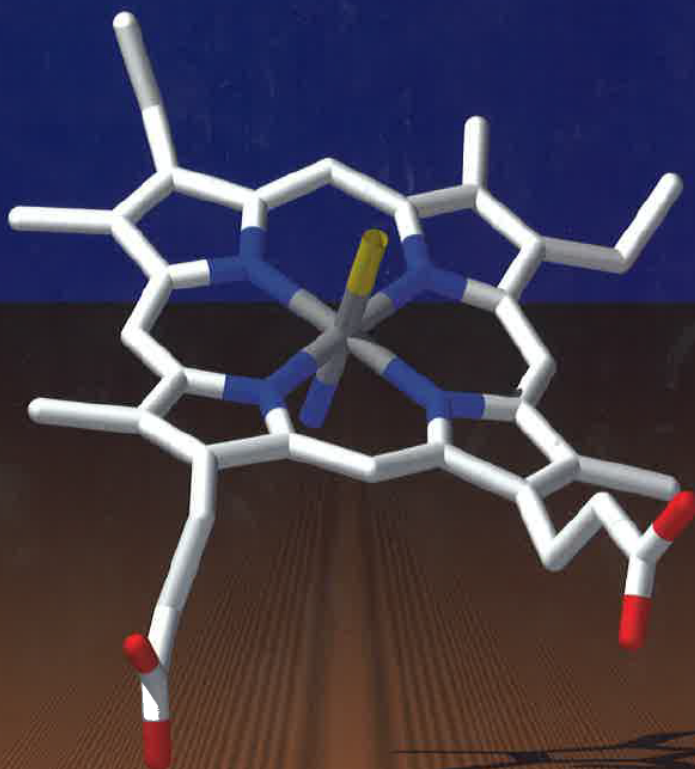


Molecular Clocks

**Deamidation of Asparaginyl
and Glutaminyl
Residues in Peptides and Proteins**



**Noah E. Robinson
Arthur B. Robinson**

MOLECULAR CLOCKS

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&

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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.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).

Table of Contents

Molecular Clocks	v
Preface	ix
Table of Contents	xi
Figures	xvii
Tables	xxi
Introduction	xxiii

CHAPTER 1

Asparagine and Glutamine

1-1. Discovery of Asparagine and Glutamine.	1
1-2. Deamidation of Asparagine and Glutamine	3

CHAPTER 2

Asn and Gln in Peptides and Proteins

2-1. Asparaginylyl Residues	7
2-2. Glutaminylyl Residues	9
2-3. C-Terminal Amides	11
2-4. Amino Acid Residue Ionization States	11

CHAPTER 3

Enzymatic Deamidation

3-1. Enzymatic Deamidation of Gln	15
3-2. Enzymatic Deamidation of Asn.	16

CHAPTER 4

Nonenzymatic Deamidation

4-1. Nonenzymatic Deamidation of Asn and Gln.	19
4-2. Deamidation Coefficient and Deamidation Index.	25

CHAPTER 5

Mechanisms of Reactions Involving Asn and Gln

5-1. Introduction	27
5-2. Current Mechanistic Model	28
5-3. Mechanistic Experiments	43

CHAPTER 6

Primary Structure Dependence of Nonenzymatic Deamidation of Asn and Gln

6-1. Peptide Experiments	51
6-2. Prediction of Primary Sequence Rates	84
6-3. Prediction of Effects of Sequence Changes on Rates	90
6-4. Primary Clocks	92
6-5. Amide Probes of Protein Structure	94
6-6. Aerobic Ascorbic Acid	95

CHAPTER 7

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

7-1. Introduction	97
7-2. Secondary Structure	99
7-3. Tertiary and Quaternary Structure	100

CHAPTER 8

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

8-1. Buffer Dependence of Deamidation	103
8-2. pH Dependence of Deamidation	110
8-3. Temperature Dependence of Deamidation	113
8-4. Ionic Strength Dependence of Deamidation	115

CHAPTER 9

Nonenzymatic Deamidation Rates of Proteins

9-1. Deamidation Prediction Calculations	117
9-2. Protein Deamidation Values	134
9-3. Protein Deamidation Distribution Functions	141

CHAPTER 10

Structural Distributions of Asn and Gln in Proteins

10-1. Primary Structure Correlations	147
10-2. Secondary Structure Correlations	152
10-3. Protein Amide Compositions.	154

CHAPTER 11

Deamidation of Peptides and Proteins in Biological Systems

11-1. Ubiquitous Biological Occurrence	157
11-2. Experimental Observations of Deamidation	158

CHAPTER 12

Particular Peptides and Proteins

12-1. Introduction	181
12-2. Adrenocorticotropin	181
12-3. Aldolase	182
12-4. Amylase	184
12-5. Apolipoprotein	187
12-6. Bcl-x _L	188
12-7. Collagen	189
12-8. Crystallin and Major Intrinsic Protein	190
12-9. Cytochrome c	201
12-10. Epidermal Growth Factor	205
12-11. Food Proteins	207
12-12. Growth Hormone	211
12-13. Growth Hormone Releasing Factor	213

12-14. Hemoglobin	214
12-15. Histone.	216
12-16. Immunoglobulin	218
12-17. Insulin	220
12-18. Interleukin	222
12-19. Lysozyme.	223
12-20. Myelin Basic Protein	225
12-21. Phenylalanine Hydroxylase.	226
12-22. Ribonuclease	227
12-23. Triosephosphate Isomerase	230
12-24. Trypsin.	234
12-25. Other Proteins	237

CHAPTER 13

Biological Molecular Clocks

13-1. Protein Dynamics	239
13-2. Asn and Gln as Individual Clocks	239
13-3. Implementation of Amide Clocks	242

CHAPTER 14

Deamidation and Aging

14-1. Introduction	245
14-2. Aging Hypotheses	251
14-3. Deamidation and Aging.	255
14-4. Correlations With Tissue Compositions	259
14-5. Diet Restriction	261

CHAPTER 15

Deamidation and Disease

15-1. Introduction	267
15-2. Celiac Disease	267
15-3. Bacterial Toxins.	268
15-4. Cancer	269

15-5. Alzheimer's Disease	270.
15-6. Other Amyloid Diseases	271
15-7. Additional Pathologies	273
15-8. Bacterial Chemotaxis and Thermotaxis.	274
15-9. Pharmaceutical Deamidation.	275

CHAPTER 16

Enzymatic Carboxyl Methylation

16-1. Methyl Transferase	277
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CHAPTER 17

Carboxyl-Terminus Amides

17-1. Enzymatic Deamidation.	283
17-2. Nonenzymatic Deamidation	285

CHAPTER 18

Isomerization, Cleavage, and Racemization

18-1. Isomerization and Cleavage	287
18-2. Racemization	290

CHAPTER 19

Analytical Techniques

19-1. General Techniques	295
19-2. Peptide Measurement.	298

CHAPTER 20

Summary	307
Index	313
References	319

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 1826² and crystallized

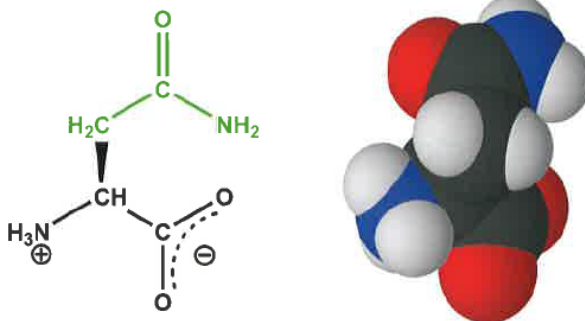


FIG. 1-1 Asparagine

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 1827⁴ 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

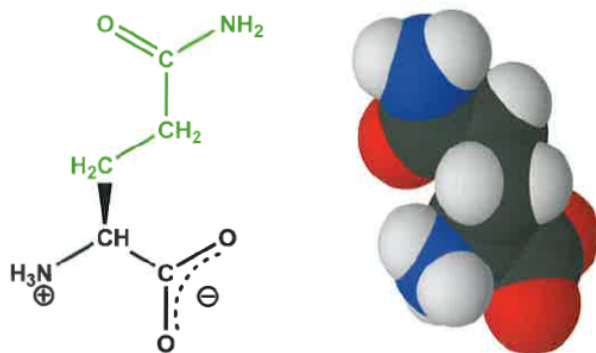


FIG. 1-2 Glutamine

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. Ber.* **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 glutamyl residues at internal or C-terminal positions in peptides.¹⁷ Glutamine deamidation produces ammonia and pyrrolidonecarboxylic acid,¹⁸ is accelerated by phosphate¹⁹ 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%

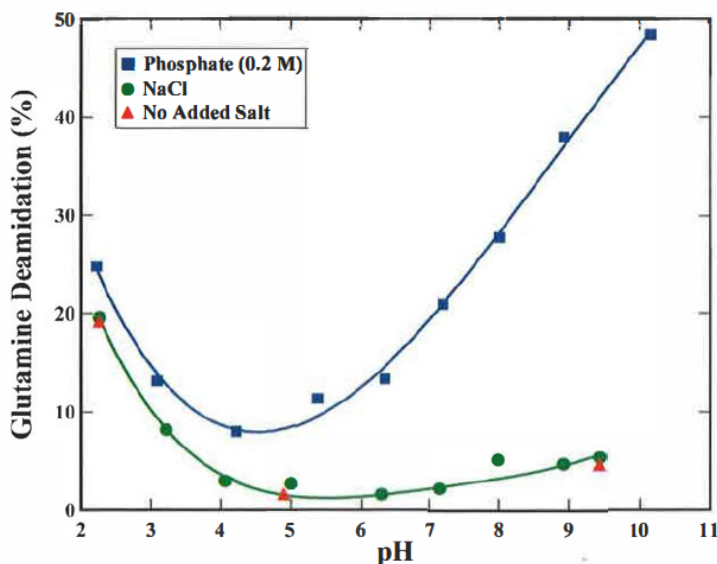


FIG. 1-3 Dependence of glutamine deamidation upon pH and phosphate. Percent deamidation in 12 hours at 37°C of 0.05 M L-glutamine in veronal buffer with 0.2 M phosphate, ■; NaCl at same ionic strength as that of phosphate, ●; and no added salt, ▲. Adapted from Gilbert, Price, and Greenstein.²⁰

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 1939²² that acid hydrolysates of proteins contain substantial quantities of D-glutamic acid. These hydrolysates can contain 5 to 10% D-glutamic acid²³ 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 Merrifield²⁷ 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 glutamyl 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 values³⁴ 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).

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% trichloroacetic 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 carbobenzoxy-succinimide methyl ester from carbobenzoxyAsn methyl ester and also the analogous glutarimide. Succinimides and glutarimides are illustrated in Chapter 5. They hydrolyzed these in H₂O 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 H₂O 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 Reynolds¹⁰ 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 Cohen¹³ 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 Fukawa¹⁷ 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 H₂O without phosphate were substantially longer at pHs 4.5, 6.8, and 9.2 – the ratio of H₂O 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 H₂O 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 residues¹⁹ 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 AlaSerAsnGlu²⁰ 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 deaminate 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 C¹⁴ 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 C¹⁴ 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 C¹⁴ 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 set²⁴ 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 $C_D \equiv (\text{deamidation half-time})/100$ in days for a single amide. $I_D \equiv [\sum(C_{Dn})^{-1}]^{-1}$, where C_{Dn} is the n th 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).

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 peptides² 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).

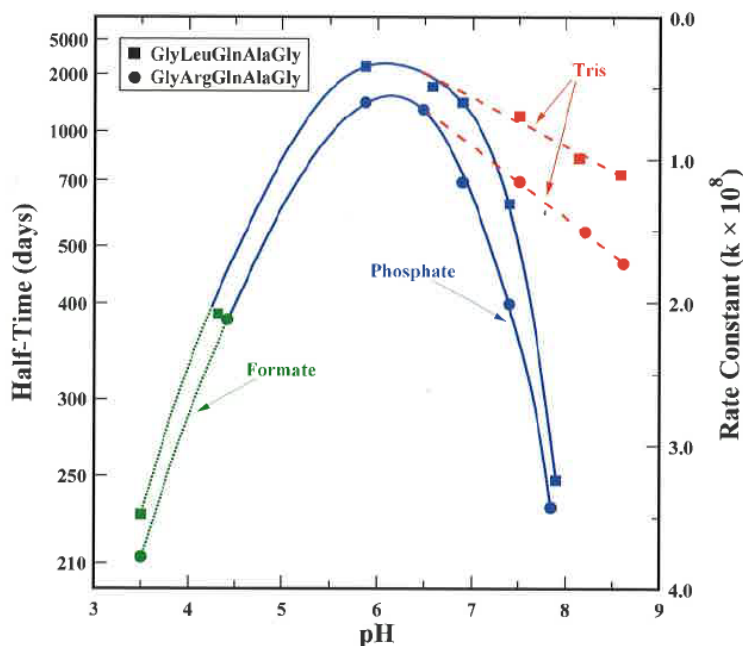


FIG. 8-1 The half-lives and first-order rate constants of deamidation vs. pH for GlyLeuGlnAlaGly, ■, and GlyArgGlnAlaGly, ●. Formic acid, sodium formate buffer was used at pH 3.6 and 4.4, ■, ●. KH_2PO_4 , Na_2HPO_4 buffer was used at pH 5.8, 6.6, 7.0, 7.4, and 7.8, ■, ●. Tris, Tris-HCl buffer was used 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 measurements⁴ 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 investigated⁵ 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 measurements, 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

$$k_{\text{observed}} = k_{H_2O} + k_{OH^-}[OH^-] + \sum(k_{Bi}[B_i])$$

(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.

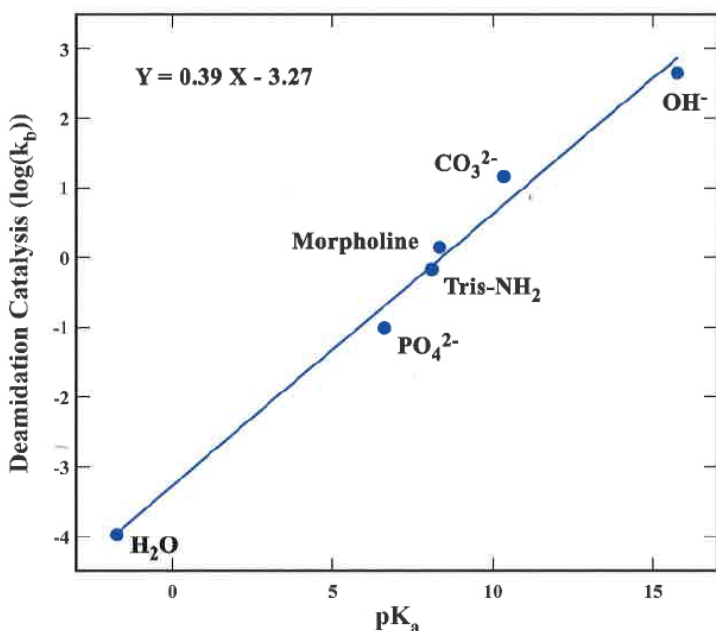


Figure 8-2 General base catalysis Brønsted plot for AcGlyAsnGlyGluNHMe deamidation. Base catalysis $\log(k_b)$ is plotted vs. pK_a , where k_b is the rate constant for catalysis and K_a is the acid equilibrium constant. Plotted from data in 1991CM.

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 BocAsnGlyGlyNH₂ 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).

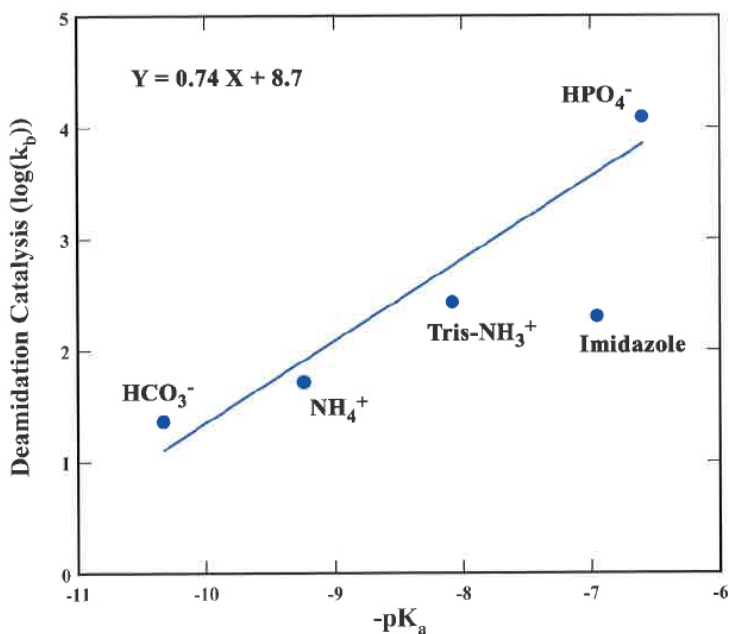


Figure 8-3 General acid Brønsted plot for BocAsnGlyGlyNH₂. Plotted from data in 1991CM.

The 35 Asn peptide deamidation rates measured in the 1970s⁹ have been combined with 32 peptide rates measured subsequently¹⁰ and the subsequent measurements have been corrected to the earlier conditions of pH 7.4, 37 °C, 0.0146 M H₂PO₄⁻, and 0.062 M HPO₄⁼.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).

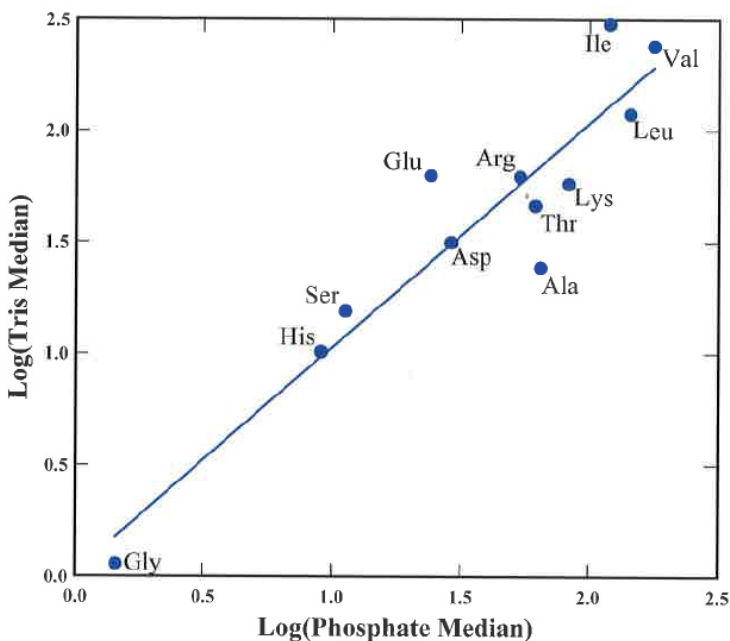


Figure 8-4 Comparison between Tris and phosphate deamidation half-times in 37 °C, pH 7.4, 0.125M Tris-HCl and 0.026 M Tris Base vs. 0.0146 M H_2PO_4^- and 0.0162 M HPO_4^- .

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 base¹² 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 $k_{\text{phosphate}}/k_{\text{water}} = 13.4$. 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 reported¹³ 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)NH₂. 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 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).

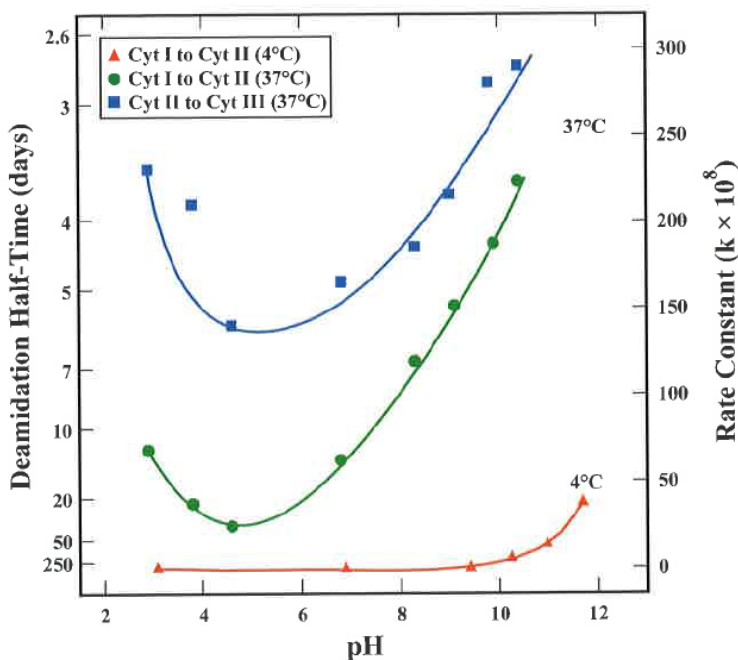


FIG. 8-5 Effect of pH and temperature on the first-order rate constant for the conversion of Cy I into Cy II, $\blacktriangle$, $\bullet$; and of Cy II into Cy III, $\blacksquare$. Formate and borate buffers of ionic strength 0.1 were used. Adapted from 1966F.

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.

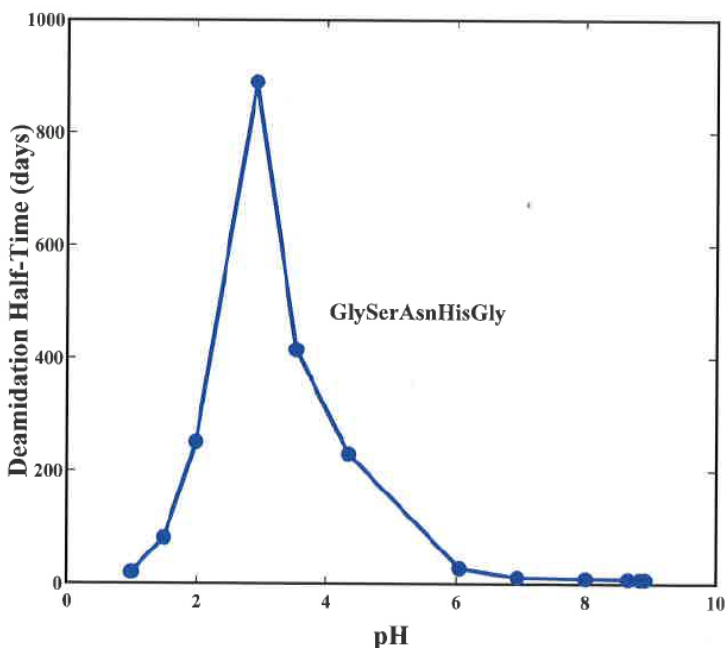


FIG. 8-6 Deamidation half-times of a 1.0×10^{-3} M solution of GlySerAsnHisGly as a function of pH in 37°C , 0.15 M Tris base adjusted to pH with 6 N HCl. The pH values shown are measured averages of the 1st, 9th and 17th point in deamidation solutions after the deamidation reactions had taken place.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

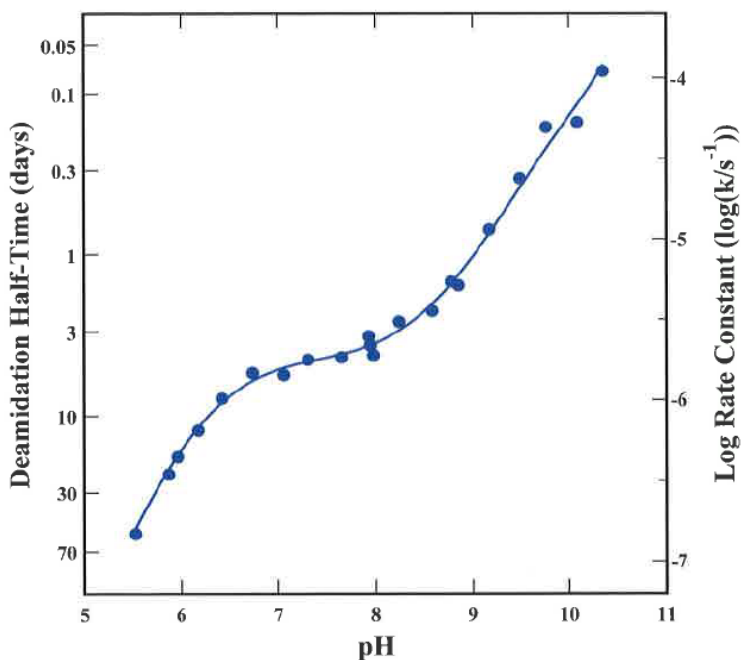


FIG. 8-7 pH dependence of AcGlyAsnGlyGlyNHMe. Adapted from 1999CS.

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 reported²⁶ 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 reported²² 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 study²⁸ of deamidation of BocAsnGlyGlyNH₂ found a substantial accelerating effect by K₂HPO₄/KH₂PO₄ and (NH₄)₂SO₄, a moderate effect from MgSO₄, and essentially no effect by NaCl and Na₂SO₄ 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).

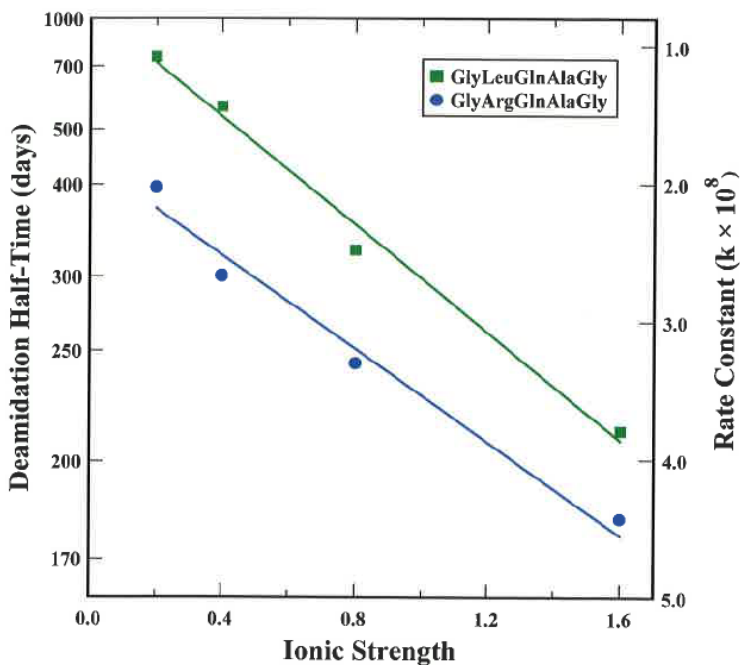


FIG. 8-8 Half-times and first-order rate constants of deamidation vs. ionic strength for GlyLeuGlnAlaGly, ■; and GlyArgGlnAlaGly, ●. The solutions were KH_2PO_4 , Na_2HPO_4 buffer at pH 7.4, ionic strength 0.2, 37.0°C, and were 0.001 M in peptide. Higher ionic strengths were obtained by addition of NaCl according to the equation $I = 1/2 \sum m z^2$, 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.

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 glutamyl residues. It is estimated that more than 0.5% of the human population suffers from this disease.

In 1985, Bruce, Bjarnason, and Peters reported¹ 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 found⁸ 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. Travaglion, L. Falzano, A. Fabbri, A. Stringaro, 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. Lern, 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 plaques¹² 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 communication (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, 1992SI, 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 Caughey¹⁹ 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).

ported²⁰ 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 out²² 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 complementarity²⁴ 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 Dahlquist³² 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(CH₃ site)Zzz(Ala/Ala(OH₂)).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. Galletti, *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 CaCl₂, 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 shown⁴³ 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. Knauft, 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, 2003SSI, and 2004XA.

⁴⁴ N. E. Robinson and A. B. Robinson, *J. Peptide Research* **63**, 437 (2004).

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
α A-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
α B-crystallin	GluSerAsp(36)LeuPhe TrpPheAsp(62)Thr Gly	1994FI
β A-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).

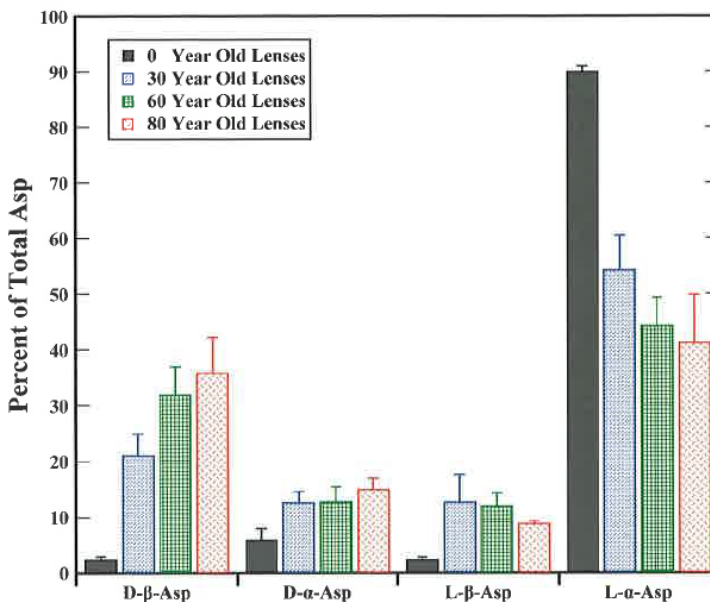


FIG. 18-1 The relative amounts of four optical isomers of Asp-151 residues, L-α-Asp, L-β-Asp, D-α-Asp, and D-β-Asp in αA-crystallins from donors of lenses in the 0, 30, 60, and 80 year ranges. Adapted from 1999FT.

in basic fibroblast growth factor⁸ 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. Shahrokhi, G. Eberlein, D. Buckley, M. V. Parandhi, 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-

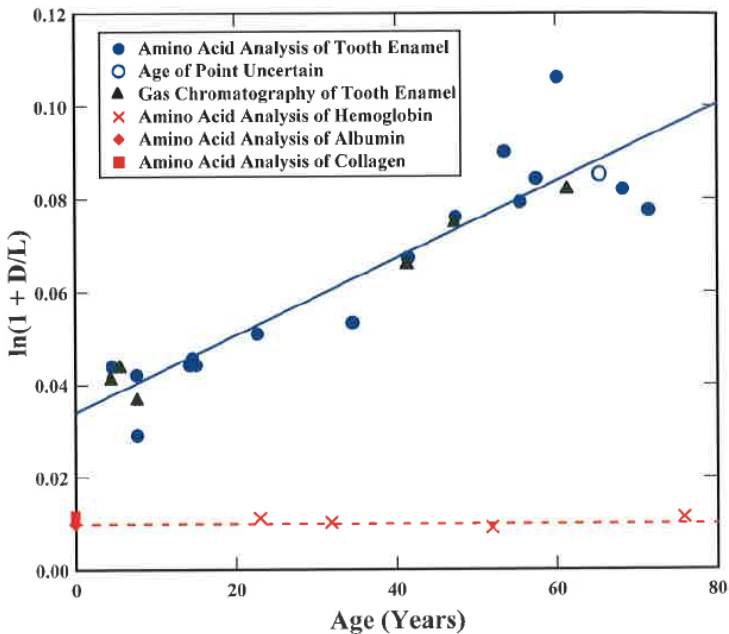


FIG. 18-2 Plot of $\ln(1 + D/L)$ for aspartic acid against age of tooth enamel and against age of the individual for the protein values. The slope ($8.29 \times 10^{-4} \text{ yr}^{-1}$) of the solid line is equal to k_{asp} for tooth enamel at about 37°C. Adapted from 1975HB.

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 1977¹³ 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 Duin¹⁵ 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 collagen 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 Collins¹⁷ 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 α A-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 α A-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 Ijssel, 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).