of the Epithelial Lens Cells, INSERM* **60**, 215 (1976).

65 P. M. M. van Haard, G. J. M. Martens, J. J. L. Mestrom, H. J. . Hoenders, and J. Wollensak, *Ophthalmic Research* **11**, 433 (1979).

66 A. A. Krichevskaya, A. I. Lukash, N. V. Pushkina, I. V. Shepotinovskaya, and K. B. Sherstnev, *Nauchnye Doki Vyss Shkoly Biologicheskie Nauki* **7**, 23 (1984); J. A. Thomson and R. C. Augusteyn, *Experimental Eye Research* **40**, 393 (1985).

67 R. Chiesa, M. A. Gawinowicz-Kolks, and A. Spector, *J. Biological Chemistry* **262**, 1438 (1987).

deamidation, which is probably largely nonenzymatic, has been observed. Emphasis has been on human lens crystallins, but other species have been investigated. Table 12-1 summarizes reports of crystallin deamidation where the deamidating residue has been reported.

Crystallins have turned out to have multiple functions. Bird and crocodile ε-crystallin is also lactate dehydrogenase.[68] Bovine α-crystallin is a heat-shock protein.[69] αB crystallin is also a heat-shock protein and an important component of ubiquinated inclusion bodies in human degenerative diseases.[70]

The most remarkable changes in human eye lens crystallin were found to occur during the first 15 to 20 years of life.[71] A protease was found in bovine lens that can, as was shown in peptide specificity experiments, cleave α-crystallin at many of its principal cleavage sites, including that at Asn(101)Glu.[72]

The hazard in using *in vivo* single point determinations of extent of deamidation of amides in the eye lenses of human adults as indications of deamidation rates has been demonstrated by Takemoto and Boyle.[73] As shown in Figures 12-2 and 12-3 adapted from their papers, Gln(50)SerLeu in αA crystallin shows no deamidation between ages 0 and 25 years and then deamidates linearly with a half-time of 28,000 days, while ThrIleGln(6)HisPro and IleGln(147)ThrGly show no deamidation in 64 years. Conversely, Asn(101) deamidates with an apparent half-time of 11,000 days for the first 25 years and then abruptly slows to a half-time of 140,000 days. The Asn(101) deamidation half-time is apparently a combination of 6,000 days in the high molecular weight and 27,000 days in the low molecular weight fractions of αA crystallin.[74]

68 G. J. Wistow, J. W. M. Mulders, and W. W. de Jong, *Nature* **326**, 622 (1987).

69 J. Horwitz, *Proc. Natl. Acad. Sci. USA* **89**, 10449 (1992); L. Takemoto, T. Emmons, and J. Horwltz, *Biochemical Journal* **294**, 435 (1993).

70 P. J. T. A. Groenen, K. B. Merck, W. W. De Jong, and H. Bloemendal, *European Journal of Biochemistry* **225**, 1 (1994).

71 L. Takemoto, *Experimental Eye Research* **60**, 721 (1995).

72 K. K. Sharma, K. Kester, and N. Elser, *Biochemical and Biophysical Research Communications* **218**, 365 (1996).

73 L. Takemoto and D. Boyle, *Biochemistry* **37**, 13681 (1998); L. J. Takemoto, *Current Eye Research* **17**, 247 (1998); L. Takemoto and D. Boyle, *Experimental Eye Research* **67**, 119 (1998); L. Takemoto and D. Boyle, *Investigative Ophthalmology and Visual Science* **39**, 4693 (1998).

74 L. Takemoto, *Experimental Eye Research* **68**, 641 (1999).

Table 12-1 Crystallins Found Deamidated

Source	Peptide or Protein	Sequence	Conditions	Apparent Deamidation Half-Time - Without Consideration of Synthesis and Turnover	Reference
Chicken - 4 month old	αA	ProSerAsn(149)MetAsp	In vivo	450 day	1987VR
Bovine	αA	HisAsn(101)GluArg	In vivo		1988VH
Chicken	αA	ProSerAsn(149)MetAsp	In vivo	511 - 120 days, 365 - 365 days, 2101 - 3,650 days	1988JM
Human	αA	SerAlaAsn(136)GlyMet	Sequencing		1975JT
Human - 20 to 27 years old	αA	Asn(101)	In vivo		1994MZ
Human - 20 to 27 years old	αA	Gln(6)	In vivo		1994MZ
Human - 20 to 27 years old	αA	TyrArgGln(50)SerThr	In vivo		1994MZ
Human - 20 to 27 years old	αA	IleGln(147)ThrGly	In vivo		1994MZ
Human - 45 years old	αA	ThrIleGln(6)HisPro	In vivo	146000	1996LS
Human - 45 years old	αA	TyrArgGln(50)SerLeu	In vivo	14000	1996LS
Human - 45 years old	αA	LysValGln(90)AspAsp	In vivo	146000	1996LS
Human - 45 years old	αA	LysHisAsn(101)GluArg	In vivo	85000	1996LS
Human - 45 years old	αA	LysIleGln(147)ThrGly	In vivo	85000	1996LS
Human - 63 and 77 years old	αA	ThrIleGln(6)HisPro	In vivo		1994YC
Human - 63 and 77 years old	αA	LysValGln(89)AspAsp	In vivo		1994YC
Humans 0 to 64 years	αA	Gln(50)SerLeu	In vivo	0 for 25 years, then 28,000	1998TB1
Humans 0 to 68 years	αA	HisAsn(101)Glu	In vivo	11,000 for 25 years, then 140,000	1998T
Humans 50 to 65 years	αA	Gln(6)	In vivo	44,000/28,000 - 13-30 kD/>30 kD	2000HH
Humans 50 to 65 years	αA	Gln(90)	In vivo	56,000/39,000 - 13-30 kD/>30 kD	2000HH
Humans 50 to 65 years	αA	Gln(104)	In vivo	78,000 - 13-30 kD	2000HH
Humans 50 to 65 years	αA	Gln(147)	In vivo	154,000/90,000 - 13-30 kD/>30 kD	2000HH
Calf lens nucleus - 22 week old	αA2	ProSerAsn(123)ValAsp	In vivo	500 days	1975KJ
Bovine	αB	ValAsn(146)Gly	In vivo	5,700 - 5-year old cows	1993GD
Human - 45 years old	αB	Asn(108)	In vivo	146000	1996LS

Source	Peptide or Protein	Sequence	Conditions	Apparent Deamidation Half-Time - Without Consideration of Synthesis and Turnover	Reference
Human - 45 years old	αB	Asn(146)	In vivo	85000	1996LS
Humans 50 to 65 years	αB	Asn(78)	In vivo	28,000/34,000 - 13-30 kD/>30 kD	2000HH
Humans 50 to 65 years	αB	Gln(108)	In vivo	62,000/34,000 - 13-30 kD/>30 kD	2000HH
Humans 60 to 80 years	αB	ThrValAsn(146)GlyPro	In vivo	Amidated and deamidated in normal/only deamidated in cataract	2003SS
Calf lens nucleus - 22 week old	αB2	Not known	In vivo	500 days	1975KJ
Bovine	αB2	ThrValAsn(146)GlyPro	Sequencing		1974OH
Human - 28 years old	βA3	Asn(54)	In vivo		2003ZS
Human - 28 years old	βA3	Gln(180)	In vivo		2003ZS
Humans 50 to 65 years	βA3	Gln(38)	In vivo		2000HH
Humans 50 to 65 years	βA3	Gln(42)	In vivo		2000HH
Human - 28 years old	βA4	Gln(62), Gln(64), Gln(65)	In vivo		2003ZS
Human - 28 years old	βB1	Asn(15)	In vivo		2003ZS
Human - 28 years old	βB1	Gln(105)	In vivo		2003ZS
Human - 28 years old	βB1	Asn(107)	In vivo		2003ZS
Human - 28 years old	βB1	Gln(222), Gln(224), Gln(226)	In vivo		2003ZS
Humans 50 to 65 years	βB1	Asn(157)	In vivo	38,000/29,000 - 13-30 kD/>30 kD	2000HH
Human - 28 years old	βB2	Asn(15)	In vivo		2003ZS
Human - 28 years old	βB2	Gln(5), Gln(7), Gln(12)	In vivo		2003ZS
Human - 28 years old	βB2	Gln(5), Gln(7), Gln(12), Gln(182), Gln(184)	In vivo		2003ZS
Bovine	βS	LeuProAsn(143)TyrArg	In vivo		1990TE
Humans - 0, 19, 55 years old	γC	Asn(24) and Gln(26)	In vivo	33,000	1998HS1
Humans - 0, 19, 55 years old	γC	Gln(66) and Gln(67)	In vivo	1st - 60% at 0 and 100% at 19 years, 2nd 15,000	1998HS1
Humans - 0, 19, 55 years old	γC	Asn(103)	In vivo	100% at 19 years	1998HS1
Humans - 0, 19, 55 years old	γC	Gln(142) and Gln(148)	In vivo	40% at 0, 60% at 19 years, and 42% at 55 years	1998HS1

Source	Peptide or Protein	Sequence	Conditions	Apparent Deamidation Half-Time - Without Consideration of Synthesis and Turnover	Reference
Humans - 4 days, 19, 45 years old	γD	HisProAsn(24)leuGln(26)ProTyr	In vivo	1st - 45% at 4 days and 100% at 19 years, 2nd 13,000	1998HS1
Humans - 4 days, 19, 45 years old	γD	TyrGluGln(47)ProAsn(49)TyrSer GlyLeuGln(54)TyrPhe	In vivo	8000	1998HS1
Humans - 4 days, 19, 45 years old	γD	AspHisGln(67)Gln(68)TrpMet	In vivo	1st - 24,000, 2nd 220,000	1998HS1
Humans - 4 days, 19, 45 years old	γD	ArgPheAsn(118)GluIleHisSerLeu Asn(124)ValLeuGluGlySerTrpVal LeuTyrGluLeuSerAsn(137)TyrArg	In vivo	1st -100% 19 years, 2nd 12,000	1998HS1
Human	γG3	LeuProAsn(138)TyrArg	In vivo		1990TE
Human - 63 and 77 years old	γS	AlaValGln(170)SerPhe	In vivo		1994YC
Humans - 32 weeks, 31, 55 years old	γS	Gln(53), Gln(63), Gln(70)	In vivo	82 days	1998HS1
Humans - 32 weeks, 31, 55 years old	γS	Gln(92)	In vivo	28000	1998HS1
Humans - 32 weeks, 31, 55 years old	γS	Gln(106), Gln(120)	In vivo	63% at 224 days, 75% at 31 years - 5,700	1998HS1
Humans - 32 weeks, 31, 55 years old	γS	Gln(170)	In vivo	32000	1998HS1
Humans 50 to 65 years	γS	Asn(76)	In vivo		2000HH
Humans 50 to 65 years	γS	Gln(92)	In vivo	59,000/51,000 - 13-30 kD/>30 kD	2000HH
Humans 50 to 65 years	γS	Gln(170)	In vivo	154,000/73,000 - 13-30 kD/>30 kD	2000HH
Humans 59 to 70 years	γS	LeuProAsn(143)TyrArg	In vivo	54% or 21,000 in cataracts vs. less than 10% in normals	2000TB
Humans 75 to 87 years	γS	Asn(14)	In vivo	190,000/22,000 - water soluble/water insoluble	2002LP1
Humans 75 to 87 years	γS	Gln(16)	In vivo		2002LP1

Source	Peptide or Protein	Sequence	Conditions	Apparent Deamidation Half-Time - Without Consideration of Synthesis and Turnover	Reference
Humans 75 to 87 years	γS	Asn(37)	In vivo	123,000/123,000 - water soluble/water insoluble	2002LP1
Humans 75 to 87 years	γS	Asn(53)	In vivo	47,000 - water insoluble	2002LP1
Humans 75 to 87 years	γS	Gln(63)	In vivo	90,000 - water insoluble	2002LP1
Humans 75 to 87 years	γS	Gln(70)	In vivo		2002LP1
Humans 75 to 87 years	γS	Asn(76)	In vivo	240,000 - water insoluble	2002LP1
Humans 75 to 87 years	γS	Gln(92)	In vivo	90,000 - water insoluble	2002LP1
Humans 75 to 87 years	γS	Gln(96)	In vivo		2002LP1
Humans 75 to 87 years	γS	Gln(106)	In vivo		2002LP1
Humans 75 to 87 years	γS	Gln(120)	In vivo	34,000/22,000 - water soluble/water insoluble	2002LP1
Humans 75 to 87 years	γS	Asn(143)	In vivo	90,000/29,000 - water soluble/water insoluble	2002LP1
Humans 75 to 87 years	γS	Gln(148)	In vivo		2002LP1
Humans 75 to 87 years	γS	Gln(170)	In vivo		2002LP1

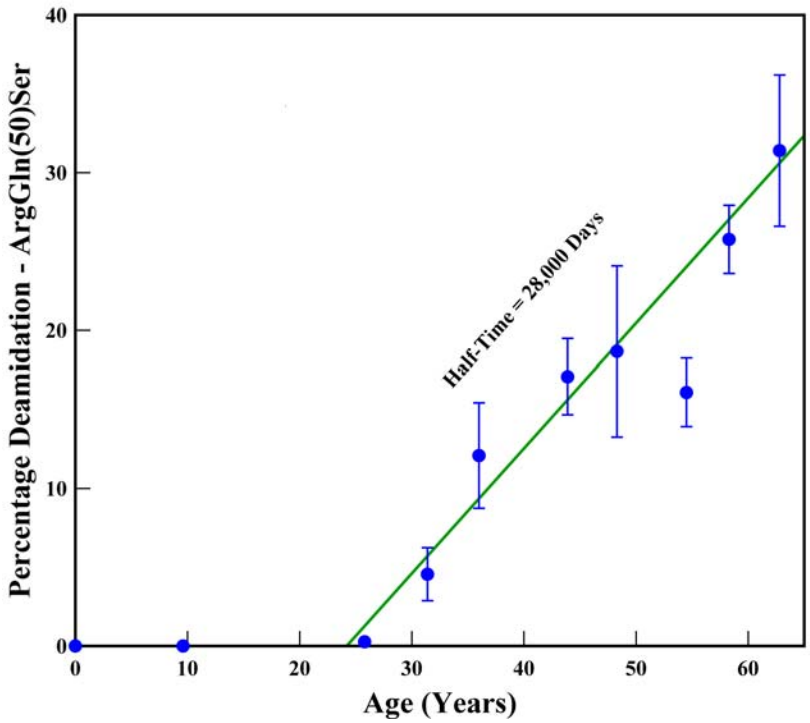

Figure 12-2 Deamidation of Gln(50) in human αA crystallin. Adapted from 1998TB1.

Hardening of human lenses begins at about 30 years.[75] These changes in deamidation at 25 years could result from 3-dimensional or other changes that affect deamidation rates, or they could be secondary to changes in protein turnover and other metabolic processes. A difference in deamidation as a function of molecular weight or aggregation has also been observed in a collection of 6 Gln and 2 Asn deamidations in αA, αB, αB_1, and γS-crystallins from 50 to 65-year old subjects where the apparent deamidation half-time was 41,000 in the >30 kD fraction and 56,000 in the 13 to 30 kD fraction.[76]

In 75 to 87-year old subjects, 3 Asn and 1 Gln deamidation half-times averaged 49,000 in water-insoluble and 109,000 in wa-

75 N. Fujii, L. J. Taikemoto, Y. Momose, S. Matsumoto, K. Hiroki, and M. Akaboshi, *Biochemical and Biophysical Research Communications* **265**, 746 (1999).

76 S. R. A. Hanson, A. Hasan, D. L. Smith, and J. B. Smith, *Experimental Eye Research* **71**, 195 (2000).

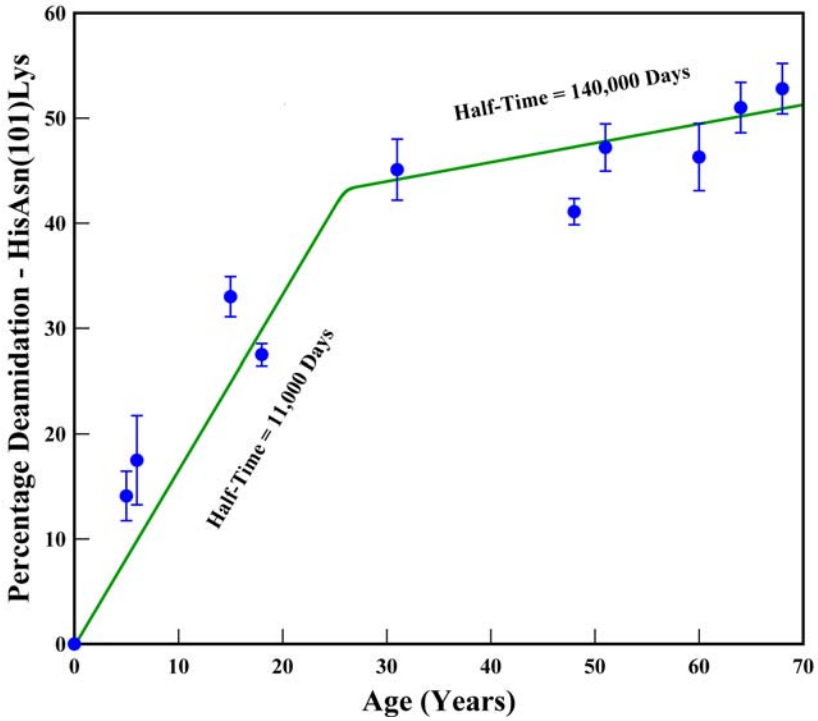

Figure 12-3 Deamidation of Asn(101) in human αA crystallin. Adapted from 1998T.

ter-soluble γS-crystallin, respectively.[77] Also, PheAsn(143)Tyr in high molecular weight aggregates with more than 5,000 molecules of γS-crystallin from 54-year old lens was found 38% deamidated, and no deamidation was observed in the low molecular weight fraction. No deamidation of Gln(92) or Gln(96) was observed in either fraction.[78]

Takemoto and Boyle found no difference in the extent of αA crystallin deamidation in lenses with cataracts and age matched controls with HisAsn(101)LysArg, Gln(50)SerPhe, and IleGln(6)HisPro deamidated 53%, 16%, and 0%, respectively in humans of ages 59 to 70 years.[79] In γS-crystallin, however, they found an average of 54% deamidation of LeuProAsn(143)TyrArg in cataracts and less than 10%

77 V. N. Lapko, A. G. Purkiss, D. L. Smith, and J. B. Smith, *Biochemistry* **41**, 8638 (2002).

78 L. Takemoto, *Current Eye Research* **22**, 148 (2001).

79 L. J. Takemoto and D. Boyle, *Investigative Ophthalmology and Visual Science* **40**, 2755 (1999); L. Takemoto and D. Boyle, *Molecular Vision* **5**, U1 (1999).

in normal lenses from 59 to 70 year-old subjects.[80] The Asp(143) deamidation product in these cataracts had an average isoAsp:Asp ratio of 1.52:1 and average D/L isomer of 0.27, demonstrating that at least part of this deamidation occurred through the imide mechanism.[81] In the αB-crystallin of 60 to 80-year old subjects, both undeamidated and deamidated ThrValAsn(146)GlyPro was found in normal lenses, whereas only the deamidated form was present in cataracts.[82]

Takemoto and Boyle found no deamidation in GlyGlyGln(92)TyrLys IleGln(96)IlePhe, LeuProAsn(143)TyrArg, and AlaValGln(170)SerPhe in γS-crystallin from the central core region of 60-year old human lenses, even though these proteins are synthesized during the fetal-embryonic period of development.[83] They cite this as support for the hypothesis that proteins with more stable amides are more resistant to protein turnover.[84]

Comparative solution studies of dimeric βB_1-crystallins that have Gln(204) vs. Glu(204) showed a less compact structure, decreased urea stability, decreased heat stability, and increased chaperone requirement for the Glu(204) form,[85] while deamidation of Gln(146) increased βB_1 aggregation[86] and diminished chaperone activity.[87] Deamidation has been observed to decrease chaperone activity in αB-crystallin[88].

Additional reports concerning crystallin deamidation include 1971CW, 1972DP, 1972C, 1972H, 1973OJ, 1973JK, 1973VH,

80 L. Takemoto and D. Boyle, *Molecular Vision* **6**, 164 (2000).

81 L. Takemoto, N. Fujii, and D. Boyle, *Experimental Eye Research* **72**, 559 (2001).

82 O. P. Srivastava and K. Srivastava, *Molecular Vision* **9**, 110 (2003).

83 L. J. Takemoto and D. Boyle, *Investigative Ophthalmology and Visual Science* **41**, S586 (2000); L. Takemoto and D. Boyle, *J. Biological Chemistry* **275**, 26109 (2000).

84 A. B. Robinson, J. H. McKerrow, and P. Cary, *Proc. Natl. Acad. Sci. USA* **66**, 753 (1970); A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **71**, 885 (1974); A. B. Robinson and L. R. Robinson, *Proc. Natl. Acad. Sci. USA* **88**, 8880 (1991); N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

85 K. J. Lampi, J. T. Oxford, H. P. Bachinger, T. R. Shearer, L. L. David, and D. K. Kapfer, *Experimental Eye Research* **72**, 279 (2001); Y. H. Kim, D. M. Kapfer, J. Boekhorst, N. H. Lubsen, H. P. Bachinger, T. S. Shearer, L. L. David, J. B. Feix, and K. J. Lampi, *Biochemistry* **41**, 14076 (2002); K. J. Lampi, Y. H. Kim, H. P. Bachinger, B. A. Boswell, R. A. Lindner, J. C. Carver, T. R. Shearer, L. L. David, and D. M. Kapfer, *Molecular Vision* **8**, 359 (2002).

86 M. J. Harms, P. A. Wilmarth, D. M. Kapfer, E. A. Steel, L. L. David, H. P. Bachinger, and K. J. Lampi, *Protein Science* **13**, 678 (2004).

87 R. Gupta and O. P. Srivastava, *Investigative Ophthalmology and Visual Science* **45**, 206 (2004).

88 R. Gupta and O. P. Srivastava, *Investigative Ophthalmology and Visual Science* **44**, 2362 (2003).

1973HK, 1974SW, 1974VJ, 1974KH, 1975DH, 1976HD, 1976VW, 1976TA, 1977VH, 1977B, 1977MB, 1977SH, 1978DB1, 1978MB, 1978KD, 1978DB, 1978O, 1981H, 1982HB, 1982S, 1983HB, 1986KK, 1990GI, 1992SV, 1994DL, 1994FI, 1995T1, 1997T, 1997FM, 1998LM, 1998MH, 1999FT, 2000WR, 2000FS, 2000WB, 2000FS1, 2001ZD, 2002CG, 2002KN, and 2003LH.

The crystallins of the human eye lens offer an opportunity for the study of a multitude of deamidating amides over very long time periods. Given the long lifetime of many lens proteins, it is likely that a detectable amount of deamidation will ultimately be observed in most of their amide residues.

It is evident, however, that *in vivo* changes in these lenses with age require quantitative measurements of deamidation as a function of time such as those illustrated in Figures 12-2 and 12-3. These studies, combined with fundamental knowledge about protein deamidation and separate deamidation measurements of purified crystallins, may make possible the thorough understanding of eye lens crystallin deamidation. This, in turn, may illuminate the effects of amide molecular clocks in other proteins that are involved in organismic development and aging.

Another protein class, Major Intrinsic Protein or MIP, makes up a large part of eye lens protein content. This is a membrane protein, which is thought to have six transmembrane domains.

Fetal bovine lens MIP includes GluSerAsn(246)GlyGln, while mature bovine lens MIP is 94% deamidated to GluSerAsp(246)GlyGln.[89] Rat MIP contains a similar sequence AspSerAsn(244)GlyGln.[90]

Human MIP has been found to contain two sequences, ValSerAsn(246)GlyGln and GluLeuAsn(259)ThrGln, that are completely deamidated by the age of 7 years.[91] Human MIP undergoes substantial cleavage at both ends of the molecule in the regions of residues 1 to 37 and 196 to 259. This cleavage proceeds linearly with age, with 100% cleavage at about age 90 years.

89 L. Takemoto and T. Emmons, *Curr Eye Res* **10**, 865 (1991); K. L. Schey, J. G. Fowler, J. C. Schwartz, M. Busman, J. Dillon, and R. C. Crouch, *Investigative Ophthalmology and Visual Science* **38**, 2508 (1997).

90 N. Dilsiz and M. J. J. Crabbe, *Biochemical Journal* **305**, 753 (1995).

91 M. C. Lai, R. L. Schowen, R. T. Borchardt, and E. M. Topp, *J. Peptide Research* **55**, 93 (2000).

12-9. CYTOCHROME C

The biochemistry of this beautiful and multifaceted protein has enriched the lives of several generations of chemists, biochemists, and biophysicists and is still the subject of intensive study. The three-dimensional structure was elucidated more than 30 years ago by Dickerson and co-workers. This structure and the early biochemistry have been reviewed.[92]

The *in vivo* turnover rate of cytochrome c in rats was first measured in 1946 and 1957.[93] In 1957, Palèus and Theorell discovered three forms in crystallized beef heart muscle cytochrome c during electrophoresis at pH 7.2. The fraction with the highest positive charge comprised about 80% of the total.[94] In 1960, Horio reported that the altered forms of cytochrome c are more easily digested with proteolytic enzymes.[95]

Studies of cow heart cytochrome c heterogeneity were reported in 1964,[96] but these reports were still attributed by many investigators to artifacts with only the primary fraction believed to exist *in vivo*.[97]

In 1966, Flatmark reported that cow heart cytochrome c deamidates sequentially[98] from I to II to III. The deamidation half-times of I and II in 37 °C, I 0.1, pH 7.4, borate were 12 days and 5 days, respectively. He found that these deamidations increase in rate with increasing ionic strength and temperature and have a pH minimum at about pH 5. After initial confusion caused by the more facile deamidation of free glutamine vs. free asparagine, AlaThrAsn(103)GluCOOH at the c-terminus end of cytochrome c was identified with conversion of I to II.

Using isoelectric focusing, Flatmark and Vesterberg resolved cytochrome II into two components[99] with one component present in much greater amount than the other. Isoelectric focusing showed a 0.23 pH unit change in isoelectric point per deamidation. Vesterberg also ap-

92 R. E. Dickerson, T. Takano, D. Eisenberg, O. B. Kallai, L. Samson, A. Cooper, and E. Margoliash, *J. Biological Chemistry* **246**, 1511 (1971); R. E. Dickerson, *Scientific American*, 58 (1972); R. E. Dickerson and I. Geis, *Harper and Row*, (1969).

93 M. W. Crandall and D. L. Drabkin, *J. Biological Chemistry* **166**, 653 (1946); J. B. Marsh and D. L. Drabkin, *J. Biological Chemistry* **224**, 909 (1957).

94 S. Paleus and H. Theorell, *Acta Chemica Scandinavica* **11**, 905 (1957).

95 J. M. Armstrong, J. H. Coates, and R. K. Morton, *Haemotin Enzymes I. V. V. Symp. Ser. Vol 19* , 385 (1960), Discussion section remarks by T. Horio.

96 T. Flatmark, *Acta Chemica Scandinavica* **18**, 1656 (1964).

97 E. Margoliash and A. Schejter, *Advances in Protein Chemistry* **21**, 113 (1966).

98 T. Flatmark, *Acta Chemica Scandinavica* **20**, 1476 (1966); T. Flatmark, *Acta Chemica Scandinavica* **20**, 1487 (1966).

99 T. Flatmark and O. Vesterberg, *Acta Chemica Scandinavica* **20**, 1497 (1966).

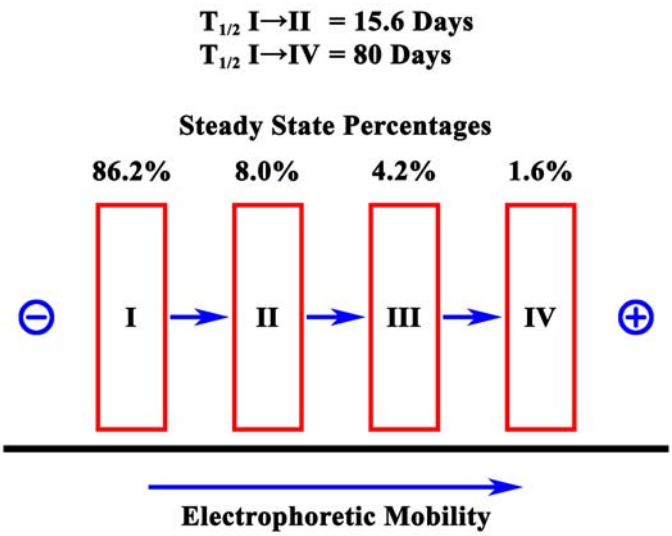

Figure 12-4 Deamidation of rat kidney cytochrome c *in vivo*. Data from 1968FS1.

plied isoelectric focusing to lactoperoxidase and myoglobin with the finding of multiple forms, which he attributed to deamidation.[100] Flatmark found that cytochromes I, II, and III have different spectroscopic optical rotation properties and that biological activity decreases with successive deamidations.[101]

Still remaining, however, was the question of whether or not cytochrome c deamidation occurs *in vivo*. In order to investigate this, Martin D. Kamen, discoverer of carbon 14 and extensive investigator of the biochemistry of cytochrome c and other heme proteins, agreed to sponsor the necessary experiments with high levels of ^{59}Fe. Flatmark moved to Kamen's laboratory at the University of California at San Diego to carry out these experiments.

Flatmark and Sletten then showed, using injections of ^{59}Fe labeled ferric chloride, that rat kidney cytochrome c deamidates *in vivo*.[102] This was the first definitive proof that deamidation of a protein occurs *in vivo*, although, as mentioned in this book with respect to several other

100 O. Vesterberg, *Acta Chemica Scandinavica* **21**, 206 (1967).

101 T. Flatmark, *J. Biological Chemistry* **242**, 2454 (1967).

102 T. Flatmark and K. Sletten, *Structure and Function of Cytochromes: Proceedings of the Symposium on Structure and Chemical Aspects of Cytochrome, Osaka (Ed. K Okunuki, M. D. Kamen, and I. Sekuzu), University Park, Baltimore*, 413 (1968); T. Flatmark and K. Sletten, *J. Biological Chemistry* **243**, 1623 (1968).

proteins, other investigators had observed deamidation in their proteins and suggested that it occurred *in vivo*.

Flatmark and Sletten further found that the half-times of conversion of cytochrome I to IV *in vitro* in pH 7.0, 37 °C, electrolyte solution corresponding to the composition of intracellular fluid, and *in vivo* in rat kidney were 95 days and 80 days, respectively. This showed that this *in vivo* deamidation is a nonenzymatic process. On this basis, they reported the *in vivo* half-life of rat kidney cytochrome c to be 80 days.

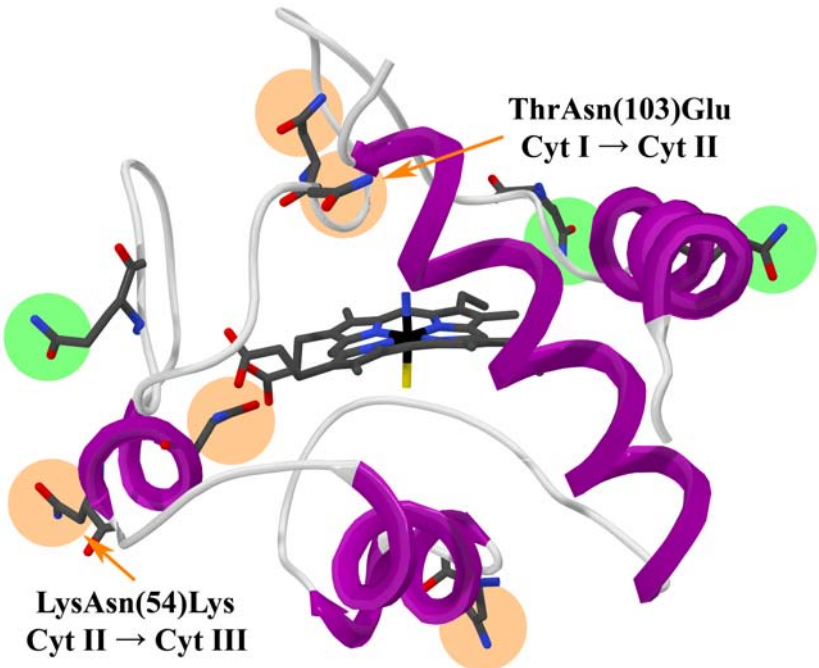

Figure 12-5 Structure of cytochrome c with amides marked. Those marked in orange are Asn and those in green are Gln. Structure data is taken from Protein Databank entry 1HRC.

Flatmark found that deamidation of cytochrome II to cytochrome III involved primarily AsnLysAsn(54)LysGly.[103]

The amides of cytochrome c are illustrated in Figure 12-5.

103 T. Flatmark - private communication.

Flatmark's findings stimulated Robinson to propose that deamidation may be a general timer of biochemical events[104] and, after an initial demonstration of sequence dependence of deamidation and protein compositional correlations, Robinson, McKerrow, and Cary proposed in 1970[105] that deamidation plays an important role as a molecular timer of "development, function, and aging of living systems."

In 1974, Robinson, McKerrow, and Legaz explored the sequence dependence of cytochrome c deamidation[106] by measuring deamidation rates of pentapeptide nearest-neighbor sequences corresponding to all 8 of the Asn and Gln in this protein. They found that the deamidation half-times for GlyThrAsn(103)Glu and GlyLysAsn(54)LysGly in I 0.2, pH 7.4, 37 °C, phosphate buffer were 16 days and 94 days, respectively. Therefore, the rate of deamidation of cytochrome I to II found by Flatmark to be 12 days under similar conditions *in vitro* and 15.6 days *in vivo* was shown to be under primary structure control. Asn(103) is located near the end of the protein chain and is not significantly restrained by protein structure.

The deamidation of cytochrome II to III, which Flatmark had identified as LysAsn(54)Lys with a measured deamidation half-time of 5 days, occurred much more rapidly in the protein than in the peptide. Moreover, this apparent acceleration of deamidation by change in three-dimensional structure could not be the result of misidentification, since, other than for GlyThrAsnGlu, the fastest deamidating cytochrome c pentapeptide sequence had a half-time of 54 days.

Since the second sequential deamidation of cytochrome c was faster than the first, it was evident that this deamidation was affected by three-dimensional structure. Comparison with the peptide indicated that this effect was an acceleration of deamidation. Conversely, all of the other amides in cytochrome c apparently deamidated more slowly than their respective peptides.

The deamidation of cytochrome c, including the observation of two forms of cytochrome II, could now be explained as simultaneous deamidation at GlyThrAsn(103)Glu and LysAsn(54)Lys in the conversion of I to II with most of the deamidation occurring at Asn(103). The small secondary variant at II apparently arises from slow deamidation

104 A. B. Robinson, *Kamen symposium at La Valencia hotel, La Jolla, California* , (1966).

105 A. B. Robinson, J. H. McKerrow, and P. Cary, *Proc. Natl. Acad. Sci. USA* **66**, 753 (1970).

106 A. B. Robinson, J. H. McKerrow, and M. Legaz, *Int. J. Peptide and Protein Research* **6**, 31 (1974).

at Asn(54). The second component of II could, alternatively arise from another amide, with the resulting heterogeneity of III unobserved. Deamidation at Asn(103), however, changes the structure of cytochrome c so that Asn(54) deamidates more rapidly to produce the change to III, which is deamidated at both positions. Cytochrome IV apparently arises from deamidation at one or more of the 6 remaining amides.

This change in structure could be intramolecular or it could involve an autocatalytic dimer or some other structure. It could also involve a different amide, since identification of Asn(54) is tentative.

Finally, Robinson, McKerrow, and Legaz pointed out[106] that an 80 day turnover rate was not compatible with the *in vivo* steady state concentrations of cytochrome c. Consideration of those concentrations and the available rate data shows that the deamidated forms of cytochrome c are more rapidly degraded *in vivo*, so the conversion of cytochrome c from I to II should be considered its *in vivo* turnover rate, which is more consistent with experimental measurements of the *in vivo* lifetime of cytochrome c.

Therefore, on the basis of *in vitro* and *in vivo* cytochrome c experiments, *in vitro* peptide experiments, and theoretical considerations reported through 1974 by Flatmark, Robinson, and their coworkers, it was known that deamidation of rat cytochrome c occurs *in vivo*; that it is subject to primary sequence control; that it can be either accelerated or decelerated by secondary or tertiary protein structure, which can even be altered by deamidation itself; and that it can control the *in vivo* turnover rate of the protein.

These investigators also discovered that the iron atoms in the hemes of cytochrome c and cytochrome c_2 can be exchanged by removal in liquid hydrogen fluoride and reinsertion in acetic acid[107] without significant deamidation.

12-10. EPIDERMAL GROWTH FACTOR

Epidermal growth factor is a 53-residue peptide hormone that binds to a receptor with protein tyrosine kinase activity.[108] It stimulates the

107 A. B. Robinson and M. D. Kamen, *Structure and Function of Cytochromes, ed. M. D. Kamen and I. Sekuzu, University of Tokyo and University of Park Press* 383 (1968); T. Flatmark and A. B. Robinson, *Structure and Function of Cytochromes, ed. M. D. Kamen and I. Sekuzu, University of Tokyo and University of Park Press,* 318 (1968).

108 R. B. Merrifield, *Peptides: Synthesis, Structures, and Applications, Academic Press, New York* , 94 (1995).

growth of epidermal and epithelial cells, inhibits gastric secretion,[109] and has a high immunosuppressive activity.

In 1984, mouse submaxillary gland epidermal growth factor was shown to have three forms. The major forms, present in a ratio of about 2:1, are amidated and singly deamidated, respectively, with deamidation occurring at Asn(1)SerTyrPro. The third form, about 5% of the total, has cleaved at AsnSer and is present as a 52-residue SerTyrPro form.[110] This was later confirmed by two other laboratories.[111] The deamidated and cleaved forms showed markedly reduced immunosuppressive activity.

Deamidation of mouse epidermal growth factor in 0.15 M ammonium bicarbonate, pH 9.0, 37 °C had a half-time of 0.86 days. Interestingly, this product showed no methyl esterification when treated with bovine brain protein carboxyl-*O*-methyl transferase, which reacts with isoAsp residues. Esterification was not improved by unfolding the peptide by reducing and alkylating the disulfide bridges. If, however, this unfolding were performed before deamidation, substantial methyl esterification occurred. This demonstrates the 3-dimensional structure dependence of the distribution of L-Asp and isoAsp during deamidation.

Mouse epidermal growth factor also includes the sequence LeuAsn(16)Gly, yet deamidation of this amide is not observed. This is an example of the fact that most amide deamidation rates are functions of primary, secondary, and tertiary structure and cannot usually be reliably predicted from one of these alone.[112]

Potential pharmaceutical use has focused attention on human epidermal growth factor, the primary degradation pathway of which is deamidation at Asn(1)SerAspSer. Lesser pathways are also important to production of very pure pharmaceutical preparations. Human epidermal growth factor also undergoes isomerization to isoAsp at AsnSerAsp(3)SerGlu and SerHisAsp(11)GlyTyr and oxidation at ValCysMet(21)TyrIle. At pH<6, succinimide Asu(11) is the most prev-

109 K. Son and C. Kwon, *Pharmaceutical Research* **12**, 451 (1995).

110 J. H. Koch, T. Fifis, V. J. Bender, and B. A. Moss, *Journal of Cellular Biochemistry* **25**, 45 (1984).

111 P. E. Petrides, P. Bohlen, and J. E. Shively, *Biochemical and Biophysical Research Communications* **125**, 218 (1984); R. P. DiAugustine, M. P. Walker, D. G. Klapper, R. I. Grove, W. D. Willis, D. H. Harvan, and O. Hernandez, *J. Biological Chemistry* **260**, 2807 (1985); R. P. DiAugustine, B. W. Gibson, W. Aberth, M. Kelly, C. M. Ferrua, Y. Tomooka, C. F. Brown, and M. Walker, *Analytical Biochemistry* **165**, 420 (1987).

112 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

alent. Chain cleavage also occurs at Asp(3). Human epidermal growth factor does not have the Asn(16) found in the mouse protein.

Deamidation half-times for human epidermal growth factor are reported as 63 days in 37 °C, 0.02 M phosphate, pH 6.0, 0.01polysorbate 80; 6 days under the same conditions at 60 °C; 29 days in 50 °C, 0.05 M phosphate, pH 7.4; 2.95 days in 60 °C, 0.05 M Tris, pH 7.0; 1.4 days in 60 °C, 0.05 M phosphate, pH 7.0; and 0.78 days in 60 °C, 0.05M physiological buffer.[113]

The latter three values have a ratio of 4.2:2:1.1 or about 4:2:1, which is in good agreement with the ratio of 3:2:1 for tris:phosphate:physiological solution found for the 10 protein types in which deamidation rates have been quantitatively measured at 37 °C.[114] Deamidation of epidermal growth factor was also faster in borate, acetate, and citrate buffers and solutions with various other additives than it was in Tris.

In vivo degradation of human epidermal growth factor in various parts of the rat digestive system varies between 0.006 and 0.15 days,[115] which poses a problem in the development of formulations for oral administration beyond that encountered from deamidation of stored formulations. Other studies have addressed the reason that large amounts epidermal growth factor are found in human urine,[116] and efforts have been made to improve its stability through variations in sequence and solvent conditions.[117]

12-11. FOOD PROTEINS

While the focus of deamidation research in biochemistry and molecular biology has been on the relatively mild chemical conditions found in living things, the harsher conditions of food processing and cooking

113 M. D. Dibiase and C. T. Rhodes, *J. Pharmacy and Pharmacology* **43**, 553 (1991); R. I. Senderoff, S. C. Wootton, A. M. Boctor, T. M. Chen, A. B. Giordani, T. J. Julian, and G. W. Radebaugh, *Pharmaceutical Research* **11**, 1712 (1994); K. Son and C. Kwon, *Pharmaceutical Research* **12**, 451 (1995).

114 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001).

115 K. Han, M. S. Choi, and Y. B. Chung, *Int. J. Pharmaceutics* **168**, 189 (1998).

116 K. Pesonen, *J. Chromatography Biomedical Applications* **568**, 226 (1991).

117 F. Araki, H. Nakamura, N. Nojima, K. Tsukuma, and S. Sakamoto, *Chemical and Pharmaceutical Bulletin (Tokyo)* **37**, 404 (1989); C. George-Nascimento, J. Lowenson, M. Borissenko, M. Calderon, A. Medina-Selby, J. Kuo, S. Clarke, and A. Randolph, *Biochemistry* **29**, 9584 (1990).

are of interest to the food industry. Under these conditions, controlled deamidation is of substantial interest in food chemistry.[118]

Deamidation changes protein structure. Extensive deamidation, as caused by the high temperatures and extremes of pH employed in food processing, generally unfolds proteins. These proteins have increased solubility due to the charges of the carboxylic acid side chains produced by deamidation and increased amounts of less polarized regions of surface as a result of exposure of their hydrophobic interiors. This amphiphilic character makes them better surface active agents and improves their usefulness as emulsifiers and foaming agents. The increased solubility from greater negative charge is also useful in other applications such as additives in acidic beverages.

In addition, the ammonia produced by deamidation produces aroma compounds and pigments primarily through participation in the Maillard reaction, which results in a very wide variety of organic compounds. This reaction also produces protein-polysaccharide compounds with antimicrobial and antiallergenic properties.[119]

These reactions occur during ordinary cooking, and they are also prevalent in common food processing procedures that employ heat. Since the properties produced by deamidation are considered useful, a substantial amount of research has been carried out to determine conditions that optimize deamidation of foods while minimizing less desirable reactions. These generally employ heat and acidic conditions. Sometimes transglutaminases, proteases, or, especially, peptidoglutaminases are employed to assist deamidation. Alkaline conditions are generally avoided because of racemization[120] and other undesirable side reactions. Crosslinking, such as the linking of Gln and Lys enzymatically by transglutaminase or nonenzymatic reactions, is also of interest.

Conversely, as the use of enzymes, both in solution and immobilized on solid supports, has found increased usefulness in the food and pharmaceutical industries, the prevention of deamidation at high temperatures has also come under active study. Since this work involves the biochemistry of enzymes and also of thermophilic organisms, it is reviewed elsewhere in this book. Although guided by the fundamental

118 J. R. Whitaker and R. E. Feeney, **19**, 173 (1983); W. E. Riha III, H. V. Izzo, J. Zhang, and C. Ho, *Critical Reviews in Food Science and Nutrition* **36**, 225 (1996); W. E. Riha III and C. Ho, *Food Reviews International* **12**, 351 (1996); J. S. Hamada, *Critical Reviews in Food Science and Nutrition* **34**, 283 (1994).

119 A. Kato, *Food Science and Technology Research* **8**, 193 (2002).

120 M. Lüpke and H. Brückner, *Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung A-Food Research and Technology* **206**, 323 (1998).

chemistry of deamidation and its dependence upon protein structure, temperature, pH, ionic strength, hydration, catalyzing substances, and suppressive agents, food deamidation research has a large practical component and is substantially empirical in nature. Detailed listing of these many practical conditions and results is beyond the scope of this book, but the discussion below provides the necessary references required to review the essential research literature.

It has been found that treatment of soy protein with 0.05N HCl at 95 °C for 30 minutes causes substantial deamidation without significant hydrolysis of peptide bonds.[121] Deamidation of soybean components has been examined in temperatures ranging as high as 150 °C, pH from 1 to 13, percentages of H_2O ranging from 0 to over 90%, and a wide range of buffers and additives. Soy deamidation has been augmented with peptidoglutaminase, protease, glutaminase, and transglutaminase.

References to these soybean product studies include 1987S, 1987SK, 1988S, 1989HH, 1989HM, 1990S, 1990S1, 1991S1, 1991H, 1992H1, 1993ZL, 1993SC, 1993ZL2, 1993ZL1, 1995WG, 1996BK, 1999CM, 1999WG, 1999WG1, 2000NM, 2000ZB, and 2000JZ.

Wheat protein deamidation, with special emphasis on gluten which is 40% Gln residues, has been studied extensively. Deamidation in 0.1N HCl or H_2SO_4 at 70 °C for 2 to 4 hours produces no significant peptide bond hydrolysis, and studies over a wide range of conditions have been carried out, including some in which deamidation is assisted by proteases. Crosslinking Gln and Lys in gluten has produced edible gluten films.

Deamidated gluten is also of special interest in the glue, paper, plywood, and print paste industries. Deamidation of gluten with NaOH is carried out to produce pigment print pastes for fabrics. These pastes are used on more than 10,000 miles of fabrics in the textile industry each day.[122]

Further research on wheat protein deamidation is reported in 1975F, 1986MO, 1986MT, 1988PB, 1989KP, 1990BV, 1992H1, 1993ZL2, 1993IL, 1993IH, 1994MR, 1996CH, 1999MA, 2000LD, 2000LL, 2002SA, and 2002NK.

Corn, maize, barley, rice, oat, canola, and sunflower proteins have also been similarly studied. Deamidation has been found to reduce the antioxidant properties of corn and barley proteins. See 1990CW,

121 N. Matsudomi, T. Sasaki, A. Kato, and K. Kobayashi, *Agricultural and Biological Chemistry* **49**, 1251 (1985).

122 L. E. Scheyer and M. Polsani, *Starch/Stärke* **52**, 420 (2000).

1991CP, 1993ID, 1993M, 1994CI, 1997CK, 1997CH, 1997WM1, 1997CK1, 1998HS, 2000KS, and 2000H.

Deamidation of milk proteins, especially casein, has been examined under a range of conditions with a greater emphasis on enzymatic processes. Deamidation under conditions of pasteurization is a special concern with casein found to be 0.5% and 9.8% deamidated after 30 minutes at temperatures of 90 °C and 130 °C, respectively.[123] Deamidation was reported during preparation of anticariogenic peptides, which include the sequences SerPSerPSerPGlu.[124] Casein may undergo deamidation *in vivo* in horse milk.[125]

Deamidation of milk protein studies include 1986MS, 1987KT1, 1992H1, 1992LV, 1993ZL2, 1994L, 1996NS, 1998MB, 1999ST, 1999SB, 1999B1, 2001GM, 2001G, 2001SL, 2002FF, 2003FF, 2003FG, 2004ND.

Deamidation of egg proteins, especially lysozyme, has been investigated over a wide range of conditions. See 1942SF, 1987KT, 1987KT1, 1993ZL1, 1993ZL2, and 1997M. Ovalbumin deamidation has been studied, 1989KL and 1997IP, and deamidation of cotton and peanut proteins has also been in investigated, 1986SK and 1987SK. Deamidation in fish proteins and crayfish byproducts has also been of interest. See 1996BC and 1997KW. Heating of herring meal results in substantial loss of available lysine.[126]

General studies emphasizing flavor changes through reactions with ammonia include 1993HH, 1995SH, 1996KH, and 1998KH.

It has been reported that dry red kidney beans are 66% deamidated and pork loin is 98% deamidated during 13 minutes of 38.1 kGy per hour irradiation from a ^{60}Co source.[127] It is likely, however, that at least part of the ammonia observed was derived elsewhere in the proteins. Although no measurements were reported for longer times, extrapolation of the reported measurements indicates that they would greatly exceed 100%. It has been shown that free radicals arising from ascorbic

123 F. Gaucheron and Y. L. Graet, *J. Chromatography A* **893**, 133 (2000).

124 N. Adamson and E. C. Reynolds, *Biotechnology and Bioengineering* **45**, 196 (1995).

125 A. S. Egito, J. -M. Girardet, C. Poirson, D. Molle, G. Humbert, L. Miclo, and J. -L. Gaillard, *International Dairy Journal* **13**, 813 (2003).

126 K. J. Carpenter, C. B. Morgan, C. H. Lea, and L. J. Parr, *British Journal of Nutrition* **16**, 451 (1962).

127 M. K. Dogbevi, C. Vachon, and M. Lacroix, *J. Food Science* **64**, 540 (1999); M. Dogbevi, C. Vachon, and M. Lacroix, *Meat Science* **51**, 349 (1999); M. K. Dogbevi, C. Vachon, and M. Lacroix, *Radiation Physics and Chemistry* **57**, 261 (2000); M. K. Dogbevi, C. Vachon, and M. Lacroix, *Radiation Physics and Chemistry* **57**, 265 (2000).

acid oxidation break peptide bonds and release ammonia through deamination.[128] A similar process may be operating in this system with free radicals arising from the radiation.

Asparagine is a major precursor for heat-induced acrylamide in foods, which is under study for health and safety reasons.[129]

12-12. GROWTH HORMONE

In 1965, Lewis and Cheever[130] reported charge heterogeneity in growth hormone with the slow formation of an acidic form at pH 7.5 accompanied by release of ammonia. This process was accelerated in 6 M urea. In 1969 they concluded that this heterogeneity resulted from two deamidations and from a mixture of monomeric, dimeric, trimeric, and tetrameric protein.[131]

In 1970, Lewis, Cheever, and Hopkins reported rates of deamidation of bovine and human growth hormone and ovine prolactin between pH 2.7 and pH 10[132] in phosphate buffer at 37 °C. At pH 7.5, the deamidation half-time for human growth hormone was 16 days. In the presence of 8 M urea, this dropped to 1.8 days. A more recent rate experiment in pH 7.4, 37 °C, physiological buffer gives a half-time of 17 days in solution[133] and 19 days in slow-release microspheres.

The computed deamidation half-time of human growth hormone at 37 °C in 0.15 M Tris at pH 7.4 is 34 days[134] and the correction factor for Tris[135] vs. phosphate is about 2. This gives a computed half-time of 17 days in phosphate at pH 7.4 as compared with the experimental value of 17 days at pH 7.4.

With only sequence control considered, in the absence of retardation by higher order structure, primary structure rates[136] can be used to compute an $I_{\text{D-primary structure}}$ for the 7 growth hormone Asn of 0.043 for a

128 S. L. Richeimer and A. B. Robinson, *Orthomolecular Psychiatry* **6**, 290 (1977).

129 M. Friedman, *J. Agricultural and Food Chemistry* **51**, 4504 (2003).

130 U. J. Lewis and E. V. Cheever, *J. Biological Chemistry* **240**, 247 (1965).

131 E. V. Cheever and U. J. Lewis, *Endocrinology* **85**, 465 (1969).

132 U. J. Lewis, E. V. Cheever, and W. C. Hopkins, *Biochimica Et Biophysica Acta* **214**, 498 (1970).

133 J. L. Cleland, A. Mac, B. Boyd, J. Yang, E. T. Duenas, D. Yeung, D. Brooks, C. Hsu, H. Chu, V. Mukku, and A. J. S. Jones, *Pharmaceutical Research* **14**, 420 (1997).

134 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001).

135 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001).

136 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

half-time of 4.3 days in Tris. Doubling the rate for phosphate gives an estimated deamidation primary structure half-time of 2.2 days at pH 7.4, in good agreement with the 8 M urea value of 1.8 days at pH 7.5.

There are three reports in the Protein Data Bank of 3-dimensional structures for human growth hormone with unambiguous positions for Asn(149) and Asn(152).[137] In an updated and computerized computation procedure,[138] Robinson computes I_{DTris} values of 0.31, 0.33, and 0.32 days for these three structures, respectively, for a phosphate average of 0.16, and a $t_{1/2}$ of 16 days.

The computed C_Ds are 0.43 for Asn(149) and 1.84 for Asn(152) and therefore predict that Asn(149) is the major deamidated form and Asn(152) is a minor form.[139] This has been experimentally shown to be the case.[139]

The measured deamidation rates of folded and unfolded growth hormone and the relative stabilities of its amides are, therefore, completely in accord with computational prediction.

Human growth hormone also shows isomerization at LeuGluAsp(130)GlySer at a slower rate than that of deamidation,[140] and has been isolated with succinimide at this position.[141] The deamidated forms of human growth hormone are reported to have unchanged biological activity but increased antibody reactivity.[142] This deamidation has also been observed *in vivo* in mice by microinjection of the protein.[143]

Deamidation has also been reported in growth hormone from cow, pig, horse, and eel in 1986SB, 1990SY, 1992LH, 1996VH, 1998MC, and 2001SB. Other growth hormone reports of interest include 1971SS,

137 These are PDB 1BP3, PDB 1HWG, and PDB 3HHR.

138 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

139 G. W. Becker, P. M. Tackitt, W. W. Bromer, D. S. Lefeber, and R. M. Riggin, *Biotechnology and Applied Biochemistry* **10**, 326 (1988); P. Gellerfors, B. Pavlu, K. Axelsson, C. Nyhlen, and S. Johansson, A*cta Paediatr. Scand. [Suppl]* **370**, 93 (1990).

140 B. A. Johnson, J. M. Shirokawa, W. S. Hancock, M. W. Spellman, L. J. Basa, and D. A. Aswad, *J. Biological Chemistry* **264**, 14262 (1989); A. Vinther, A. Holm, T. Hoeg-Jensen, A. M. Jespersen, N. K. Klausen, T. Christensen, and H. H. Sorensen, *European Journal of Biochemistry* **235**, 304 (1996).

141 G. Teshima, J. T. Stults, V. Ling, and E. Canova-Davis, *J. Biological Chemistry* **266**, 13544 (1991).

142 L. A. Retegui and A. C. Paladini, *Molecular Immunology* **23**, 119 (1986); A. Skottner, A. Forsman, B. Skoog, J. L. Kostyo, C. M. Cameron, N. A. Adamafio, K. Thorngren, and M. Hagerman, *Acta Endocrinologica (Copenh)* **118**, 14 (1988).

143 J. E. Battersby, V. R. Mukku, R. G. Clark, and W. S. Hancock, *Analytical Chemistry* **67**, 447 (1995).

1977B2, 1979LS, 1981LS, 1983SS, 1989FW, 1990HB, 1991B, 1991PD, 1991PD1, 1991SB, 1995KS, 1999H, 1999B, and 2000GS2.

12-13. GROWTH HORMONE RELEASING FACTOR

In 1990, Bongers, Heimer, Pan, Hulmes, Campbell, and Felix reported[144] that human growth hormone releasing factor, a 44-residue peptide with an amidated carboxyl terminal, deamidates in pH > 7 at PheThrAsn(8)SerTyr and is isomerized at TyrAlaAsp(3)AlaIle in pH 3.5, and that both reactions markedly reduce biological activity.

This was confirmed in 1991,[145] with the additional finding of deamidation at IleLeuAsn(28)ArgGln and almost total loss of biological activity upon deamidation at Asn(8). These investigators measured the rates of degradation in 37 °C, pH 7.4, 0.02 M sodium phosphate, 0.15 M NaCl, 0.01% NaN_3 of four 32-residue analogues with Ser alternatively substituted for Asn. They found degradation half-times of 1550 hours, 746 hours, 202 hours, and 150 hours for no Asn, with Asn(28), with Asn(8), and with both Asn(28) and Asn(8), respectively. With their 95% confidence limits included, this gives a mean half-time for Asn(8) of 10 days with a range of 9 to 11 and for Asn(28) of 60 days with a range of 39 to 194.

Tris primary structure rates in 37 °C, pH 7.4, 0.15 M Tris-HCl for GlyThrAsnSerArg and GlyLeuAsnArgGly are 17 and 62 days, respectively.[146] Therefore, these deamidations are essentially primary structure controlled in GHRF. Asn(8) is 1.7-fold accelerated by phosphate as expected. Asn(28) may also be similarly accelerated, but the confidence range is too wide to determine this.[147]

It has been reported that, at pH 9.2, the induction of increased helical structure in this peptide with 40% methanol approximately doubles its

144 J. Bongers, E. P. Heimer, Y. -. Pan, J. Hulmes, R. M. Campbell, and A. F. Felix, *Abstracts of Papers of the American Chemical Society* **200**, 77 (1990); J. Bongers, E. P. Heimer, T. Lambros, Y. E. Pan, R. M. Campbell, and A. F. Felix, *Int. J. Peptide and Protein Research* **39**, 364 (1992); J. Bongers, T. Lambros, A. M. Felix, and E. P. Heimer, *J. Liquid Chromatography* **15**, 1115 (1992).

145 A. R. Friedman, A. K. Ichhpurani, D. M. Brown, R. M. Hillman, L. F. Krabill, R. M. Martin, H. A. Zurcher-neely, and D. M. Guido, *Int. J. Peptide and Protein Research* **37**, 14 (1991).

146 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

147 N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001).

deamidation half-time. Substitution of Ala at position 15 increases this effect by 50%, while substitution of Pro eliminates it.[148]

Substitution of Asn(8) with Ser, Thr, or Gln increases the biological activity *in vitro* by 3-fold and *in vivo* by 11 to 13-fold, while decreasing aqueous and plasma instability.[149]

Bovine growth hormone releasing factor also deamidates at Asn(8),[150] and additional work has been done on techniques to measure its deamidation products.[151]

12-14. HEMOGLOBIN

In 1949, Harvey Itano and John Singer discovered that the hemoglobin of patients with sickle-cell anemia has a higher electric charge.[152] The charge change is caused by a genetic mutation in which Glu(6) is changed to Val.[153] The changed hemoglobin molecules aggregate in long fibers. This causes red blood cells to distort in shape and block capillaries and also leads to cell lysis and anemia. This discovery showed the profound effect on protein structure that can be caused by changing the charge at one location – a change that accompanies the deamidation of every Asn or Gln.

It was later shown that the density of red blood cells increases with cell age.[154] This has proved valuable in studying the deamidation of red blood cell proteins.

In 1972, it was reported that the human genetic variant hemoglobin J Singapore is caused by a double mutation with Asn(α78) changing to

148 C. L. Stevenson, M. E. Donlan, T. M. Kubiak, A. R. Friedman, and R. T. Borchardt, *Pharmaceutical Research [Suppl.]* **9**, 2053 (1992); C. L. Stevenson, M. E. Donlan, A. R. Friedman, and R. T. Borchardt, *Int. J. Peptide and Protein Research* **42**, 24 (1993).

149 R. M. Campbell, P. Stricker, R. Miller, J. Bongers, W. Liu, T. Lambros, M. Ahmad, A. M. Felix, and E. P. Heimer, *Peptides* **15**, 489 (1994).

150 R. A. Martin, D. L. Cleary, D. M. Guido, H. A. Zurcher-Neely, and T. M. Kubiak, *Biochimica et Biophysica Acta* **1164**, 252 (1993).

151 C. L. Stevenson, R. J. Anderegg, and R. T. Borchardt, *Journal of Pharmaceutical and Biomedical Analysis* **11**, 367 (1993); L. J. Gonzalez, T. Shimizu, Y. Satomi, L. Betancourt, V. Besada, G. Padron, R. Orlando, T. Shirasawa, Y. Shimonishi, and T. Takao, *Rapid Communications in Mass Spectrometry* **14**, 2092 (2000).

152 L. Pauling, H. Itano, J. Singer, and I. Wells, *Science* (1949) **110**, 543

153 S. R. Dickman and I. H. Moncrief, *Proceedings of the Society for Experimental Biology and Medicine* **77**, 631 (1951).

154 R. C. Leif and J. Vinograd, *Proc. Natl. Acad. Sci. USA* **51**, 520 (1964); C. Bishop and T. C. Prentice, *J. Cell Physiology* **67**, 197 (1966).

Asp(α78) and Ala(α79) changing to Gly(α79).[155] In fact, this variant had only one mutation at Ala(α79). This accelerates postsynthetic deamidation of Asn(α78).

Wild-type human hemoglobin has 10 Asn. By 1998, a total of 44 human mutations had been found that change various hemoglobin residues into Asn and 16 that change the carboxyl-side residue of one of the 10 Asn into another residue.[156]

Reports of some of the most interesting of these mutants include 1975GW, 1975KW, 1975CB, 1976SW, 1976MJ, 1976BB1, 1977HW, 1991WV, 1996HD, 1997KK, and 1998PG. Some articles on hemoglobin biochemistry include 1976P, 1986BF, 1990P, 1990RS, 1995N1, and 1996JB.

Seven of the mutant forms of human hemoglobin have been found to deamidate, including SerAsn(α50)Gly, ProAsn(α78)Gly, SerAsn(α139)Thr, AspAsn(β80)His, LeuAsn(β82)Gly, LeuAsn(β92)Cys, and LysAsn(β145)His. Of these, SerAsn(α139)Thr and LeuAsn(β92)Cys involve a frame-shift mutation and a heme-loss mutation, respectively, so their structures cannot be assumed to be equivalent to wild-type hemoglobin. Deamidation of the other five mutants has been successfully predicted from primary, secondary, and tertiary structure.[157] The observed[158] deamidation rates of SerAsn(α50)Gly, AspAsn(β80)His, and LeuAsn(β82)Gly have been correctly determined from their computed C_Ds.[159]

These five mutants involve three AsnGly and two AsnHis sequences. The 70 hemoglobin AsnXxx variants include an additional four AsnGly and one AsnHis variants. Computational consideration of secondary and tertiary structure correctly assigned all ten, with higher C_Ds for those five that have not been observed to deamidate.[157]

155 R. Q. Blackwell, W. H. Boon, C. S. Liu, and M. I. Weng, *Biochimica et Biophysica Acta* **278**, 482 (1972).

156 T. H. J. Huisman, M. F. H. Carver, and G. D. Efremov, *A Syllabus of Human Hemoglobin Variants, The Sickle Cell Anemia Foundation, Augusta, GA, USA,* (1996); R. Hardison, D. H. K. Chui, C. Riemer, W. Miller, M. Carver, T. Molchanova, G. Efremov, and T. H. J. Huisman, *Hemoglobin* **22**, 113 (1996)

157 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson, *California Institute of Technology Report* (2000).

158 R. Paleari, E. Paglietti, A. Mosca, M. Mortarino, L. Maccioni, S. Satta, A. Cao, and R. Galanello, *Clinical Chemistry* **45**, 21 (1999).

159 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001).

In diabetics with 5-fold increased blood glucose, hemoglobin glycosylation increased by 42%, and deamidation of hemoglobin by 13%.[160]

More deamidated hemoglobin is found in older red blood cells as compared with younger, and the susceptibility of this hemoglobin to proteolytic cleavage[161] is increased. Deamidation of the LeuAsn(β82)Gly hemoglobin mutant, Hemoglobin Providence, decreases red blood cell *in vivo* half-life to 23.5 days.[162]

Additional reports of interest to hemoglobin deamidation are 1977CF1, 1977S, 1980PF, 1984T1, 1985BL, 1991LC, 1992J, and 1992WK.

12-15. HISTONE

In 1974, deamidation rates were measured in pH 7.4, I 0.2, 37 °C, phosphate buffer for the sequences present in histone IV, and it was suggested that deamidation is also likely in histones I, IIb_1, IIb_2, and III.[163] Histone deamidation was proposed as a molecular timer for development, function, turnover, and aging of cells and organisms.[163]

The most unstable amide with respect to primary structure in histone IV was found in 1974 to be AspAsn(25)Ile.[163] In 1982, AspAsn(25)Ile in human spleen histone was observed to be deamidated *in vivo* by 5 to 10%.[164]

Two forms of both histone I and histone IIb were discovered in 1983,[165] and deamidation at AlaGluAsn(3)SerAla in histone V was reported in 1994.[166]

160 N. V. Pushkina, I. E. Tsybul'Skii, and A. I. Lukash, *Voprosy Meditsinskoi Khimii* **33**, 52 (1987).

161 A. L. Kizilshtein, A. M. Levin, and I. E. Tsybul'Skii, *Ukrainskii Biokhimicheskii Zhurnal* **60**, 14 (1988).

162 P. R. McCurdy, J. Fox, and W. Moo-Penn, *J. Human Gerontology* **27**, 62A (1975).

163 A. B. Robinson and J. W. Scotchler, *Int. J. Peptide and Protein Research* **6**, 279 (1974).

164 T. Hayashi, Y. Ohe, H. Hayashi, and K. Iwai, *J. Biochemistry* **92**, 1995 (1982).

165 J. A. Hardin and J. O. Thomas, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7410 (1983).

166 M. Kouach, D. Belaihe, M. Jaquinod, M. Couppez, D. Kmiecik, G. Ricart, A. V. Dorsselaer, P. Sautiere, and G. Briand, *Biological Mass Spectrometry* **23**, 283 (1994).

Histone I°, a new form of histone in non-replicating tissue was found in 1969.[167] This histone decreases in regenerating pancrease and liver and increases with animal age.[168]

Deamidation of histone I° at AcThrGluAsn(3)SerThr was reported in 1998 and 1999 with the extent of deamidation in rats increasing with age to levels at 450 days of 38% and 49% deamidated in liver and brain, respectively. Additional values for age-dependent deamidation of rat and mouse histone I° were determined and found to have increased to levels at 300 days of 50%, 43%, and 53% for rat kidney, liver, and brain and 29%, 20%, and 18% for mouse kidney, liver, and brain, respectively.[169]

More than 40 years ago, James Bonner suggested that histones regulate the reading of the genetic code and thereby the development and differentiation of living cells. His presentation of this in lectures to his students at Caltech in 1961, including A. B. Robinson, led to the experiments and suggestions about histone deamidation made in 1974.[163] Bonner's hypothesis and related research have been reviewed.[170]

Knowledge about histones has vastly increased and many new things have been learned in the past 30 years. It is, however, still reasonable to suggest that the extensive *in vivo* deamidation of these proteins, which are basic building blocks of chromatin, may have profound effects on nucleic acid utilization.

A new complement of histones is made at cell division, with the old histones substantially retained,[171] providing the time required for *in vivo* deamidation as a function of organismic age.

Additional relevant histone papers include 1964H, 1969H, 1969DF, 1970S, 1975TR, 1992MK, 2000MD, and 2001AA.

167 Panyim, S. and Chalkley, R., *Biochemical and Biophysical Research Communications* **37**, 1042 (1969).

168 M. A. Lea, *Cancer Biochemistry Biophysics* **9**, 199 (1987).

169 H. Lindner, B. Sarg, B. Hoertnagl, and W. Helliger, *J. Biological Chemistry* **273**, 13324 (1998); H. Lindner, B. Sarg, H. Grunicke, and W. Hellinger, *J. Cancer Research and Clinical Oncology March-April,* **125**, 182 (1999).

170 S. C. R. Elgin, S. C. Froehner, J. E. Smart, and J. Bonner, *Advances in Cell and Molecular Biology, (Ed. E. J. DuPraw), Academic Press, New York* **1**, 1 (1971); J. Bonner, *California Institute of Technology*, 1 (1973); I. Isenberg, *The Cell Nucleus* **4**, 135 (1978).

171 P. Byvoet, *J. Molecular Biology* **17**, 311 (1966); R. S. Piha, M. Cuenod, and H. Waelsch, *J. Biological Chemistry* **241**, 2397 (1966).

12-16. IMMUNOGLOBULIN

In 1967 and 1968, Reisfeld and Parkhouse-Slade proved that immunoglobulin light chains and heavy chains undergo postsynthetic deamidation.[172]

Many observations of mouse immunoglobulin deamidation have been made since then. A recent study of nine forms of immunoglobulin illustrates its deamidation at GlnAsn(161)Gly in the light chain and ThrAsn(141)Ser in the heavy chain. These two deamidations give rise to 9 variants as illustrated in Figure 12-6.[173]

Other observations of charge heterogeneity probably arising from deamidation of immunoglobulins include 1967AA, 1968RI, 1969LW, 1970AW, 1971PF, 1972GP2, 1972MP, 1973WS, 1975BM, 1977H, 1978KL, 1988EM, 1990WR, 1992H, 1992G, 1993TB, 1994PP, 1995MK, 1996UO, 1998HK, 1998MN, and 2003ZC. Reports of immunoglobulin deamidation during sequencing work include 1972SM, 1981LA, 1975CK, and 1996W.

Deamidation has been reported to reduce the thermostability and fluorescence of human immunoglobulin.[174]

Efforts to produce and store immunoglobulin without deamidation have been successful.[175] Solutions in 60% glycerol or 40% ethylene glycol decreased deamidation of blood immunoglobulin, while 40% glucose or 40% sucrose increased it.[176] Human monoclonal antibody was found to be stable with respect to deamidation at 40 °C if it was freeze-dried in 0.06 M sucrose or trehalose at a sugar:protein ratio of 360:1.[177]

A mouse monoclonal antibody has been reported to deamidate in the light chain at ArgGlnAsn(156)GlyVal and in the heavy chain at

172 R. A. Reisfeld, *1967 Cold Spring Harbor Symposium Quant. Biol.* **32**, 291 (1967); B. Parkhouse (Slade), *PhD Thesis, University of London* (1968).

173 M. Perkins, R. Theiler, S. Lunte, and M. Jeschke, *Pharmaceutical Research* **17**, (2000).

174 A. I. Lukash, A. P. Shepelev, N. V. Pushkina, I. E. Tsybul'skii, and D. V. Al'perovich, *Vopr Med Khim* **31**, 104 (1985); A. I. Lukash, A. P. Shepelev, D. V. Al'perovich, N. V. Pushkina, and K. B. Sherstnev, *Antibiotiki I Meditsinskaya Biotekhnologiya* **31**, 848 (1986).

175 M. A. Schenerman, J. N. Hope, C. Kletke, J. K. Singh, R. Kimura, E. T. Tsao, and G. Folena-Wasserman, *Biologicals* **27**, 203 (1999).

176 N. V. Pushkina, I. E. Tsybul'Skii, and A. I. Lukash, *Prikladnaya Biokhimiya I Mikrobiologiya* **22**, 198 (1986).

177 J. L. Cleland, X. Lam, B. Kendrick, J. Yang, T. Yang, D. Overcashier, D. Brooks, C. Hsu, and J. F. Carpenter, *J. Pharmaceutical Sciences* **90**, 310 (2001).

ThrAsnAsn(386)Gly. In pH 7.0 phosphate, saline, and polysorbate 80 at 37 °C, Asn(386) had a deamidation half-time of 18 days.[178]

An antibody used for the treatment of breast cancer was found to be 16% deamidated at AspValAsn(30)ThrAla in the light chain, 1% deamidated at PheThrAsn(55)GlyTyr in the heavy chain, 10%

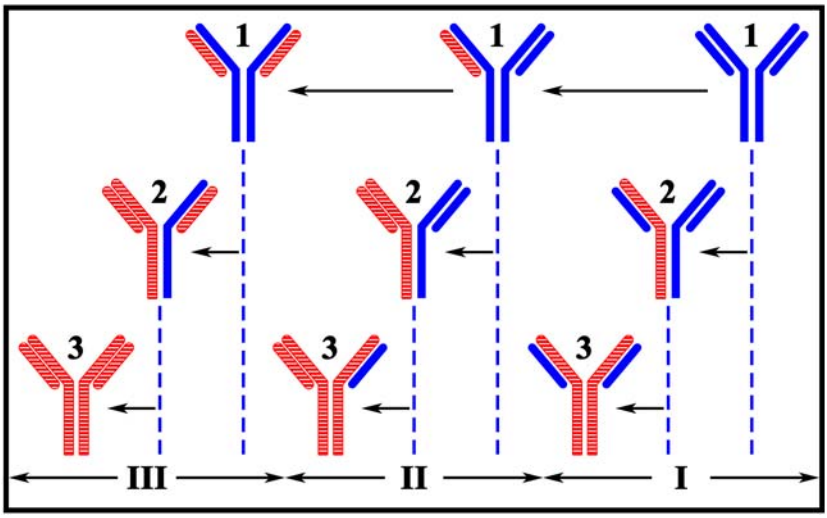

Figure 12-6 Illustration of two deamidated forms of immunoglobulin. Chains shown in blue are undeamidated and those in striped red are deamidated. The numbers refer to heavy chain variants and the Roman numerals refer to light chain variants. Adapted from 2000PT.

isomerized at GlyGlyAsp(102)GlyPhe in the heavy chain, and 1% succinimide at Asp(102).[179] These three residues are all in complementarity forming regions. In another instance, Asp(32)Gly in the light chain was found isomerized in the complementarity region.

Deamidation provides a potential means of introducing biologically useful *in vivo* diversity into the complementarity region of immunoglobulin.

Immunoglobulins are quite variable, so matching a particular 3-dimensional structure with a specific immunoglobulin deamidation is problematic. However, 3-dimensional Asn computation for PBD entry 1IGT[47], a 3-dimensional structure of mouse immunoglobulin, gives GlnAsn(157)Gly as the most unstable amide in the light chain.

178 D. J. Kroon, A. Baldwin-Ferro, and P. Lalan, *Pharmaceutical Research* **9**, 1386 (1992).

179 R. J. Harris, B. Kabakoff, F. D. Macchi, F. J. Shen, M. Kwong, J. D. Andya, S. J. Shire, N. Bjork, K. Totpal, and A. B. Chen, *J. Chromatography B* **752**, 233 (2001).

GlnAsn(161)Gly and GlnAsn(156)Gly have, as referenced above, been reported deamidated in mouse immunoglobulins.

12-17. INSULIN

Bovine insulin, the first large peptide purified in deamidated form, was purified by Harfenist and Craig in Flexner Hall at Rockefeller University by countercurrent distribution in 1952.[180] In the first demonstration of synthesis of a large peptide by automated Merrifield solid-phase peptide synthesis, insulin was synthesized by Marglin and Merrifield in Flexner Hall in 1966.[181]

Insulin is comprised of two peptide chains with the A chain having 21 residues and the B chain 30 residues. It has two inter-chain and one intra-chain disulfide bonds and is subject to several postsynthetic modifications, of which deamidation is the most prevalent.

Deamidation occurs at TyrCysAsn(A21) in acidic solution and PheValAsn(B3)GlnHis in neutral solution, apparently by anhydride and imide mechanisms, respectively. Insulin also forms α- or β-linked dimers through transamidation of Asn(A21) with Gly(A1) or Phe(B1). It has been suggested that this dimerization proceeds through the same anhydride that mediates deamidation at Asn(21).[182] The dimer normally constitutes about 3% of insulin preparations, but up to 30% of biologically circulating insulin because it is degraded more slowly *in vivo*[183].

During x-ray structure determination, ambiguities arose at Asn(B3), possibly as a result of deamidation.[184]

In addition to these amide reactions, which are the principal *in vitro* degradation processes, insulin also undergoes chain cleavage at Ala(A8)Ser(A9), Schiff-base dimerization between Gly(A1) and Phe(B1), and dimerization by disulfide exchange at Cys(A7) and

180 E. J. Harfenist and L. C. Craig, *J. American Chemical Society* **74**, 3083 (1952); E. J. Harfenist, *J. American Chemical Society* **75**, 5528 (1953).

181 A. Marglin and R. B. Merrifield, *J. American Chemical Society* **88**, 5052 (1966).

182 R. G. Strickley and B. D. Anderson, *Pharmaceutical Research* **13**, 1142 (1996); R. G. Strickley and B. D. Anderson, *J. Pharmaceutical Sciences* **86**, 645 (1997).

183 R. T. Darrington and B. D. Anderson, *J. Pharmaceutical Sciences* **84**, 275 (1995).

184 T. L. Blundell, J. F. Cutfield, E. J. Dodson, G. .. G. Dodson, D. C. Hodgkin, and D. M. Mercola, *Cold Spring Harbor Symposia on Quantitative Biology* **36**, 233 (1972).

Cys(B7). Crystallized as a hexamer, insulin disassociates into dimers and monomers in solution. The monomers are subject to partial unfolding, aggregation, and then fibril growth and precipitation. These reactions have been reviewed.[185]

Insulin retains almost full biological activity after deamidation at Asn(A21) or Asn(B3), but loses its activity upon cleavage or dimerization.[186]

Deamidation of Asn(B3) in pH 7.4 sodium acetate, 0.1% methyl paraben, 0.7% NaCl at 37 °C has a half-time of 136 days.[187] The computed value[188] from primary, secondary, and tertiary structure is 117 days in 0.15 M Tris, pH 7.4, 37 °C, which is in good agreement with the experimental value.

Deamidation of Asn(A21) has been used to probe the structure of insulin amyloid fibrils with the conclusion that these Asn occupy two different packing positions because only half of the Asn(A21) residues are found to deamidate.[189]

As a result of its pharmaceutical importance, the stability of insulin has been investigated as a function of primary sequence,[190] concentration, and solution properties 1992BL, 1993RP, 1994DA, 1995DA, 1997PR, and 2002DL.

Additional insulin deamidation studies include 1962S, 1962CC, 1963CH, 1963SC, 1966BY, 1966S, 1975SP, 1975KB, 1979DM, 1981FP, 1983SE, 1992M, 1992DA1, 1994DA, 1996OF, 1997SA, 2002JH and 2003SL.

185 J. Brange and L. Langkjoer, *Pharmaceutical Biotechnology* **5**, 315 (1993); J. Brange, *Acta Pharmaceutica Nordica* **4**, 209 (1992); M. R. Ladisch and K. L. Kohlmann, *Biotechnology Progress* **8**, 469 (1992); J. Schlichtkrull, M. Pingel, L. G. Heding, J. Brange, and K. H. Jorgensen, *HDB Expt. Pharm., Handbook of Experimental Pharmacology* **32**, 729 (1975).

186 J. Brange, O. Hallund, and E. Sarensen, *Acta Pharmaceutica Nordica* **4**, 223 (1992).

187 J. Brange, L. Langkjaer, S. Havelund, and A. Volund, *Pharmaceutical Research* **9**, 715 (1992).

188 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001).

189 M. R. Nilsson and C. M. Dobson, *Protein Science* **12**, 2637 (2003).

190 J. Brange, U. Ribel, J. F. Hansen, G. Dodson, M. T. Hansen, S. Havelund, S. G. Melberg, F. Norris, K. Norris, L. Snel, A. R. Sorensen, and H. O. Voigt, *Nature (London)* **333**, 679 (1988); J. Markussen, I. Diers, P. Hougaard, L. Langkjaer, K. Norris, L. Snel, A. R. Sorensen, E. Sorensen, and H. O. Voigt, *Protein Engineering* **2**, 157 (1988).

12-18. INTERLEUKIN

The 159 residue protein human interleukin-1α deamidates at ArgAlaAsn(36)AspGln. It is 25% deamidated as expressed in and isolated from E. coli.[191] This is the amount of deamidation found in 16 days in Tris at pH 7.4, 37 °C for the peptide GlyAlaAsnAspGly.[192] Purification was carried out at pH ≤ 8 and T ≤ 8 °C. Moreover, during capillary electrophoresis for less than 20 minutes in 0.05 M tetraborate at pH 8.3, deamidation of interleukin-1α was reported to increase to 25% at 30 °C as compared to 8% at 20 °C.[193] Therefore deamidation of this residue is surely not impeded and may even be accelerated by secondary or tertiary structure. Both the amidated and deamidated forms have good biological activity as does a mutant with Ser(36) substituted for Asn(36).

The 153 residue protein human interleukin-1β is only about 25% homologous with interleukin-1α, although they both bind to the same receptor and have some 3-dimensional similarities.

Mouse interleukin-1β deamidates at HisLeuAsn(32)GlyGln with a half-time in pH 8.5, 0.1 M Tris at 37 °C of 1.5 days and a 3 to 4-fold reduction of biological activity.[194] Human interleukin-1β, however, has a deamidation half-time in pH 7.0, 0.01 M Tris at 30 °C of between 160 and 330 days.[195] The human sequence is HisLeuGln(32)GlyGln.

This interesting case in which replacement of Asn by Gln increases deamidation rate by two orders of magnitude is reviewed in Chapter 9.

Interleukin 2, a 133 residue protein, has 3 increasingly negative components in isoelectric focusing with almost no loss of biological activity. The most abundant of these was identified as interleukin 2

191 S. Clarke, *Biological Methylation and Drug Design (Ed. R. T. Borchardt, C.R. Creveling, and P. M. Ueland), Humana Press, Clifton NJ*, 3 (1986); P. T. Wingfield, R. J. Mattaliano, H. R. MacDonald, S. Craig, G. M. Clore, A. G. Gronenborn, and U. Schmeissner, *Protein Engineering* **1**, 413 (1987); P. Wingfield, M. Payton, P. Graber, K. Rose, J. Dayer, A. S. Shaw, and U. Schmeissner, *European Journal of Biochemistry* **165**, 537 (1987); K. Hong and G. J. Jhon, *Korean Biochemical Journal* **22**, 209 (1989); S. A. Carr, M. F. Bean, M. E. Hemling, and G. D. Roberts, *Biological Mass Spectrometry (Ed. A. L. Burlingame and J. A. McCloskey), Elsevier, Amsterdam*, 621 (1990); S. P. Monkarsh, E. A. Russoman, and S. K. Roy, *J. Chromatography* **631**, 277 (1993).

192 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

193 N. A. Guzman, H. Ali, J. Moschera, K. Iqbal, and A. W. Malick, *J. Chromatography* **559**, 307 (1991).

194 E. M. Cassidy, B. T. Wakim, A. G. Ferguson, and A. M. Samarel, *J. Molecular and Cellular Cardiology* **23**, 589 (1991).

195 L. C. Gu, E. A. Erdös, H. Chiang, T. Calderwood, K. Tsai, G. V. Visor, J. Duffy, W. -C. Hsu, and L. C. Foster, *Pharmaceutical Research* **8**, 485 (1991).

deamidated at IleSerAsn(88)IleAsn. The deamidation half-time in 0.005 M acetate at pH 5, 40 ° was 500 days, and no isoAsp was detected in the product.[196]

The calculated C_D is 2362[197] for imide deamidation and 80 for hydrolysis in pH 7.4, 0.15 M Tris at 37 °C while the experimental value is 5, corresponding to the lower pH and higher temperature.

Therefore, the computations predict that this deamidation proceeds by hydrolysis rather than imide, and the reaction product isomer confirms this.

A 271-residue fusion protein with granulocyte-macrophage colony stimulating factor linked to 133 residue interleukin 3 by an 11-residue linker was found to be partially deamidated in interleukin 3 at AspPheAsn(38)AsnLeu after synthesis. LeuAsn(41)GlyGlu was shown to be not deamidated.[198]

Interleukin 11, a 177-residue protein, was found to deamidate at AlaAspGlyAspHisAsn(49)LeuAspSer with a deamidation half-time of 275 days in 0.01 M phosphate, 0.3 M glycine, pH 7.0 at 30 °C and was followed by peptide bond cleavage. Isomerization of all three Asp in this sequence was also found with the average of the amount of isoAsp at each position being about the same as the amount of deamidated Asn(49). This peptide is thought to be in a loop between two helices.[199]

12-19. LYSOZYME

Charge heterogeneity was first noticed in chicken egg-white lysozyme in 1952.[200] In 1972, a deamidated form of human lysozyme was reported in leucocytes from patients with chronic myelogenous leukemia, but not in normal patients.[201] Substantial heterogeneity, partially attributed to deamidation, was observed in 1973 in the product of the Merrifield solid phase synthesis of chicken egg-white lysozyme, the second chemical synthesis of a protein.[202]

196 K. Sasaoki, T. Hiroshima, S. Kusumoto, and K. Nishi, *Chemical and Pharmaceutical Bulletin* **40**, 976 (1992).

197 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

198 A. Balland, D. A. Krasts, K. L. Hoch, M. J. Gerhart, K. E. Stremler, and S. W. Waugh, *European Journal Biochemistry* **251**, 812 (1998).

199 W. Zhang, M. J. Czupryn, P. T. Boyle Jr., and J. Amari, *Pharmaceutical Research* **19**, 1223 (2002).

200 H. H. Tallan and W. H. Stein, *J. Biological Chemistry* **200**, 507 (1952).

201 J. -P. Perin and P. Jolles, *Clinica Chimica Acta* **42**, 77 (1972).

202 J. J. Sharp, A. B. Robinson, and M. D. Kamen, *J. American Chemical Society* **95**, 6097 (1973).

In 1973, deamidation rates were measured in pH 7.5, 0.15 M phosphate at 37 °C for 13 of the Asn and the 3 Gln sequences of chicken egg-white lysozyme in GlyXxxAsn/GlnYyyGly peptides.[203] The analogues GlyThrAsn(48)GlyGly and GlyGlyAsn(103)GlyGly were not included because the sequence assignments for residues 48 and 103 of this lysozyme were Asp (48) and Asp(103), apparently as a result of their rapid deamidation.[204] The correct assignment of Asn(48) and Asn(103) was made[205] and proved in 1981.[206] AspGlyAsn(103)GlyMet is also found in chicken, quail, and pheasant lysozyme.[207] The other 13 Asn were found to have deamidation half-times between 40 and 120 days, and the 3 Gln all had deamidation half-times of more than 3,000 days, so ample cause for heterogeneity was found without consideration of the AsnGly sequences. Mosquito lysozyme was found deamidated at AsnArgAsn(69)GlySer and GlnIleAsn(80)AspArg.[208]

A. M. Klibanov and coworkers have conducted, for industrial purposes, a substantial amount of research on the thermal inactivation of enzymes at high temperatures. They found that the irreversible inactivation of lysozyme at 100 °C results from deamidation at pHs 4, 6, and 8. The percent deamidation is 90%, 100%, and 35% deamidated, respectively.[209] Concomitantly, the biological activity of lysozyme was 100%, 55%, and 21% for undeamidated, monodeamidated, and dideamidated lysozyme.[210] High temperature stability was greater in the anhydrous organic solvents nonane, 1-butanol, and dimethyl formamide,[211] and addition of 1.5 M sucrose or trehalose also diminishes the deamidation rate of lysozyme at 100 °C, pH 6.[212]

203 A. B. Robinson and S. Tedro, *Int. J. Peptide and Protein Research* **5**, 275 (1973).

204 M. O. Dayhoff, *National Biomedical Research Foundation* , D138 (1972); R. Canfield, *J. Biological Chemistry* **238**, 2698 (1963).

205 J. Hermann and J. Jollès, *Biochimica Et Biophysica Acta* **200**, 178 (1970).

206 T. Imoto, K. Okazaki, H. Yamada, K. Fujita, T. Yamato, and D. Koga, *J. Biochemistry* **90**, 991 (1981).

207 I. M. Ibrahimi,, E. M. Prager, T. J. White, and A. C. Wilson, *Biochemistry* **18**, 2736 (1979); J. Jolles, I. M. Ibrahimi, E. M. Prager, F. Schoentgen, P. Jolles, and A. W. Wilson, *Biochemistry* **18**, 2744 (1979).

208 V. P. Hernandex, L. Higgins, and A. M. Fallon, *Developmental and Comparative Immunology* **27**, 11 (2003).

209 T. J. Ahern and A. M. Klibanov, *Science* **228**, 1280 (1985).

210 T. J. Ahern and A. M. Kilbanov, *Methods of Biochemical Analysis* **33**, 91 (1987).

211 D. B. Volkin, A. Staubli, R. Langer, and A. M. Klibanov, *Biotechnology and Bioengineering* **37**, 843 (1991).

212 T. Ueda, M. Nagata, and T. Imoto, *J. Biochemistry* **130**, 491 (2001).

Protease susceptibility of lysozyme *in vitro* and *in vivo* increases with deamidation, with 100%, 232%, and 271% for undeamidated, deamidated at Asn(103), and deamidated at Asn(106), respectively.[213]

Lysozyme is antibacterial for gram-positive bacteria. Deamidation of 2.7 amides per lysozyme molecule at 80 °C, pH 6.0, for 20 minutes causes lysozyme to become antibacterial for gram-negative bacteria, while still retaining its gram-positive activity.[214]

In a study of mouse T-cells immunized to lysozyme peptide Asp(48)-Trp(62), it was found that these T-cells recognized the peptide deamidated at GlnIleAsn(59)SerArg, but not the undeamidated peptide.[215] It was found that this peptide had a deamidation half-time of about 10 days in pH 7.5, 37 °C, PBS buffer, which in agreement with the literature values for pentapeptide standards.[216] In the intact protein, Asn(59) was not observed to deamidate, which is consistent with its computed C_D of 4.6.[217] The computed C_Ds for Asn(103) and Asn(106) are 0.06 and 0.58,[217] entirely in accord with these being the first and second most unstable amides in lysozyme.

Additional reports of interest concerning lysozyme deamidation include: 1988KS, 1988P1, 1992AH, 1994MU, 1994TY1, 1994TY, 1995TY2, 1995TY, 1996KN, 1995TY1, 1997AU, 1998NM, 1999MU, 2001CP, 2003HH1, and 2003KU.

12-20. MYELIN BASIC PROTEIN

Myelin basic protein constitutes 30% of the protein in human central nervous system myelin. It has been of special interest in the study of demyelinating diseases, such as the autoimmune disease experimental allergic encephalomyelitis, EAE. In EAE, the animal's immune system attacks its own myelin basic protein at a specific site on the protein. This leads to illness and death. EAE is considered to be a useful model for the study of diseases such as multiple sclerosis. A 9-residue

213 A. Kato, S. Tanimoto, Y. Muraki, K. Kobayashi, and I. Kumagai, *Bioscience Biotechnology and Biochemistry* **56**, 1424 (1992).

214 H. R. Ibrahim, S. HigasMguchi, Y. Sugimoto, and T. Aoki, *Food Research International* **29**, 771 (1996); H. R. Ibrahim, *Nahrung* **42**, 187 (1998).

215 S. N. McAdam, B. Fleckenstein, I. B. Rasmussen, D. G. Schmid, I. Sandlie, B. Bogen, N. J. Viner, and L. M. Sollid, *J. Experimental Medicine* **193**, 1239 (2001).

216 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

217 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

encephalitogenic peptide has been isolated and characterized[218] as PheSerTrpGlyAlaGluGlyGlnArg.

In 1971, deamidation of myelin basic protein was reported at ProSerGln(103)GlyLys, and, in 1973, deamidation of the encephalogenic peptide itself was found at Gln(8).[219] This was confirmed in 1976.[220] It was suggested that deamidation-mediated protein turnover is a part of the disease process.[221]

Myelin basic protein *in vivo* turnover is apparently slow, so substantial amounts of Asp isomerization and Ser phosphorylation have also been observed, along with deamidation at PheLysAsn(92)IleVal and AspGluAsn(84)ProVal.[222]

In 1987, it was reported that human brain myelin basic protein disassociates from brain membrane upon exposure to physiological ionic strength and is immediately digested into three parts, which then undergo rapid deamidation.[223] This deamidation may be a part of the normal proteolytic process, but one or more of the many peptide species produced may also play a role in neurological diseases.

Additional studies related to deamidation of myelin basic protein include 1971EB, 1975DM, 1977CC, 1977MD, 1980JE, 1984CM, 1985CB, 1987SC, 1997BY, 1998ZL, 1999CG, 1999BR, and 2001ZJ.

12-21. PHENYLALANINE HYDROXYLASE

Phenylalanine hydroxylase, PAH, catalyzes the conversion of phenylalanine to tyrosine. It must be carefully regulated because vertebrates do not synthesize phenylalanine, and well-controlled pools of phenylalanine and tyrosine are necessary for protein synthesis. More

218 E. H. Eylar, F. C. Westall, and S. Brostoff, *J. Biological Chemistry* **246**, 3418 (1971); F. C. Westall, A. B. Robinson, J. Caccam, J. Jackson, and E. H. Eylar, *Nature* **229**, 22 (1971).

219 A. Hagopian, F. C. Westall, J. S. Whitehead, and E. H. Eylar, *J. Biological Chemistry* **246**, 2519 (1971); F. C. Westall, *J.theor.Biol.* **38**, 139 (1973).

220 F. C. -H. Chou, C. -. Jen Chou, R. Shapira, and R. F. Kibler, *J. Biological Chemistry* **251**, 2671 (1976).

221 F. C. Westall, *Immunochemistry* **11**, 513 (1974).

222 R. E. Martenson, M. J. Law, and G. E. Deibler, *J. Biological Chemistry* **258**, 930 (1983); R. Shapira, K. D. Wilkinson, and G. Shapira, *J. Neurochemistry* **50**, 649 (1988); O. Z. Sellinger and M. F. Wolfson, *Biochimica et Biophysica Acta* **1080**, 110 (1991).

223 P. Glynn, A. Chantry, N. Groome, and M. L. Cuzner, *J. Neurochemistry* **48**, 752 (1987).

than 400 mutant forms of PAH are known,[224] with many leading to severe metabolic disease.

In 1996, Doskeland and Flatmark found that PHA, which has an *in vivo* half-life in rat liver of 2 days, is metabolized through the ubiquitin-proteasome pathway.[225]

Flatmark and co-workers have carried out extensive studies of the structure and function of PHA, which include 1996KF, 1997EF, 1997EM, 1998FE, 1998EF, 1998CT, 2000EB, 2000FE, 2001HS, 2001BC, 2001FS, and 2001AF.

In 0.015 M pH 7.0 sodium HEPES buffer at 37 °C, PHA deamidates at AsnGlnAsn(32)GlyAla with a half-time of 1.9 days, which triples its catalytic efficiency.[226] In pH 7.4 0.15 M Tris-HCl at 37 °C, this amide has a predicted deamidation C_D of 0.0145. $100C_D$ = 1.45 days.[227] Deamidation has, therefore, been suggested as controlling the *in vivo* turnover of PAH.[228]

Eight additional asparaginyl residues in the PAH catalytic domain have been shown to deamidate with half-times between 10 and 300 days at pH 7, 37 °C. These rates also correlate well with those computed from 3-dimensional structure.[229]

12-22. RIBONUCLEASE

In 1970, Bornstein and Balian reported the specific cleavage by 2 M hydroxyl amine at pH 9.0 of Asn(67)Gly in bovine ribonuclease and suggested that this reaction proceeds by a cyclic imide mechanism.[230]

Subsequently, there were several reports of deamidation of ribonuclease. See 1976MA, 1977MA, 1978WR, 1978WR1, 1978DV, 1981DD, and 1984VV.

224 A. P. Doskeland and T. Flatmark, *Biochimica et Biophysica Acta* **36439**, 1 (2001).

225 A. P. Doskeland and T. Flatmark, *Biochemical Journal* **319**, 941 (1996).

226 T. Solstad, R. N. Carvalho, O. A. Andersen, D. Waidelich, and T. Flatmark, *European Journal of Biochemistry* **270**, 929 (2003).

227 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

228 T. Solstad and T. Flatmark, *European Journal of Biochemistry* **267**, 6302 (2000).

229 R. N. Carvalho, T. Solstad, E. Bjorgo, J. F. Barrosot, and T. Flatmark, *J. Biological Chemistry* **278**, 15142 (2003); R. M. N. Carvalho, T. Solstad, N. E. Robinson, A. B. Robinson, and T. Flatmark, *Proceedings of the 12th International Symposium on the Chemistry and Biology of Pteridines and Folates* (2002); N. E. Robinson, ***Proc. Natl. Acad. Sci. USA*** **99**, 5283 (2002).

230 P. Bornstein and G. Balian, *J. Biological Chemistry* **245**, 4854 (1970).

In 1985, Thannhauser and Sheraga confirmed the deamidating sequence of ribonuclease A as CysLysAsn(67)GlyGln. With the 8 Cys sulfonated and, therefore, the 3-dimensional structure disrupted, they observed a deamidation half-time for Asn(67) in 0.02 M Tris, pH 7.5 at 38 °C of 3 days.[231] In 1993, ribonuclease A deamidated in pH 8.2, 1% ammonium carbonate was shown to produce both Asp(67) and isoAsp(67) products.[232]

Human ribonuclease 4 deamidates at CysLysAsn(66)GlyLys,[233] and smut fungus ribonuclease U_2 deamidates at ValAlaAsn(32)GlyAsp.[234] The latter is odd in that, while deamidation occurred *in vivo* in culture broth at Asn(32), no deamidation was observed at ValTyrAsn(68)GlyPro. Asn(68) is predicted, on the basis of 3-dimensional structure, to have a lower C_D than Asn (32).[235] Only two other proteins out of 28, epidermal growth factor and fibroblast growth factor, have computed C_D values that fail to correctly predict the most unstable amide.[235] In all three cases, the second-most unstable and the most unstable are reversed. It is possible that the solution structure of ribonuclease U_2 is different from that in the crystals used for this structure determination.

Monodeamidated ribonuclease folds more slowly than the undeamidated form,[236] but regains full enzymatic activity. When deamidated at 90 °C, however, the non-, mono-, di-, and tri-deamidated forms have 100%, 65%, 38%, and 19% biological activity, respectively.[237] At temperatures over 100 °C, ribonuclease deamidates more slowly in anhydrous nonane, 1-butanol, and DMF.[238]

The crystal structure of the isoAsp(67) form has been determined.[239] The thermal denaturation temperature of RNAase-Asp(67) is slightly

231 T. W. Thannhauser and H. A. Scheraga, *Biochemistry* **24**, 7681 (1985).

232 A. DiDonato, M. A. Ciardiello, M. d. Nigris, R. Piccoli, L. Mazzarella, and G. D'Alessio, *J. Biological Chemistry* **268**, 4745 (1993).

233 H. Zhou and D. J. Strydom, *European Journal of Biochemistry* **217**, 401 (1993).

234 S. Kanaya and T. Uchida, *Biochemical Journal* **240**, 163 (1986).

235 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

236 Y. P. Venkatesh and P. J. Vithayathil, *Int. J. Peptide and Protein Research* **25**, 27 (1985); S. Orru, L. Vitagliano, L. Esposito, L. Mazzarella, G. Marino, and M. Ruoppolo, *Protein Science* **9**, 2577 (2000).

237 S. E. Zale and A. M. Klibanov, *Biochemistry* **25**, 5432 (1986).

238 D. B. Volkin, A. Staubli, R. Langer, and A. M. Klibanov, *Biotechnology and Bioengineering* **37**, 843 (1991).

239 S. Capasso, A. D. Donato, L. Esposito, F. Sica, G. Sorrentino, L. Vitaglianol, A. Zagari, and L. Mazzarella, *J. Molecular Biology* **257**, 492 (1996); L. Esposito, L.

higher than that of RNAase-Asn(67), but is very much lower for RNAase-isoAsp with the enthalpy change suggesting the loss of two hydrogen bonds.[240]

This provides an interesting demonstration of the fact that equilibrium thermodynamics cannot predict reaction rates, nor vice versa. During the deamidation of Asn(67) in RNAase-A at 37 °C in pH 8.0, 0.01 M tris, the RNAase-isoAsp form initially predominates because hydrolysis of the succinimide to isoAsp is faster than for Asp. These reactions eventually achieve equilibrium, however, by exchange through the succinimide. At equilibrium, the greater stability of RNAase-Asp(67) causes it to predominate over RNAase-isoAsp.[241]

The ratio isoAsp(67):Asp(67) is initially as high as in peptides, where it is generally 3:1, but, as equilibrium is achieved, this ratio drops to 1:2 in RNAase. Formation of the succinimide is rate limiting at neutral pH.[242] This preponderance of Asp over isoAsp will, of course, be true of any amide for which the Asp configuration is of lower energy than the isoAsp. This is likely to be the case in many proteins. The nonenzymatic equilibration of Asp and isoAsp takes place through the imide and at a rate that is usually much slower than deamidation. In protein preparations that have had time to equilibrate, however, this can markedly change the isoAsp:Asp ratio.

The deamidation half-time of Asn(67) of ribonuclease A measured in several concentrations of pH 7.4 Tris at 37 °C and then extrapolated to zero buffer concentration is 67 days.[243] The computed C_D is 0.696.[244] This C_D corresponds to a half-time of 70 days. Half-times for 8-residue model peptides of ribonuclease A sequences with and without a disulfide bond were 3.75 and 2.54 days, respectively. The 0.15 M pH 7.4 Tris rate at 37 °C for GlyLysAsnGlyGly is 1.02. Reduced and unfolded ribonuclease A was reported to have a deamidation half-time in 0.1 M pH 7.9 Tris at 37 °C of 0.96 days.[245] Clearly, secondary and tertiary structure largely determines the deamidation of Asn(67).

Vitagliano, F. Sica, G. Sorrentino, A. Zagari, and L. Mazzarella, *J. Molecular Biology* **297**, 713 (2000).

240 G. Baronea, F. Catanzano, P. D. Vecchio, C. Giancola, and G. Graziano, *Pure and Applied Chemistry* **69**, 2307 (1997); F. Catanzano, G. Graziano, S. Capasso, and G. Barone, *Protein Science* **6**, 1682 (1997).

241 S. Capasso and P. D. Cerbo, *J. Peptide Research* **56**, 382 (2000).

242 S. Capasso, G. Balboni, and P. D. Cerbo, *Biopolymers* **53**, 213 (2000).

243 S. Capasso and S. Salvadori, *J. Peptide Research* **54**, 377 (1999).

244 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

245 S. J. Wearne and T. E. Creighton, *Proteins: Structure, Function, and Genetics* **5**, 8 (1989).

Tris is a mild base catalyst of deamidation, so the computed Tris half-time should be lower than the experimental zero buffer half-time. In the case of ribonuclease A, this question can be resolved by a difference between the crystal structure and the solution structure. C_D computed for Asn(67) in crystal structure 1AFK is 0.696, but for solution NMR structure 2AAS is 0.402. This difference is caused by the presence of a hydrogen bond to the amide nitrogen of Asn(67) that is present in the crystal structure, but not present in the NMR solution structure. So, increase of the computed Tris C_D of 0.402 to correct for buffer catalysis brings the computed deamidation half-time and the zero buffer experimental half-time into good agreement.

Agreement between C_D computation and qualitative observations has also been obtained for human ribonuclease 4.

Other interesting studies related to ribonuclease deamidation include: 1974S, 1975DR, 1978SB, 1982AC, 1982BK, 1985OP, 1986DG, 1988GC, 1993RS, 1997LX, 1999GB, 2000CC, 2001C1, and 2003GL.

12-23. TRIOSEPHOSPHATE ISOMERASE

Heterogeneity of triosephosphate isomerase in human erythrocytes was reported in 1974,[246] in human eye lenses in 1976,[247] and in human lymphoblasts and fibroblasts in 1977.[248] This heterogeneity was found to be greater in old human lenses than in young lenses. In 1980, Gracy and Yaun discovered that human TPI deamidation occurs preferentially at LysMetAsn(15)GlyArg and ValThrAsn(71)GlyAla, which are located at the interface between the two subunits of this dimeric enzyme.[249] The complete primary and 3-dimensional crystal structure of chicken TPI, which does not have Asn(71), was determined in 1975.[250]

Gracy and coworkers then undertook extensive studies of triosephosphate isomerase, which have revealed a detailed description of this remarkable enzyme.

246 B. M. Turner, R. A. Fisher, and H. Harris, *Isozymes, (Ed. C. L. Market), Academic Press, New York* **1**, 781 (1974).

247 H. Skala-Rubinson, M. Vibert, and J. C. Dreyfus, *Clinica Chimica Acta* **70**, 385 (1976).

248 M. V. Kester, E. L. Jacobson, and R. W. Gracy, *Archives of Biochemistry and Biophysics* **180**, 562 (1977).

249 R. W. Gracy and P. M. Yuan, *Federation Proceedings*, **39**, 1690 (1980).

250 D. W. Banner, A. C. Bloomer, G. A. Petsko, D. C. Phillips, C. I. Pogson, I. A. Wilson, P. H. Corran, A. J. Furth, J. D. Milman, R. E. Offord, J. D. Priddle, and S. G. Waley, *Nature* **255**, 609 (1975).

Characterization of TPI deamidation products gave Asp(71): Asp(15) of 2.54:1 for human and 1.73:1 for rabbit TPI.[251] The computed C_D values give 2.27:1,[252] so these could be independent deamidations. With no computed C_D values available at that time, however, the investigators proposed that Asn(71) deamidates before Asn(15), in a sequential process resembling that of cytochrome c. This proposal was partially based upon the fact that deamidation was not observed in chicken TPI at Asn(15), so deamidated Asn(71) might be a prerequisite for deamidation of Asn(15). They further proposed that this deamidation, like that previously shown in cytochrome c and aldolase, controls TPI turnover and, with the assistance of decreased catabolic activity with age, is responsible for the accumulation of degraded TPI in older tissues such as in older human erythrocytes.[253] Increased deamidated forms of TPI were found in skin fibroblasts from humans with the premature aging diseases, progeria and Werner's syndrome.[254]

In a series of studies between 1982 and 1991, Gracy, Yüksel, coworkers, and colleagues concluded that, as described in their publications and summarized[255] that:

1. Mammalian TPI deamidates at Asn(71), which then permits the deamidation of Asn(15).

2. Deamidated TPI accumulates in a wide variety of aging cells, apparently as a result of decreased catabolic activity.

3. A hinged lid at the active site of TPI closes during catalysis, which enhances the deamidation of Asn(71). So, the probability of deamidation of an individual TPI molecule is a function of the number of times it is used as a catalyst.

4. Deamidation loosens the association of the monomers in ordinarily dimeric TPI and enhances catabolism, which begins in fibroblasts with hydrolysis of the bond between Thr(139)Glu(140).

5. Glucosephosphate isomerase may behave in a similar manner.

251 P. M. Yuan, J. M. Talent, and R. W. Gracy, *Mechanisms of Aging and Development* **17**, 151 (1981).

252 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001).

253 R. W. Gracy, H. S. Lu, P. M. Yuan, and J. M. Talent, *Altered Proteins and Aging, (Ed. R. C. Adelman and G. S. Roth), CRC Press, Boca Raton, FL* **Chapt. 2**, 9 (1983).

254 T. O. Tollefsbol, M. R. Zaun, and R. W. Gracy, *Mechanisms of Aging and Development* **20**, 93 (1982).

255 R. W. Gracy, private communication to ABR, August 19, 1991.

The relevant publications are 1983GL, 1982G, 1983G, 1984LY, 1984NT, 1985AI, 1985GC, 1985YJ, 1986YJ, 1986YG, 1986YG1, 1986YG, 1987YJ, 1987YG, 1987AC, 1990JY, 1990SK1, 1991WN, 1991SG, 1992SY1, 1992SY2, 1992SY, 1994MM1, 1994GG, 1995A1, 1995GY, and 1997TZ.

Yüksel and Gracy found the single deamidation half-time of TPI in pH 7.0, 37 °C in 0.05 M phosphate and triethanolamine buffers to be 37.8 and 21.7 days, respectively. Computed C_Ds in pH 7.4, 37 °C 0.15 M Tris for Asn(71) and Asn(15) are 0.273 and 0.635 or $t_{1/2}$ = 27 and 63 days, respectively. I_D, assuming independence of the amide residues, is 0.187 for a single deamidation half-time of 19 days. At pH 10, deamidation was up to 7-fold increased in the presence of substrate with an average of 3-fold in 8 different buffers.[256]

In the absence of substrate, the 8 buffers generally obeyed a Brønsted plot of pK_a vs. deamidation rate. Tris buffer deviates significantly from the plot with much lower catalysis than the other buffers. The *in vivo* TPI deamidation half-time in human fibroblasts is between 0.5 and 2 days.[257]

The *in vitro* deamidation rates of human TPI are, therefore, entirely consistent with computed C_Ds, except that these C_Ds, based on crystal structure, suggest that Asn(15) should show the measured deamidation regardless of Asn(71). It is possible that the quaternary structure is different in the crystals. Alternatively, the interpretation of the hydridization experiments might be incomplete.

A genetic variant of human TPI has been reported that deamidates more rapidly; causes, therefore, a TPI deficiency; and leads to hemolytic anemia, myopathy, and mental retardation has been reported.[258]

Human, rabbit, chicken, and yeast TPIs all have Asn(15), but chicken and yeast lack Asn(71) with Lys and Ser, respectively, replacing it.[259] Chicken TPI follows a similar *in vivo* degradation apparently triggered by oxidation of IleAlaCys(126)IleGly with increased de-

256 K. Ü. Yüksel and R. W. Gracy, *Archives of Biochemistry and Biophysics* **248**, 452 (1986).

257 K. Yüksel, M. Jahani, M. L. Chapman, and R. Gracy, *Isozyme Bulletin* **19**, 20 (1986).

258 S. W. Eber, A. Pekrun, A. Bardosi, M. Gahr, W. K. G. Krietsch, J. Krüger, R. Matthei, and W. Schröter, *European Journal of Pediatrics* **150**, 761 (1991).

259 A. Sun, K. U. Yuksel, and R. W. Gracy, *J. Biological Chemistry* **267**, 20168 (1992).

graded forms in older cells, and turkey TPI is thought be similar.[260] Both chicken and yeast TPI have a hinged-lid active site similar to that of human TPI.[261]

Studies of yeast TPI have also shown that deamidation or mutation of Asn(14) and Asn(78) in the dimer interface decreases TPI activity and increases susceptibility to proteases.[262] Deamidation of Asn(78) reduces activity to 66% of that of undeamidated TPI. Replacement of Asn(78) in yeast TPI with Thr or Ile increases thermal stability.[263] His(12)Lys(13) is found instead of Asn(12)Gly(13) in TPI of the thermophilic bacteria *Bacillus stearothermophilus*, which helps preserve function but not thermal stability.[264]

The sequential model of deamidation of mammalian TPI wherein deamidation of Asn(71) is required before deamidation of Asn(15) is based on two experimental systems. First, hybrid dimers of rabbit and yeast TPI, which lacks Asn(71), show that rabbit Asn(71) accelerates the deamidation of yeast Asn(15), but that rabbit Asn(15) cannot deamidate when opposite yeast Ser(71).[265] Second, the oxidation at Cys(126) of avian TPI, which lacks Asn(71), leads to the deamidation of Asn(15).[266] The effect of substrate on the TPI reaction method has been theoretically modeled.[267]

Human fibroblast TPI is also an excellent example of the widely observed fact that altered, especially deamidated, proteins accumulate in older cells. The prevalent hypothesis is that this is the result of de-

260 K. N. Gracy, C. Y. Tang, K. Ü. Yüksel, and R. W. Gracy, *Mechanisms of Aging and Development* **56**, 179 (1990); C. Tang, K. Ü. Yüksel, T. M. Jacobson, and R. W. Gracy, *Archives of Biochemistry and Biophysics* **283**, 12 (1990); Y. Zhang, K. Ü. Yüksel, and R. W. Gracy, *Archives of Biochemistry and Biophysics* **317**, 112 (1995).

261 Z. Zhang, S. Sugio, E. A. Komives, K. D. Liu, J. R. Knowles, G. A. Petsko, and D. Ringe, *Biochemistry* **33**, 2830 (1994); K. U. Yuksel, A. Sun, R. W. Gracy, and K. D. Schnackerz, *J. Biological Chemistry* **269**, 5005 (1994).

262 J. I. Casal, T. J. Ahern, R. C. Davenport, G. A. Petsko, and A. M. Klibanov, *Biochemistry* **26**, 1258 (1987).

263 T. J. Ahern, J. I. Casal, G. A. Petsko, and A. M. Klibanov, *Proc. Natl. Acad. Sci. USA* **84**, 675 (1987).

264 L. F. Delboni, S. C. Mande, F. Rentier-Delrue, V. Mainfroid, S. Turley, F. V. Vellieux, J. A. Martial, and W. G. Hol, *Protein Science* **4**, 2594 (1995); M. Alvarez, J. Wouters, D. Maes, V. Mainfroid, F. Rentier-Delrue, L. Wyns, E. Depiereux, and J. A. Martial, *J. Biological Chemistry* **274**, 19181 (1999).

265 A. Sun, K. U. Yüksel, and R. W. Gracy, *Archives of Biochemistry and Biophysics* **322**, 361 (1995).

266 R. W. Gracy, J. M. Talent, and A. I. Zvaigzne, *J. Experimental Zoology* **282**, 18 (1998).

267 F. A. S. Konuklar, V. Aviyente, G. Monard, and M. F. Ruiz Lopez, *J. Physical Chemistry B* **108**, 3925 (2004).

creased catabolism in these cells. Starvation apparently activates the ordinary catabolic machinery or auxiliary machinery. Older human fibroblasts starved for 0, 48, 96, and 240 hours, showed percentages of deamidated TPI of 23.5, 14.5, 12.0, and 2.5%, respectively.[268]

The conclusions of Gracy and coworkers are as follows:

Triosephosphate isomerase, an enzyme that is widely distributed in organisms from bacteria to mammals, is a dimer with four charged or potentially charged residues paired with each other in the dimer interface. In mammals, these are all Asn residues.

As the enzyme functions, a "hinged lid" over the active site closes, excluding water and preventing hydrolysis of triosephosphates to toxic products. When the lid is closed, Asn(71) in that monomer deamidates more readily. This deamidation facilitates deamidation of the paired Asn(15) on the other monomer. These deamidations weaken the dimer association, open the enzyme, and lead, through increased catabolism, to protein turnover.

The net result is that amide clocks inside each TPI molecule count the number of catalytic cycles by increasing the probability of deamidation of that molecule during the catalytic process.

Structural change caused by deamidation opens the enzyme to degradation, but, as cells age, the degradative machinery weakens, so deamidated TPI accumulates in aging cells. This accumulation can be reversed by fasting.

This amplified deamidation wherein a first amide clock facilitates a second deamidation that then participates in the protein structure change mediated by the first clock has been reported for two proteins, cytochrome c and TPI.

12-24. TRYPSIN

In 1981, Kossiakoff and Spencer reported a 2.2 Å resolution neutron diffraction structure for bovine trypsin.[269] This structure was refined in the presence of D_2O to 1.9 Å. Crystal growth was carried out over a period of one year in 8% MgSO4 at pH 7.4, and soaking in D_2O and data collection required another 6 months.

268 R. W. Gracy, M. L. Chapman, J. K. Cini, M. Jahani, T. O. Tollefsbol, and K. Y. Yüksel, *Molecular Biology of Aging (Ed. A. D. Woodhead and A. Blackett), Plenum Press, New York*, 427 (1985); R. W. Gracy, J. M. Talent, and A. I. Zvaigzne, *J. Experimental Zoology* **282**, 18 (1998).

269 A. A. Kossiakoff and S. A. Spencer, *Biochemistry* **20**, 6462 (1981).

Examining this structural data seven years later, Kossiakoff found that IleAsn(48)Ser, TyrAsn(95)Ser, and LeuAsn(115)Ser were deamidated in the crystals with all three present as L-Asp. Since the protein was exposed to high ionic strength and neutral pH for 1.5 years, it is not surprising that deamidation was observed.

In 1988, Kossiakoff published a paper entitled "Tertiary Structure is a Principal Determinant of Protein Deamidation."[270] The data presented does not actually demonstrate tertiary structure dependence of deamidation, which was, in any case, well-established in experiments 15 to 20 years earlier. See, in review, Robinson and Rudd.[271] It depends instead upon a purported absence of primary structure dependence for its proof. No deamidation rate data is presented.

Kossiakoff states that, in his crystallographic study of trypsin, "No correlation was found between preference to deamidate and the chemical character of residues flanking the site, as had been proposed from previous peptide studies," and that, "All three deamidated groups have the sequence X-Asn-Ser, where X is Ile[47], Tyr[94], and Leu[114], respectively. According to the peptide predictions, these large hydrophobic residues should retard deamidation."

In fact, the 3 AsnSer sequences that he found to be deamidated among the 16 Asn in trypsin were among the 5 fastest primary sequence-predicted Asn residues in trypsin based on measurements published prior to 1974. Considering measurements available in 2001, they are among the 4 fastest.

The fifth most rapidly deamidating peptide ever measured at the time Kossiakoff published these words was GlyIleAsnSerGly, which had a measured deamidation half-time in pH 7.4, 0.15 I, phosphate buffer at 37 °C of 18 days.[272] His trypsin was in solution for a year during crystallization. This peptide has a primary sequence identical to one of the three deamidating sequences in trypsin. It is an early example of the dominance of the carboxyl-side residue over the amino-side in determining deamidation rate, and is in the 1974 peptide deamidation list in Robinson and Rudd.[271]

Carboxyl-side Ser sequences are now known to have median Tris deamidation half-times of 16 days, in good agreement with this early

270 A. A. Kossiakoff, *Science* **240**, 191 (1988).

271 A. B. Robinson and C. J. Rudd, *Current Topics in Cellular Regulation* **8**, 247 (1974).

272 A. B. Robinson and S. Tedro, *Int. J. Peptide and Protein Research* **5**, 275 (1973).

determination.[273] The three most rapidly deamidating Asn in trypsin are correctly predicted by calculations that depend upon the protein primary, secondary, and tertiary structure.[274] Without inclusion of the primary structure dependence, this computation would fail.

It has been suggested that the deamidation of the three AsnSer residues in trypsin proceeds by a unique mechanism because Kossiakoff reports that the reaction products are in the L-Asp form. It has been pointed out that this hypothesis lacks verification.[275]

No experiments on the deamidation of trypsin in solution were reported. The long period of time during crystal growth permitted the selective inclusion of one isomeric form in the crystals and the exclusion of others. Moreover, the equilibrium concentrations of the final deamidation products can, over this long time interval, be shifted toward the L-Asp forms by the relative stabilities of the protein in the L-Asp and L-isoAsp configurations.[276] Both of these effects have since been experimentally demonstrated in other proteins.

While additional undiscovered deamidation mechanisms besides imide and direct hydrolysis may indeed exist, these experiments do not show this. The strong correlation in trypsin with primary structure rates also suggests the imide mechanism.

Recent investigations[277] have experimentally demonstrated the differentiation effect for LeuAsn(115)Ser in trypsin, one of the three labile Asn in trypsin. During a 27-month period of crystal growth in Tris at pH 8.5, a crystal form was created that had only D-Asp at position 115. This D form would not co-crystallize with ordinary trypsin. Other crystals were obtained after only one month, but these gave poor resolution, probably because they were a mixture of altered proteins, so crystals that first appeared 27-months after setting up the crystallization were used.

273 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

274 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001).

275 W. J. Chazin and A. A. Kossiakoff, *Deamidation and Isoaspartate Formation in Peptides and Proteins (Ed. D. W. Aswad, CRC Press, Boca Raton, FL)*, 193 (1995).

276 S. Capasso and P. D. Cerbo, *J. Peptide Research* **56**, 382 (2000).

277 S. D. Marco and J. P. Priestle, *Structure* **5**, 1465 (1997); U. Rester, M. Moser, R. Huber, and W. Bode, *Acta Crystallographica Section D, Biological Crystallography* **56**, 581 (2000).

Moreover, additional investigations of porcine trypsin have found an isoAsp form at Asp(115) that is selected by crystallization.[278] These crystals required 17 to 27 months in pH 6.5, 0.02 M MES, 0.15 M NaCl at 21 °C for growth.

Computed C_D values[279] for bovine trypsin based upon a more recent structure determination[280] give C_D = 60.2, 1.70. 1.57. and 6.23 for LeuAsn(34)Ser, IleAsn(48)Ser, TyrAsn(95)Ser, and LeuAsn(114)Ser, respectively. Thus, the three AsnSer sequences calculated from primary, secondary, and tertiary structure to be most unstable are, in fact, the three found to deamidate. The expected deamidation half-times of 170, 157, and 623 days are consistent with the long period for crystallization.

The deamidation of trypsin depends upon primary structure and higher order structure. The inherently fast AsnSer sequences are computationally predicted to be differentially slowed in the ordinary manner. The reported steric homogeneity in the crystals was probably the result of differential crystallization, although it could have arisen in other ways.

12-25. OTHER PROTEINS

The proteins listed in Table 11-1, including the 23 discussed in this chapter, comprise all observations of protein deamidation found in this review. In view of the ubiquitous nature of deamidation, frequent reports of additional such observations in other proteins are expected.

Table 11-1 is restricted to proteins for which the investigators suggested that deamidation was responsible for their experimental observations. In many cases, this deamidation was very thoroughly characterized. In others, charge heterogeneity and other general observations led to the investigator's conclusions. It is to be expected that, in some cases, these conclusions were incorrect. All have been included in the table, so that researchers interested in individual proteins can easily find references that may be relevant to their studies.

Deamidation reviews of interest include 1974RR, 1980R, 1985H, 1992CS, 1992L, 1995A1, 1995A2, 1996P, 1999XS, 2000Y, 2001LH, 2002MH, 2003RA, 2003C, and 2003Y.

278 U. Rester, M. Moser, R. Huber, and W. Bode, *Acta Crystallographica Section D, Biological Crystallography* **56**, 581 (2000).

279 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

280 M. T. Stubbs, R. Huber, and W. Bode, *FEBS Letters* **375**, 103 (1995).

CHAPTER 13

Biological Molecular Clocks

13-1. PROTEIN DYNAMICS

The molecular clock hypothesis has been extant for many years. As more data accumulates this hypothesis becomes stronger. While this alone is interesting, the recent discovery[1] of the pervasiveness of deamidation has changed our view of proteins.

Proteins are usually thought of as small fixed machines. Each one is designed with a specific structure for a specific function or set of functions. As proteins become old they are replaced with new proteins in order to keep them from deteriorating too much or causing negative effects. Additionally, some proteins are only needed briefly for a specific function and are then discarded.

With the demonstration that a large fraction of proteins are deamidating over biologically relevant time periods, it now appears that the protein pool is in a much more dynamic amide-determined, time-dependent state of change than previously supposed.

The additional possibilities for biological systems are extensive. A protein is not necessarily a single species. It can be a whole family of molecules that are generated from the original structure. This provides additional flexibility in biological systems.

13-2. ASN AND GLN AS INDIVIDUAL CLOCKS

After synthesis and folding, peptides and proteins undergo changes in charge and conformation through nonenzymatic deamidation of asparaginyl and glutaminyl residues. Each amide has a specific deamidation rate that is genetically determined by the sequence of residues immediately adjacent in the peptide chain and by secondary, tertiary, and quaternary structure.

Deamidation introduces a negatively charged carboxylic acid side chain and also causes isomerization. In effect, every amide in a protein

1 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002); N. E. Robinson, *PhD Thesis, California Institute of Technology, Chemistry* (2003); See also Chapters 9 and 11.

molecule is a miniature molecular clock that changes the structure of the protein in accordance with its deamidation rate and the structural consequences of a change in charge and conformation at its location.

Proteins are altered by many *in vivo* post-synthetic modifications, including chain cleavage, crosslinking, deamidation, glycosylation, oxidation, phosphorylation, racemization, and other enzymatic and nonenzymatic processes. Nonenzymatic deamidation of Asn and Gln is, however, unique. It is the most prevalent; it is an inherent characteristic of every amide residue, including therefore 10% of the 20 ordinary building blocks of proteins and 8% of those actually used in proteins; and it is under precise genetic control.

Most biochemical processes are enzyme mediated. The number of potential chemical reactions involving the substances in living things is very large. Most of these reactions occur very slowly under physiological conditions, so living things can select the reactions required for life simply by producing enzymatic catalysts only for those that are desirable. Deamidation is unusual because it occurs nonenzymatically and ubiquitously at biologically important rates and because it produces fundamental changes in the structures of proteins, which are the essential functional elements in living things.

Enzymatic deamidation of specific Gln residues is known to occur in some proteins, but enzymatic deamidation of Asn has, as yet, not been found. Since Asn deamidation is generally much faster than Gln, Asn is likely to be involved in more timing processes than is Gln.

Moreover, since deamidation is under precise genetic control and can easily be genetically suppressed, it is evident that the widespread presence of rapidly deamidating Asn residues in biological peptides and proteins has a beneficial purpose. Since it operates without enzyme mediation, changes protein structure markedly when it occurs, and can be genetically programmed at any location in a protein and for any timed interval from hours to centuries, deamidation is an ideal molecular clock.

Is deamidation a general biomolecular clock or rather an odd curiosity that regulates only occasional biological processes? First, it is present. Second, it is ideal for this purpose. Third, it is built into almost every protein. Why, with deamidation available, would any other clock be necessary? Fourth, deamidation is obviously being used for some widespread and fundamental biological purpose. If this were not so, it would be genetically suppressed because it is otherwise very disruptive to protein structures and would not be tolerated in living systems.

When these arguments were published 30 years ago and the structure dependence of deamidation over a wide range suitable for a molecular clock was discovered, there remained the possibility that amide residues had other essential functions requiring their presence regardless of their disruptive character. It has now been shown, however, that most amide residue structures are stable over biological time periods, but that a relatively small percentage are both unstable with respect to deamidation and genetically selected to be present in a large percentage of proteins.[2] It is evident, therefore, that this instability is biologically functional. If it were not, stable amide structures, which make up most of those available, could easily be genetically specified.

It is also known that the sequences of residues near amides are unusually non-random.[3] Nonenzymatic deamidation of Asn has, so far, been shown to regulate three types of processes, including the timing of the turnover rates of cytochrome c[4] and aldolase,[5] the counting of individual enzyme catalytic cycles in triosephosphate isomerase,[6] and the time-dependent monitoring of DNA repair and the regulating of apoptosis in Bcl-X_L.[7] Asn deamidation has been observed in more than 200 types of biological peptides and proteins.[8] In many cases, deamidation has a significant affect on biological activity. Some of these cases are reviewed in Chapter 12. See also in review 1995TH.

2 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001); N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001); N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001); N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002); N. E. Robinson and A. B. Robinson, *Mechanisms of Ageing and Development* **125**, 259 (2004); N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

3 A. B. Robinson and L. R. Robinson, *Proceedings of the National Academy of Sciences of the United States of America* **88**, 8880 (1991).

4 T. Flatmark and K. Sletten, *J. Biological Chemistry* **243**, 1623 (1968); A. B. Robinson, J. H. McKerrow, and M. Legaz, *Int. J. Peptide and Protein Research* **6**, 31 (1974)..

5 C. Y. Lai, C. Chen, and B. L. Horecker, *Biochemical and Biophysical Research Communications* **40**, 461 (1970); C. F. Midelfort and A. H. Mehler, *Proc. Nat. Acad. Sci. USA* **69**, 1816 (1972); J. H. McKerrow and A. B. Robinson, *Science* **183**, 85 (1974).

6 A. Sun, K. U. Yüksel, and R. W. Gracy, *Archives of Biochemistry and Biophysics* **322**, 361 (1995); R. W. Gracy, J. M. Talent, and A. I. Zvaigzne, *J. Experimental Zoology* **282**, 18 (1998).

7 B. E. Deverman, B. L. Cook, S. R. Manson, R. A. Niderhoff, E. M. Langer, I. rosova, L. A. Kulans, X. Fu, J. S. Weinberg, J. W. Heinecke, K. A. Roth, and S. J. Weintraub, *Cell* **111**, 51 (2002).

8 See Chapter 11, Table 11-1.

In the case of protein turnover, in addition to the three specific known cases and some additional proposed cases a rough correlation between *in vivo* half-life and protein amide content has been reported and most deamidated proteins are found to be more easily digestible by proteolytic enzymes. It is possible that deamidated proteins are recognized and processed by the same ubiquitin system that mediates the very rapid turnover of N-rule marked proteins.[9] The N-rule system has been extensively studied.[10] Protein turnover can, of course, also be implemented by deamidation-timed turnover of organelles

In the case of development and metabolic regulation, numerous deamidation mechanisms are probably present. Two, those for triosephosphate isomerase and Bcl-X_L, have been discovered.

The distribution functions for deamidation half-times of proteins in humans, *Drosophila*, and most other organisms show that deamidation of many proteins occurs during the biological lifetimes of the organelles, cells, and organisms in which they are imbedded. Amide clocks are present, available, and able to time almost all biological processes with timing requirements from hours to centuries, including virtually any biological process from protein turnover to aging.

13-3. IMPLEMENTATION OF AMIDE CLOCKS

In the case of deamidation-produced active proteins, only a small amount of deamidation may be required to initiate a biological change, while, in processes that depend upon loss of protein function caused by deamidation, larger percentages of deamidation are probably necessary.

Great diversity may be expected in the implementation of amide clocks. The clocks themselves, however, have the substantial advantage that they deamidate at genetically determined rates. While these rates are subject to metabolic control, especially by protein-protein interactions as in Bcl-X_L, they are intrinsic properties of individual protein structures and do not require additional factors such as oxidizing or phosphorylating agents to function.

How then are these clocking functions implemented? Since Harvey Itano's discovery of the cause of sickle-cell anemia and the introduction of the concept of molecular disease in 1949,[11] the potentially profound structural effects of a change of one charge in a protein molecule have

9 N. E. Robinson, *Submitted to J. Theoretical Biology* (2003).

10 A. Varshavsky, *Cold Spring Harbor Symposia on Quantitative Biology* **60**, 461 (1995).

11 L. P. Pauling, H. A. Itano, S. J. Singer, and I. C. Wells, *Science* **110**, 543 (1949).

been evident. Deamidation changes the charge at its location and can induce some isomerization as well. These effects then manifest themselves in a multitude of ways.

There are many examples of proteins in which deamidation increases susceptibility to proteolytic digestion, decreases thermal stability, and changes other characteristics. This is unsurprising. It would be more surprising if such changes did not occur.

A virtually unlimited number of research papers could be written by observing peptide and protein structural changes upon deamidation. These changes are no more than would be expected by most protein chemists. They do not, in themselves, confer any special character to the deamidation reactions under study. Deamidation can potentially and profoundly alter peptide and protein structure in a multitude of ways. The full extent of the biological consequences of such alterations is, at present, unknown.

CHAPTER 14

Deamidation and Aging

14-1. INTRODUCTION

Biological aging can be defined as increased age-specific mortality with increase in time. A multitude of biochemical changes occur as a function of time in all living things, but it is those that lead to debility and death that, for obvious reasons, have preoccupied human attention throughout recorded history.

These biochemical changes are extensive. For example, at least one-third of the thousands of substances in human and mouse urine change quantitatively with increase in physiological and chronological age.[1] Some of these substances increase in quantity with time, while others decrease. These changes in the molecules cast off into the urine reflect the pervasive age-dependent changes occurring in the biochemical metabolisms by which they are produced and regulated.

The fundamental molecular basis of the aging of living things is unknown, although it is quite possible that the cause or causes are among the large number of hypothetical mechanisms that have been proposed.

These hypotheses can be divided into two classes, which are conceptually and even philosophically different. One holds that aging occurs primarily through the abandonment of the organism to biochemical degradation through mechanisms that comprise inherent weaknesses in living systems. The other holds that aging is deliberately built into organisms – that there are specific, genetically programmed biochemical aging clocks running inside each one.

It is, of course, not necessary that the mechanisms of aging of all organisms be the same, but, in the absence of knowledge of these mechanisms, this assumption is the simplest. When aging is better understood, this can be examined.

Of the two classes of hypotheses, the second is the more likely. The life spans of living things vary over a range of more than 100,000 at ordinary temperatures, yet the fundamental biochemistry of most living things is similar. It seems improbable that an abandonment of these or-

1 A. B. Robinson and L. R. Robinson, *Mechanisms of Aging and Development* **59**, 47 (1991); A. B. Robinson, H. Dirren, A. Sheets, J. Miquel, and P. R. Lundgren, *Experimental Gerontology* **11**, 11 (1976).

ganisms to the inherent vicissitudes of their biochemistry would occur over such a wide range of time intervals.

Rather, as also appears likely from the apparent advantages of observed life spans, it is probable that aging is deliberately programmed into living systems.

The hypothesis that deamidation may serve as a molecular clock for organismic aging was published by Robinson, McKerrow, and Cary in 1970. Thereafter, this hypothesis became known as the "deamidation theory of aging" and took its place among the many other biochemical processes that have been proposed as aging mechanisms.[2]

The parallel hypothesis that deamidation is a mechanism for genetically programmed time-dependent alteration of protein molecules for positive biological purposes, other than organismic aging, within living systems[3] has proved to be correct, as is discussed elsewhere in this book.

Increased percentages of deamidated proteins with increased age have been extensively observed. These observations are neither necessary nor sufficient evidence for deamidation as a timer of aging. There is no reason that a timer of aging must manifest itself widely in the protein pool, nor is a simultaneous change of two parameters evidence for cause and effect.

Aging research is inevitably linked to life extension research. There are three aspects of life extension as illustrated[4] in Figures 14-1 to 14-4.

Figure 14-1 shows the ordinary distribution of life span for American men. The first objective of life extension research is the squaring of this curve as shown in Figure 14-2, so that each person experiences an ordinary full life without premature death. This work consists primarily of identifying and counteracting the causes of premature human death.

The second objective is the moderate extension of the length of an ordinary full life as illustrated in Figure 14-3. This work involves the study of systematic changes that can be made in human living conditions in order to achieve longer lives, without markedly altering the

2 K. Moffat, *Int. J. Peptide and Protein Research* **15**, 149 (1980).

3 A. B. Robinson, J. H. McKerrow, and P. Cary, *Proc. Natl. Acad. Sci. USA* **66**, 753 (1970); A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **71**, 885 (1974); A. B. Robinson and J. W. Scotchler, *Int. J. Peptide and Protein Research* **6**, 279 (1974); A. B. Robinson and L. R. Robinson, *Proc. Natl. Acad. Sci. USA 88*, 8880 (1991); N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001);N. E. Robinson and A. B. Robinson, *Mechanisms of Ageing and Development* **125**, 259 (2004) and other papers by these authors between 1971 and 2004.

4 A. B. Robinson and L. R. Robinson, *Mechanisms of Aging and Development* **59**, 47 (1991).

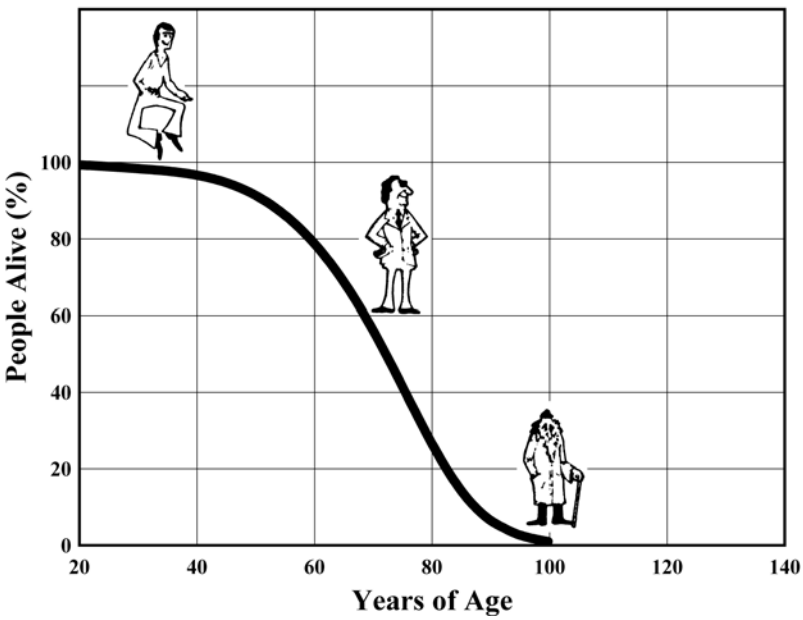

FIG. 14-1 Aging curve for a population comprised of American males in 1974 as calculated from life expectancy compilations of the U.S. Public Health Service. Adapted from 1991RR.

nature of the aging clock. These would include, for examples, diet restriction or body temperature reduction.

A recent study illustrated these two effects on the age-specific mortality of *Drosophila*.[5] In this case it was shown that reduction of body temperature decreases the rate of physiological aging, whereas diet restriction, while markedly extending life span, does not change the rate of aging of *Drosophila*. Diet restriction merely changes the probability of death at any specific physiological age.

Similar human diet restriction is possible, as are small changes in human body temperature. By means of these changes, human life span can probably be increased, as has been observed in many animals. It has been estimated that a decrease of human body temperature by 2 °C could increase human life span by 20 years.[6]

Figure 14-4 illustrates very large increases in life span that may be possible once the timers for aging are discovered. If these clocks are amenable to human intervention, Figure 14-4 might become reality.

5 W. Mair, P. Goymer, S. D. Pletcher, and L. Partridge, *Science* **301**, 1731-1733 (2003).

6 B. L. Strehler, *Gerontology* **8**, 14 (1967).

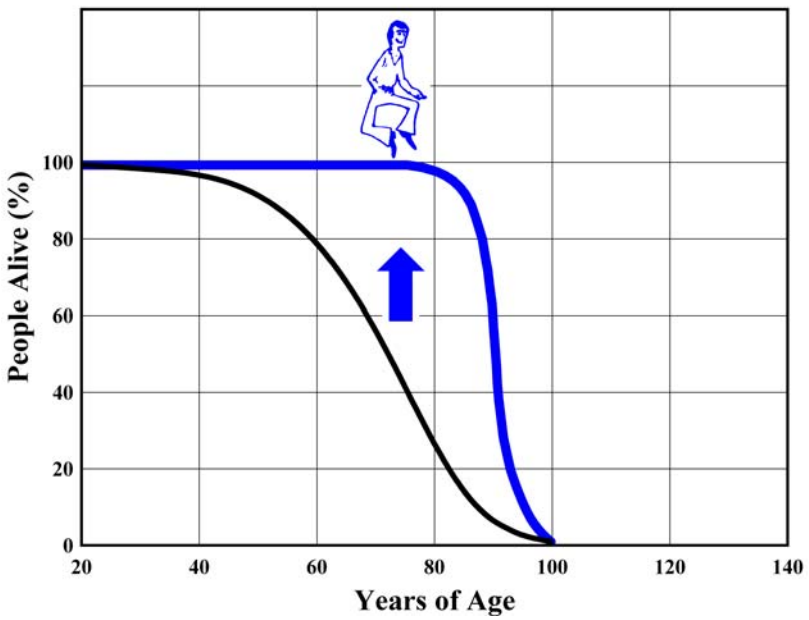

FIG. 14-2 Change in the human aging curve through improvements in diet and other living conditions, but with no change in intrinsic life span. Adapted from 1991RR.

Until the clocks are discovered, however, it will be unknown whether or not the marked slowing or resetting of those clocks will be possible.

We will designate the processes illustrated in Figures 14-2, 14-3 and 14-4 as "Type I, Type II, and Type III life extension." The comprehensive study of aging involves research into the factors and circumstances that must be overcome to achieve these life extensions.

As an organism moves along the one-dimensional line extending from 0% of life elapsed to 100% of life elapsed, as illustrated in Figure 14-5, the probability of death at each point determines the rate of that movement. Increases in those probabilities from any cause diminish life span.

Much progress has already been made in Type I life extension. Improved sanitation, better nutrition, antibiotics, and other advances have markedly increased the average length of human life among those people who have access to these benefits. Excellent living conditions have been discovered for a wide variety of organisms, primarily in the context of their use in industry, as pets, or for scientific research. Still, Figure 14-2 shows that much additional improvement is needed.

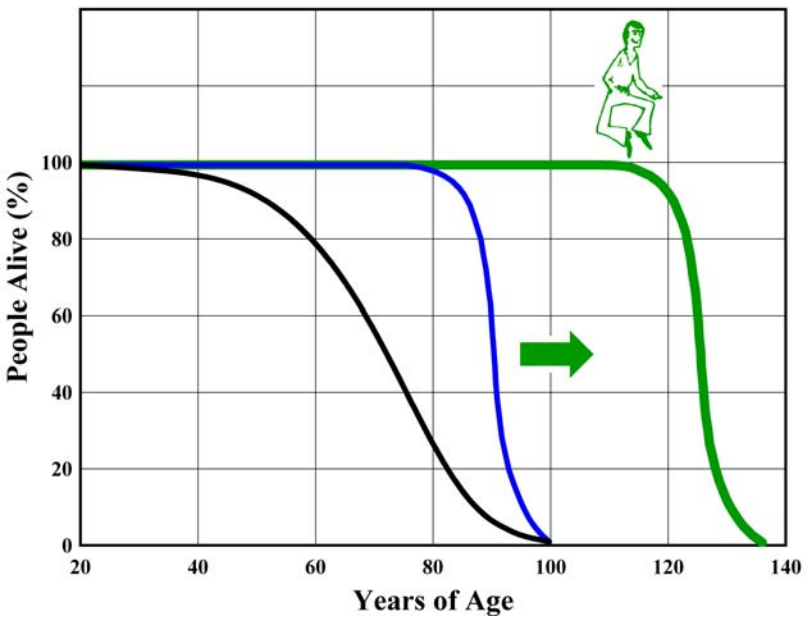

FIG. 14-3 Change in the human aging curve through improvements in diet and other living conditions that provide a modest increase in the intrinsic life span. Adapted from 1991RR.

Type II life extension of about the amount indicated in Figure 14-3 may also be possible. Diet restriction is a very promising technique for this as is indicated by current research.

The fundamental cause of aging is, as yet, not elucidated. When this has been done, the possibility for Type III life extension can be evaluated.

Deamidation may have a role to play in all three processes.

First, Chapter 15, reviews current research involving deamidation in some life-shortening diseases. The number of such examples will probably increase as investigations continue, since proteins are intimately involved in all living processes and deamidation is taking place in most proteins.

Second, it is possible that direct reversal of biochemical changes that occur with age can, to some extent, aid in the achieving of the objective illustrated in Figure 14-3. Reviewed herein, for example, is work showing that deamidated proteins accumulate in older cells. This accumulation can be reversed by fasting or diet restriction. While, as has been suggested, this accumulation may result from diminution of protein catabolic machinery with age and therefore not be an aging

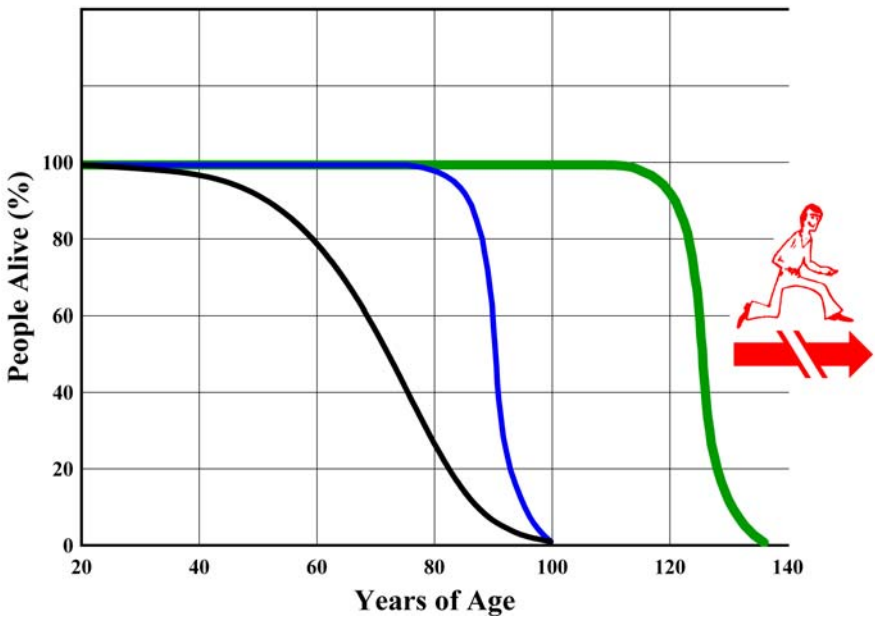

FIG. 14-4 A large increase in life span that may be possible from understanding the timers of aging. Adapted from 1991RR.

clock but rather the consequences of such a clock, removal of this accumulating cellular debris may provide Type II life extension.

Third, it is possible that deamidation is a fundamental molecular clock that times organismic aging. If so and if this clock is built into one or a relatively small number of proteins, it might be possible to change its rate. The merits of this hypothesis rest, at present, primarily on the fact that amide molecular clocks are present in the proteins of all living things and that a significant fraction of these amide clocks have half-time settings appropriate to the timing of the aging of the organ-

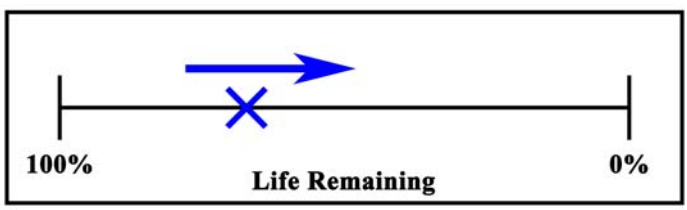

FIG. 14-5 Representative axes of physiological age. Adapted from 1991RR.

isms in which they are found. These simple, genetically controlled clocks are available and capable of timing aging. Why use others?

14-2. AGING HYPOTHESES

A multitude of hypotheses have been offered to explain aging. These are often referred to as "theories" of aging, but, in view of the fact that experimental confirmation is not available for any of them, they are more correctly termed "hypotheses." All of these hypotheses involve observed biochemical deteriorations of one sort or another.

Examples include the free radical hypothesis,[7] the oxidation hypothesis,[8] the protein synthesis error catastrophe hypothesis,[9] the crosslinking hypothesis,[10] the nucleic acid replication hypothesis,[11] the nucleic acid rate and extent of repair hypothesis,[12] the accumulation of somatic chromosome damage hypothesis,[13] the immunological hypothesis,[14] the racemization hypothesis,[15] the autoimmunity hypothesis,[16] the virus hypothesis,[17] the chemical balance hypothesis,[18] the loss of redundant genes hypothesis,[19] and the deamidation hypothesis.[20] There

7 D. Harman, *J. Gerontology* **11**, 298 (1956); J. Bjorksten, *J. American Geriatrics Society* **6**, 740 (1958); D. Harman, *The Lancet* **277**, 200 (1961).

8 E. R. Stadtman, *Experimental Gerontology* **23**, 327 (1988); E. R. Stadtman, *J. Gerontology* **43**, B112 (1988).

9 L. Orgel, *Proc. Natl. Acad. Sci. USA* **49**, 517 (1963); L. E. Orgel, *J. American Geriatrics Society* **67**, 1476 (1970); M. Laughrea, *Experimental Gerontology* **17**, 305 (1982).

10 J. Bjorksten, *J. American Geriatrics Society* **16**, 408 (1968); J. Bjorksten, E. R. Weyer, and S. M. Ashman, *Finska Kemists. Medd.* **80**, 70 (1971); J. Bjorksten, *Clinical Biochemistry* **2**, 209 (1982).

11 Z. A. Medvedev, *Adv. Gerontol. Res.* **1**, 181 (1964); B. L. Strehler, *Proc. 7th Congr. Gerontol. Vienna* , 177 (1966); R. W. Hart and R. B. Setlow, *Proc. Nat. Acad. Sci. USA* **71**, 2169 (1974).

12 R. W. Hart and R. B. Setlow, *Proc. Nat. Acad. Sci. USA* **71**, 2169 (1974).

13 L. Szilard, *Proc. Nat. Acad. Sci. (Wash.)* **45**, 30 (1959).

14 L. Walford, *The Immunological Theory of Aging, Williams and Wilkins, Baltimore* , 70 (1969).

15 J. L. Bada, *Proc. Natl. Acad. Sci. USA* **72**, 2891 (1975).

16 R. L. Walford, *J. Gerontology* **17**, 281 (1962).

17 J. E. Hotchin, *Tolerence, Auto-Immunity and Aging, (Ed. M. M. Sigel, and R. A. Good), Charles C. Thomas Publisher Springfield, IL*, 132 (1972).

18 L. V. Komarov, *J. Theoretical Biology* **4**, 147 (1963).

19 B. L. Strehler and M. R. Freeman, *Mechanisms of Aging and Development* **14**, 15 (1980).

20 A. B. Robinson, J. H. McKerrow, and P. Cary, *Proc. Natl. Acad. Sci. USA* **66**, 753 (1970); A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **71**, 885 (1974); A. B.

are dozens of additonal categories and subcategories of such hypotheses.

Interest in the oxidation hypothesis has been especially great in recent years. The work of Stadtman and coworkers on mixed-function oxidation is especially prominent. Reviews and examples of this large research literature include 1985L, 1985FS, 1986S, 1987D, 1987OL, 1992S, 1993S, 1996DF1, 1997PL, and 2000SL.

Type I and Type II life extensions include the multitude of therapeutic and lifestyle changes proposed by numerous advocates. In many of these cases, a single proposal is rhetorically supported by many different hypotheses, even including tenuous links to Type III extension. Diet restriction advocates, for example, cite free radicals,[7] accumulation of altered proteins in aged cells,[21] and reduced rate of cancer growth with diet restriction[22] in addition to correlational observations concerning the incidence of major degenerative diseases such as heart deterioration, cancer, and diabetes.

The aging literature contains many fascinating experiments, some of which have become classics.

Clive McCay showed that diet restricted animals live much longer than well-fed animals.[23] This finding has been extended by other investigators to a wide variety of organisms.[24]

T. M. Sonneborn showed that, when flatworms divide into two, the less differentiated section then grows more rapidly than the other and lives longer and more vigorously.

Albert I. Lansing showed that rotifers born from the eggs of young mothers had much longer lives than those from older mothers and that, if the older eggs were used for repeated generations, the rotifer lines died out altogether. Conversely, when the eggs of young 5-day-old mother rotifers were continually used for 54 generations, the lifespan of the rotifers was increased from 24 days to 104 days.[25]

Robinson, *Mechanisms of Aging and Development* **9**, 225 (1979); N. E. Robinson and A. B. Robinson, *Mechanisms of Ageing and Development* **125**, 259 (2004).

21 R. W. Gracy, M. L. Chapman, J. K. Cini, M. Jahani, T. O. Tollefsbol, and K. Y. Yüksel, *Molecular Biology of Aging* 427 (1985).

22 A. B. Robinson, A. Hunsberger, and F. C. Westall, *Mechanisms of Ageing and Development* **76**, 201 (1994).

23 C. M. MacCay and F. Crowell, *Sci. Mon.* **39**, 405 (1934); C. M. McCay, , 139 (1952).

24 A. M. Holehan and B. J. Merry, *Biol. Rev.* **61**, 329 (1986).

25 A. I. Lansing, *Scientific American* **188**, 39 (1953).

Muggleton and Danielli found[26] that, by preventing growth through nutrition for 3 to 5 weeks, they could transform amoeba that had an unlimited "immortal" life span into two types with limited life spans. Thereafter, immortal amoeba could be transformed into age-limited types by transfer of cytoplasm or nuclei. Transfer of cytoplasm conferred logarithmic growth and transfer of nuclei conferred stemline growth, with both types of amoeba colonies now having limited life spans.

In 1961, L. Hayflick and P. S. Moorhead, markedly extending observations by other investigators, showed that there is an upper limit on the number of generations that human diploid cells can be maintained in tissue culture and that, after about 50 generations, the cells either transformed into chromosomal heteroploid cells or else the cell line died out. Figure 14-6 illustrates this work.[27]

The first growth phase of these diploid human cells ends with formation of the first confluent sheet of cells; during the second phase, rapid cell growth occurs; and, in the third phase, the cells decline and the culture is lost. The cells can, however, at any time convert into haploid cells. If this happens, the culture does not die out.

Hayflick and Morehead concluded that this limit in generations should be explained either "by postulating a factor, necessary for cell survival, whose rate of duplication is less than that of the cell" or by postulating a factor for which "the rate of synthesis may be unchanged, but a slightly higher rate of loss (through some unknown *in vitro* condition) would eventually yield the same result." Synthesis needs to take place because the number of generations is too great to be explained by molecules that occur in fetal cells and are then not further synthesized. Deamidating protein molecules would, of course, be ideal candidates for this factor with their "higher rate of loss" controlled by the deamidation rate. A parallel argument can be constructed for an inhibiting factor.

Later investigators have hypothesized that this limit is imposed by inherent limitations in the nucleic acids that allow only a fixed number of replications. Presumably, the chromosomal changes in haploid cells avoid this limitation.

26 A. Muggleton and J. F. Danielli, *Experimental Cell Research* **49**, 116 (1968).

27 L. Hayflick and P. S. Moorhead, *Experimental Cell Research* **25**, 585 (1961); L. Hayflick, *Experimental Cell Research* **37**, 614 (1965); L. Hayflick, *Exp. Geront.* **5**, 291 (1970); C. W. Daniel and L. J. T. Young, *Experimental Cell Research* **65**, 27 (1971).

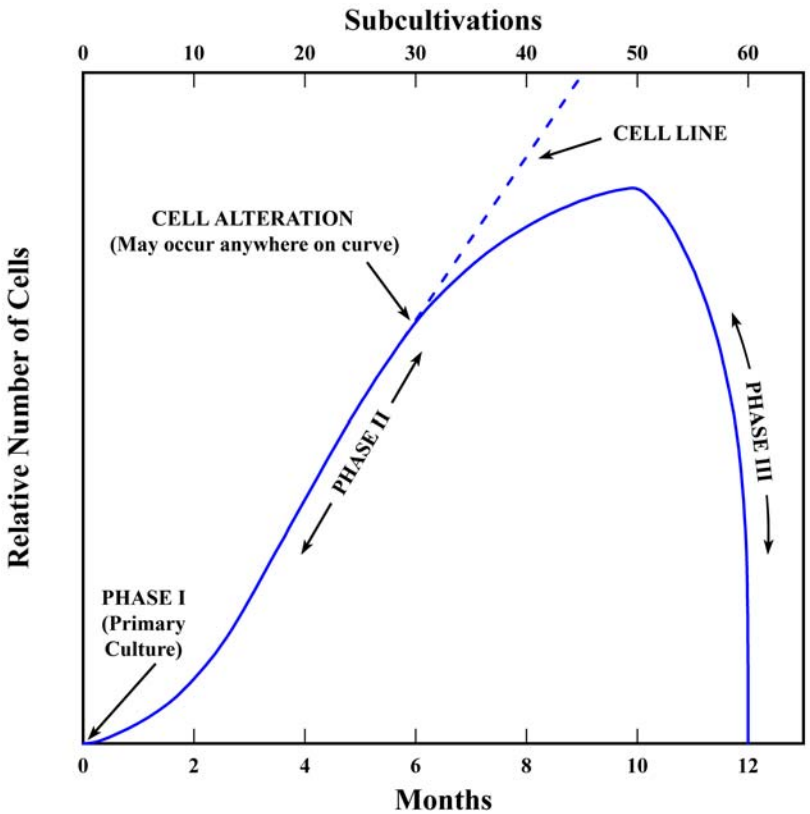

FIG. 14-6 Human cell growth in culture. Phase I, or the primary culture, terminates with the formation of the first confluent sheet. Phase II is characterized by luxuriant growth necessitating many subcultivations. Cells in this region are termed "cell strains". An alteration may occur at any time giving rise to a "cell line" whose potential life is infinite. Conversely, cell strains characteristically enter Phase III and are lost after a finite period of time. Adapted from 1961HM.

Human skin fibroblasts were found to be capable of fewer doublings as a function of donor age, with the linear decrease with age extrapolating to zero at 250 years.[28] Skin fibroblasts from cystic fibrosis subjects, are capable of fewer doublings than those from normal subjects.[29]

In a 1972 review of published experiments on cell lifetimes in tissue culture and implanted cultures,[30] Daniel stated, "In several cases, both *in vivo* and *in vitro* limitation upon ultimate life span appears to be related to a built-in number of cell divisions. In other instances, the passage of metabolic time, the age of the donor organism, or a variety of

28 G. M. Martin, C. A. Sprague, and C. J. Epstein, *Cell Senescence* **23**, 86 (1970).

29 B. L. Shapiro, L. F. -H. Lam, and L. H. Fast, *Science* **203**, 1251 (1979).

30 C. W. Daniel, *Adv. Geront. Res.* **4**, 167 (1972).

physical and chemical variables in the cells' environment, affect the aging process." This situation would also fit well with amide clocks.

Shock points out[31] that death and disappearance of cells throughout body tissues correlates strongly with human aging. He concludes that it is likely "that changes in the internal metabolism of a cell damage its capacity for self-repair and reproduction."

An interesting approach to molecular aging has been reported in which a peptide library of more than 500,000 octapeptides was exposed to oxygen and light in pH 7.4, phosphate buffer at 40 to 50° while still on a Merrifield solid-phase peptide synthesis resin. Peptides from resin beads that developed strong UV fluorescence were sequenced. Five "aging prone" peptides were isolated, each having a Trp and at least one Lys.[32]

Type I and Type II life extension can be achieved by elimination of deleterious factors as well as by positive changes. Figure 14-7 shows a Gompertz mortality curve for United States residents in 1960. The probability of death increases logarithmically throughout adult life. Figure 14-8 shows the shift in this Gompertz curve for just heart disease deaths as a function of cigarette consumption. The shift shown in Figure 14-8 corresponds to a seven-year decrease in life expectancy per pack per day of cigarettes smoked. If all causes of death are included, the shift is 8 years per pack per day.[33]

Pauling pointed out that if, in 1960, all American smokers ceased smoking cigarettes, the increase in national life expectancy would be about 4 years, whereas, if 100% of all deaths from cancer were eliminated, the increase would be only 2.8 years.

14-3. DEAMIDATION AND AGING

It is now well established that the amounts of deamidation found for many proteins are increased *in vivo* as a function of age. This age dependence includes both whole organisms and individual tissues such as blood red cells. Additional relevant references to age-dependent protein changes in general and deamidation in particular include 1975GM,

31 N. W. Shock, *Scientific American* **206**, 100 (1962).

32 G. L. Juskowiak, S. J. Stachel, P. Tivitmahaisoon, and D. L. Van Vranken, *J. American Chemical Society* **126**, 550 (2004).

33 L. Pauling, *Engineering and Science Magazine (California Institute of Technology)* **May**, (1960); E. C. Hammond and D. Horn, *J. American Medical Association* **166**, 1159 (1958); L. Pauling, *New Dynamics of Preventive Medicine, ed Leon R. Pomeroy, pub Symposium Specialists, Miami, FL*, (1974).

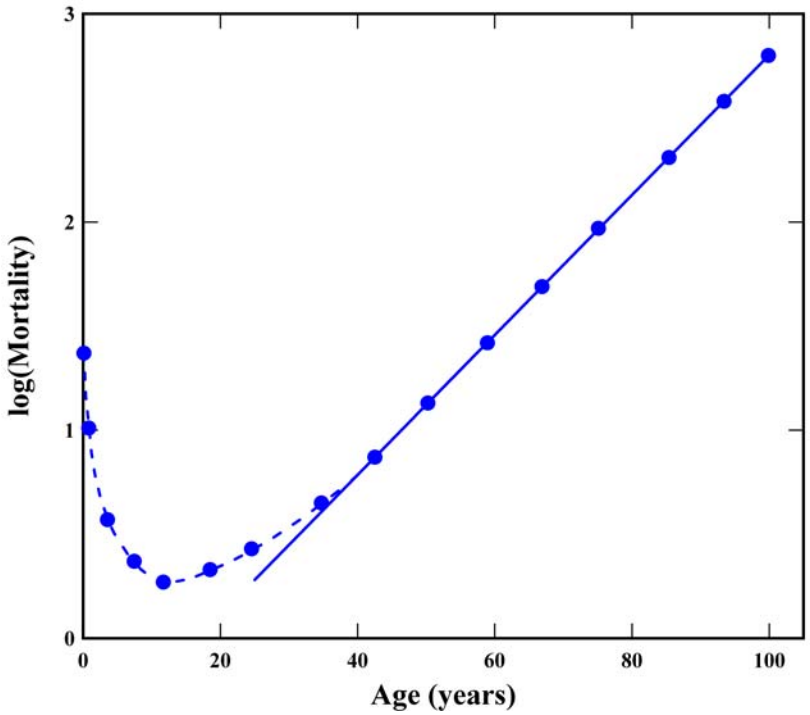

FIG. 14-7 Gompertz mortality diagram for U. S. residents. The vertical coordinate represents the common logarithm of the age-specific mortality (number of deaths per thousand people of that age), and the horizontal coordinate represents the age (diagram by Professor R. M. Sutton). Adapted from 1960P.

1976SV, 1977KB1, 1977R, 1980S, 1982DC1, 1982BS, 1983M, 1984AR, 1986TC, 1988LC, 1989HB, 1990G, 1992RD, 1997G, 2001MC, and 2001LH.

Deamidation of human proteins with age affects short and long-lived proteins,[34] both of which are found in abundance in human tissues.[35] For example, erythrocyte membrane protein 4.1b deamidates *in vivo* with a half-time of 41 days. This is similar to the erythrocyte life-time and may be involved in determining that life-time.[36]

Gershon conducted extensive studies of the enzymes of nematodes, which have a lifetime of about 25 days during which DNA synthesis

34 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001).

35 S. Ritz-Timme, *Z. Gernontol. Geriat.* **34**, 452 (2001).

36 M. Inaba and Y. Maede, *Biochimica et Biophysica Acta* **944**, 256 (1988).

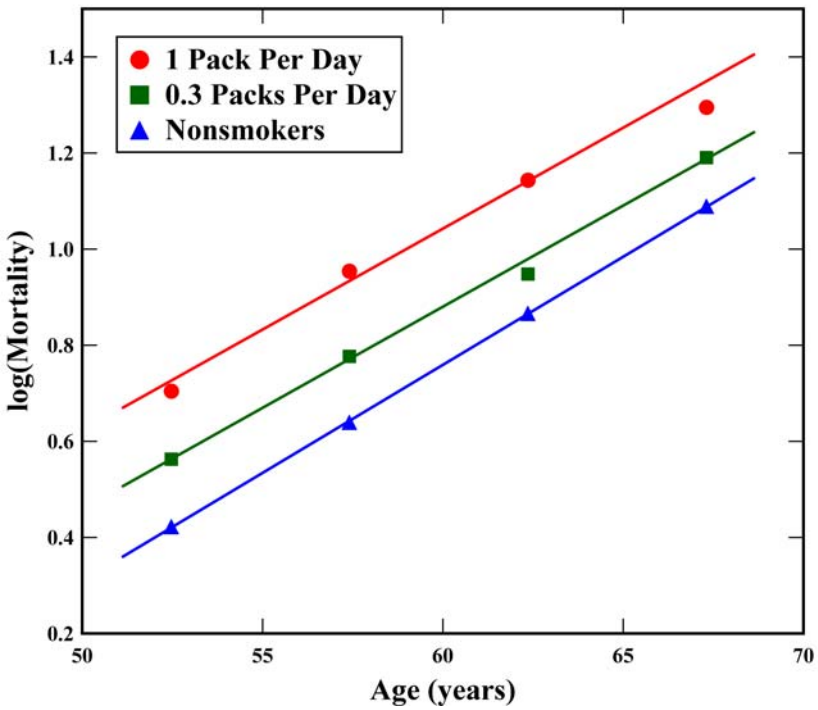

FIG. 14-8 The logarithm of the age-specific mortality from coronary heart disease (deaths per year per thousand persons), as given by Hammond and Horn, from a study of 187,783 men. Values are for three populations: non-smokers, ▲, smokers who average 0.3 packs per day, ■, and smokers who average 1 pack per day, ●. Adapted from 1960P.

and cell division are believed to be absent.[37] These experiments included 17 enzymes that apparently underwent changes in protein structure as a function of age. Gershon, however, did not observe charge changes associated with these altered enzymes, so he concluded that "modifications involving charge differences apparently do not contribute significantly to this phenomenon."[38] Nematode protein synthesis and catabolism both slow with age.[39]

It may be that Gerson was correct in his conclusions about the alterations in these particular enzyme changes in nematodes or, alterna-

37 M. Erlanger and D. Gershon, *Experimental Gerontology* **5**, 13 (1970).

38 H. Gershon and D. Gershon, Nature **227**, 1214 (1970); D. Gershon, *Experimental Gerontology* **5**, 7 (1970); U. Reiss and D. Gershon, *European Journal of Biochemistry* **63**, 617 (1976); D. Gershon and H. Gershon, *Gerontology* **22**, 212 (1976); D. Gershon, *Mechanisms of Ageing and Development* **9**, 189 (1979).

39 H. K. Sharma, H. R. Prasanna, R. S. Lane, and M. Rothstein, *Archives of Biochemistry and Biophysics* **194**, 275 (1979).

tively, he may have lacked sufficiently high resolution techniques at this early date to discover the deamidated forms.

Conversely, Pushkina, Lukash, and coworkers carried out extensive studies of proteins in mammalian systems and concluded that deamidation and chain cleavage were the primary age-dependent changes observed. These changes led to increased protein turnover which was, however, inhibited by increased disulfide bridges and decreased activity of catabolic enzymes with age.[40]

Gracy and coworkers have carried out extensive studies of triosephosphate isomerase, as reviewed in Chapter 12. These studies have included special emphasis on the age-dependent properties of TPI in various organisms and tissues.[41]

Deamidated TPI accumulates in older cells as do many other deamidated enzymes. These accumulations are enhanced in diseases of premature aging such as progeria and Werner's syndrome and have been ascribed to decreased efficiency of cell catabolic activity with age.[42] These cellular accumulations of altered enzymes can be reduced by diet restriction or fasting.

Additional references to Gracy's work, other than those in Chapter 12, include 1985GC, 1983G, 1985GY, 1990GY, and 1991G.

A substantial amount of research was carried out in the 1970s on the properties of glucose-6-phosphate dehydrogenase as a function of age in normal and prematurely aged individuals. Numerous electrophoretically more negatively charged forms were observed and frequently attributed to deamidation, although this was not definitively proved. Recent computations[43] indicate that human glucose-6-phosphate 1-dehydrogenase has an I_D of about 1.4 and therefore a deamidation half-time at pH 7.4, 37 °C of about 140 days, which might, especially with turnover, place its deamidation products beyond the

40 Personal communication to A. B. Robinson from N. Pushkina, May 15, 1992; A. A. Krichevskaya, A. I. Lukash, N. V. Pushkina, and K. B. Sherstnev, *Voprosy Meditsinskoi Khimii* **24**, 160 (1978); A. A. Krichevskaia, A. I. Lukash, N. V. Pushkina, K. B. Sherstnev, and A. M. Mendzheritskii, *Vopr Biokhim Mozga* **13**, 127 (1978); N. V. Pushkina, *Ukr Biokhim Zh* **51**, 680 (1979); A. L. Kizilshtein, A. M. Levin, and I. E. Tsybul'Skii, *Ukrainskii Biokhimicheskii Zhurnal* **60**, 14 (1988); A. Y. Goncharov, A. L. Kisilshtein, I. E. Tsibulsky, and A. I. Lukash, 47 (1990); A. Y. Goncharov, A. L. Kizil'shtein, I. E. Tsibul'skii, and A. I. Lukash, *Ukrainskii Biokhimicheskii Zhurnal* **62**, 43 (1990); A. I. Lukash, N. V. Pushkina, I. A. Klimova, and I. N. Nasarova, *Biopolimery-i-Kletka* **10**, 53 (1994).

41 R. W. Gracy, M. L. Chapman, J. K. Cini, M. Jahani, T. O. Tollefsbol, and K. Y. Yüksel, *Molecular Biology of Aging* , 427 (1985).

42 T. O. Tollefsbol and R. W. Gracy, *Bioscience* **33**, 634 (1983).

43 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001).

levels easily verified with the research tools of that time. Relevant studies include 1969FL, 1972HT, 1974HP, 1975KC, 1975GM, 1975WC, 1976KB2, 1976KB, 1976SV, 1976KB1, 1977KG, 1977KB, 1979L1, 1983DK, and 2002IC.

That deamidation is found, *in vivo* in a wide variety of proteins in many organisms, to be a function of tissue and organismic age is not surprising in view of the computed deamidation half-times for proteins shown in Figures 9-11, 9-15, and 9-16.

In proteins in general and in human and *Drosophila* proteins in particular it has been shown that a substantial-fraction of these proteins have deamidation half-times within the life-times of those organisms and their tissues.[44]

Since deamidated proteins are often more rapidly catabolized than undeamidated proteins, the extent of *in vivo* deamidation is often underestimated by measurements of steady-state values. Nevertheless, deamidation in the steady state and deamidation as a function of age has been observed in a wide variety of proteins, organelles, and organisms.

Extensive observations of deamidation as a function of age do not, of course, verify the hypothesis that deamidation may be a molecular clock for aging. These observations are neither necessary nor sufficient tests of that hypothesis.

Conversely, the lack of evidence that verifies the amide organismic aging hypothesis has no bearing upon the general amide molecular clock hypothesis, for which there is substantial and steadily increasing theoretical and experimental support.

Additional references of interest include 1892W, 1934PP, 1942S, 1956CB, 1957S, 1959B, 1962K, 1966K, 1970K, 1978WH, 1979M, 1980SP1, 1981S, 1981G, 1981B2, 1982KS, 1982R, 1983KG, 1983WP, 1986VJ, 1986B, 1986FQ, 1989PO, 1990M, 2002HB1, and 2003H1.

14-4. CORRELATIONS WITH TISSUE COMPOSITIONS

In 1976, it was found that 12 out of 24 ninhydrin-positive compounds measured in proteolytic-enzyme-digested whole-body homogenates of *Drosophlia melanogaster* were age correlated.[45] A total of 6 of these substances, which included 7 of the ordinary 20 amino

44 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002); N. E. Robinson and A. B. Robinson, *Mechanisms of Ageing and Development* **125**, 259 (2004).

45 A. B. Robinson, R. Willoughby, and L. R. Robinson, *Experimental Gerontology* **11**, 113 (1976).

acids, increased with age and 6 decreased. By far the strongest correlations, which were significant at $p \leq 0.0002$, were a decrease in glutamine and an increase in glutamic acid with age. No such correlation was present for asparagine and aspartic acid. Figures 14-9 and 14-10 show these correlations. If this correlation of Gln and Glu reflects deamidation, then it is probably catalysed because most nonenzymatic Gln deamidation would be too slow.

A similar but less pronounced pattern of diminished glutamine and increased glutamic acid without a similar change in asparagine and aspartic acid was observed in whole body digests without proteolytic digestion in Bombyx mori silkworms.[46]

In measurements of 51 human urinary ninhydrin-positive constituents as a function of age in men,[47] the strongest correlation found was an increase in aspartic acid with age with $p \leq 0.000001$. The fourth strongest correlation was for the sum of asparagine and glutamine, which decreased with age with $p \leq 0.0002$. Glutamic acid showed no correlation with age. Figure 14-11 shows the correlation of the sum of asparagine and glutamine with age.

Considering only the direction of the correlations and not their high statistical significance, with about 24 substances measured in *Drosophila*, the one-tailed probability of the observed outcome being in the direction of deamidation in either asparagine or glutamine is $(2)/(2)(24) = 0.04$. For the 51 substances in human urine it is 0.02. If the correlated aspartic acid and glutamic acid are considered to be independent, these values are 0.0016 and .0004, respectively. In silk worms, 17 substances were measured, but the investigators did not publish quantitative relative probabilities.

Both the *Drosophila* and human urine experiments were conducted in the context of research in diagnostic medicine, and the investigators did not expect results in any way relevant to deamidation. The correlations themselves are of high statistical significance.

Interestingly, the two systems that show no Asn correlation with age involve short-lived organisms, while the one that does show Asn vs. age correlation is mammalian, with a longer life span. Similarly, in Chapter 14-3 are reviewed experiments where age-dependent deamidation was observed in humans but not in flat worms. Perhaps these aging processes are fundamentally different for short and long-lived organisms.

46 M. Osanai and S. Kikuta, *Experimental Gerontology* **16**, 445 (1981).

47 A. B. Robinson and L. R. Robinson, *Mechanisms of Aging and Development* **59**, 47 (1991).

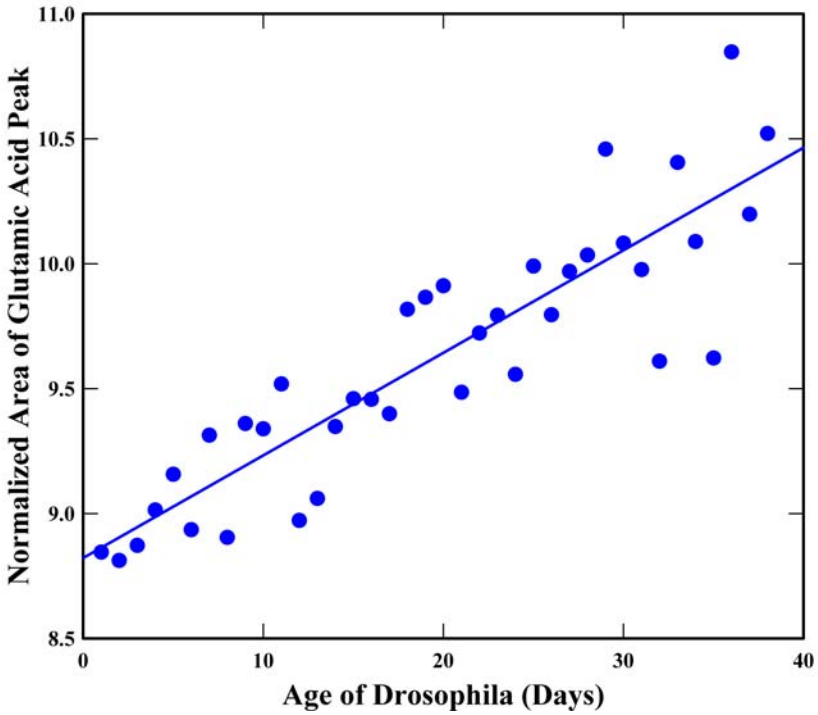

FIG. 14-9 Normalized area of the peak with retention time identical to that of glutamic acid vs. time. The straight line was calculated for the values by the method of least squares. Adapted from 1976RW.

14-5. DIET RESTRICTION

Since the pioneering work of McCay, referenced above, a great many observations of diet restriction and reduced mortality have been made in a wide variety of organisms. A recent example is provided by the work of Mair and coworkers in 2003 in *Drosophila*.[5] There is a large anecdotal literature indicating that diet restriction reduces degenerative disease and provides human Type I and Type II life extension, but there is no such literature indicating that Type III life extension is affected.

Many investigators, most notably Gracy and Pushkina and coworkers, have considered the possibility that diet restriction reduces age-specific mortality by causing accumulated old proteins, especially deamidated proteins, to be cleared from older cells. This probably results from an adaptive ability to reuse protein more efficiently when diet is restricted.

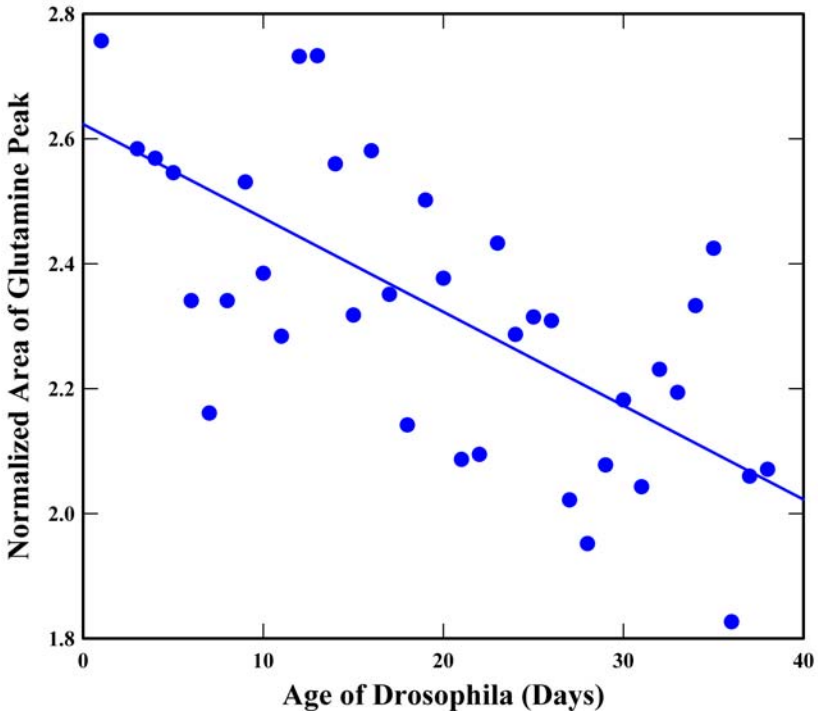

FIG. 14-10 Normalized area of the peak with retention time identical to that of glutamine vs. time. The straight line was calculated for the values by the method of least squares. Adapted from 1976RW.

As exemplified in Figure 14-8, Type I and Type II life extension can be degenerative disease specific. Moreover, these effects can reduce either disease incidence or disease mortality.

Figure 14-12 illustrates the effect of diet restriction on growth rate of cancer.[48] Fruit and vegetable diets sharply restrict nutrient intake. In these experiments, the growth rate of squamous cell carcinoma in mice was observed to vary over a range of *20-fold* as a function of diet alone. The richer the diet, the faster the cancer growth. Conversely, the more restricted the diet, the slower the cancer growth.

Diets extremely high in a single type of nutrient, such as protein or vitamin C, also restricted cancer growth, but the animals were unhealthy at these doses, which may have effectively restricted nutrition by distorting biochemical processes.

48 A. B. Robinson, A. Hunsberger, and F. C. Westall, *Mechanisms of Aging and Development* **76**, 201 (1994).

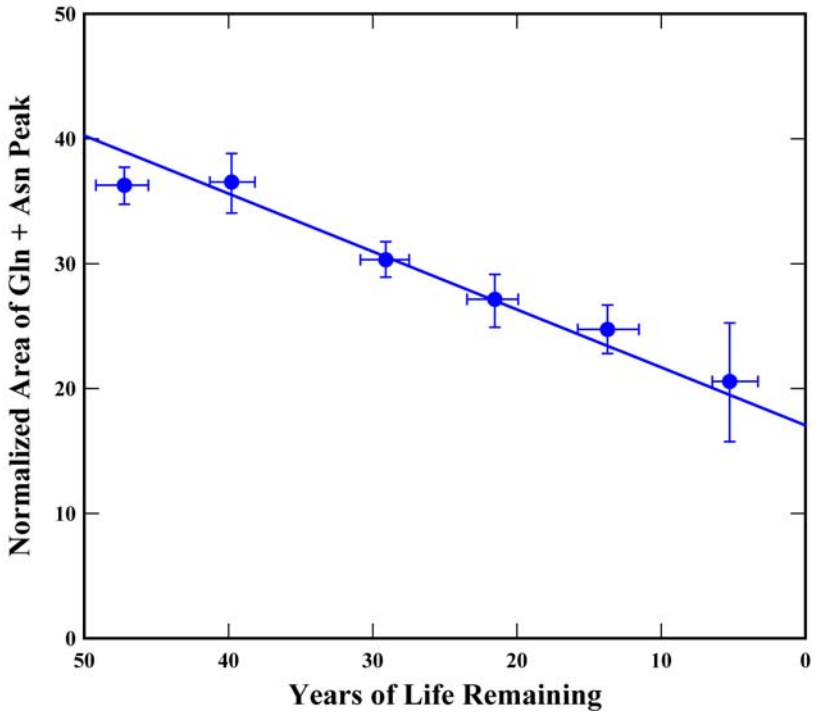

FIG. 14-11 Normalized urine amide values for 205 men grouped and averaged in six groups of similar age. The groups contained 38, 60, 53, 32, 13 and 9 men from young to old respectively. The years of remaining life were calculated from actual ages of the men and U. S. Public Health Service mortality compilations. Adapted from 1991RR.

Figure 14-12 shows that vitamin C in ordinary doses that are probably conducive to the best of health doubled cancer growth rate. Note that the *incidence* of cancer was not studied and may even be opposite in effect. Raw fruit and vegetable diets, however, sharply reduced cancer growth rate. A wide variety of diets was used to test the possibility that this reduction was the result of special anti-cancer agents rather general restriction. While more research is needed, all results so far indicate that diet restriction is the cancer growth reducing component.

Figure 14-12 also shows that very high, health impairing doses of vitamin C enhanced the effects of diet restriction. The highest doses of vitamin C shown in Figure 14-12 are just below the lethal dose of vitamin C. At doses of vitamin C just 2-fold higher, the death rate of mice was so great that the experiments could not be completed.

This effect of diet restriction upon cancer growth rate may be simply the result of starving the cancer. Alternatively, the clearing of

deamidated and otherwise altered proteins from cells may strengthen natural anti-cancer defenses. Many other reasons can be hypothesized for this phenomenon.

In these experiments, squamous cell carcinoma was induced by UV light and was pathologically identical to the human form of this type of cancer. Since diet alone can restrict growth rate of this cancer by 20-fold in this system and since there are extensive accounts of individuals who have observed a similar effect on their own cancers, research on diet restriction and cancer therapy should be very actively pursued.

Diet restriction and longevity is currently a very active field of research. See, for example, 2003WC1 and 2003AG.

Interestingly, ingestion of amounts of aluminum salts in quantities often used by humans increased the racemization of Asp in rat brains by

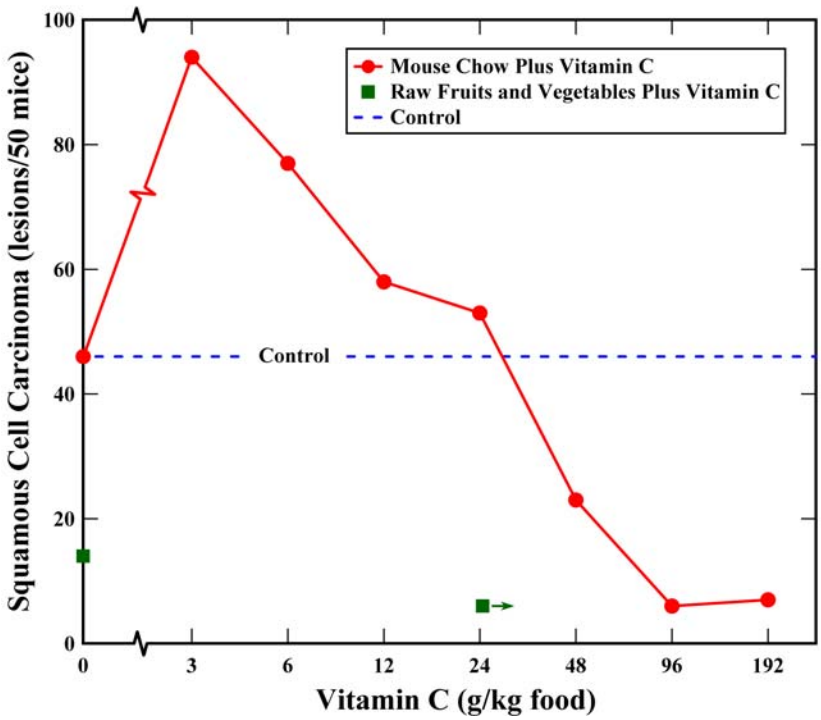

FIG. 14-12 Sum of severity 2-5 squamous cell carcinoma lesions 2 months after UV irradiation and after normalization to 50 mice for dry Wayne Lab Blox mouse chow plus vitamin C; and for raw fruit and vegetable diet plus vitamin C. Since the mixture of fruits and vegetables was 850 g/kg water, the dry food comparative value of the raw fruit and vegetable diet plus 25 g/kg vitamin C value is about 165 g/kg vitamin C. The maximum cancer growth for the dry mouse chow is at 3 g/kg vitamin C. Adapted from 1994RH.

about 25%.[49] The concentrations of aluminum in those brains increased by about 100%. The Asp values were for Asp + Asn, since hydrolysis was used in the analysis.

Many environmental, dietary, and physiological factors affect aging and are beyond the scope of this book.

49 K. K. Anderson, G. L. Perez, G. H. Fisher, and E. H. Man, *Neuroscience Research Communications* **6**, 45 (1990).

CHAPTER 15

Deamidation and Disease

15-1. INTRODUCTION

After synthesis and folding, peptides and proteins undergo changes in charge and conformation through nonenzymatic deamidation of Asn and Gln. These changes are under precise genetic control through primary, secondary, tertiary, and quaternary structure. Under physiological conditions, deamidation introduces a negative charge at the reaction site and partial geometric isomerization. Deamidation occurs during the *in vivo* biological lifetimes of many protein molecules.

Since deamidation is, therefore, an intrinsic dynamic property of peptides and proteins, it is reasonable to anticipate that enzymatic and nonenzymatic deamidation will be found to play a part in many pathological processes.

The discovery of these roles of deamidation in disease processes is, at present, still in its infancy. In this chapter, we summarize some of the initial findings and hypotheses.

15-2. CELIAC DISEASE

Celiac disease involves an apparent genetic predisposition to intolerance for the food proteins gluten, gliadin, and glutenin, which are usually consumed in wheat, rye, and barley products. These proteins contain unusually high amounts of prolyl and glutaminyl residues. It is estimated that more than 0.5% of the human population suffers from this disease.

In 1985, Bruce, Bjarnason, and Peters reported[1] transglutaminase activity in human jejunal mucosa. They found that this activity was elevated in human subjects with untreated celiac disease and in patients with celiac disease in remission. It had been reported by earlier investigators that deamidated gliadin did not cause celiac symptoms. It is now well established that deamidation by transglutaminase is a key component in celiac disease.

1 S. E. Bruce, I. Bjarnason, and T. J. Peters, *Clinical Science* **68**, 573 (1985).

Some Pro and Gln-rich peptides are resistant to ordinary digestion. For example, such a 33-residue peptide from gluten has been identified.[2] After deamidation by tissue transglutaminase in the intestine, this peptide is recognized by the immune system, stimulates a T-cell response, and leads to an intestinal immune response. This results in the symptoms of the disease. A genetically determined immune defect in the immune recognition system is thought to be one of several causative factors in the disease. There is evidence that at least several such peptides are involved.

Celiac disease is currently treated by restrictive diets that avoid wheat, rye, and barley. Some investigators are attempting to develop palatable and affordable food processing methods that would render these grains non-immunogenic for celiac disease victims. Other efforts toward therapy involve transglutaminase blockers and substances that interfere with the immune response.

Recent articles, of interest include: 1998MM, 1998MM1, 1998SL, 1998TW, 1998WK, 1999O, 1999QM, 1999MS, 2000AD, 2000S, 2000OG, 2000MS, 2000AK, 2001MM, 2001AD, 2001SM, 2001OU, 2001PH, 2002VK, 2002SN, 2002TE, 2002KE, 2002PH, 2002FM, 2002AM, 2002NM, 2003M, 2003K, 2003H, 2003MF, 2003EP, 2003MG, 2003VS, 2003DE, 2003DT, 2003CD, 2004KQ, 2004FQ, and 2004MF.

15-3. BACTERIAL TOXINS

Although *E. Coli* is a benevolent and helpful ordinary resident of the human gastrointestinal tract, pathologic strains of *E. coli* occasionally arise, perhaps through ordinary mutation or viral transformation. Some of these pathologic forms produce 1,014-residue toxins CNF1 and CNF2, which have glutaminase and transglutaminase activity. *Bordetella bronchiseptica*, *B. pertussis*, and *B. parapertusis* produce DNT, a similar 1,451-residue toxin. These enzymes have similar consequences.

E. coli toxin CNF1 enzymatically deamidates Rho family proteins RhoA, Cdc42, and Rac at Gln(63), Gln(61), and Gln(61), respectively. These are GTPases that are involved in regulation of the actin cytoskeleton and in various signal transduction processes. Deamidation of these GTPases changes their activity and leads to urinary and digestive cytotoxic necrosis and illness. This deamidation also causes

2 L. Shan, O. Molberg, O. Parrot, and Et. Al., *Science* **297**, 2275 (2002).

macropinocytosis wherein normal epithelial cells acquire phagocytic properties, thereby increasing apoptosis.[3] DNT causes dermonecrosis.[4]

It has been reported that the RhoA deamidation site for CNF1 also requires Arg(68) and Leu(72) as well as Gln(63) and that for DNT requires Arg(68), Leu(72), and Thr(37).[5] Other investigators found an 11-residue sequence that is deamidated by RhoA, but lacks Leu(72).[6]

This process has been associated with more than 50% of human cases of urinary tract infection or prostatitis.[7]

Articles of interest include: 1997SS, 1997HI, 1997FL, 1997A, 1998SL2, 1998LS, 1998GS, 1998EH, 1998CM, 1998BF, 1998SS1, 1998SA, 1998B, 1999RM, 1999LS2, 1999RP, 1999BO, 1999KK, 1999PV, 1999SG, 1999SH2, 2000MB, 2000LS, 2000HN, 2001MM1, 2001H, 2001FR, 2001FF, 2001B, 2002FF1, 2002DM, 2002BF, 2002LP, 2002BR, 2002FR, 2003BM, 2003FQ, 2003AS, 2004HP, 2003BG, and 2004PA.

15-4. CANCER

Many human cancer cells lack proteins p53 and Rb. DNA-damaging chemical and radiation therapies for cancer depend upon a different response of cancer cells and normal cells. DNA damage induces cell death (apoptosis) in many cancer cells, while normal cells survive. Yet, many cancer cells have increased resistance to apoptosis.

It has been found[8] that DNA damage initiates deamidation of the protein Bcl-X_L at AlaIleAsn(52)GlyAsn and AlaValAsn(66)GlyAla. In normal cells, DNA damage sends both a pro-apoptotic signal and an anti-apoptotic signal through DNA damage induction of p53. The anti-apoptotic signal involves production of protein Rb, which then suppresses deamidation of Bcl-X_L. Undeamidated Bcl-X_L inhibits

3 S. Travaglione, L. Falzanoa, A. Fabbri, A. Stringaroa, S. Faisb, and C. Fiorentinia, *Toxicology in Vitro* **16**, 405 (2002).

4 G. Schmidt and K. Aktories, *Methods in Enzymology* **325**, 125 (2000).

5 M. Lerm, G. Schmidt, U. Goehring, J. Schirmer, and K. Aktories, *J. Biological Chemistry* **274**, 28999 (1999).

6 G. Flatau, L. Landraud, P. Boquet, M. Bruzzone, and P. M. P, *Biochemical and Biophysical Research Communications* **267**, 588 (2000).

7 K. E. Rippere-Lampe, M. Lang, H. Ceri, M. Olson, H. A. Lockman, and A. O. O'Brien, *Infection and Immunity* **69**, 6515 (2001).

8 B. E. Deverman, B. L. Cook, S. R. Manson, R. A. Niderhoff, E. M. Langer, I. rosova, L. A. Kulans, X. Fu, J. S. Weinberg, J. W. Heinecke, K. A. Roth, and S. J. Weintraub, *Cell* **111**, 51 (2002); S. J. Weintraub and S. R. Manson, *Mechanisms of Aging and Development* **125**, 255 (2004).

apoptosis. Therefore, since these cancer cells lack both p53 and Rb, DNA damage-induced deamidation of Bcl-X_L leads to cell death.

While the mechanism of Bcl-X_L deamidation suppression by Rb is unknown, it probably involves changes in the higher order protein structure near Asn(52) and Asn(66), since both amides in ordinary Bcl-X_L have computed deamidation half-times of about 1 day.[9]

It has been suggested that Bcl-X_L serves as a "chronometric buffer" allowing time for cells to respond to low-level genotoxic stress induced events.[8] The biochemical events that initiate apoptosis occur with a delay of 1 day because they require deamidation of Bcl-X_L.[10]

Some tumor cells acquire resistance to apoptosis by increasing production of Bcl-X_L or by reducing its rate of deamidation.[11]

Additional articles of interest concerning deamidation of Bcl-X_L include: 1996MS, 1996SM, 1997AK, 2000TT, 2002LT, and 2003Y. See also Chapter 12.

15-5. ALZHEIMER'S DISEASE

Alzheimer's Disease, a specific age-dependent form of brain degeneration, usually destroys smell, memory, orientation, and other similar abilities, while leaving motor functions unchanged. Blood flow to the brain is reduced, and the brain gradually shrinks in size. Characteristic amyloid plaques[12] are found outside of brain cells, while filamentous deposits called "tangles" appear inside the cells. Down's syndrome subjects have an extra chromosome, which increases their production of the amyloid precursor protein. They usually develop Alzheimer's symptoms at 30 to 40 years of age. About 5 to 10% of Alzheimer's victims have genetic markers for the disease and develop symptoms between 50 and 60 years of age. The remaining 90 to 95% become ill in their late 60s or older.

The neurofibrillary tangles are made up of paired helical filaments of which the microtubule forming protein tau is a primary component. In 1986, it was reported that *in vitro* assembly of tau into filaments requires prior enzymatic deamidation of Gln in tau by glutaminase.[13] In 1992, it was reported that the neurofibrillary tangles of some Alzhei-

9 N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

10 S. Weintraub, Personal cummunication (2001).

11 T. Takehara and H. Takahashi, *Cancer Research* **63**, 3054 (2002).

12 A. Alzheimer, *Allg. Z. Psychiatrie (Berlin)* **64**, 146 (1907).

13 E. M. de Garcini,, L. Serrano, and J. Avila, *Biochemical and Biophysical Research Communications* **141**, 790 (1986).

mer's patients contained 1.5 to 2-fold more isoAsp and D-Asp than comparable controls.[14]

Several investigators have reported isomerization of Asp and deamidation of Asn in peptides from Alzheimer's plaque or from model peptides with increased filamentary structure. These include isomerization of tau at Asp(193), ArgGluAsn(381)AlaLys, and ThrAsp(387)HisGly; isomerization of the 39 to 42-residue amyloid-β peptides that are found abundantly in amyloid plaque at Asp(1), Asp(7), Asp(23); and Gln deamidation to pyrrolidone at the N-terminal. These modifications have been associated with increases in β sheet structures that are found more abundantly in the filaments and plaque.

Deamidation of GlnAlaAsn(167)AlaTrp and IleIleAsn(279)LysLys has been reported in tau from both normal and Alzheimer's disease brains.[15]

See 1993RL, 1994FS, 1994SF, 1996WT, 1999OB, 1999WT, 2000SW, and 2002SF. Reviews include 1991S, 1991YM, 1992S1, 2003I, and 2004KK.

15-6. OTHER AMYLOID DISEASES

Alzheimer's disease appears to be one of a class of diseases characterized by peptide, protein fragment, and protein aggregation. Given the tens of thousands of different proteins in various stages of catabolism and the many variants available, especially in molecules that have existed long enough to undergo postsynthetic modifications, it is not surprising that some of these molecules would aggregate in pathological formations.

In fact, it is remarkable that the surfaces of the tens of thousands of different proteins in a human or other organism are so designed that deleterious protein-protein interactions are apparently not present except as occasional distinct pathologies.

In view of the decrease in catabolic efficiency with age and the relatively longer times that long-lived proteins exist in older people, it is reasonable to expect that the incidence of these pathologies would increase with age.

Since amide residues are present in most proteins and are deamidating at genetically predetermined rates, deamidation can be ex-

14 I. L. Payan, S. Chou, G. H. Fisher, E. H. Man, C. Emory, and W. F. Frey, II, *Neurochemical Research* **17**, 187 (1992).

15 M. Hasegawa, M. Morishima-Kawashima, K. Takios, M. Suzuki, K. Titani, and Y. Ihara, *J. Biological Chemistry* **267**, 17047 (1992).

pected to be greater in abnormal accumulations of altered peptides and proteins. That deamidation and related Asp isomerization might, in fact, be causative of such accumulations is suggested by the work on Alzheimer's disease.

Many of the essential molecular structures in living things are exquisite aggregates of different peptides and proteins. Some of the functional signals from amide clocks probably involve special aggregate structures. These effects are generally beneficial and essential to life and health, but harmful versions may also occur.

Ten disease-involved amyloid precursor peptides and proteins were documented in 1988.[16] These and their associated conditions include: serum amyloid AA protein – reactive systemic secondary amyloidosis; immunoglobulin light chains – monoclonal gammopathy- and myeloma-associated amyloidosis; prealbumin – familial and senile systemic amyloidosis; β_2-microglobulin – amyloidosis associated with long-term haemodialysis; procalcitonin – amyloid in thyroid medullary carcinoma; cystatin C – Icelandic familial cerebrovascular amyloidosis; β-protein – Alzheimer's disease, Down's syndrome, and senile dementia; islet amyloid polypeptide – islets of Langerhans in Type 2 diabetes; atrial natriuretic peptide – isolated atrial amyloid; and injected porcine insulin – iatrogenic amyloid. Articles about these conditions include: 1976SW1, 1983SM, 1987CC, 1987WW, 1990OO, 1990LC, 1991PW, 1994LR, and 1997KE.

In Type 2 diabetes, amyloid deposits are formed by the peptide hormone amylin. Nilsson, Driscoll, and Raleigh have found,[17] in studies on an amylin fragment SerAsnAsnPheProAlaIleLeuSerSer, that as little as 5% deamidation causes amyloid-like aggregates to form from the whole preparation, including the undeamidated peptide. The deamidated peptide functions to initiate aggregates like a seed crystal in crystallization.

Parkinson's disease involves the aggregation of α-synuclein, a 140-residue protein.[18]

Prion disease involves the aggregation of prion proteins. Weber, McFadden, and Caughey[19] have found L-isoAsp and D-Asp in scrapie prion protein. Sandmeier, Hunziker, Kunz, Sack, and Christen have re-

16 F. E. Dische, C. Wernstedt, G. T. Westermark, P. Westermark, M. B. Pepys, J. R. Rennie, S. G. Gilbey, and P. J. Watkins, *Diabetologia* **31**, 158 (1988).

17 M. R. Nilsson, M. Driscoll, and D. P. Raleigh, *Protein Science* **11**, 342 (2002).

18 M. G. Spillantini, *Proc. Natl. Acad. Sci. USA* **95**, 6469 (1998).

19 D. J. Weber, P. N. McFadden, and B. Caughey, *Biochemical and Biophysical Research Communications* **246**, 606 (1998).

ported[20] deamidation in 253-residue human prion protein at ThrAsn(108)Met in 0.10M sodium phosphate, pH 7.4, 37 °C with a half-time of 33 days, and in the corresponding 21 residue peptide, with a half-time of 12 days. This is probably sequence controlled, since GlyThrAsnMetGly has a half-time of 48 days in 0.10M Tris-HCl, pH 7.4, 37 °C.[21]

Perutz has pointed out[22] that 8 neurodegenerative diseases involve Gln repeats. These proteins are partially catabolized, and the repeat segments enter the cell nuclei of neurons and form aggregates. These diseases include Huntington's disease, Kennedy's disease, and various types of spinocerebellar ataxia. Gln repeats are common and repeats of 37 residues or less are apparently harmless, but repeats of 41 residues or more form toxic neuronal nuclear aggregates. Spontaneous aggregation of model proteins with Gln repeats in a flexible region have been demonstrated *in vitro*.[23] It has been suggested that Gln-repeat aggregation may result from structural complementarity[24] or from transglutaminase crosslinking of Gln and Lys.[25]

15-7. ADDITIONAL PATHOLOGIES

Heterogeneous forms of amylase thought to be caused by deamidation were found in the saliva of patients with cystic fibrosis. Deamidated amylase is diagnostic for pancreatitis. See 1976CA, 1977DA, 1977CD, and the discussion of amylase in Chapter 12.

Experiments indicating that deamidation plays a role in cataract formation in eye lenses are reviewed in Chapter 12-8.

Isomerization of Asp has been found to initiate autoimmunity to cytochrome c in mice,[26] and deamidation has been proposed as involved in autoimmunity in humans.[27] Deamidation of

20 E. Sandmeier, P. Hunziker, B. Kunz, R. Sack, and P. Christen, *Biochemical and Biophysical Research Communications* **261**, 578 (1999).

21 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

22 M. F. Perutz, *Trends in Biochemical Sciences* **24**, 58 (1999).

23 K. Stott, J. M. Blackburn, P. J. G. Butler, and M. F. Perutz, *Proc. Natl. Acad. Sci. USA* **92**, 6509 (1995).

24 M. F. Perutz, T. Johnston, M. Suzuki, and J. T. Finch, *Proc. Natl. Acad. Sci. USA* **91**, 5355 (1994).

25 H. Green, *Cell* **74**, 955 (1993).

26 M. J. Mamula, R. J. Gee, J. I. Elliott, A. Sette, S. Southwood, P. Jones, and P. R. Blier, *J. Biological Chemistry* **274**, 22321 (1999).

27 F. C. Westall, *J. Theoretical Biology* **38**, 139 (1973).

TyrMetAsn(3)GlyThr initiates recognition by melanoma-specific T-cells.[28]

Protein-accessible L-isoAsp and D-Asp sites on erythrocyte membranes increase with cell age in humans, but are found to be even greater in patients with hereditary spherocytosis, which causes cytoskeletal disorganization.[29]

A protease essential to the replication of human rhinoviruse-14 C3, which specifically cleaves internal GlnGly peptide bonds, was found to be 55% deamidated *in vivo* at GlyGlyAsn(164)GlyArg. This reduced its biological activity by 80%.[30] Hepatitis C virus envelope glycoprotein E1 is 1000-fold more active after deamidation of GlyAsn(234)AlaSer, which may be an essential step in binding and cell lysis during infection.[31]

15-8. BACTERIAL CHEMOTAXIS AND THERMOTAXIS

The methyl-accepting chemotaxis proteins of *E. coli* undergo reversible methylation of Glu residues in response to chemical attractants. In 1981, Rollins and Dahlquist[32] demonstrated that an additional covalent modification was involved, which decreases the charge of these proteins and suggested that this might be enzymatic deamidation of Gln, thereby providing additional methylation sites. Related publications include: 1975A, 1983KE, 1983KB, 1983BK, 1985BH, 1995SS, 2004BM, and 2004HK.

The methylating enzyme recognizes a 9-residue consensus sequence (Ala/Ser)XxxYyyGluGlu(CH_3 site)Zzz(Ala/Ala(OH_2).[33] There are six methylation sites, including four Glu and two Gln that are enzymatically deamidated. In sensory transducer protein Tsr, these are Gln(297) and Gln(311).[34] Further studies are: 1986PH, 1988OM,

28 J. C. A. Skipper, R. C. Hendrickson, P. H. Gulden, V. Brichard, A. V. Pel, Y. Chen, J. Shabanowitz, T. Wolfel, C. L. Slingluff Jr., T. Boon, D. F. Hunt, and V. H. Engelhard, *J. Experimental Medicine* **183**, 527 (1996).

29 D. Ingrosso, S. D'Angelo, A. F. Perna, A. Iolascon, E. M. Del Giudice, S. Perrotta, V. Zappia, and P. Galleti, *European Journal of Biochemistry* **228**, 894 (1995).

30 G. A. Cox, R. B. Johnson, J. A. Cook, M. Wakulchik, M. G. Johnson, E. V. Villarreal, and Q. M. Wang, *J. Biological Chemistry* **274**, 13211 (1999).

31 M. Selby, A. Erickson, C. Dong, S. Cooper, P. P, M. Houghton, and C. M. Walker, *J. Immunology* **162**, 669 (1999).

32 C. Rollins and F. W. Dahlquist, *Cell* **25**, 333 (1981).

33 D. M. Nowlin, J. Bollinger, and G. L. Hazelbauer, *J. Biological Chemistry* **262**, 6039 (1987).

34 M. S. Rice and F. W. Dahlquist, *J. Biological Chemistry* **266**, 9746 (1991).

1988SS, 1990PD, and 1996SS. A similar system of Gln deamidation is also required for chemotaxis transducer proteins in *Bacillus subtilis*.[35]

Thermotaxis transducer protein TAR in *E. Coli* is a warm sensor when deamidated and unmethylated and a cold sensor when deamidated and methylated. It does not function as a thermosensor at all unless Gln(295) and Gln(309) are enzymatically deamidated.[36]

15-9. PHARMACEUTICAL DEAMIDATION

As the utilization of peptides and proteins for pharmaceutical uses rises, undesirable impurities from nonenzymatic deamidation is an increasing problem. In Chapter 12, we reviewed this problem with respect to insulin and some other proteins. Additional examples include somatotropin,[37] interferon,[38] tumor necrosis factor,[39] and blood albumins and gamma globulins.[40]

Treatment of cystic fibrosis with DNAse is complicated by the deamidation of GlyArgAsn(74)SerTyr. This deamidation half-time is 23 days in 0.005 M Tris, pH 7, 0.15 M NaCl, 0.001 M $CaCl_2$, 37 °C.[41] The deamidation half-time of GlyArgAsnSerGly is 14.3 days in 0.15 M Tris, pH 7.4, 37 °C.[42] Allowing for the pH and buffer concentration difference, this deamidation of DNAse is apparently essentially primary sequence controlled.

While many of these deamidations pose purity problems associated with drug approval and shelf life, deamidation is potentially more dangerous. Chen, Ede, Jackson, McCluskey, and Purcell have shown[43] that the succinimide intermediate in the deamidation of IleMetIleLysPheAsnArgLeu is immunologically recognized as a dis-

35 C. J. Kristich and G. W. Ordal, *J. Biological Chemistry* **277**, 25356 (2002).

36 S. Nishiyama, N. T. Nara, M. Homma, Y. Imae, and I. Kawagishi, *J. Bacteriology* **179**, 6573 (1997).

37 M. J. Hageman, J. M. Bauer, P. L. Possert, and R. T. Darrington, *J. Agricultural and Food Chemistry* **40**, 348 (1992).

38 S. Evans and P. Grassam, J. Parenteral Sci. Tech. **40**, 83 (1986); D. Russellharde, M. Knauf, and E. Croze, *J. Interferon and Cytokine Research* **15**, 31 (1995).

39 J. Geigert, *J. Parenteral Science and Technology* **43**, 220 (1989).

40 N. V. Pushkina, I. E. Tsybul'Skii, and A. I. Lukash, *Prikladnaya Biokhimiya I Mikrobiologiya* **22**, 198 (1986).

41 D. C. Cipolla, I. Gonda, K. C. Meserve, S. Weck, and S. J. Shire, *ACS Symposium Series* **567**, 322 (1994).

42 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001).

43 W. Chen, N. J. Ede, D. C. Jackson, J. McCluskey, and A. W. Purcell, *J. Immunology* **157**, 1000 (1996).

tinct entity, while the Asn peptide is not recognized. Stable succinimides are easily synthesized, and could be inadvertently present in pharmaceutical preparations.

In view of this, recent development of a method that allows prediction of primary sequence deamidation rates for Asn as a function of neighboring natural and potential synthetic amino acid residues may be especially useful.[44] This procedure predicts, for example, that the deamidation rate of insulin can be reduced by 10-fold through subtle modifications that may not affect its biological activity. Thus, it is becoming more feasible to engineer minor alterations in pharmaceutical peptides and proteins that reduce their deamidation rates.

Conversely, instances will probably arise in which deamidation will be engineered into specific locations in drugs in order to achieve desirable time-dependent properties.

See Chapter 6-3, which describes the design of new peptide and protein natural or synthetic primary sequences with computationally predicted deamidation rates.

Further articles of interest in pharmaceutical deamidation include: 1981LT, 1992L, 1992PN, 1993L, 2001BX, 2003SS1, and 2004XA.

44 N. E. Robinson and A. B. Robinson, *J. Peptide Research* **63**, 437 (2004).

CHAPTER 16

Enzymatic Carboxyl Methylation

16-1. METHYL TRANSFERASE

S-adenosyl-L-methionine:protein *O*-methyltransferase, which produces labile methyl esters of Asp residues *in vivo*, was first observed and generally understood between 1965 and 1971.[1] Early discoveries concerning this enzyme have been reviewed.[2] It is widely distributed in living things. A protein O-methyltransferase that methylates Glu residues during bacterial chemical sensing is also known.[3]

S-adenosyl-L-methionine:protein *O*-methyltransferase appeared, however, to be remarkably inefficient, with a variation of nearly three orders of magnitude in the percentage of Asp residues methylated in different proteins.[4] In 1982, McFadden and Clarke found[5] that human erythrocyte membrane S-adenosyl-L-methionine:protein *O*-methyltransferase preferentially methylated D-Asp residues, and these findings were extended in 1983.[6] It was also found that methylation of human erythrocyte membrane proteins increases 3 to 4-fold in older erythrocytes.[7]

Studies of adrenocorticotropin, ACTH, and hexapeptide analogues of ACTH subsequently showed that methylation by S-adenosyl-L-methionine:protein *O*-methyltransferase, PCMT, occurred primarily at the ValTyrProAsn(25)GlyAla position following

1 J. Axelrod and J. Daly, *Science* **150**, 892 (1965), M. Liss and L. M. Edelstein, *Biochemical and Biophysical Research Communications* **26**, 497 (1967), S. Kim and W. K. Paik, *J. Biological Chemistry* **240**, 4629 ((1965), S. Kim and W.K. Paik, *Biochemistry* **10**, 3141 (1971).

2 W. K. Paik and S. Kim, *Biochemistry* **1**, 202 (1980).

3 S. Clarke, K. Sparrow, S. Panasenko, and D. E. Koshland, Jr., *J. Supramolecular Structure* **13**, 315-328 (1980).

4 C. Gagnon, S. Kelly, V. Manganiello, M. Vaughan, C. Odya, W. Strittmatter, A. Hoffman, and F. Hirata, *Nature* **291**, 515 (1981).

5 P. N. McFadden and S. Clarke, *Proc. Natl. Acad. Sci. USA* **79**, 2460 (1982).

6 C. M. O'Connor and S. Clarke, *J. Biological Chemistry* **258**, 8485 (1983).

7 J. R. Barber and S. Clarke, *J. Biological Chemistry* **258**, 1189 (1983); P. Galletti, D. Ingrosso, A. Nappi, V. Gragnaniello, A. Iolascon, and L. Pinto, *European Journal of Biochemistry* **13**, 25 (1983).

deamidation.[8] These peptide studies showed that this enzyme is specific for isoAsp and does not react with L-Asp. In the case of these hexapeptides, the enzyme did not react with D-Asp. Studies of four additional peptides also showed no reactivity with D-Asp.[9] Additional early studies include 1981TC, 1981FC, and 1982RJ. More recently, it has been shown that the affinity of human PCMT for D-Asp peptides is sequence specific and 700 to 10,000-fold lower than for isoAsp peptides. PCMT from the thermophilic bacteria *Pyrococcus furiosus* reacts much more easily with D-Asp as compared with human PCMT.[10]

Experiments carried out, between 1985 and 1990, especially those by Clarke and coworkers and Aswad and coworkers, explored the properties of PCMT. It was found that:

1. PCMT is widely distributed and present in many, perhaps most tissues and species.

2. The primary substrates for PCMT are isoAsp residues produced by deamidation of Asn in peptides and proteins. IsoAsp is also produced from Asp, but this reaction is about two orders of magnitude less prevalent. Older proteins which have more deamidation, generally react more strongly with PCMT. In 1990, there was still confusion concerning the reaction of PCMT with D-Asp residues. Short model peptides had shown no reactivity with D-Asp, but some D-Asp-containing proteins apparently did react.

3. Most proteins showed low reactivity with PCMT, which was attributed to low amounts of deamidation. In addition, steric hindrance can reduce PCMT reactivity as was demonstrated with ribonuclease.[11]

4. The methyl esters of Asp formed by PCMT are unusually labile because they demethylate through the same succinimide mechanism by which Asn deamidates. Therefore, since the isoAsp substrates form at especially labile Asn, these also form especially labile CH_3-Asp.

5. Since both L-Asp and L-isoAsp are formed from succinimide and PCMT acts only on isoAsp, the continued cycling of this methylation and demethylation process leads to a net conversion of isoAsp to L-Asp. An analogous situation can apparently occur with D-Asp. Therefore, PCMT, to the extent that it is not inhibited by three-dimen-

8 E. D. Murray, Jr. and S. Clarke, *J. Biological Chemistry* **259**, 10722 (1984); D. W. Aswad, *J. Biological Chemistry* **259**, 10714 (1984).

9 J. Lowenson and S. Clarke, *Federation Proceedings* **46**, 2090 (1987).

10 J. D. Lowenson and S. Clarke, *Gerontology* **37**, 128 (1991); J. Lowenson and S. Clarke, *J. Biological Chemistry* **267**, 5985 (1992); N. Thapar, S. C. Griffith, T. O. Yeates, and S. Clarke, *J. Biological Chemistry* **277**, 1058 (2002).

11 P. Galletti, A. Ciardiello, D. Ingrosso, A. D. Donato, and G. D'Alessio, *Biochemistry* **27**, 1752 (1988).

sional structure, removes the D-Asp and IsoAsp isomerization that accompanies Asn deamidation. The reaction is relatively inefficient, and it cannot, of course, reverse the change in charge. This process has been clearly demonstrated as has its steric inhibition.[12]

6. In addition to its potential biological significance, PCMT provides a means of testing for Asn deamidation and Asp isomerization in peptides and proteins. PCMT is, however, only a qualitative indicator of deamidation and not a general method for reliable quantitative analysis because:

a. PCMT is susceptible to steric interference

b. PCMT responds to Asp isomerization as well as Asn deamidation.

c.The deamidation-produced L-Asp:isoAsp ratio depends upon protein three-dimensional structure.

In very well characterized systems, PCMT can be used quantitatively, but the prior characterization requires some other means of quantitative analysis of deamidation. PCMT is very useful in detecting isoAsp in systems where more quantitative means are not applicable.

7. Some additional speculations were made. For examples, it was hypothesized that PCMT also reversed errors in DNA transcription and that methylation might be an intermediate in protein turnover.

This work is summarized in 1990 reviews.[13] Specific references are 1985OC1, 1985OC, 1985C, 1986C, 1986MC1, 1986MC, 1987O, 1987LC, 1987OD, 1988OY, 1989OC, 1989OG, and 1990LO; 1985JA1, 1985JF, 1985JA, 1987JM, 1987AJ, 1987JL, 1987AJ1, 1989JS1, and 1990A; and 1988VH, 1988VC, 1988SK, and 1989HJ.

As is the case with numerous other enzymes, PCMT is required for organismic good health, although reduced quality life is possible in its absence. PCMT-deficient mice have diminished growth, show increased levels of PCMT substrates, and exhibit fatal brain seizures and markedly reduced lifespans of about 45 days.[14] If PCMT is present only in the mouse brain at 6.5-13% of normal levels and not present in other

12 J. A. Lindquist and P. N. Mcfadden, *J. Protein Chemistry* **13**, 553 (1994); D. J. Weber and P. N. McFadden, *J. Protein Chemistry* **16**, 269 (1997).

13 I. M. Ota and S. Clarke, *Protein Methylation (Ed. W. K. Paik and S. Kim), CRC Press, Boca Raton, FL*, 179 (1990); B. A. Johnson and D. W. Aswad, *Protein Methylation, (Ed. W. K. Paulk and S. Kim), CRC Press, Boca Raton, FL*, 195 (1990).

14 E. Kim, J. D. Lowenson, D. C. MacLaren, S. Clarke, and S. G. Young, *Proc. Natl. Acad. Sci. USA* **94**, 6132 (1997); A. Yamamoto, H. Takagi, D. Kitamura, H. Tatsuoka, H. Nakano, H. Kawano, H. Kuroyanagi, Y. Yahagi, S. Kobayashi, K. Koizumi, T. Sakai, K. Saito, T. Chiba, K. Kawamura, K. Suzuki, T. Watanabe, H. Mori, and T. Shirasawa, *Journal of Neuroscience* **18**, 2063 (1998); T. Shirasawa, *Seikagaku* 71, 134 (1999); E. Kim, J. D. Lowenson, S. Clarke, and S. G. Young, *J. Biological Chemistry* **274**, 20671 (1999).

tissues, lifespan is about 250 days as compared with 700 days in control.[15] PCMT-reactive isoAsp is 80-fold higher in histone H2B in the brains of PCMT-deficient mice.[16]

Total activity of human erythrocyte PCMT varies in a unimodal and apparently gaussian distribution over about a 1.5-fold range, and two human forms have been discovered involving an Ile/Val substitution, which have differing heat stabilities.[17] PCMT-deficient nematodes have a 3.5-fold reduction in lifespan, and *E. coli* are also weakened by PCMT deficiency.[18] PCMT inhibits Bax-induced apoptosis in mouse neurons.[19]

Over-expression of PCMT in *Drosophila* has been reported to increase lifespan by about 30% at 29 °C, but not at 25 °C.[20] It has been suggested, however, that this is a manifestation of heat-shock survival and not of reduced rate of aging and that isoAsp and D-Asp conversion to L-Asp may not be the primary function of PCMT.[21]

Lanthier and Desrosiers have succeeded in restoring a substantial amount of age-decreased biological activity *in vivo* in type-I collagen and fibronectin by means of PCMT.[22]

While PCMT reacts readily with isoAsp if three-dimensional structure permits, its reactivity with D-Asp is much less. Recently a mammalian D-aspartyl endopeptidase has been discovered, which may fill this gap.[23]

15 J. D. Lowenson, E. Kim, S. G. Young, and S. Clarke, *J. Biological Chemistry* **276**, 20695 (2001).

16 A. L. Young, W. G. Cartea, H. A. Doyle, M. J. Mamula, and D. W. Aswad, *J. Biological Chemistry* **276**, 37161 (2001).

17 C. L. David, C. L. Szumlanski, C. G. DeVry, J. O. Park-Hah, S. Clarke, R. W. Weinshilboum, and D. W. Aswad, *Archives of Biochemistry and Biophysics* **346**, 277 (1997); C. G. DeVry and S. Clarke, *J. Human Genetics* **44**, 275 (1999).

18 C. Li and S. Clarke, *Proc. Natl. Acad. Sci. USA* **89**, 9885 (1992); R. M. Kagan, A. Niewmierzycka, and S. Clarke, *Archives of Biochemistry and Biophysics* **348**, 320 (1997); J. Visick, H. Cai, and S. Clarke, *J. Bacteriology* **180**, 2623 (1998).

19 K. J. Huebscher, J. Lee, G. Rovelli, B. Ludin, A. Matus, D. Stauffer, and P. Fürst, *Gene* **240**, 333 (1999).

20 D. A. Chavous, F. R. Jackson, and C. M. O'Conner, *Proc. Natl. Acad. Sci. USA* **98**, 14814 (2001).

21 J. Kindrachuk, J. Parent, G. F. Davies, M. Dinsomore, S. Attah-Poku, and S. Napper, *J. Biological Chemistry* **278**, 50880 (2003).

22 J. Lanthier and R. R. Desrosiers, *Experimental Cell Research* **293**, 96 (2004).

23 T. Kinouchi, S. Ishiura, Y. Mabuchi, Y. Urakami-Manaka, H. Nishio, Y. Nishiuchi, M. Tsunemi, K. Takada, M. Watanabe, M. Ikeda, H. Matsui, S. Tomioka, H. Kawahara, T. Hamamoto, K. Suzuki, and Y. Kagawa, *Biochemical and Biophysical Research Communications* **314**, 730 (2004).

High plasma homocysteine levels have been reported to inhibit PCMT, and it was suggested that folate could lower these levels and reduce this effect.[24]

While the biological role of this interesting enzyme is not yet fully understood, its presence and possible ubiquitous role as an eliminator of D-Asp and isoAsp may provide further support for the amide molecular clock hypothesis.

Most genetically specifiable Asn structures in proteins are stable, with only a small percentage deamidated during the *in vivo* lifetime of the proteins and tissues in which they are genetically specified.[25] Therefore, by simply specifying stable amide structures, living things could not only avoid the changes in charge and conformation that accompany deamidation, but they could also avoid operation of the burdensome and inefficient PCMT enzyme systems. That they instead specify unstable amides and then go to the further effort of reversing the resulting isomerization further implicates deamidation as a beneficial reaction extensively required for life.

Additional articles about PCMT include 1991JS, 1991LO, 1991IK, 1991JA, 1991JN, 1992LO, 1992MG, 1992RL, 1992MO, 1993JN, 1993PI, 1993MC, 1993JA, 1993BG, 1994MM, 1994LM, 1994GL, 1994GB, 1994GB1, 1995AG, 1995A1, 1995BC, 1995JA, 1995LC, 1995KC, 1995DA2, 1995A, 1996OK, 1996PD, 1996OA, 1996DT, 1997OG, 1997ML, 1997KG, 1997IC, 1998SL1, 1998IC, 1998VI, 1999KH, 2000AP, 2000KK, 2000CR, 2000TC, 2000SP1, 2000SA1, 2001DI, 2002SC, 2002ZC, 2002GB, 2003JK, 2003DG, and 2003BB.

24 A. F. Perna, N. G. DeSanto, and D. Ingrosso, *Mineral and Electrolyte Metabolism* **23**, 174 (1997).

25 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002); N. E. Robinson and A. B. Robinson, *Mechanisms of Ageing and Development* **125**, 259 (2004).

CHAPTER 17

Carboxyl-Terminus Amides

17-1. ENZYMATIC DEAMIDATION

There are many biologically occurring peptides for which *in vivo* enzymatic deamidation of the amidated carboxyl terminus has been reported. These deamidations usually cause almost complete loss of biological activity. The most studied has been thyrotrophin-releasing hormone, TRH, pyroGluHisProNH_2. The amino end is pyroglutamic acid and the carboxyl end is amidated.

TRH deamidation has been observed in pig brain; rat and human plasma and spinal cord; rabbit and rat intestines; cow brain; cow pituitary; and rat brain, pituitary, liver, mammary glands, and kidney.[1] See also 1979DR, 1979KB, 1980BK, 1982SW, 1982GM, 1987HW, 1987CM, 1991SG1, and 1993TS. The cow brain enzyme also deamidates luteinizing hormone releasing hormone, LHRH, pyroGluHisTrpSerTyrGluLeuArgProGlyNH_2, by cleaving the ProGly bond, so it has Pro endopeptidase activity.[2] TRH has been used therapeutically in the treatment of amyotrophic lateral sclerosis, wherein this deamidation has been observed to increase in patients receiving the drug.[3]

In a study of 13 naturally occurring peptide hormones, it was shown that the cow brain enzyme cleaves after Pro even several residues from the carboxyl-terminal if the residue on the amino side of Pro is His, Lys, or Arg, so the carboxyl-terminal deamidation activity results from a specific peptide bond cleavage enzyme. Cleavage occurred in luliberin, tuftsin, angiotensin II, melanotropin, and neurotensin.[4]

The 17-residue peptide gastrin is enzymatically deamidated at the carboxylterminal –PheNH_2 by deamidases in the liver, intestines, stom-

1 W. Taylor and J. E. Dixon, *Biochimica Et Biophysica Acta* **444**, 428 (1976); K. Bauer and F. Lipmann, *Endocrinology* **99**, 230 (1976); A. Dupont, F. Labrie, L. Levasseur, J. Dussault, and A. V. Schally, *Clinical Endocrinology* **5**, 323 (1976).

2 L. B. Hersh and J. F. McKelvy, *Brain Research* **168**, 553 (1979).

3 J. Turner, T. Schwartz, W. W. Tourtellotte, and B. R. Brooks, *Neurology* **38** Suppl. 1, 326 (1988).

4 S. S. Tate, *European Journal of Biochemistry* **118**, 17 (1981).

ach, pancrease, and kidneys of rats, dogs, cats, and humans.[5] This is probably due to carboxypeptidase, which catalyzes the deamidation of $-PheNH_2$ in addition to cleavage of PheXxx peptide bonds.[6] A similar enzyme has been found in human platelets.[7] A 6-residue neuropeptide growth factor antagonist being tested as a treatment for human small-cell lung cancer was found to be deamidated at the carboxyl terminus by a carboxypeptidase in the spleen and kidney.[8] A similar enzyme deamidates opioid tretrapeptide agonists in rats and a cardioexcitatory tetrapeptide in seaslugs.[9]

Pig calpain cysteine proteases also catalyze carboxyl terminus deamidation[10] as do amidases in frog skin[11] and nematode muscle.[12]

Additional related research, especially on loss of biological activity through carboxyl terminus deamidation, includes 1981Z, 1985RR, 1991MN, 1992BM, 1997BM, and 2001CT.

Carboxypeptidases and carboxyl terminus amidases obtained from various species or from synthetic modifications have been used in efforts to develop enzyme-based peptide synthesis procedures. Examples include 1987CG1, 1989CH, 1990SK, and 1993KS.

Peptide amino acid carboxyl terminus amidases from bacteria and oranges that are stereospecific for L-amino acids have been used to prepare high purity L-amino acids and L-amino acid derivatives.[13]

So far as is known, the carboxyl terminus amidases described in this chapter have no ability to deamidate the side chains of Asn.

5 J. H. Walsh and L. Laster, *Biochemical Medicine* **8**, 432 (1973).

6 K. Breddam, *Carlsberg Research Communications* **49**, 535 (1984).

7 H. L. Jackman, F. Tan, H. Tamei, C. Beurling-Harbury, X. Li, R. S. Skidgel, and E. G. Erdos, *J. Biological Chemistry* **265**, 11265 (1990).

8 D. A. Jones, J. Cummings, S. P. Langdon, A. Maclellan, and J. F. Smyth, *Biochemical Pharmacology* **50**, 585 (1995); D. A. Jones, J. Cummings, S. P. Langdon, and J. F. Smyth, *General Pharmacology* **28**, 183 (1997).

9 E. Krondahl, H. v. Euler-Chelpin, A. Orzechowski, G. Ekström, and H. Lennernäs, *Peptides* **22**, 613 (2001); F. Morishita, O. Matsushima, Y. Furukawa, and H. Minakata, *Peptides* **24**, 45 (2003).

10 M. Hatanaka, T. Sasaki, T. Kikuchi, and T. Murachi, *Archives of Biochemistry and Biophysics* **242**, 557 (1985).

11 G. C. Camarao, K. M. Carvalho, and P. Cohen, *Brazilian Journal of Medical and Biological Research* **27**, 2863 (1994).

12 M. Sajid, C. Keating, L. Holden-Dye, I. D. Harrow, and R. E. Isaac, *Molecular and Biochemical Parasitology* **75**, 159 (1996); J. W. Bowman, A. R. Friedman, D. P. Thompson, A. K. Ichhpuran, M. F. Kellman, N. Marks, A. G. Maule, and T. G. Geary, *Peptides* **17**, 381 (1996).

13 U. Stelkes-Ritter, G. Beckers, A. Bommarius, K. Drauz, K. Günther, M. Kottenhahn, M. Schwarm, and M. Kula, *Biocatalysis and Biotransformation* **15**, 205 (1997).

17-2. NONENZYMATIC DEAMIDATION

Peptides stored in the presence of a strong acid are found to deamidate rapidly at their carboxyl terminus amides at 4°C.[14]

It has been reported that small amounts of deamidation of carboxyl terminus amides of PheTrpArgNH_2 and ArgTrpPheNH_2 were qualitatively observed in acid and basic pHs at 80°C.[15] These amides are, however, quite stable to nonenzymatic deamidation and apparently deamidate only by acid or base hydrolysis.

The deamidation half-time of AlaAlaAlaAlaNH_2 was found to be 6,600 days in pH 7.4, 37 °C, 0.15 M Tris-HCl. This is in good agreement with the reported median deamidation half-time of GlyXxxAsnProGly peptides of 8,000 days, where the imide mechanism is suppressed.[16]

14 P. B. W. Ten Kortenaar, B. M. M. Hendrix, and J. W. Van Nispen, Int. J. Pept. Protein Res. **36**, 231 (1990); R. G. Strickley, M. Brandl, K. W. Chan, K. Straub, and L. Gu, *Pharmaceutical Research* **7**, 530 (1990).

15 J. L. E. Reubsaet, J. H. Beijnen, E. H. Belshof, M. Bouyakhrichan, A. Bult, E. Hop, Y. Kellekule, R. J. van Maanen, J. Teeuwsen, and W. J. Underberg, *J. Pharmaceutical and Biomedical Analysis* **19**, 277 (1999).

16 N. E. Robinson, Z. W. Robinson, B. R. Robinson, A. L. Robinson, J. A. Robinson, M. R. Robinson, and A. B. Robinson, *J. Peptide Research* **63**, 426 (2004).

CHAPTER 18

Isomerization, Cleavage, and Racemization

18-1. ISOMERIZATION AND CLEAVAGE

As discussed in previous chapters, isomerization to a mixture of L-Asp, L-isoAsp, D-Asp, and D-isoAsp often occurs at Asn and Asp because these products are characteristic of the imide mechanism.

Sometimes, the imide itself is seen in a protein, usually at low pH. See, for examples, 1994SE, 1994TY1, 1998GA, and 2002SV.

It has been shown, in studies of AcGlyAspGlyGlyMe, that the isomerization of Asp to isoAsp is entirely reversible. The reaction requires that the Asp side chain be in the neutral state and that the carboxyl side backbone nitrogen be deprotonated.[1]

The amounts of isomerization in most short peptides are semi-quantitatively similar, with L-isoAsp:L-Asp ratio usually having a value of about 3:1. Protein structure can, however, markedly affect this ratio. For example, isoAsp formation at Asp(15) in a His15Asp mutant of histidine-containing protein HPr is apparently prevented by the intrusion of Glu(85), so only the L-Asp form is observed. Removal of Glu(85) allows the formation of isoAsp.[2]

Since L-Asp is often favored by thermodynamics and isoAsp by kinetics, long exposures to solvent that allow the attainment of equilibrium can also affect this ratio.

Table 18-1 lists some examples of Asp isomerization.

The isomerization of Asp usually proceeds more slowly than the deamidation of Asn, so the acceleration by carboxyl side Gly is more noticeable.

Since the mechanisms are similar, the relative rates of Asp isomerization within a protein can be successfully predicted by the computation method devised for the estimation of Asn deamidation

1 S. Capasso, A. J. Kirby, S. Salvadori, F. Sica, and A. Zagari, *J. Chemical Society-Perkin Transactions* **2**, 437 (1995).

2 L. Athmer, J. Kindrachuk, F. Georges, and S. Napper, *J. Biological Chemistry* **277** 30502 (2002).

Table 18-1 Instances of Asp Isomerization.

Peptide or Protein	Residues	Reference
Daptomycin	AspGly	1989KM
αA-crystallin	ValLeuAsp(58)SerGly PheGluAsp(84)LeuThr GlyValAsp(151)AlaThr	1990GI, 1997FM, 1999FT, 2000FS, 2002KN
Somatotropin	LeuGluAsp(129)GlySer	1992VS
Pro-opiomelanocortin	Asp(16)Gly	1993TB1
αB-crystallin	GluSerAsp(36)LeuPhe TrpPheAsp(62)Thr Gly	1994FI
βA-Amyloid 42 Residue Peptide	Asp(1)AlaGlu ArgHisAsp(7)SerGly	1993RL
Fibroblast Growth Factor	ProGluAsp(15)GlyGly	1994SE
Caeridin	LeuLeuAsp(4)GlyLeu	1995WS1
Hirudin	GlySerAsp(33)GlyGlu HisAsnAsp(53)GlyAsp	1998GA
Neurotrophic Factor	ValSerAsp(96)LysVal	2001MH

rates.[3] Simonovic and Volz have reported the finding of a stable succinimide ring during structure determination of CheY protein from *E. coli*.[4] They find this ring at Asp(75)Gly. By application of the Asn computation method,[5] Simonovic and Volz verified that this Asp is the most likely to isomerize as compared with, for example, Asp(38)Gly and Asp(64)Gly in CheY. When Asp(57) in CheY is changed to Asn, it deamidates rapidly to Asp.[6]

Figure 18-1 shows the gradual isomerization of GlyValAsp(151)AlaThr with age in human eye lens α-A-crystallin.[7] By age 60, Asp(151) is 44% L-Asp, 32% D-isoAsp, 12% L-isoAsp, and 12% D-Asp.

Cleavage of the peptide bond following Asp also occurs in analogous fashion to that following Asn. It has been observed, for example,

3 M. Simonovic and K. Volz, *J. Molecular Biology* **322**, 663 (2002); N. E. Robinson, *Proc. Natl. Acad. Sci. USA* **99**, 5283 (2002).

4 M. Simonovic and K. Volz, *J. Molecular Biology* **322**, 663 (2002).

5 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 12409 (2001).

6 P. M. Wolanin, D. J. Webre, and J. B. Stock, *Biochemistry* **42**, 14075 (2003).

7 N. Fujii, L. J. Takemoto, Y. Momose, S. Matsumoto, K. Hiroki, and M. Akaboshi, *Biochemical and Biophysical Research Communications* **265**, 746 (1999).

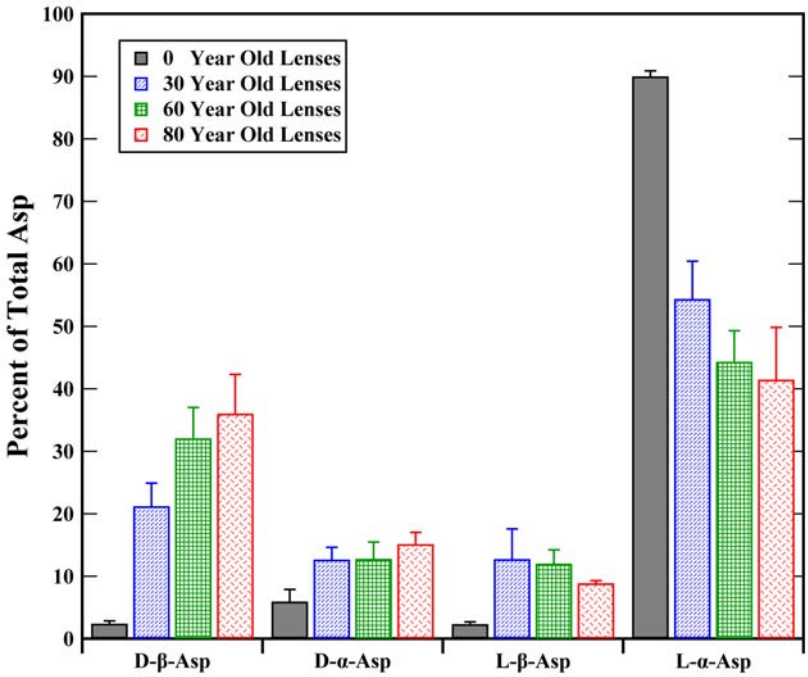

FIG. 18-1 The relative amounts of four optical isomers of Asp-151 residues, L-α-Asp, L-β-Asp, L-α-Asp, and D-β-Asp in αA-crystallins from donors of lenses in the 0, 30, 60, and 80 year ranges. Adapted from 1999FT.

in basic fibroblast growth factor[8] at ProGluAsp(15)GlyGly and PheLysAsp(28)ProLys. This cleavage is accelerated by phosphate buffer and by hydroxylamine and exhibits a sequence dependence similar to deamidation. Glu undergoes the same reaction, but 20 to 40-fold more slowly.[9]

Cleavage after Asn is found as a biologically occurring protein-splicing mechanism. Splicing cleavage usually occurs after Asn in the sequence XxxHisAsnYyy and another peptide is spliced to the c-terminal end. Splicing also occurs in other motifs.[10] See for examples 1992SG, 1993CC, 1994XC, 1995SX, and 1995K.

8 Z. Shahrokh, G. Eberlein, D. Buckley, M. V. Paranandi, D. W. Aswad, P. Stratton, R. Mischak, and Y. J. Wang, *Pharmaceutical Research* **11**, 936 (1994).

9 J. K. Blodgett, G. M. Loudon, and K. D. Collins, *J. American Chemical Society* **107**, 4305 (1985).

10 G. Amitai, B. Dassa, and S. Pietrokovski, *J. Biological Chemistry* **279**, 3121 (2004).

18-2. RACEMIZATION

Research on protein racemization increased in the early 1970s, especially in the laboratories of Bada and coworkers.[11]

This work focused first on fossil dating using racemization and then proceeded to the question of its potential effect on long-lived organisms. Since D-Asp was the most prevalent D residue observed, studies of Asp were emphasized. Figure 18-2 is adapted from Helfman and Bada 1975.[12] It was found that tooth enamel racemizes at a rate of about 0.1 % per year. On the basis of measurements of dentine, which is pri-

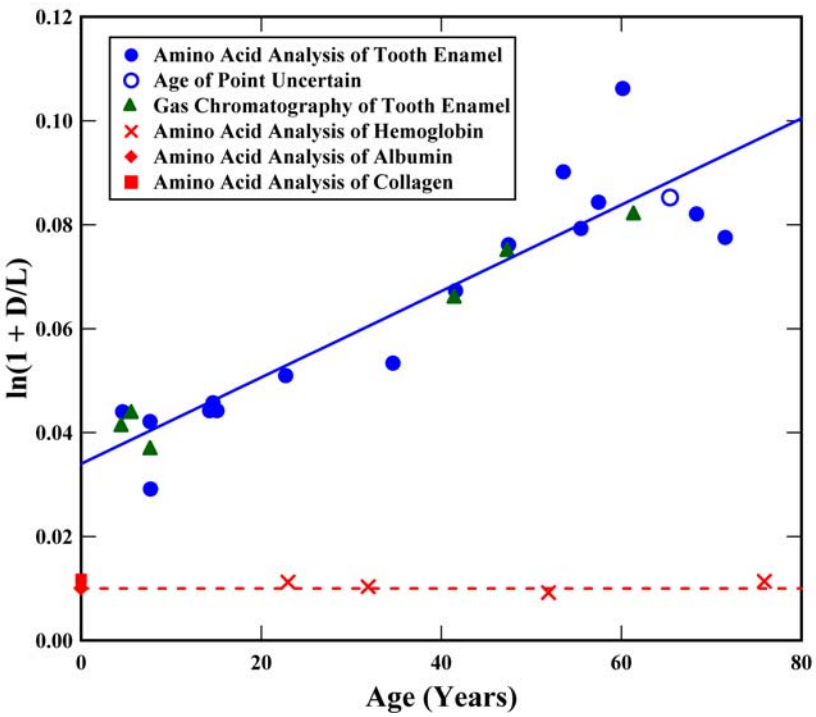

FIG. 18-2 Plot of ln(1 + D/L) for aspartic acid against age of tooth enamel and against age of the individual for the protein values. The slope (8.29×10^{-4} yr^{-1}) of the solid line is equal to k_{asp} for tooth enamel at about 37°C. Adapted from 1975HB.

11 J. L. Bada, B. P. Luyendyk, and J. B. Maynard, *Science* **170**, 730 (1970); J. L. Bada, *Adv. Chem. Ser.* **106**, 309 (1971); J. L. Bada and R. Protsch, *Proc. Natl. Acad. Sci. USA* **70**, 1331 (1973); J. L. Bada, R. A. Schroeder, R. Protsch, and R. Berger, *Proc. Natl. Acad. Sci. USA* **71**, 914 (1974); And other publications.

12 P. M. Helfman and J. L. Bada, *Proc. Natl. Acad. Sci. USA* **72**, 2891 (1975); P. M. Helfman and J. L. Bada, *Nature* **262**, 279 (1976).

marily collagen, they estimated that about 6% of stable body protein would be expected to be racemized in 60 years *in vivo* at 37 °C. This work was extended to eye lens proteins in 1977[13] with the finding that eye lens proteins racemized about 50% faster than those in teeth, probably owing to temperature or protein configurations. Those in cataracts racemized somewhat faster. It was pointed out that metal ions or other solvent components have potential to increase these rates.[14]

Most of this racemization apparently occurs by means of imide mechanisms. Collins, Waite, and van Duin[15] have shown that the imide model can be used to fit the racemization data at high temperatures, but that this fails at 37 °C in collagen. They conclude that the three-dimensional structure of collegen markedly inhibits the reaction. The same situation prevails for deamidation of collagen as discussed in Chapter 12-7. Their review of the entire peptide and protein racemization literature at a wide variety of temperatures, with peptides and unfolded proteins utilized at lower temperatures, results in a racemization half-time of about 5,000 days at 37 °C.

This 5,000 days is about 50-fold longer than the mean for Asn deamidation and approximately equal to that for Gln deamidation. It represents a sort of global average of the accumulation of D-isomers through action of the imide mechanism on Asn and Asp minus repair or loss of D-isomer by the various mechanisms. Nonenzymatic Gln deamidation and Asp and Asn racemization appear, therefore, to be of comparable potential physiological impact. Gln deamidation produces, however, a change in charge, while both potentially produce conformation changes, so Gln deamidation might be expected to have a greater average impact on protein structure than would racemization. Of course, the Asn deamidation that accompanies Asn racemization is much more pervasive.

The research literature and current understanding of the production of D-Asp and D-isoAsp *in vivo* in proteins has been reviewed by Ritz-Timme and Collins.[16] Ritz-Timme, Laumeier, and Collins[17] have measured the racemization rates of human skin elastin and have suc-

13 P. M. Masters, J. L. Bada, and J. S. Zigler, Jr., *Nature* **268**, 71 (1977).

14 P. M. Helfman, J. L. Bada, and M. Shou, *Gerontology* **23**, 419 (1977).

15 M. J. Collins, E. R. Waite, and A. C. T. van Duin, *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **354**, 51 (1999).

16 S. Ritz-Timme and M. J. Collins, *Ageing Research Reviews* **1**, 43 (2002).

17 S. Ritz-Timme, I. Laumeier, and M. J. Collins, *British Journal of Dermatology* **149**, 951 (2003).

cessfully applied the three dimensional Asn prediction model [18] to the understanding of these rates.

Some additional references of interest from the substantial literature about racemization, which is beyond the scope of comprehensive review in this book, include 1980P, 1980BB, 1982FM, 1983M1, 1983MS, 1984B1, 1987SC, 1989SR, 1992GH, 1992PV, 1993GD, 1994FI, 1996RZ, and 1998LB.

Racemization is a special problem in food processing procedures, especially those employing strong base. See for examples 1979MF, 1980MF, and 1987LF. Very substantial amounts of D amino acids and D amino acid residues can be introduced by food processing and food preparation. The potential health effects of the presence of these racemization products are unknown.

In the αA-crystallin of 5 to 8-year old cows, the D/L ratio at GlyValAsp(151)AlaGly was found to be 0.49, while that for other Asp residues ranged between 0.025 and 0.080,[19] which demonstrates the specificity conferred by three-dimensional structure. In human eye lens, the preferential racemization to D-Asp at Asp(151) was found to diminish by about 3-fold when tertiary structure was disrupted.[20]

Peptides, IleGlnThrGlyLeuAsp(151)AlaThrHisAlaGluArg, ThrValLeuAsp(58)SerGlyIleSerGluValArg, and HisPheSerProGluAsp(84)LeuThrValLys from αA-crystallin, were estimated to have half-times of racemization at 37 °C of 3.6, 12.8, and 20.6 years, respectively in 0.1 M phosphate, pH 7.0.[21] These were estimated from measurements at 50 °C to 90 °C and extrapolation to 37 °C. This suggests primary structure effects on racemization, which are, as yet, largely unexplored. The presence of acetate or guanidinium ions has been reported to increase racemization of the succinimide intermediate.[22]

18 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 4367 (2001).

19 P. J. T. A. Groenen, P. R. L. A. Avan den ljssel, C. E. M. Voorter, H. Bloemdal, and W. W. de Jong, *FEBS Letters* **269**, 109 (1990).

20 N. Fujii, K. Harada, Y. Momose, N. Ishii, and M. Akaboshi, *Biochemical and Biophysical Research Communications* **263**, 322 (1999).

21 N. Fujii, Y. Momose, and K. Harada, *Int. J. Peptide and Protein Research* **48**, 118 (1996).

22 I. Schon, T. Szirtes, A. Rill, G. Balogh, Z. Vadasz, J. Seprodi, I. Teplan, N. Chino, K. Y. Kumogaye, and S. Sakakibara, *J. Chemical Society-Perkin Transactions* **1**, 3213 (1991).

About 1% of human red blood cell membrane Asp was found to be in the D form after 38 days, corresponding to a cell lifetime expectation of 3%.[23]

Thus, while racemization studies began as a parallel but separate investigation of another post-synthetic alteration of protein structure and continue in this way for other residues which racemize at slower rates, the dependence of D-Asp production upon Asn deamidation as well as Asp racemization and their common imide mechanism has brought these subjects together.

Early investigators measured D-Asp and thought that all of this racemic product originated from L-Asp. It is now known that it originates from both L-Asn and L-Asp, primarily through the imide mechanism.

The relative *in vivo* rates of racemization vs. deamidation, the precise genetic control over deamidation, and the charge change accompanying deamidation, render deamidation a much more likely candidate as a molecular clock for most *in vivo* processes, but the potential relevance of racemization as a factor in the degenerative aging of long-lived organisms is significant.

23 L. S. Brunauer and S. Clarke, *J. Biological Chemistry* **261**, 12538 (1986).

CHAPTER 19

Analytical Techniques

19-1. GENERAL TECHNIQUES

Early investigations of deamidation utilized thin layer and paper chromatography and the usual tools of the organic chemist. The first investigations of larger peptides and proteins were carried out by countercurrent distribution. As analytical techniques improved, deamidation of Asn and Gln began to be recognized as a widespread phenomenon.

The introduction of paper electrophoresis permitted better separations, so most peptide studies in the 1970s were conducted with this technique in combination with isotopic labeling. In these lower resolution systems, verification of the reaction products was difficult and lack thereof occasionally led to errors. The best verification at the time was enzymatic digestion followed by amino acid analysis. Presence of the enzyme in the reaction mixture was a complication, but this can be circumvented by immobilized enzymes.[1]

The principal analytical advance that allowed relatively simple separation of proteins in different states of deamidation was gel electrophoresis. While this technique has sufficient resolution to separate most proteins on the basis of a single change in charge, it is also highly dependent upon protein configuration. This can be beneficial in devising separations wherein multiple deamidations are occurring simultaneously, so that resolution of two or more forms carrying the same charge is necessary. Electrophoretic conditions are, however, highly empirical and vary from protein to protein.

The introduction of isoelectric focusing in the late 1960s was a substantial help in deamidation studies, since separations on the basis of charge are usually predictable in this system. Charge changes can be masked by structural factors, but this appears to be more the exception than the rule.

Isoelectric focusing techniques have advanced to such an extent that commercially available immobilized gradients with resolving power as

1 G. P. Royer and J. P. Andrews, *J. Biological Chemistry* **248**, CU33 (1973).

sharp as 0.001 pH units are now available.[2] This exceeds the minimum resolution required for single charge separations in small proteins by two orders of magnitude and permits investigation of subtle structural effects. Indeed, isoelectric focusing has advanced to such high resolution that one of the principal disadvantages is precipitation of the protein at its isoelectric point because such sharp bands are produced. If precipitation is avoided, direct gel scanning provides good quantitative values.

Two-dimensional maps by isoelectric focusing and electrophoresis have been widely used in protein separations,[3] but have not been employed as much in deamidation studies as might have been expected. In a recent study using this technique followed by mass spectrometry,[4] all of the observed AsnGly peptides in a set of 64 nuclear proteins from lung cancer cells were found to be partially deamidated. Part of this deamidation may have occurred during sample handling and purification.

Deamidation artifacts produced during isolation, purification, and analysis must be carefully guarded against in all deamidation experiments. As much as possible, preparations should avoid solution conditions known to accelerate deamidation. Storage at -80 °C is a very good precaution. In general, controls should be utilized in which the molecules of interest are exposed to differing and extended periods of time in the various experimental conditions followed by verification that these do not significantly increase observed deamidation.

In the case of small peptides and peptide mixtures from proteolytic digestion of proteins, high-pressure liquid chromatography in small bore columns is widely used. This has the advantage of in-line quantitative analysis and also direct injection into mass spectrometers for structural identification. HPLC has, however, the disadvantage that amidated and deamidated peptides can be differentially absorbed in the tubing and chromatographic column. This leads to errors in quantitation. This problem is exacerbated in HPLC-mass spectrometry systems where sample concentrations can be very low. In these cases, peptides are often entirely lost, and unrecognized partial losses lead to erroneous results.

2 Z. N. Farwig, A. V. Campbell, and R. D. Macfarlane, *Analytical Chemistry* **75**, 3823 (2003).

3 P. H. O'Farrell, *J. Biological Chemistry* **250**, 4007 (1975).

4 L. J. Gonzalez, L. Castellanos-Serra, V. Badock, M. Diaz, A. Moro, S. Perea, A. Santos, D. Paz-Lago, A. Otto, E. Muller, S. Kostka, B. Wittmann-Liebold, and G. Padron, *Electrophoresis* **24**, 237 (2003).

Peptide absorption on container walls begins to become a problem at 10^{-3} M for some especially susceptible peptides, and is worse at lower concentrations. At concentrations of 10^{-6} M, virtually all peptides require exquisite attention to apparatus surface extent and composition.

The advance of mass spectrometry, especially the development of electrospray and matrix-assisted laser desorption ionization sources, has markedly improved deamidation measurements.

It is now possible to carry out quantitative single-peptide deamidation experiments by direct loop injection into a mass spectrometer without prior chromatographic separation. This can also be accomplished for peptides produced by enzymatic digestion of small proteins. Elimination of the chromatographic step for such digests is, however, not generally applicable at present because rich mixtures of peptide types interfere with each other in the mass spectrometer. This and other problems are rapidly being solved, so direct injection, high-resolution mass spectrometry will likely become increasingly utilized in simple systems. Also, very high-resolution mass spectrometry makes possible the direct measurement of deamidation in peptides at neutral pH, without protonation of the Asp or Glu carboxyl group.[5]

Increasingly, deamidated Asn is being identified during three-dimensional structure determinations by high resolution nuclear magnetic resonance.[6] IsoAsp, where Asn is expected, is detected during backbone configuration determination with these methods.

For complicated mixtures, chromatography followed by mass spectrometry is currently often the method of choice. Capillary electrophoresis or isoelectric focusing are also used as pre-separations for the mass spectrometer.

The small mass change of 1 amu upon deamidation is an impediment to measuring deamidation rates of macromolecules. In large molecules, the presence of naturally occurring isotopes markedly reduces effective resolution. One solution to this is to react the amides or carboxylic acids of the protein with a larger and more easily distinguishable species. Methylation is commonly used. Recently, a fluorescent 22 base pair DNA oligomer was used for this purpose.[7]

5 D. G. Schmid, F. v. der Mulbe, B. Fleckenstein, T. Weinschenk, and G. Jung, *Analytical Chemistry* **73**, 6008 (2001).

6 V. Tugarinov, R. Muhandiram, A. Ayed, and L. E. Kay, *J. American Chemical Society***124**, 10025 (2002); V. V. Rogov, C. Lucke, L. Muresanu, H. Wienk, I. Kleinhaus, K. Werner, F. Lohr, P. Pristovsek, and H. Ruterjans, *European Journal of Biochemistry* **270**, 4846 (2003).

7 J. Won, R. J. Meagher, and A. E. Barron, *Biomacromolecules* **5**, 618 (2004).

Although time consuming, the highest resolution method for the study of deamidation in peptides and proteins is currently immobilized gradient isoelectric focusing followed by band elution and mass spectrometry.[2] With resolution of 0.001 in the pH gradient and a high resolution mass spectrometer, there are probably very few deamidation processes of current interest that cannot be followed quantitatively by this combination of techniques. A second-dimension electrophoresis separation provides, of course, further resolution.

Inside biological systems, the situation is different. Various reactions are available for tagging Asp and Glu residues, such as chemical or enzymatic methylation. See, for example, Chapter 16. These methods are, however, primarily qualitative because the reactions are affected by both the structures of the proteins and of the organelles in which they are found. Where quantitative results are necessary, these methods can be used in very well characterized systems, but the efforts involved in that characterization and the caveats necessary in interpretation of the results are substantial.

The details of analytical techniques for deamidation are described in the deamidation studies reviewed and referenced in the preceding chapters and will not be repeated here. The analytical procedures chosen by these investigators generally speak for themselves. Additional analytical references of interest include 1935VP, 1938KH, 1958CR, 1958RN, 1961EL, 1966MG, 1972HG, 1973B, 1973B, 1989FW, 1995G, 1995WS, 1997S, 1997FT, 1998HA, 1999WG2, 2000LS2, 2000LK, 2000BB, 2001TM, 2002JS, 2003BN, 2003KU, 2004DO, and 2004EZ.

19-2. PEPTIDE MEASUREMENT

Where peptide reactants or products are of the same masses, chromatography is currently the method of choice. This is used, for example, in studies in which the various isomeric products of deamidation are measured separately. In these studies, all of the synthetic products must be prepared for use as quantitative chromatographic standards.

While mass spectrometric methods for distinguishing these isomers have been reported, these are presently dependent upon sophisticated equipment and interpretation and are qualitative at best.

Where quantitative measurement of deamidation alone is required, direct mass spectrometry is by far the best method. This can be extended to proteins by proteolytic digestion with or without pre-separation by chromatography. Current mass spectrometers diminish in

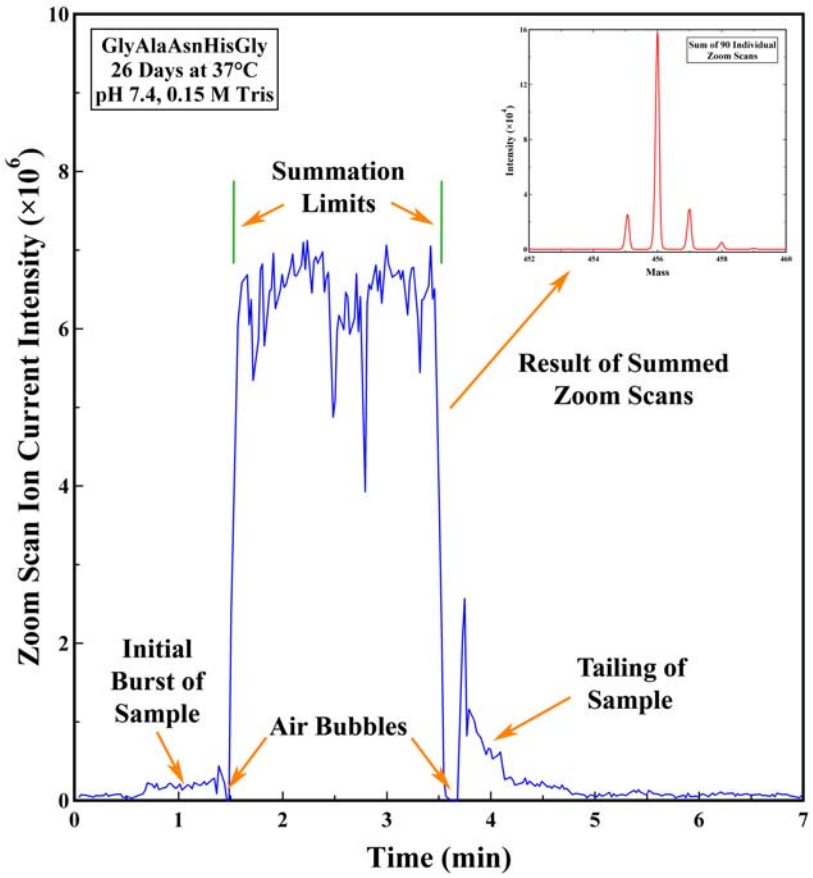

FIG. 19-1 Total 10 amu zoom scan ion current as a function of time for GlyAlaAsnHisGly after 26 days of deamidation (2004R).

capability as the number of peptide species being simultaneously measured increases, so pre-chromatography improves results in these cases.

Direct measurement of deamidation by loop injection into an ion trap mass spectrometer[8] provides an example. This is the method used for most of the peptide deamidation rates listed in Table 6-1.

For example, the deamidation rate of 0.001 M GlyAlaAsnHisGly was measured in pH 7.4, 37.0 °C, 0.15 M Tris-HCl buffer. Special precautions were taken to assure reliable incubation conditions, to prevent

8 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001); N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001); N. E. Robinson, *PhD Thesis, California Institute of Technology, Chemistry* (2003).

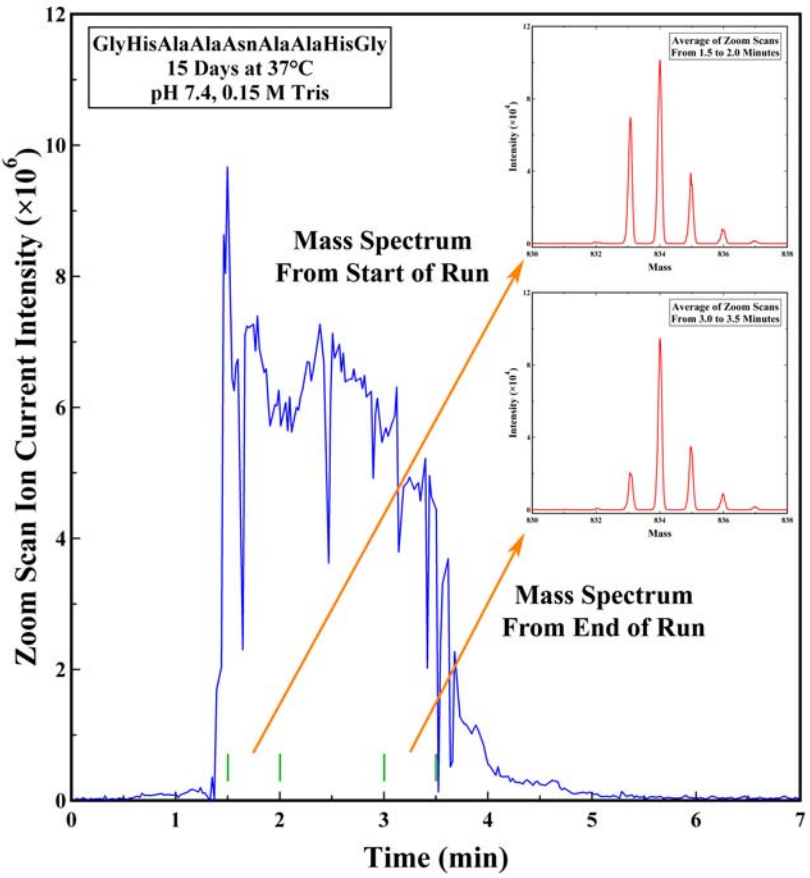

FIG. 19-2 Example of a badly tailing sample. Deamidation of GlyHisAlaAlaAsnAlaAlaHisGly after 15 days (2004R).

peptide loss on the surfaces of the apparatus, and to assure quantitative reproducibility of the mass spectrometer[8], which was a Thermoquest LCQ. Dilution of the sample to 10^{-6} to 10^{-7} M placed the concentration in the linear dynamic range of the mass spectrometer and diminished the effects of buffer ions. Use of the LCQ ion trap in zoom scan mode allowed Tris polymers to dissociate, so that they did not create interference at the peptide masses and also allowed averaging at higher resolution to markedly improve the data.

Periodically during the deamidation reaction, samples were taken, and frozen at -80 °C. When all 18 samples had been collected, they were diluted 500-fold and alternated with each other and with samples

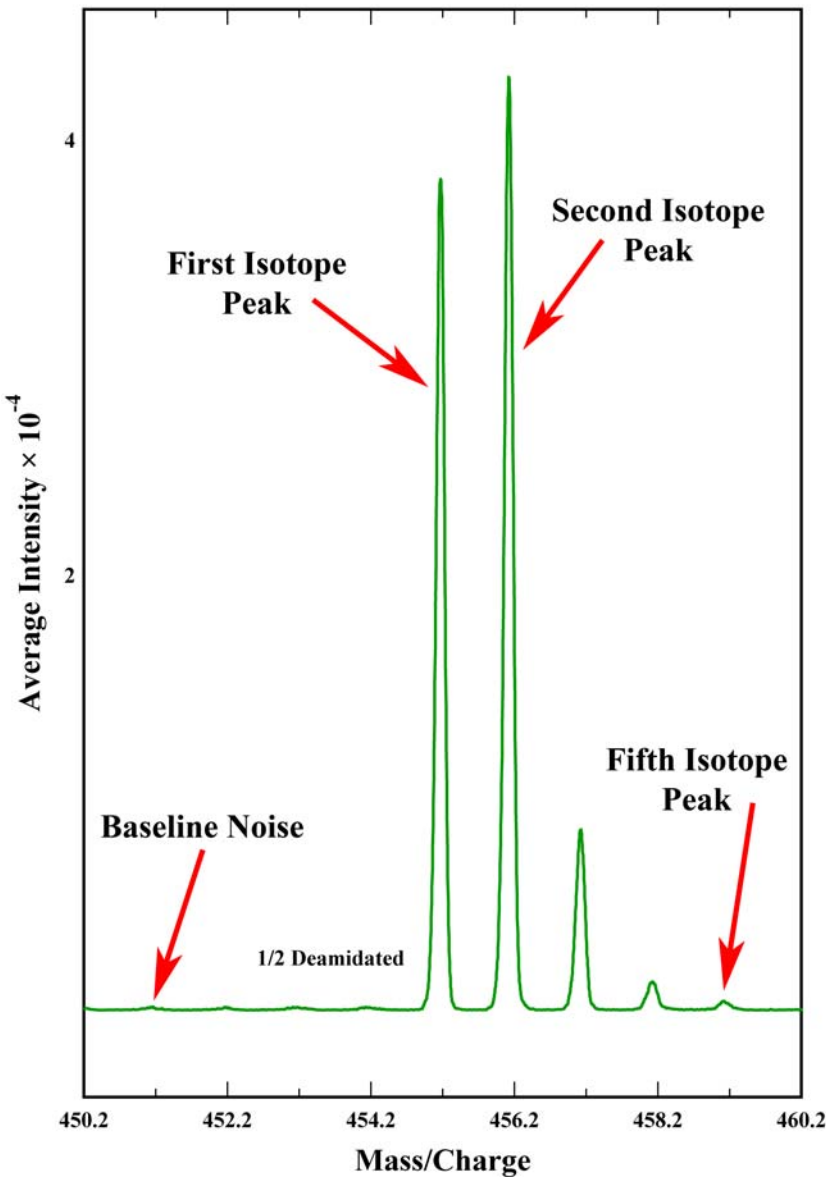

FIG. 19-3 Sample average zoom scans from a half deamidated sample of GlyAlaAsnHisGly. Adapted from 2001RR.

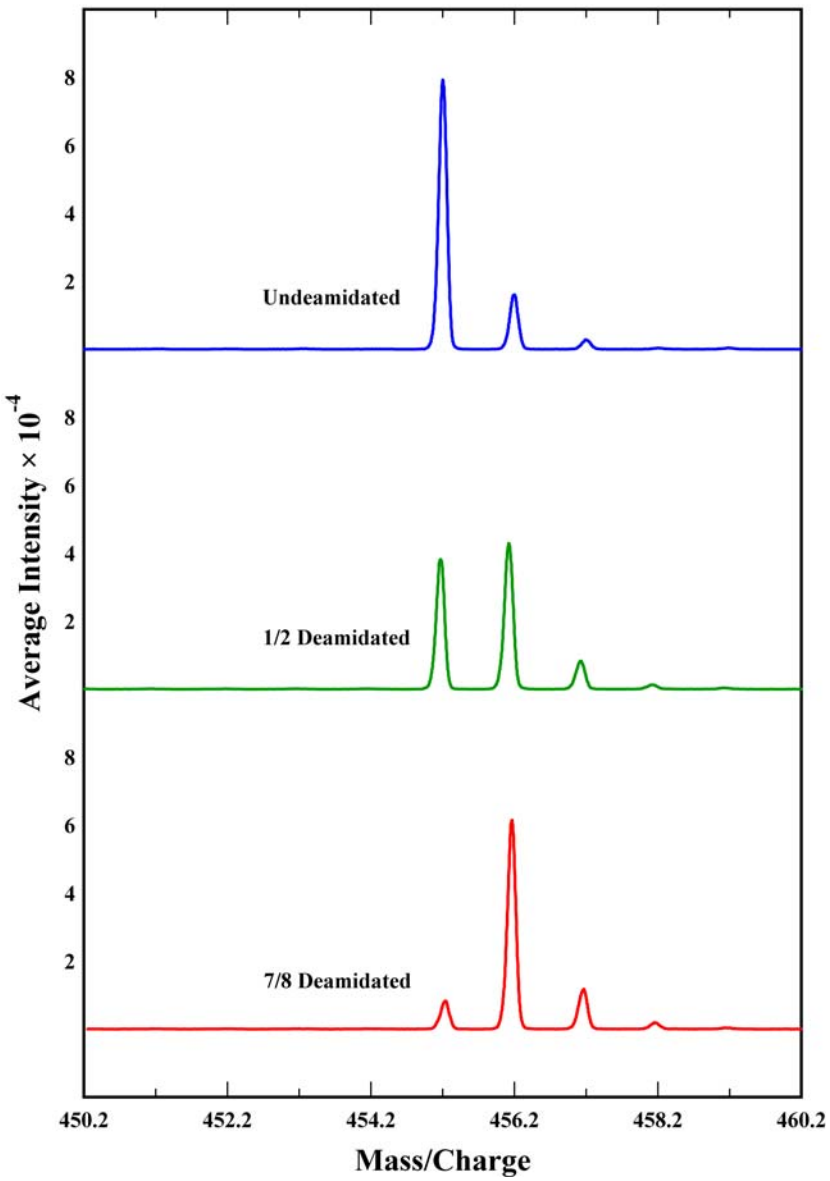

FIG. 19-4 Change in peak intensities as deamidation of GlyAlaAsnHisGly progresses. Adapted from 2001RR.

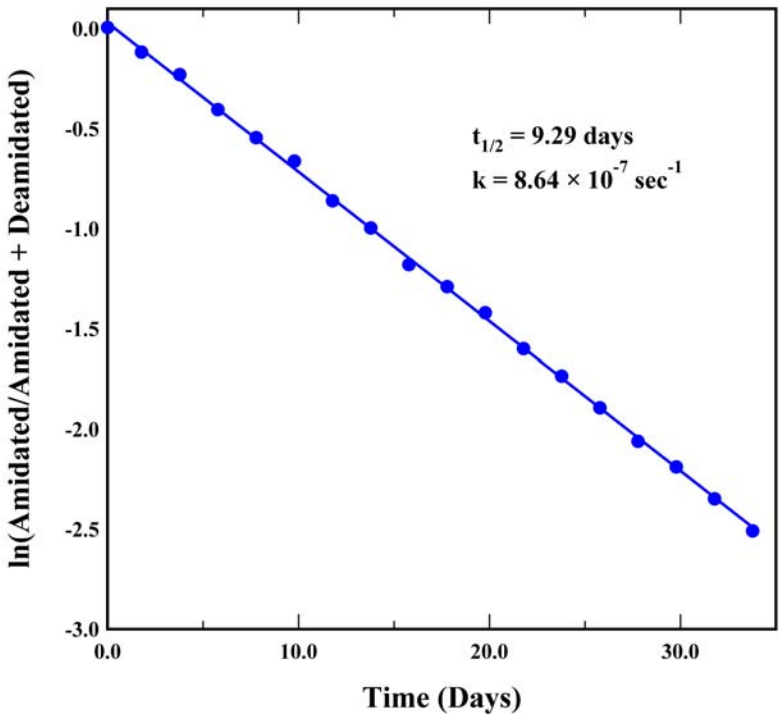

FIG. 19-5 Deamidation of GlyAlaAsnHisGly (2004R).

from another peptide rate experiment. All 36 measurements were made sequentially at a rate of 7 minutes per measurement.

Figure 19-1 shows the mass spectrometer total ion current for the 10 mass unit range being scanned during the injection of GlyAlaAsnHisGly after 26 days of deamidation. Air bubbles placed by the sample injector minimize diffusion in the tubing. This analysis shows a satisfactory rectangular curve with minimal sample tailing. Tailing is caused by peptide binding to tubing and valve surfaces and by tiny regions of dead space in the injection system. Tailing is minimized by numerous precautions that have been reviewed in detail.[9] All of the approximately 100 zoom scans between the indicated summation limits were summed to obtain the experimental result.

Figure 19-2, however, shows a similar measurement of GlyHisAlaAlaAsnAlaAlaHisGly which has tailed badly due to peptide binding to tubing walls in the injection system. The binding is peptide

9 N. E. Robinson, *PhD Thesis, California Institute of Technology, Chemistry* (2003).

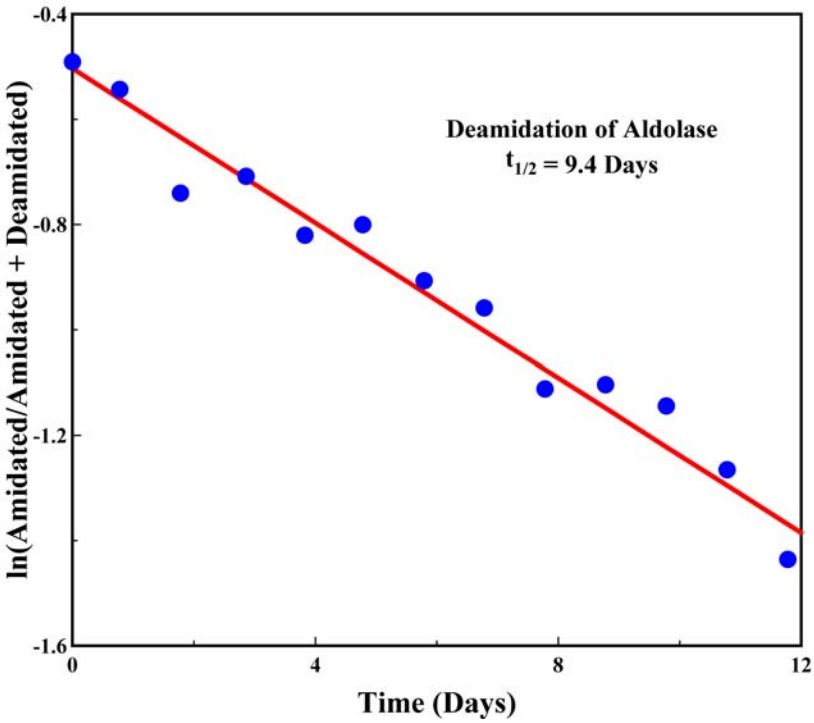

FIG. 19-6 Deamidation of SerAsn(360)His in rabbit muscle aldolase. Adapted from 2001RR2.

dependent. In this case, the deamidated peptide has bound more strongly than the undeamidated. While this is an unusually severe example, careful data analysis can extract a deamidation rate from 18 points with reasonable but diminished accuracy. Without such care, the calculated value will be seriously in error.

Figure 19-3 shows the summed zoom scans for GlyAlaAsnHisGly after the peptide has been one-half deamidated. Integration of this curve, correction for baseline noise and naturally occurring isotope ratios, and computation including the other 17 deamidation points gives the final result.

Figure 19-4 shows the gradual increase of the average peptide mass during a rate experiment of GlyAlaAsnHisGly. Figures 19-3 and 19-4 illustrate the high quality of quantitative data available with this technique. No data smoothing or other computational improvements have been applied to the illustrated results, which show the raw averaged values from the approximately 100 zoom scans.

Figure 19-5 shows the computed first-order rate plot of the 18 measurements of GlyAlaAsnHisGly, which give a deamidation half-time of 9.29 days in pH 7.4, 37 °C, 0.15 M Tris-HCl.

The 18-point curve provides a good detector of extraneous factors that might be overlooked. So long as this curve is perfectly straight with minimal scatter of values from the least-squares line, it is unlikely that errors such as peptide absorption on tubing or peptide cleavage are significantly affecting the result. Cleavage products and other reaction products can, of course, be simultaneously monitored in the ion trap mass spectrometer during the deamidation measurements.

Figure 19-6 shows a similar experiment measuring the first deamidation of rabbit muscle aldolase, which occurs in the carboxyl-terminal peptide IleSerAsnHisAlaTyr.[10] The deamidation half-time is 9.4 days, in close agreement with the sequence-determined half-time of 9.0 days for GlySerAsnHisGly. These measurements were at 0.001 M aldolase, pH 7.4, 37.0 °C, 0.015 M Tris-HCl.

Measurement of the aldolase peptide was made possible by digestion of the sample with a proteolytic enzyme immediately before analysis. This method, however, releases many additional peptides into the solution, which compromises accuracy. The greater scatter of the data in Figure 19-6 reflects this. Moreover, this peptide is the first released during enzymatic digestion. If the digestion had been allowed to go to completion, these measurements would have been much more difficult. For small proteins, complete analysis in this way is sometimes possible with current techniques.

It is this analytical method that has permitted the modern systematic and reliable measurement of peptide deamidation rates.[10] These rates form an essential element in the computation of protein amide deamidation rates from protein three-dimensional structures.[11]

10 N. E. Robinson and A. B. Robinson, *Proc. Natl. Acad. Sci. USA* **98**, 944 (2001); N. E. Robinson, A. B. Robinson, and R. B. Merrifield, *J. Peptide Research* **57**, 483 (2001); N. E. Robinson, *PhD Thesis, California Institute of Technology, Chemistry* (2003).

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CHAPTER 20

Summary

Two of the twenty naturally occurring amino acids that comprise the building blocks of proteins have the unusual property that they are fundamentally unstable in aqueous solutions at the ordinary temperatures and pHs of living things. Asparagine reacts with water to produce aspartic acid, and glutamine produces primarily pyrrolidonecarboxylic acid.

More importantly, the amino acid residues produced when these two amino acids are incorporated into peptides and proteins as the asparaginyl and glutaminyl residues Asn and Gln are also unstable in aqueous solutions under physiological conditions. Their deamidation produces a family of isomers. These isomers have carboxylic acid side chains that are negatively charged at physiological pH. Since the side chains of Asn and Gln are not charged, this reaction produces a pronounced change in the structures of the peptides and proteins in which they are incorporated.

Asparagine was discovered two centuries ago, and the presence of Asn and Gln in proteins has been known for more than 130 years. Since then, more than 1,500 research reports related to the deamidation of Asn and Gln have been published. The deamidation of Gln proceeds both enzymatically and nonenzymatically in physiological systems, while only the nonenzymatic deamidation of Asn has been reported.

Until 1967, nonenzymatic deamidation of Asn and Gln was thought by many investigators to be entirely an *in vitro* rather than an *in vivo* phenomenon and, until 1970, this deamidation was thought to have little or no physiological significance. Between 1967 and 1974 it was discovered that deamidation occurs *in vivo* and that genetic control of deamidation provides *in vivo* deamidation rates over a very wide, physiologically relevant range. These discoveries and the proposal of the hypothesis, with some confirming examples, that nonenzymatic deamidation serves as a molecular clock for the regulation of physiological processes, initiated more interest in deamidation and an increased amount of experimental research. Subsequently, much has been learned, especially about Asn, which usually deamidates more rapidly than Gln.

It is now known that the genetically specified primary sequence control of nonenzymatic deamidation of Asn and Gln provides a range of deamidation half-times under physiological conditions between about 1 day and 50 years. Combination of multiple amides in proteins and modulation of deamidation by secondary, tertiary, and quaternary structure provides a currently known range of physiological protein deamidation half-times between a few hours and more than a century, with the actual range probably even greater. Primary-structure-determined deamidation rates are exquisitely modulated by higher order protein structure and by the protein's interactions with the molecules and organelles of living things.

The reaction mechanisms of nonenzymatic deamidation have been extensively studied. It is now thought, with substantial experimental justification, that Asn deamidation proceeds primarily through a succinimide mechanism with also a small amount of direct hydrolysis. Similarly, Gln deamidation proceeds through a glutarimide, but, since this reaction is relatively slow, direct hydrolysis is more significant. Where imide formation is not possible or is suppressed by structure or reaction conditions, hydrolysis prevails.

The wide range of deamidation rates under physiological conditions results primarily from the sensitivity of nonenzymatic deamidation to steric hindrance and to apparent catalysis by functional amino acid residue side chains.

Nonenzymatic deamidation rates in aqueous solutions depend upon pH, temperature, ionic strength, the nature and concentrations of other molecules present, and other solvent properties.

To date, nonenzymatic deamidation has been reported in more than 200 types of naturally occurring peptides and proteins and will surely be found in most peptides and proteins as analytical techniques improve. Specific rapidly deamidating residues have been identified in about 30 proteins for which three-dimensional structures are also known. Deamidation rate measurements are available for 10 of these proteins.

The primary structure dependence of deamidation in peptides has been extensively investigated, and a semi-empirical and theoretical framework has been developed that allows reliable prediction of the deamidation rates of most small peptides. Secondary structure often affects the deamidation rates of larger peptides in ways that have been qualitatively demonstrated, but are not quantitatively understood.

A quantitative computation procedure has been developed for estimating the effect of protein secondary, tertiary, and quaternary structure

on Asn deamidation rates. Combination of these estimates with experimental primary structure rates for peptides permits computerized prediction of the deamidation rate of any Asn in a protein for which the three-dimensional structure is known. These computed protein deamidation rates are usually correct within a factor of 2 or better. Refinement to higher accuracy awaits improved and extended experimental measurement of protein deamidation rates.

This method has been used to compute the individual Asn and overall protein deamidation rates of about 18,000 proteins, which contain approximately 230,000 individual asparaginyl residues. These computations are available and regularly updated at the Internet site www.deamidation.org. Other deamidation information and customized calculations of deamidation of other proteins by request are also available at this site. Deamidation has been carefully studied in about 10% of the protein types in which it has been reported.

Deamidation of Asn usually produces, in addition to L-Asp and depending upon the structure of the deamidating molecule, substantial amounts of isoAsp and some D-Asp and D-isoAsp. Enzymes that convert this isoAsp and D-Asp to L-Asp have been found to be widespread *in vivo* and necessary to good health in living things.

Enzymatic deamidation of Gln has been specifically implicated in several human diseases, and nonenzymatic deamidation of Asn is thought to play a role in other pathologies. Deamidation of Asn and Gln has been found to be useful in the food processing industries. As peptides and proteins are increasingly used in the pharmaceutical industry, deamidation of Asn has become an obstacle to preparation and storage of purified pharmaceutical agents.

Awareness and measurement of deamidation should be a routine concern for most peptide and protein chemists. Regardless of its biological significance in their specific peptides and proteins of interest, these miniature molecular clocks are running in most peptides and proteins and thereby changing the structures of these molecules as a function of time and conditions.

The hypothesis that nonenzymatic deamidation serves as a ubiquitous molecular clock for the regulation of biological processes has been strengthened by ongoing research on the range and precise genetic control of deamidation and discovery of the widespread genetic specification of biologically relevant deamidation rates in peptides and proteins in living things. This hypothesis has also been supported by studies showing that nonenzymatic deamidation controls the *in vivo* turnover rates of cytochrome c and aldolase; serves as a counter of enzyme cata-

lytic cycles in triosephosphate isomerase; and regulates apoptosis by means of deamidation of Bcl-X_L.

The hypothesis that biological regulation by means of nonenzymatic deamidation is a ubiquitous phenomenon still rests upon the logic that an easily genetically suppressed process that is so disruptive to the structures of peptides and proteins would not be so generally present unless it were of widespread use. It is now strongly confirmed by the finding that a large percentage of the 18,000 proteins for which deamidation rates have been computed have been found to deamidate within biologically relevant time intervals. The molecular clock hypothesis has correctly predicted the use of nonenzymatic deamidation as a timer of biological processes.

Wherever an amide residue is present in a peptide or protein, it is definitively a miniature molecular clock that runs at a predetermined rate and changes the peptide or protein structure in specific ways. The consequences of most of these clocks, both molecularly and biologically, remain to be determined. We hope that the information reviewed in this book will prove useful to scientists engaged in extending knowledge about deamidation. A more succinct and less detailed book is also available.[1]

1 N. E. Robinson and A. B. Robinson, *Protein Deamidation*, Althouse Press (2004).

INDEX

References

Research related to the deamidation of asparaginyl and glutaminyl residues in peptides and proteins spans two centuries. The research papers listed below comprise those that we have used in the preparation of this book. We would greatly appreciate being informed of any relevant research reports that we have overlooked.

The red line in Figure R-1 shows the annual frequency of publication of research papers related to this subject, while the dotted blue line shows the number of authors per research paper of these publications.

We have chosen unique reference specification numbers for use in this book. These numbers include the year of publication and the first

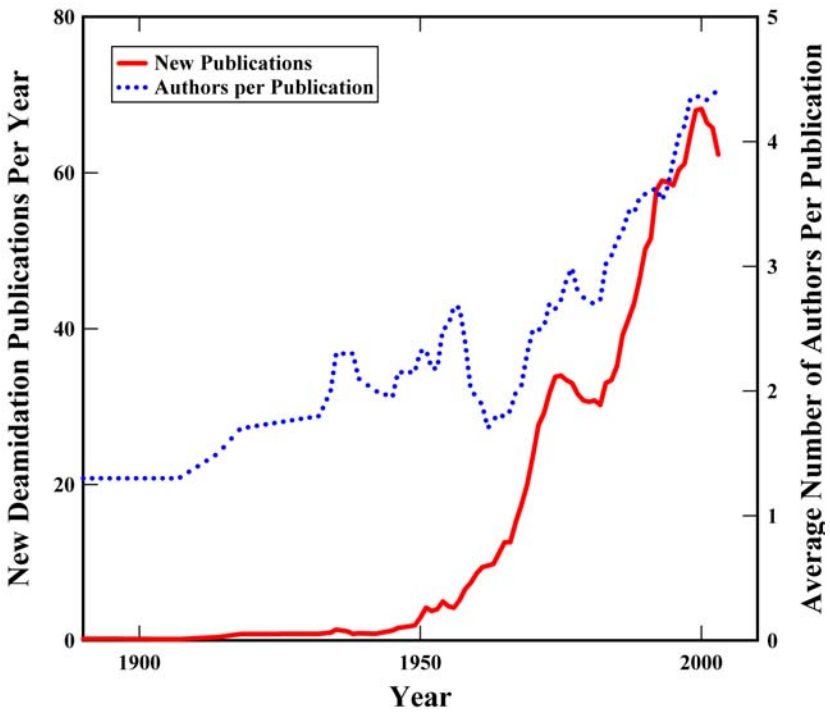

FIG. R-1 Publication frequency per year of research papers related to deamidation (red line - left axis) and average number of authors per paper (dotted blue line - right axis). Both curves have been smoothed with a five-year moving average.

letters of the first two authors' last names followed by a number when necessary to accomodate redundancy. It is intended that this format will facilitate the accurate location of references in the reference list and also mental cross-referencing during reading of the text.

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