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Nonenzymatic Deamidation of Asparaginyl and Glutaminyl Residues in Proteins

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ABSTRACT: Some asparagine and glutamine residues in proteins undergo deamidation to aspartate and glutamate with rates that depend upon the sequence and higher-order structure of the protein. Functional groups within the protein can catalyze this reaction, acting as general acids, bases, or stabilizers of the transition state. Information from specific proteins that deamidate and analysis of protein sequence and structure data bases suggest that asparagine and glutamine lability has been a selective pressure in the evolution of protein sequence and folding. Asparagine and glutamine deamidation can affect protein structure and function in natural and engineered mutant sequences, and may play a role in the regulation of protein folding, protein breakdown, and aging.

KEY WORDS: proteins, nonenzymatic deamidation, asparagine, glutamine, mechanisms, posttranslational.

I. INTRODUCTION

Of the many posttranslational modifications of proteins observed to occur under physiological conditions, the nonenzymatic deamidation of asparagine (Asn) and glutamine (Gln) residues to aspartate (Asp) and glutamate (Glu) is unique in several ways. In contrast to other such modifications, which are either the result of enzymatic action or environmental insult, deamidation is a hydrolytic reaction requiring only water to form products. The products of this hydrolytic reaction are both natural amino acids (Asp and Glu), which is one of the reasons why the occurrence of deamidation in proteins is often difficult to detect. The variability of deamidation rates in both peptides and proteins, and the diversity of proteins in which it has so far been documented to occur, suggest that both sequence and structure of each specific protein play an important role in regulating the deamidation rate of specific Asn or Gln residues.

Robinson and Rudd[1] reviewed peptide and protein deamidation, and discussed possible roles for this reaction *in vivo*. Since that time, a number of specific cases of deamidation in proteins have been documented. Their number and diversity suggest that deamidation of Asn and Gln may be more widespread than previously thought. New insights into the mechanisms of Asn and Gln deamidation have also been gained, and they begin to provide some categories for the classification and prediction of deamidation sites in proteins and peptides. The ubiquity of this reaction and the existence of an enzyme that recognizes and modifies one of the products of nonenzymatic deamidation in proteins (see below) support earlier proposals that this posttranslational modification plays a role in physiological processes.

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In this review, I examine proteins in which deamidation has been shown to occur. Instances where sequence information or a three-dimensional structure exists have been tabulated and the structure examined using molecular graphics. These new data, taken together with earlier studies of Robinson and co-workers[1] on the deamidation of peptides, provide a basis for understanding the mechanisms of nonenzymatic deamidation in proteins. The relationship between these mechanisms of deamidation and the sequences and structures of proteins and peptides is discussed. The influence of deamidation on protein folding and its implications for natural and engineered mutant proteins, and possible physiological roles for nonenzymatic deamidation of peptides and proteins, are considered.

II. PROTEINS THAT DEAMIDATE

The extent to which deamidation actually occurs in proteins is hard to assess. This is in part a methodological problem in that the strongest evidence for deamidation is the determination of a difference in amino acid sequence between the deamidated form and another form, presumed to be the primary translation product. The sequence of the latter can be obtained from either a protein or a DNA sequence, but the putative deamidated form must be sequenced as a protein and the newly formed Asp or Glu detected against the large background of Asp or Glu residues occurring naturally in the protein.

The conditions used in the purification of proteins and in their sequence determinations often promote deamidation and can introduce artifacts.[2] The rate of Asn or Gln deamidation in a peptide or protein is influenced by the conditions of pH, temperature, and ionic strength,[3] extremes of which are often employed in purification of proteins. This rate also depends upon both the sequence immediately around the Asn or Gln residue[2-11] and on higher order structure of the peptide or protein.[11,12] As will be discussed in detail below, the influences of secondary, tertiary, and quaternary structure on the rates of Asn and Gln deamidation are complex and can act to both accelerate and decelerate the deamidation reaction.

Table 1 lists proteins in which deamidation has been demonstrated or inferred to occur. This table is divided into three sections, demarcated by horizontal lines. The uppermost section consists of those proteins of known three-dimensional structure that have been shown to undergo deamidation. The middle section is proteins for which strong evidence of deamidation has been obtained and for which the amino acid sequence around the deamidating residue has been determined or inferred. The bottom section shows proteins for which some data supporting the occurrence of deamidation exists, but for which direct demonstration by sequencing or other means is lacking. Where available, the sequence immediately surrounding the deamidating residue is listed. If evidence for or against isoAsp formation is available, this is noted, and for those cases in which the deamidation half life of the protein and/or the peptide with the same sequence have been measured, these half-lives are listed.

An unknown number of entries in Table 1 will be cases of deamidation inferred from a protein sequence without knowledge of whether the deamidation occurred in the native protein or in a peptide prepared during the sequencing. These are particularly difficult instances to characterize, since they require separation of protein species differing in charge (if such species exist) and determination of the amino acid sequence of each for comparison.

III. MECHANISMS OF PROTEIN AND PEPTIDE DEAMIDATION

Figure 1 shows the frequency with which each amino acid occurs immediately before and after the deamidating Asn residues listed in Table 1.[179] Only those instances where the deamidating residue has been documented are included in this tabulation. It should be borne in mind in all generalizations made from a selected subset of a total population, such as this, that this subset may represent those proteins that can be isolated and sequenced, and that can survive deamidation without entering degradative pathways *in vivo*. It is clear from Figure 1 that the frequency with which specific amino acids occur immediately adjacent to labile Asn residues is nonrandom.

TABLE 1
Proteins that Deamidate Nonenzymatically

Protein	Sequence	Deamidation half-times		IsoAsp	Ref.
		Peptide	Protein		
Three-dimensional structure available					
Aspartate aminotransferase	AsnGly* + others*	—	22 d		13-21
Calmodulin	GlyAsnGly	—	—	+	22, 23
Carbonic anhydrase (B, C)	HisAsnGly* + others*	—	—	+	24-27
	AsnAsnGly	—	—	+	
	PheAsnGly	—	—	+	
Cytochrome C (horse heart)	ThrAsnGlu	16 d	81 d		28-35
	LysAsnLys	94 d	—		
	ProAsnLeu*	277 d	—		
Dihydrofolate reductase (recombinant)	SerAsnArg	—	—	–	36
Glucagon	MetAsnThr	—	—	+	37
Hemoglobin Providence (human)	LeuAsnGly	—	—	–	38-42
Hemoglobin Singapore (human)	ProAsnGly	—	—		43
Hemoglobin Wayne (human)	SerAsnThr	—	—		44
Insulin (human, bovine)	ValAsnGln	—	—	+	45-51
	CysAsnCOO	—	—	–	
Lysozyme	ArgAsnThr*	28 d	—		52-60
	ThrAsnGly*	—	—		
	CysAsnAsp*	28 d	—		
	AsnAsnGly*	—	—		
	GlyAsnGly*	—	—		
Ribonuclease A (bovine)	LysAsnGlyGlnThr-AsnCysThrGlnSer	—	—	+	61-69
	ProAsnCys	—	—		
Ribonuclease (bovine seminal)	LysAsnGly	—	—		70-74
Triosephosphate isomerase (human)	MetAsnGly	—	—		75-86
	ThrAsnGly	—	—		
Trypsin (bovine)	IleAsnSer	18 d	—		87
	TyrAsnSer	—	—		
	LeuAsnSer	—	—		
Trypsin inhibitor (bovine)	ValAsnGly* + others	—	—		88
(human)	LeuAsnGly*	—	—		89
Tryptophan synthase	MetGlnArg	—	—		90
Sequence available					
Adrenocorticotropin (porcine, ovine, human)	ProAsnGlyAla	—	—	+	93-97
Alcohol dehydrogenase (*Drosophila*)	AsnGlnAsnGly*	—	—		98-102
Aldolase (rabbit muscle)	SerAsnHis	6 d	8 d	+	103-111

TABLE 1 (continued)
Proteins that Deamidate Nonenzymatically

Protein	Sequence	Deamidation half-times: Peptide	Deamidation half-times: Protein	IsoAsp	Ref.
Amyloid serum protein (human)	SerAsnMet	—	—		112
	SerAsnAla	52 d	—		
	GluAsnSer	—	—		
Calbindin (recombinant)	LysAsnGly	—	—	+	113
Chloroperoxidase (*Caldariomyces fumago*)	AsnAsnThr	—	—	–	114
	ProAsnAsn	—	—	–	
	ValGlnSer	—	—	–	
Cholera toxin B chain	LysAsnGly	—	—	+	115-120
	LysAsnThr	—	—		
	PheGlnVal	—	—		
Crystallin-αA (human)	HisAsnGlu	—	—		121-127
Crystallin-αA (chicken)	SerAsnMet	—	—		128, 129
Crystallin-αB_2 (bovine)	LeuAsnVal	—	—		130
	ValAsnGlyPro	—	—		
Crystallin-βB_p (bovine)	SerAsnHis	6 d	—		131
Crystallin-ϵ (duck)	-AsnLeu	—	—		132
	CysAsnLeu	—	—		
Epidermal growth factor	NH_2AsnSer	—	—	+	133, 134
F_2 coat protein	AlaAsnGly	—	—	+	135
Growth hormone (human)	ThrAsnSerHisAsnAsp	—	—	+	136-140
	GlyGlnIle	—	—		
Growth hormone (bovine)	AlaAsnAla	95 d	—		141
	ThrAsnMet	—	—		
	LysGlnThr	—	—		
Histone H4 (human)	AspAsnIle	75 d	—	–	142
Hypoxanthine-guanine phosphoribosyltransferase	CysAsnAsp	28 d	—	–	143
Immunoglobulin κ chain (mouse)	-AsnIle	—	—		144
	LeuAsnSer	—	—		
	SerAsnThr	—	—		
	ThrGlnTrp	—	—		
	TrpAsnSer	—	—		
Interleukin-1α (human)	AlaAsnAsp	—	—	–	145, 146
Myelin basic protein (bovine)	SerGlnGly	—	—		147
	AlaGlnGly	—	—		
Neocarzinostatin	GlyAsnGly	—	—		148
Ovalbumin	ThrAsnGly*	—	—	+	149
Parathyroid hormone (human)	ValAsnVal	—	—	–	150-152
Prolactin (ovine, bovine)	ProAsnGly*	—	—		153-160
	TyrAsnAsnAsnCys*	—	—		
Ribonuclease U_2 (*Ustilago sphaerogena*)	AlaAsnGly	—	—	+	161
Serine hydroxymethyltransferase	ValAsnGly	3.3 d	19 d	+	162, 163
Somatotropin (human)	ThrAsnSerHisAsnHis*	—	—		164-166
Substance P (human)	ProGlnGlnPhe*	—	—		167
	GlyLeuMetamide*	—	—		

TABLE 1 (continued)
Proteins that Deamidate Nonenzymatically

Protein	Sequence	Deamidation half-times		IsoAsp	Ref.
		Peptide	Protein		
No sequence or structure available					
Acetylcholinesterase (cobra venom)	—	—	—		168, 169
Amylase (human salivary)	—	—	—		170-175
Enterotoxin B (*staphylococcus*)	—	—	—		176
Phosphoryl carrier protein protein	—	—	—		177
Proteinase (alkaline)	—	—	—		178

Note: The upper section of the table lists those proteins for which a three-dimensional structure is known. The middle section lists those for which the deamidating residue is known or can be inferred with reasonable certainty. The bottom section lists those proteins for which there is evidence of deamidation but no data or inference can be drawn regarding the residue(s). The sequence column lists the sequence, where known, in which the deamidating residue lies. Asterisks denote attributed sequences at which deamidation is likely, but not proven, to occur. The + and − signs indicate cases where the presence or absence of an isoAsp (+) or a normal L-Asp (−) residue can be inferred with some confidence (e.g., blockage of Edman amino-terminal analysis by isoAsp or incorporation of methyl groups in the presence of methyltransferase II enzyme, or the absence of these effects). Deamidation half-times for the protein and corresponding peptide of the same sequence are listed where available.

Ser, Thr, and Lys occur disproportionately, frequently immediately before a deamidating Asn, while Ser, Gly, Thr, and His occur with high frequencies after a labile Asn, respectively.

In earlier studies on model tetra and pentapeptides of different sequence by Robinson and co-workers,[1] a broad range of deamidation rates (6 to 3278 d) was observed. Several generalizations were made from the sequence dependence of these rate data:

- Polar residues preceding Asn and Gln increase deamidation rates.
- Neighboring Ser and Thr increase deamidation rates.
- Bulky, hydrophobic residues preceding Asn and Gln correlate with low deamidation rates.

More recent measurements of the deamidation rates of peptides in which Asn is followed by a Gly residue have extended the lower range of half-lives for Asn deamidation to 1.4 d.[180] These observations are generally consistent with the data in Table 1 for the deamidation of proteins and can be understood on the basis of the mechanisms elucidated for amide hydrolysis,[181] which are discussed below. However, there are a number of exceptions to these generalizations, which clearly implicate higher order structure in modulating deamidation rates. The role of secondary and tertiary structure in protein deamidation will be discussed below.

A. General Acid-Base Catalysis

Deamidation is a hydrolytic reaction, formally similar to the peptide-bond cleavage reaction, which is catalyzed by proteases.[181] It is catalyzed by acids and bases (nucleophiles), and requires a water molecule. Figure 2 shows a composite mechanism for acid- and base-catalyzed deamidation reactions. The general acid, HA, catalyzes the reaction by protonating the amido –NH– leaving group of the Asn side chain. A

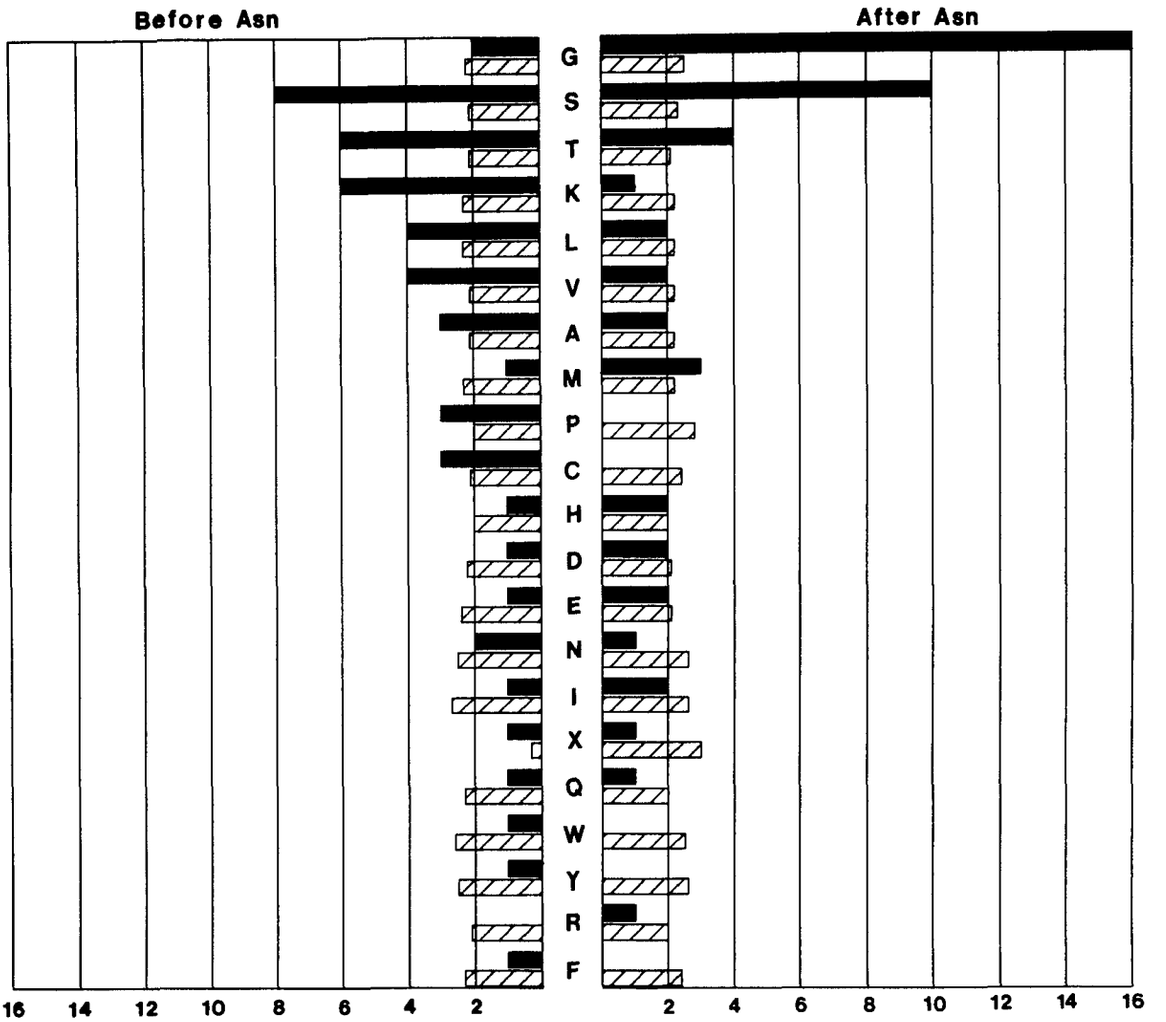

FIGURE 1. Frequency with which each amino acid occurs before (left solid bars) and after (right solid bars) labile amide residues of proteins in Table 1 vs. the expected frequency (striped bars). (Adapted from Wright, H. T., *Protein Engineer,* 4, 283. With permission.)

general base (the conjugate base, A^- or hydroxide ion) can attack the carbonyl carbon of the amido group or activate another nucleophile by abstraction of a proton for attack on the amide carbon. The transition state is inferred to be an oxyanion tetrahedral intermediate, whose stabilization by proton donors will increase the rate of the reaction. The order of acid- and base-catalyzed steps in Figure 2 varies with reaction conditions, particularly pH.

The pH of maximum stability of Asn and Gln in peptides[3] and for cytochrome c[11] (i.e., minimum spontaneous deamidation rate) is around pH 6.0. This pH dependence is consistent with the opposing pH dependences of specific base- and acid-catalyzed reactions, resulting in a crossover point for minimum deamidation rate at a pH that is suboptimum for both mechanisms.

Using the earlier data for peptide deamidation rates and a few instances of proteins with known deamidating sequences, Wright and Robinson[12] showed how specific amino acid side chains are likely to function in catalyzing the deamidation of Asn and Gln in peptides and proteins. These and other cases were discussed by Wright.[179] The Ser and Thr side chains can function as general acid groups, providing a proton to the leaving group or stabilizing the transition state. It is pos-

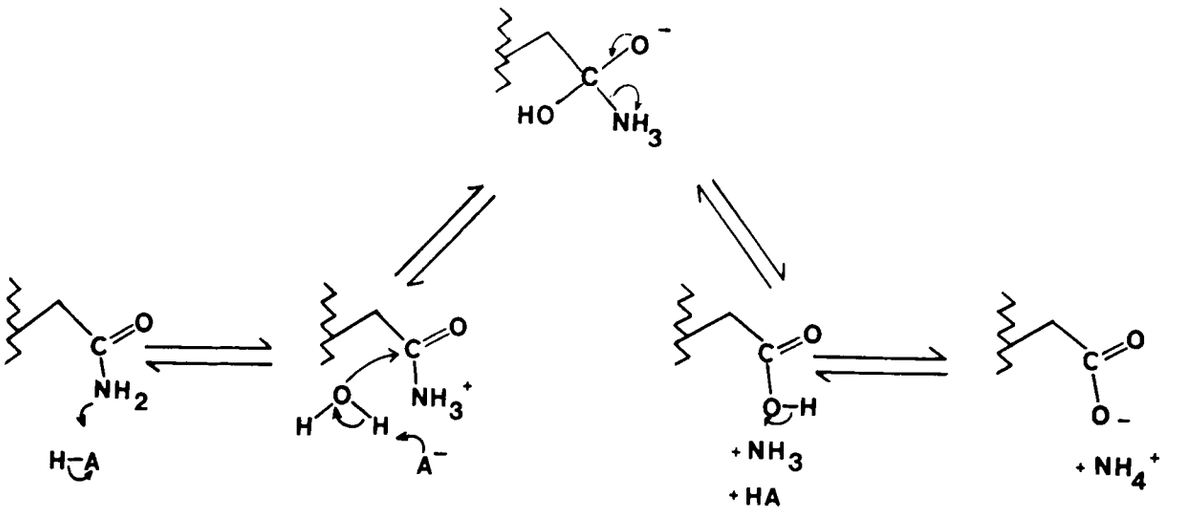

FIGURE 2. General mechanism of deamidation in which HA is a general acid. A conjugate base, A^-, could also function as nucleophile in place of water or as an activator of OH^- ion or another nucleophile. (Adapted from Wright, H. T., *Protein Engineer*, 4, 283. With permission.)

sible, though less likely, that in special cases Ser and Thr could also act as general bases to activate hydroxyl ion or as nucleophiles to attack the amide side chain. Asp, Glu, and His side chains are all nucleophiles at neutral pH, which can attack the carbonyl carbon of the amide side chain or function as general bases to activate nucleophiles. Lys and Arg, which correlate with high deamidation rates when next to Asn and Gln in sequence, may stabilize the oxyanion intermediate. Figure 3 shows a hypothetical deamidating configuration for the first labile Asn to deamidate in cytochrome C, in which general acidic groups and nucleophiles could function to accelerate these deamidation reactions.[12]

1. Amino-Terminal Amide Deamidation

There are two special cases of nucleophile-catalyzed deamidation of amides at or near the amino terminus of proteins. It has been known for many years that amino-terminal Gln readily deamidates by cyclization with its own terminal amino group to form pyrrolidone carboxylic acid.[182] This deamidation occurs much more rapidly than that of Gln residues internal to peptide chains (Figure 4).

Deamidation with cyclization does not occur at amino-terminal Asn because of the unfavorable size of the resultant ring structure, but an analogous reaction has recently been shown to occur in a peptide in which Asn is one residue removed from the amino terminus. In an NMR study of deamidation of the tetrapeptides Val-Asn-Gly-Ala (VNGA) and acetyl-Val-Asn-Gly-Ala (ac-VNGA), Lura and Schirch[162] observed several different mechanisms of deamidation. At pH 7.0, ac-VNGA deamidated to give a 3:1 ratio of ac-Val-isoAsp-Gly-Ala to ac-Val-Asp-Gly-Ala. However, unacetylated VNGA did not give these products. Instead, a cyclic product formed by attack of the terminal amino group on the Asn side chain. At pH higher than 7.5, VNGA deamidated to yield the same products in the same 3:1 ratio as was observed at pH 7.0 for the ac-VNGA. This mechanism depends upon the Asn (or Gln) residue being one residue removed from the free amino-terminal group. It also demonstrates that the amino terminus can act as a nucleophile in deamidation of peptides or proteins where an Asn or Gln residue is in proximity to it. Below pH 7.5, the ac-VNGA and VNGA had different conformations, consistent with the different mechanisms observed for their deamidation reactions.[162]

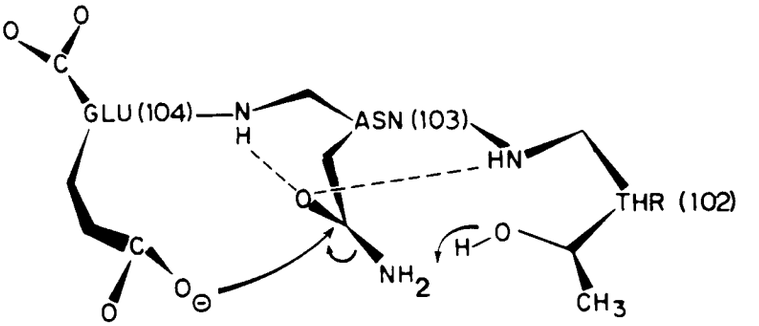

FIGURE 3. Hypothetical mechanism for the deamidation of Asn(103) in horse-heart cytochrome C catalyzed by adjacent amino acids. (From Wright, H. T. and Robinson, A. B., *From Cyclotrons to Cytochromes,* Kaplan, N. O. and Robinson, A. B., Eds., Academic Press, New York, 1982, 727. With permission.)

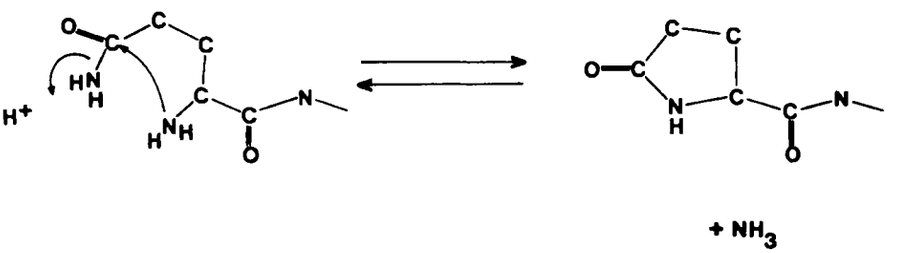

FIGURE 4. Mechanism for the cyclization of amino-terminal glutamine to form pyrrolidone carboxylic acid.

2. Deamidation at the Carboxyl Terminus

Like Gln at the amino terminus of a peptide or protein, Asn at the carboxyl terminus undergoes deamidation. Two possible mechanisms for this reaction have been proposed. One is a cyclization with the terminal carboxylate group to form an anhydride (Figure 5). The cyclization reaction is a nucleophilic attack of the carboxylate group on the amide side chain and results in the deamidation of a carboxyl terminal Asn.[183-185] Alternatively, it has been suggested that under acid conditions, the protonated terminal α-carboxylate group stabilizes the side chain of Asn by hydrogen bonding in a conformation that is susceptible to attack by solvent nucleophile.[51]

B. Deamidation via the β-Aspartyl Shift Mechanism

Asn-Gly, Asn-Ser, and Asn-Ala sequences in peptides and proteins can undergo deamidation by a mechanism that has been found to be quite common.[186-189] The high frequency with which Gly occurs after a labile Asn residue in Table 1 is due to its capacity to undergo a β-aspartyl shift reaction with the side chain of the preceding Asn. For many years it has been known that this reaction occurs in peptides.[190-198] The β-aspartyl shift mechanism is a special case of nucleophilic attack on the Asn side chain amide group, in which the main chain peptide nitrogen of the succeeding residue, usually glycine, functions as the nucleophile (Figure 6). The relative specificity for Gly, and to a lesser extent, Ser and

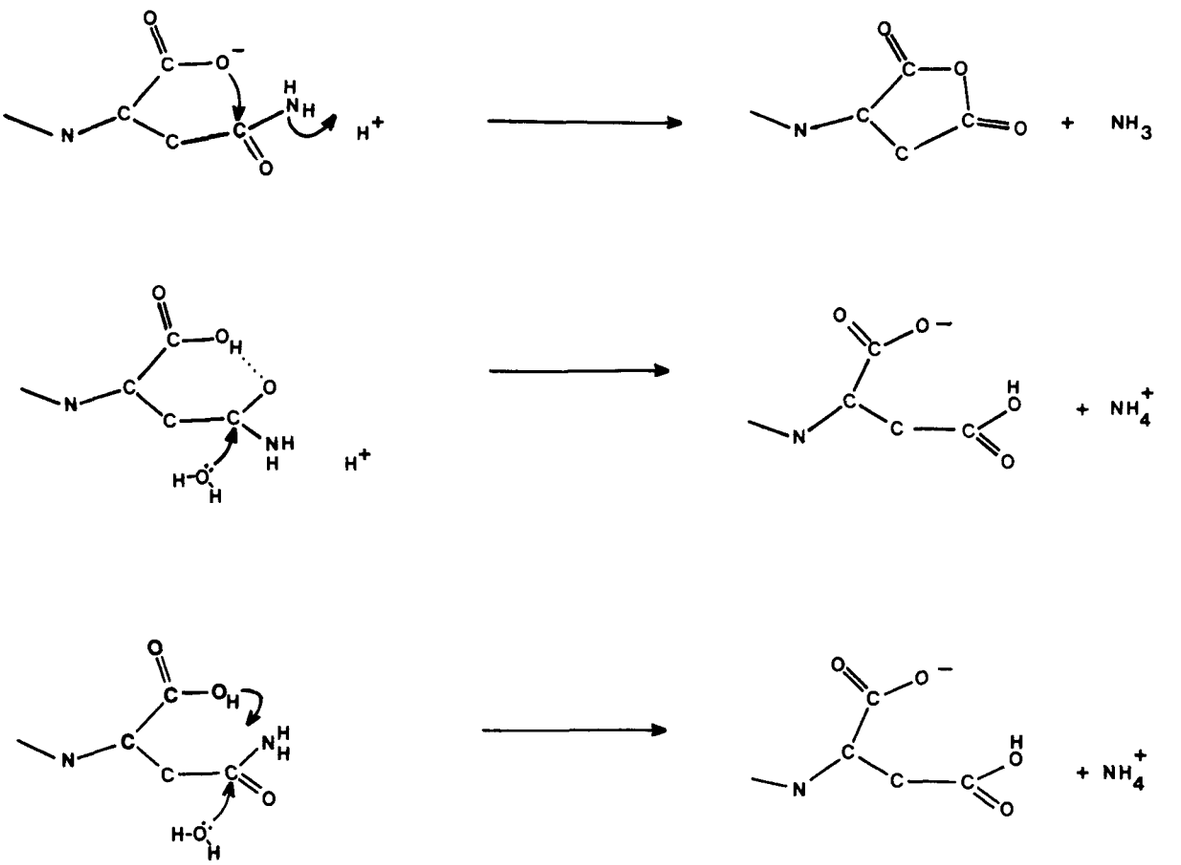

FIGURE 5. Mechanisms for the cyclization of carboxyl-terminal asparagine. The cyclic anhydride is probably unstable and breaks down to deamidated aspartate. (Adapted from Wright, H. T., *Protein Engineer,* 4, 283. With permission.)

Ala,[199,200] in the position after the labile Asn is in part (but see below) due to their small side chains, which do not obstruct the cyclization reaction to the succinimide intermediate. The reaction is likely to be base catalyzed, with proton removal at the amide –NH–. The succinimide intermediate has been demonstrated[188,189] and can break down by either of two pathways to yield an α-linked or a β-linked Asp-Gly sequence. Under approximate physiological conditions, the ratio of β- to α-linked product is about 3:1.

The pH dependence for deamidation by the β-aspartyl shift mechanism of the hexapeptide Val-Tyr-Pro-Asn-Gly-Ala, a fragment of ACTH, is consistent with base catalysis from pH 5 to pH 12.[201,202] The pH of maximum stability for this peptide is lower (pH 3 to 4) than that observed for peptides and cytochrome c (previous section), suggesting that the mechanisms differ. The increasing rate of deamidation to Asp and isoAsp products in a 1:3 ratio with pH in the range of 5 to 12 is consistent with deprotonation of the peptide –NH– as the rate-limiting step for this mechanism. At pH below 4, the rate of deamidation is much lower, and appears to occur primarily by acid-catalyzed hydrolysis, with only a small fraction of succinimide intermediate formed. This succinimide intermediate is stable to further hydrolysis to Asp and isoAsp products at low pH.

The discovery and characterization of the protein carboxyl methyltransferase enzyme,[149,186,203-208] which specifically methylates isoaspartyl (β-Asp) residues, one of the products of the β-aspartyl shift reaction,[97,205] has facilitated the detection of deamidation arising from this reaction in proteins. However, studies using protein carboxyl methyltransferase to identify the isoaspartyl product, which forms as a result of

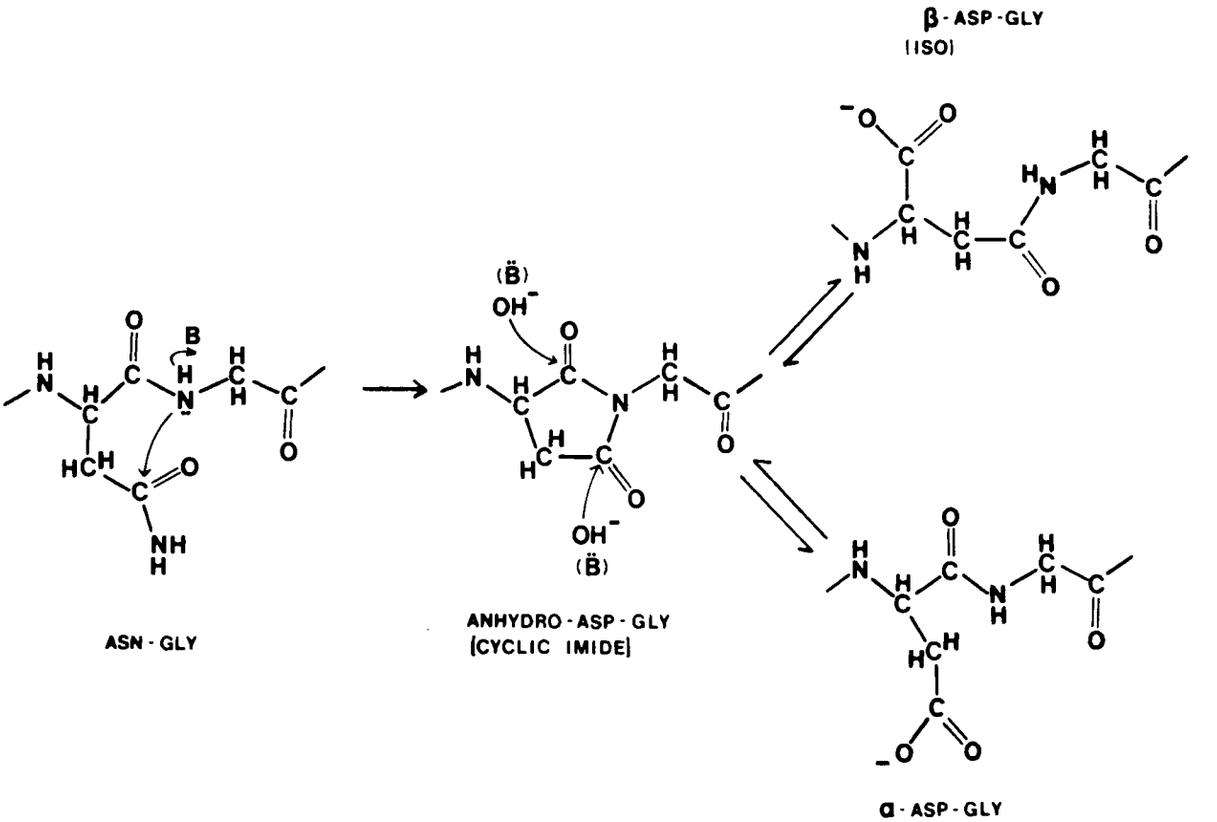

FIGURE 6. Mechanism for the cyclization of asparagine by the β-aspartyl shift mechanism to form a succinimide intermediate. This intermediate breaks down to form either the α- or β-isomeric aspartate products in the ratio of about 1:3.

deamidation by the β-aspartyl shift mechanism, have yielded confusing results. Observations that incubation of calmodulin under conditions that promote deamidation (10 h at pH 11.0 to 11.5 and 37°) increases the incorporation of methyl groups catalyzed by protein carboxylmethyltransferase led to the inference that deamidation of Asn was occurring under these conditions.[206,207] However, other studies of methyl-group incorporation in glucagon[37] and calmodulin[208] identify the sites of methylation as aspartate residues in the original sequence, which have presumably undergone isomerization to β-isoAsp. In calmodulin Asp(2) and Asp(78) and/or Asp(80) are methylated. However, another study[23] shows a strong correlation between ammonia generation and methyl-group incorporation, and supports deamidation at Asn(60) and/or Asn(97) by a β-aspartyl shift mechanism. Since Asn in Asn-Gly sequences has an intrinsically higher rate of deamidation by the β-aspartyl shift mechanism than Asp in Asp-Gly sequences, it is to be expected that the former will be sites of methyl-group incorporation by carboxyl methyltransferase more often than the latter. However, we cannot exclude conformational effects that could reverse these relative rates, and the incorporation of methyl groups catalyzed by protein carboxyl methyltransferase cannot be used as a specific assay for deamidation of Asn residues.

C. Peptide Chain Cleavage

A mechanism similar to the β-aspartyl shift mechanism has recently been shown to occur, in which peptide chain cleavage as well as deamidation occurs immediately after an Asn residue.[129,209] Geiger and Clark[180] have also shown limited cleavage reactions in model peptides. The

nucleophile in this reaction is the side chain amido $-NH_2$ group of Asn, which attacks its own main chain peptide carbonyl carbon, with displacement of the peptide chain on the carboxyl side of the labile Asn (Figure 7). While this is not a deamidation of the side chain of an Asn or Gln, its mechanism is similar.

D. Asparagine Deamidates More Rapidly than Glutamine

Table 1 shows that deamidation of Asn is observed more frequently than that of Gln, confirming the observation made earlier by Robinson and co-workers[1] that the rates of deamidation of Asn in model peptides are faster than those for deamidation of Gln in peptides of comparable or similar sequence. On the basis of model building, Wright and Robinson[12] proposed that this difference was due to the greater distance from adjacent main-chain amido –NH– groups to the Gln side-chain amide group compared with that of Asn. The proximity of these amido –NH– groups can increase the rate of deamidation of Asn residues in at least two ways. As hydrogen bond donors, the –NH– groups can stabilize the tetrahedral oxyanion transition state (see Figure 3) of Asn, but make only weak contacts with that of Gln. The extra methylene group of the Gln side chain makes the distance from these –NH– groups to the negatively charged oxygen of the oxyanion too long to form good hydrogen bonds. In the β-aspartyl shift mechanism, Asn preceding a Gly deamidates rapidly in peptides, but this reaction does not occur for Gln preceding Gly. Deamidation of amino-terminal Gln is thus far the only case where Gln deamidates more rapidly than Asn in the same position.

E. What is the Intrinsic, Uncatalyzed Rate of Asn and Gln Deamidation in Peptide Chains?

The large range of deamidation rates of the model peptides studied by Robinson and co-workers established that there are primary and probably secondary structure effects upon Asn and Gln deamidation rates. However, it was not clear whether these effects on the deamidation rate are all increasing the rate above its uncatalyzed value, or whether some were decreasing it below its spontaneous value.

To estimate the magnitude and sign of these effects, Wright and Robinson[12] calculated the expected spontaneous rate of deamidation of acetamide from measured values. Using this value as a reference and correcting for the different reaction order for acetamide and peptide deamidation reactions, they showed that the rates of deamidation of Asn and Gln in the peptides studied were enhanced by factors of 220 to 18,000 for the Asn-containing peptides and 34 to 1200 for the Gln-containing peptides over the rate for acetamide. Therefore, incorporation of Asn and Gln into a peptide increases their rates of deamidation, most probably due to the transition-state stabilization mentioned above and to the introduction of a potential nucleophile in the amide nitrogen of the succeeding residue. The variation in enhanced deamidation rates of the different peptides is a result of further modulating effects arising from specific sequences and higher order structure.

F. Water Participation in Deamidation

Almost all incidences of deamidation in peptides and proteins require the participation of a water molecule to go to completion. In peptides, there are minimal obstructions to water access to the labile amide. However, the more stable protein structures may limit access of water to amide groups and so influence the rates of any deamidation reactions. Deamidation rates of Asn and Gln residues on the surfaces of proteins will not be limited by water access, while those that occur in the interior of proteins may be. Such a limitation will be determined by the static protein structure and by the frequency with which buried Asn and Gln are exposed to solvent during rapid dynamic changes in the structure due to thermal motion.

In most cases, the tetrahedral intermediate of the deamidation reaction is unstable and breaks down rapidly to products. In the case of the β-aspartyl shift mechanism, deamidation of Asn could occur without participation of water, to

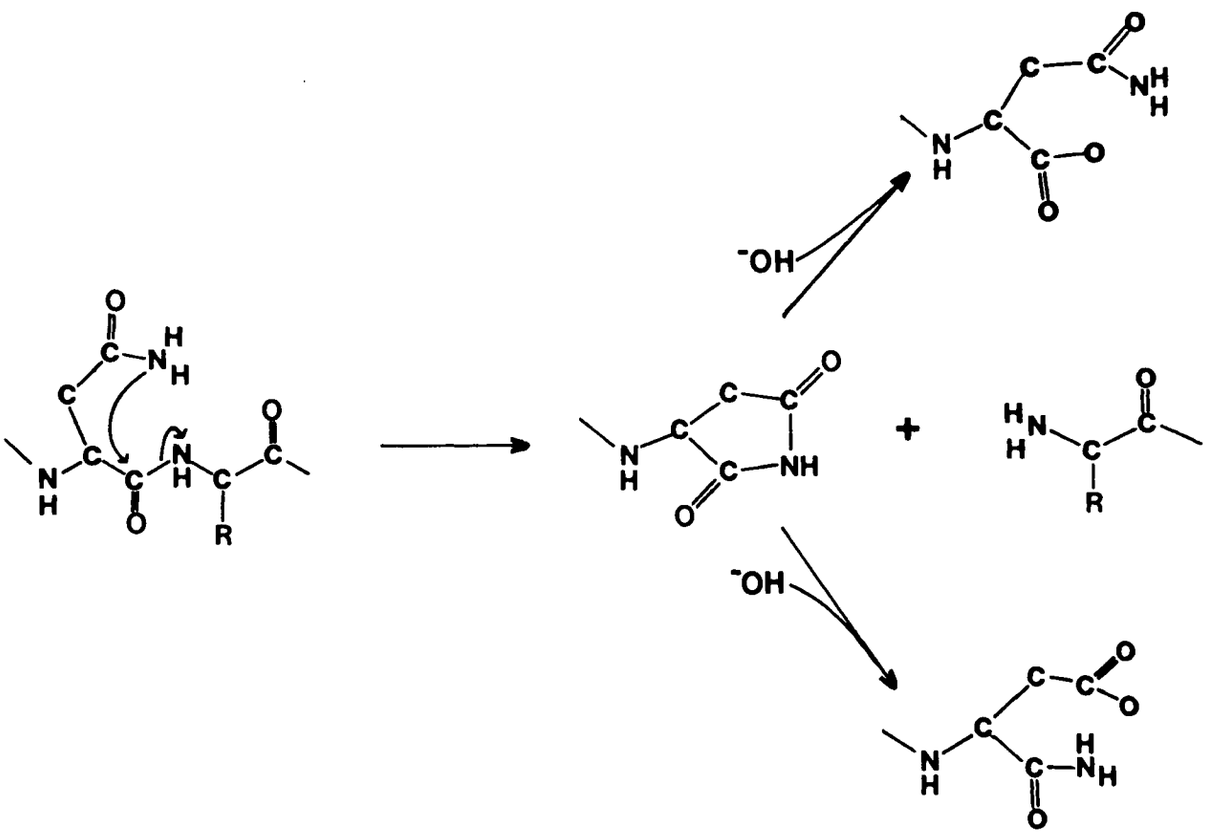

FIGURE 7. Mechanism for the cleavage of peptide bonds through cyclization of the asparagine side-chain amido $-NH_2$ with the carbonyl carbon of the peptide bond.

yield the succinimide intermediate, which in the absence of water might be stable. These conditions might be realized in special structures such as membranes.

IV. DEAMIDATION OF SPECIFIC PROTEINS

Some of the entries in Table 1 are discussed in detail here in light of the above discussion of the mechanisms of deamidation. They have been selected in many cases to illustrate specific mechanistic or biological phenomena that may be associated with deamidation of Asn and Gln in proteins.

A. Adrenocorticotropin

Adrenocorticotropin (ACTH) exists *in vivo* in two forms, one of which is the deamidation product of the other.[93] Deamidation occurs at Asn 25 in the sequence . . . Pro-Asn-Gly-Ala . . . [93-97] The reaction yields an Asp in a β-aspartyl linkage to the succeeding Gly and can be methylated by methyltransferase II.[97] Geiger and Clarke[180] studied the deamidation of model peptides homologous with the deamidating region of ACTH. Replacement of Leu for the Gly residue following the Asn results in cleavage of the peptide chain immediately after the Asp to an extent of 14% of the product. Replacement of Pro for the Gly reduced the rate of reaction, but still gave tetrapeptide cleavage products. These cleavages could be the result of the nucleophilic attack by the Asn side chain or of already deamidated Asp on the carbonyl carbon of the Asn-X peptide bond. The possibility of cleavage of main chain peptide bonds as a result of deamidation reactions was proposed by Wright and Robinson[12] based on the observed lability of Asn-Gly bonds in the pres-

ence of nucleophiles[210] and mild acids,[211] and of Asp-Pro bonds through the participation of the side chain carboxyl group as a nucleophile.[212]

B. Alcohol Dehydrogenase

Alcohol dehydrogenase from *Drosophila melanogaster* has been shown to exist in multiple forms, some of which have been suggested to be the products of deamidation.[101] There are four allelic variants of this enzyme, and all show multiple bands in electrophoresis. Furthermore, it was shown that forms with higher net negative charge arise from others of lower negative charge. Some of these changes are due to transformations related to NAD^+ and substrate or inhibitor binding, but others are likely to be the result of deamidation.

The protein[99] and DNA sequence[100] of one of these alleles show there to be two Asn-Gly sequences, at residues 91-92 and at 231-232. Circumstantial evidence that the Asn-Gly sequence at residues 91-92 undergoes deamidation comes from the fact that one of the subforms binds NAD^+ poorly. Although there is almost no sequence homology between the *Drosophila* and horse-liver LADHs,[100] alignment of the putative NAD^+ binding domains of the two enzymes shows Gly(92) to be at an invariant position in the NAD^+ binding pocket. Asn(91), which would correspond to Ile(269) of the horse-liver enzyme, would also be in the NAD^+ binding pocket, and inspection of this region of the horse-liver LADH structure[102] shows that this residue is in contact with the adenine ribose part of the NAD^+ cofactor. Introduction of a negative charge at this site in the *Drosophila* enzyme as a result of deamidation of Asn(91) would very likely affect the binding of the cationic NAD^+ and could be the reason that one of the multiple forms of the *Drosophila* LADH is observed to have diminished affinity for its cofactor.

Gln(230), in the sequence Asn-Gln-Asn-Gly, is also labile. Evidence already existed for deamidation of Asn(229) in this sequence,[98] and the deamidation of Gln(230) may occur as a consequence of the creation of a nucleophilic Asp at the adjacent residue or vice versa.

C. Aldolase

Koida et al.[105] and Lai et al.[106] showed age-dependent heterogeneity in rabbit muscle aldolase and proved that this resulted from deamidation of an Asn in the sequence Ser-Asn-His, where the Asn is four residues from the carboxyl terminus. The half-time for deamidation of this residue *in vivo* has been measured by Midelfort and Mehler as 8 d.[107] McKerrow and Robinson[10] synthesized the model peptide Gly-Ser-Asn-His-Gly and measured its half-time of deamidation to be 6.4 d *in vitro*. They proposed from this that the rate of deamidation of the corresponding labile Asn in aldolase is controlled by the nearest neighbor residues and suggested that turnover of aldolase *in vivo* is controlled by the deamidation reaction.[10] McKerrow and Robinson also suggested mechanisms for this deamidation reaction in which the adjacent serine and histidine residues function as general acid and nucleophile, respectively. These postulated mechanisms were elaborated on by Wright and Robinson,[12] and a hypothetical configuration around this labile Asn is illustrated in Figure 8. Although this mechanism may be a minor pathway of deamidation, the resistance of the Asn-His bond to carboxypeptidase digestion[106] supports a β-aspartyl shift mechanism as the major pathway.

D. Aspartate Aminotransferase

Multiple forms of cytoplasmic and mitochondrial aspartate aminotransferase from several different tissues and organisms have been observed,[13-19] and in at least one case, evidence supports their existence *in vivo*.[14,15]

There are conflicting data on the causes of the heterogeneity in this enzyme, but a correspondence between ammonia generation and the appearance of more negatively charged forms in electrophoresis of the pig-heart enzyme is consistent with deamidation being one of the causes.[19] These measurements found a half-time of 22 d for the apparent deamidation of two amide groups. There was little or no effect on the specific activity of these forms as a result of the change in charge.

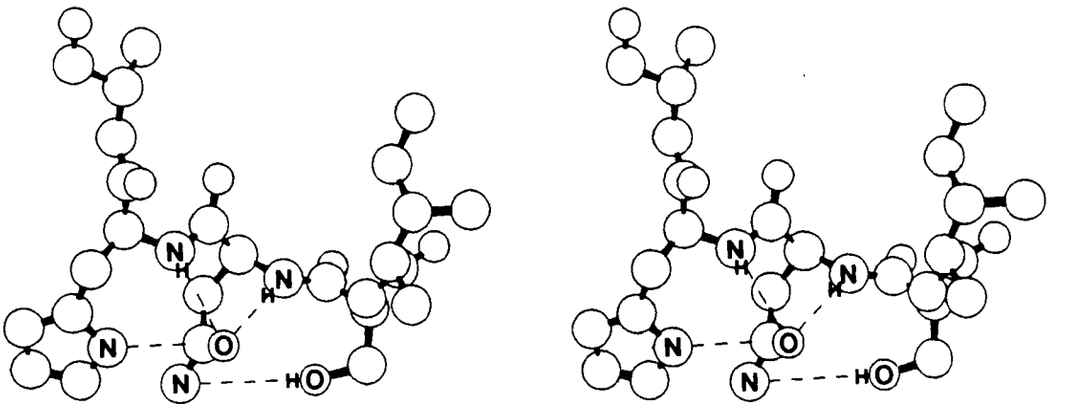

FIGURE 8. Hypothetical conformation for the deamidation of Asn(358) in rabbit muscle aldolase, in which neighboring residue side chains catalyze the reaction.

The crystal structure of cytoplasmic aspartate aminotransferase[20,21] shows that several Asn residues are in configurations that favor deamidation. Asn(144) is in an Asn-Gly sequence. The main chain ($\psi = -52°$) and side chain ($\chi^1 = -76°$, $\chi^2 = 88°$) torsional angles are not optimal for a β-shift deamidation reaction (see below), but limited changes in local conformation could attain a conformation favorable for formation of the succinimide intermediate. Asn(179) is positioned so that only small changes in the orientation of Glu(178) and Ser(175) could orient them as catalysts for deamidation. Asn(351) is oriented close to the side chains of Thr(349) and His(352), and Asn(375) is approached by Glu(371), Glu(376), and His(378). Minimal changes in the positions and orientations of these groups are necessary for them to participate as nucleophiles or general acids in the deamidation reaction.

E. Calmodulin

There have been conflicting reports of deamidation in calmodulin, both from amino acid sequence analysis and from measurements of methyl group incorporation catalyzed by carboxyl methyltransferase (see above). Direct measurement of ammonia release and its correlation with methyl acceptor activity[23] support the identification of Asn residue(s) 60 and/or 97 as labile amide groups. Both are in Gly-Asn-Gly sequences and are part of different calcium-binding domains. In the presence of calcium at concentrations above 0.1 μ*M*, enzyme-catalyzed methyl group incorporation, and by implication deamidation at these sites, is greatly decreased. Since physiological calcium concentrations are around 0.1 μ*M*, it is possible that deamidation of calmodulin occurs *in vivo*.

F. Carbonic Anhydrase

Funakoshi and Deutsch[24] showed that carbonic anhydrases B and C consist of sets of isozymes that can be distinguished both immunologically and electrophoretically. Carbonic anhydrase B consists of at least five and possibly seven isozymes, while carbonic anhydrase C consists of at least three and possibly five isozymes. Conversion to more negatively charged forms was observed *in vitro*, occurred with change in amide content, and appeared to increase with age.

Carbonic anhydrase B contains two Asn-Gly sequences near the amino terminus, and carbonic anhydrase C contains one of these same two near the amino terminus plus two others. In the crystal structures of these two carbonic anhydrase isozymes, only one of the five Asn-Gly sequences has a main-chain conformation approaching that in which formation of the succinimide intermediate is favored. Changes in conformation would be necessary for these sites to undergo deamidation by the β-aspartyl shift mechanism.

The crystal structure of carbonic anhydrase B[27] shows three other Asns in environments likely to catalyze deamidation, in addition to the Asn-Gly sequences. Asn(69), Asn(224), and Asn(245) all lie close to functional groups like His and Glu, which could catalyze their deamidation. These potential deamidating configurations around the Asns in carbonic anhydrase B are the result of tertiary interactions of the protein structure. The neighboring Asn(244) and Asn(245) occur close together in the sequence, and could act synergistically. In carbonic anhydrase C, Asn(67), Asn(230), and Asn(251) are all in environments with potential catalytic groups for deamidation, and a deamidated Asn(67) could contribute to the deamidation of Gln(92).

Some of the histidines suggested as possible functional groups in the deamidation reaction are ligands of the Zn, and they would be available to catalyze deamidation in the apoenzyme, but not in the holoenzyme. Similarly, changes of conformation around the Asn-Gly bonds are more probable in the apo- than in the holoenzyme. This differential stability may be similar to the effect of calcium on deamidation of calmodulin and could be a mechanism by which the apo form is selectively deamidated as a prelude to degradation.

G. Chloroperoxidase

Of the three residues that deamidate in this enzyme,[114] one is a Gln and another is an Asn in the sequence Pro-Asn-Asn. A third Asn that deamidates is in the sequence Asn-Asn-Thr, where the first Asn is glycosylated. A possible relationship between *N*-glycosylation of Asn residues and their susceptibility to deamidation during biosynthesis is discussed below.

H. Cholera Toxin

Cholera toxin consists of two subunits, A and B, with a stoichiometry of one A subunit to 5 B subunits per toxin molecule. The A subunit activates adenylcyclase, and the B subunit binds the toxin to the cell surface. Purified cholera toxin has been separated into three forms by electrophoresis.[116] Deamidation of cholera toxin at high pH with generation of ammonia yields three isozymes, which are identical electrophoretically to those observed in the native purified preparations. This conversion follows an irreversible order, resulting in increasingly negative electrophoretic products. Sequence analysis of the cholera toxin B chain[118,119] found two deamidated Asns, which were thought to arise during the sequence analysis. The Asns are Asn(44) and Asn(70) which are in the sequences Lys-Asn-Gly and Lys-Asn-Thr, respectively.

Spangler and Westbrook[120] observed multiple bands for cholera toxin on analytical isoelectric focusing gels and found two charge variants of isolated B chain in a ratio of about 3:1. For a single deamidation event, which is consistent with the two forms of the B chain, a binomial distribution model of the abundances of the different combinations of A chain and deamidated B chains gave a population composition closely resembling the elution profile of cholera toxin from a high-performance ion-exchange column. This suggests that the isoforms of cholera toxin arise as a result of a single deamidation with random distribution of the two forms of the B chain into the pentamer according to their abundances. It is not known why the deamidation does not go to completion in all subunits. The rate of this deamidation is quite rapid, since the multiple bands are observed in preparations that are less than 10 d old. These data point to the Asn-Gly as the most likely site of deamidation in the B chain, with the other two deamidations possibly arising during sequencing.

I. Crystallins from Eye Lens

Lens crystallins have been the object of a number of studies directed at determining the chemical changes that occur during development. They are also of interest because of their exceedingly slow or nonexistent turnover during the lifetime of an organism. It had been suggested that bovine α crystallins A2 and B2 convert to A1 and B1 as a result of the deamidation of Asn(123)[123,124,130] and of the nearby Gln(126).[121,122] It has now been shown that phosphorylation of Ser(122), and not deamidation, is

the cause of these changes in charge in bovine α crystallin A2.[127] Phosphorylation of bovine crystallin B2 is also the likely cause for charge heterogeneity in that protein. Charge heterogeneity alone is obviously insufficient to prove deamidation.

Although these results eliminated deamidation as the source of observed charge heterogeneity in these specific cases, deamidation has been shown to occur in other crystallins. In chicken α_A-crystallin, Asn(149) in the sequence Ser-Asn-Met deamidates,[128] and aged human αA_x-crystallin is deamidated at Asn(101) in the sequence His-Asn-Glu.[126] Sequence analysis of bovine β-crystallin B_p[131] showed a deamidated Asn residue, though it is not established whether this occurs *in vivo* or during purification and sequencing. This Asn is at the amino terminus (residue 3) in the sequence Ser-Asn-His, which is the same as that of the rapidly deamidating Asn at the carboxyl terminus of rabbit muscle aldolase. Bovine αB_2 crystallin deamidates at Asn(80) in the sequence Leu-Asn-Val and at Asn(142) in the sequence Val-Asn-Gly.[130] The ϵ crystallin from duck appears to deamidate at two different Asn residues (residues 163 and 265) in the sequences Cys-Asn-Leu and X-Asn-Leu.[132] Whether these deamidations occur *in vivo* as posttranslational modifications or during the sequencing has not been established.

J. Cytochrome C

The deamidation of horse-heart cytochrome C was the first carefully studied case of protein deamidation and was discussed in detail by Robinson and Rudd.[1] No new work on this protein has been done since that review, but several features of these deamidation reactions merit recapitulation here.

Flatmark and co-workers[28,29] showed *in vitro* that singly, doubly, and triply deamidated forms of cytochrome C occur in rat, horse, and beef heart, and that the singly deamidated form exists as two isomers.[32] Flatmark and Sletten[34] showed that three deamidated forms occur *in vivo* and that they are the result of successive deamidations. The first-order rates of deamidation *in vitro* and *in vivo* in rats were found to be identical, as were the reaction products.

The first of the three deamidations studied occurs in the Thr-Asn-Glu sequence at the carboxyl terminus of cytochrome C. Inspection of the crystal structure of horse-heart cytochrome C[35] shows that changes in the conformation of the last two residues of the sequence, as well as a rotation of the threonine side chain, would create a configuration in which the side chain carboxyl group of the Glu could be a nucleophile and threonine a general acid. The main chain dihedral angles of this tripeptide are close to those of a 3_{10} helix. The configuration around Asn(103) in the native structure and in the proposed deamidating state are shown in Figure 9.[12]

The half-life for the deamidation of the model tetrapeptide with the same sequence as that of the carboxyl-terminal tetrapeptide of horse-heart cytochrome C is 16 d, in close agreement with the estimated half-life of 15.6 d for deamidation of this Asn in cytochrome C.[34] This supports the hypothesis[1,11] that this deamidation is controlled by the sequence immediately around the labile Asn. Furthermore, the preponderance of undeamidated over deamidated forms (8:1) *in vivo* suggests that deamidated forms are rapidly degraded and that a single deamidation is sufficient to transfer a molecule into the population of rapidly degrading ones. The rate at which the Gly-Thr-Asn-Glu tetrapeptide deamidates is 10^4 greater than the rate inferred for the standard acetamide.[12]

Robinson and co-workers[11] measured the rates of deamidation of model tetra- and pentapeptides for all Asn-containing sequences in cytochrome C. Using these rates and the results of Flatmark,[29] they concluded that a model for the deamidation of cytochrome C based upon multiple first-order, independent deamidation events controlled only by local sequence was inconsistent with the abundances of deamidated forms observed *in vitro*. They proposed, instead, that the first deamidation near the carboxyl terminus, which is controlled only by local sequence, precipitates a conformational change in the structure of the molecule that accelerates the deamidation of the second labile amide relative to the deamidation rate for the model pentapeptide of the same sequence.

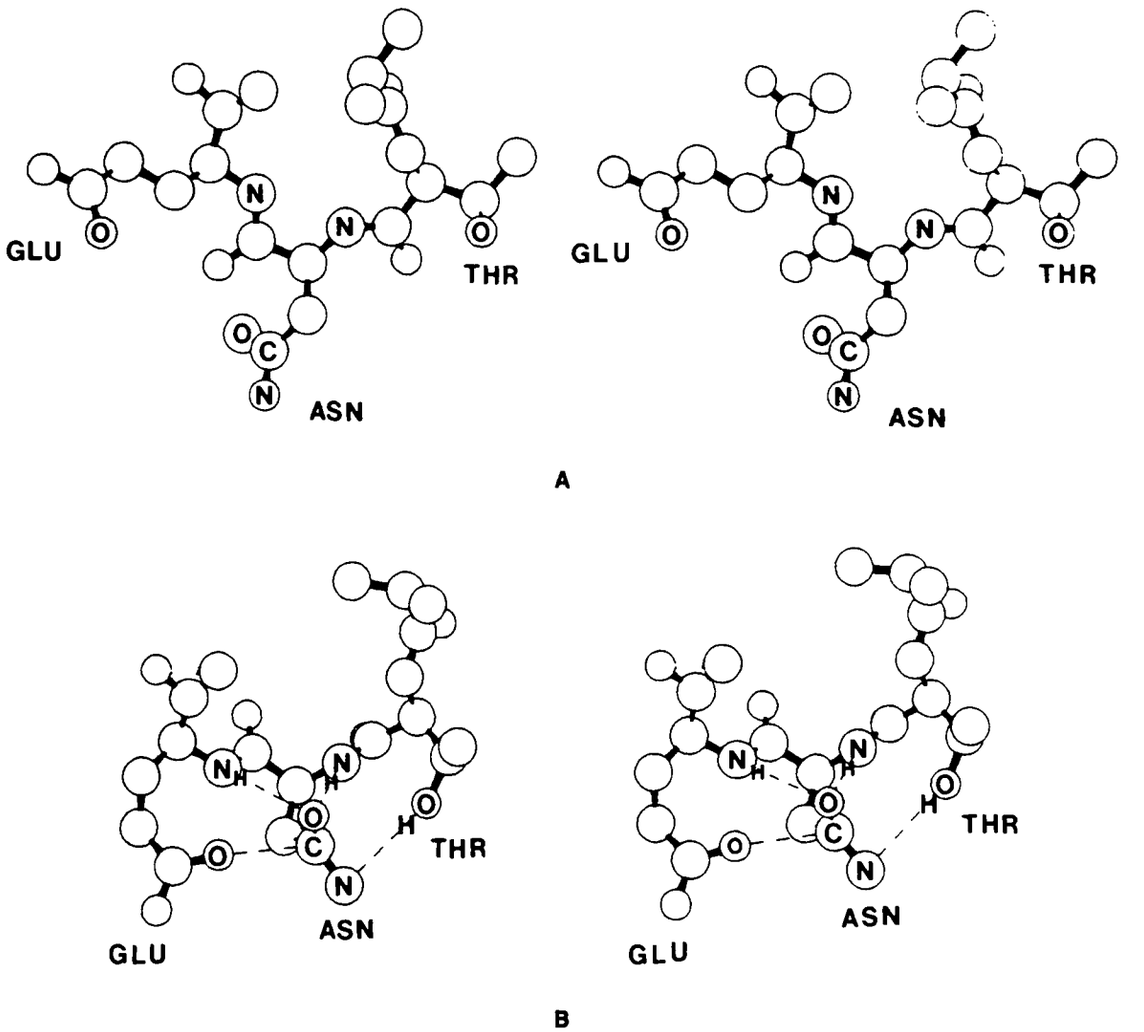

FIGURE 9. (a) Conformation of the carboxyl-terminal three residues of horse-heart cytochrome c as determined in the crystal structure. (b) Conformation of the same residues after local changes in conformation, which bring the adjacent side chains into contact distance with the labile amide side chain of Asn(103). The inferred reaction in this conformation is depicted in Figure 3. (Adapted from Wright, H. T., *Protein Engineer,* 4, 283. With permission.)

Asn(54) (horse-heart cytochrome C numbering) was subsequently identified as the second deamidating residue.[33] Its position in the structure of albacore cytochrome C shows that the side chains of Asp(50) and Lys(53) could come within contact distance of the Asn(54) amide side chain. Asp(50) could function as a nucleophile, and Lys(53) may stabilize the oxyanion transition state in catalyzing this second deamidation (Figure 10).

The identity of the third deamidating residue has not been established, but examination of the albacore cytochrome C structure suggests that Asn(31) is a good candidate.[179] The side chain of this residue lies between the side chains of His(26) and Thr(19), and rotation of the Asn(31) side chain alone brings it into contact distance with the potential general acid and nucleophile side chains of these two residues (Figure 11). This conformation may only be accessible after the deamidation of the other two labile Asns in the molecule.

Cytochrome C is the first example of multiple

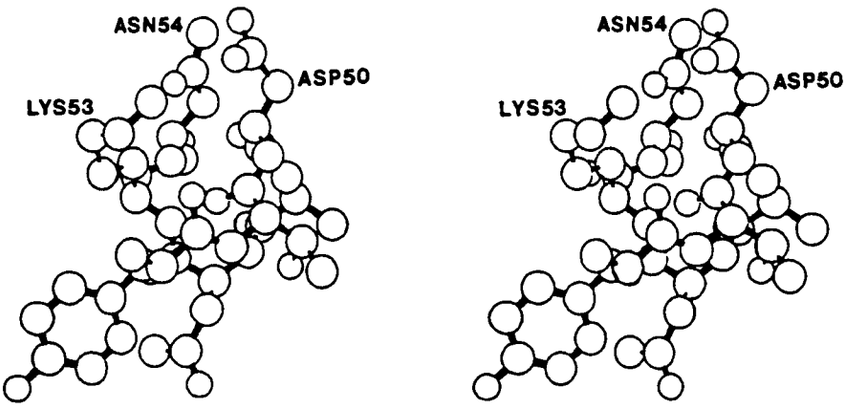

FIGURE 10. Stereo view of environment of Asn(54) in cytochrome C. (Adapted from Wright, H. T., *Protein Engineer*, 4, 283. With permission.)

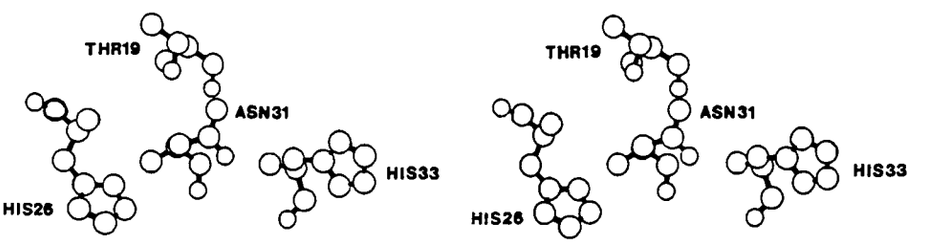

FIGURE 11. Stereo view of environment of Asn(31) in cytochrome C. (Adapted from Wright, H. T., *Protein Engineer*, 4, 283. With permission.)

deamidations in which one deamidation effects changes in the molecule that influence the rate of a subsequent deamidation. The considerable distance between Asn(103) and Asn(54) also shows that these changes have effects on potential deamidation sites that are far away (Figure 12).

K. Epidermal Growth Factor

One of the low-abundance forms of mouse epidermal growth factor has been shown to be deamidated at the amino-terminal Asn. This Asn is followed in sequence by a Ser, which probably catalyzes the deamidation. The deamidated form of epidermal growth factor has the same receptor binding and mitogenic activity as the undeamidated form,[134] but the single deamidation destroys its immunosuppressive biological activity.[135]

L. Growth Hormone

Fresh pituitary extracts show fast electrophoretic forms of human growth hormone, which were attributed to deamidation at residues Asn(152) and Gln(137).[136] A subsequent study showed that Asn(149) is also deamidated.[138] These forms appear spontaneously *in vitro* and are accompanied by other more anodic forms present in lower amounts. Recent results on the deamidation of recombinant human growth hormone (rHGH) have forced revision of some these inferences about the sites of deamidation in HGH.[140] This work shows that deamidation occurs exclusively at residue Asn(149) in the sequence Asp-Thr-Asn-Ser-His under physiological conditions. Some deamidation at Asn(152) in the sequence Ser-His-Asn-Asp-Asp under conditions of high pH has also been reported.[139] It is somewhat surprising that Asn(149) deamidates not by a nearest

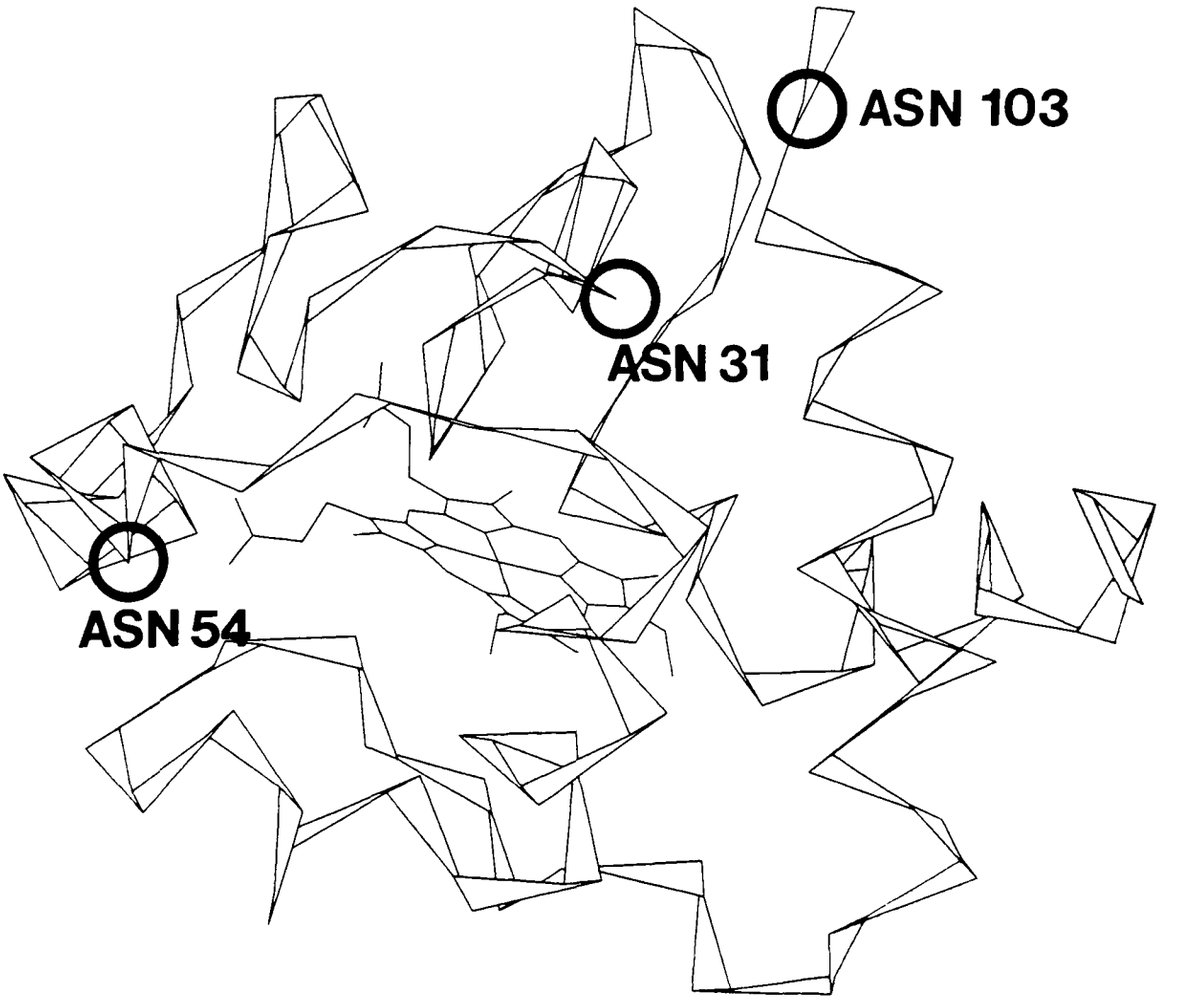

FIGURE 12. Backbone diagram of the structure of horse-heart cytochrome C showing the locations of the labile amide groups [inferred for Asn(31)]. (From Wright, H. T., *Protein Engineer*, 4, 283. With permission.)

neighbor catalyzed mechanism, but by a β-aspartyl shift mechanism. The possible general acid catalysis by the neighboring Thr or Ser side chains and possible local conformations favorable to this mechanism may accelerate the reaction. The altered specificity of subtilisin cleavage at Asn(149), which was observed in earlier work,[136] can now be understood as due to the protease-resistant isoAsp(149)-Ser(150) bond. Although a detailed structure has not been reported, the crystal structure of the highly similar porcine growth hormone has been described to be poorly ordered in the region of residues 128 to 151.[137] This flexibility may facilitate deamidation in this region of the sequence.

Bovine growth hormone also exists in multiple forms, and these have been shown by tryptic hydrolysis and Edman sequencing to differ in the degree of amidation of three residues: Asn(13), Gln(140), and Asn(148).[141] The differences in deamidation sites between bovine and human growth hormones reflect differences in sequence adjacent to these residues in the two forms.

M. Hemoglobin

1. Hemoglobin Providence

Three forms of hemoglobin were detected in

the individual carrying the mutant Providence form: wild-type hemoglobin-A, hemoglobin [Asn(B82)], and hemoglobin [Asp(B82)].[38,42] This is the first mutant to show a change at what was thought to be an invariant lysine at position B82 of the β-chain. In the wild type, this lysine forms an ion-pair that neutralizes the 2,3-diphosphoglycerate bound in the internal pocket of deoxy-hemoglobin. Characterization of the properties of hemoglobin Providence shows that cooperativity is unaffected by the mutation at residue B82, but anion and pH effects are greatly reduced.[39] It is suggested that the pK of His(B143) close to Asn(B82) in the mutant form of the enzyme is perturbed and that this operates against the other prototropic groups responsible for the Bohr effect. Replacement of the Lys by Asn in the cavity in which 2,3-diphosphoglycerate binds decreases the oxygen affinity of hemoglobin Providence at neutral pH and increases dissociation of the subunits.

The origin of the different forms *in vivo* was investigated by *in vivo* labeling with ^{14}C-glycine. These experiments showed that the Asn(B82) form constitutes 90% of the total hemoglobin at the beginning of labeling, but the Asn(B82) and Asp(B82) forms become equal in abundance after 5 weeks.[40] These results suggest that the Asn(B82) is deamidating with time to Asp.

An *in vitro* study of the deamidation of this mutant hemoglobin, with reference to the crystal structure, was done by Perutz et al.[41] They measured the deamidation in the presence and absence of heme ligands and of 2,3-diphosphoglycerate. Carbon monoxyhemoglobin Providence, lacking bound 2,3-diphosphoglycerate, deamidated completely in 69 d at 37°C. Deoxyhemoglobin Providence, with bound 2,3-diphosphoglycerate, had a lower rate of deamidation, which was attributed to the binding of His(B143) to the 2,3-diphosphoglycerate ligand. This binding would prevent the histidine side chain from participating in the deamidation reaction.

It is pointed out in this work that there are eight other abnormal hemoglobins with Asn next to His, none of which are known to be deamidated. It was concluded from this that His(B143) alone is probably not sufficient to catalyze this observed deamidation and that Gly(B83) may also be implicated in the deamidation of hemoglobin Providence.

It is possible that the deamidation of Asn(B82) occurs through a β-aspartyl shift mechanism, since residue B83 is a Gly. However, the structure of human deoxyhemoglobin shows a value of $-39°$ for ψ of Lys(B82), the residue that mutates to an Asn in hemoglobin Providence, considerably different from the optimal value of $-120°$ proposed by Clarke.[213] Unless there is a change in this main chain conformation as a result of the mutation, a β-aspartyl shift mechanism seems unlikely. His(B143) and Asn(B80) can all come within hydrogen-bonding distance of Asn(B82) if changes in side chain conformation occur (Figure 13). These residues within contact distance of Asn(B82) could catalyze the deamidation by protonation of the leaving amide $-NH_2$ group (His or Asn) and/or nucleophilic attack (His) with stabilization of the transition state by one of the hydrogen-bond donors. Asn(B82) occurs at the end of a helix, a regularity that has been noted in a number of structures (see below).[214]

The general acid function provided by His or Asn may be important in this instance, since the deamidating residue is not completely exposed, and the supply of solvent protons to it may be limited, especially in the deoxy form.

2. Hemoglobin Singapore

Hemoglobin Singapore is a mutant hemoglobin in which two amino acids are apparently changed.[43] Ala(α79) is replaced by a Gly and Asn(α78) is replaced by an Asp. The authors suggest that the change from Ala to Gly is a single point mutation at the DNA level, and the change from Asn to Asp is postsynthetic as the result of deamidation by the β-aspartyl shift mechanism. The occurrence of a β-aspartyl linkage in this mutant hemoglobin was not established, so the mechanism of deamidation is open to question. It is also possible that the Ala ⇒ Gly replacement introduces flexibility to this segment of the polypeptide that permits the nearby Asp(α75) and/or His(α72) to catalyze the deamidation of Asn(α78). Only changes in side-chain conformation are nec-

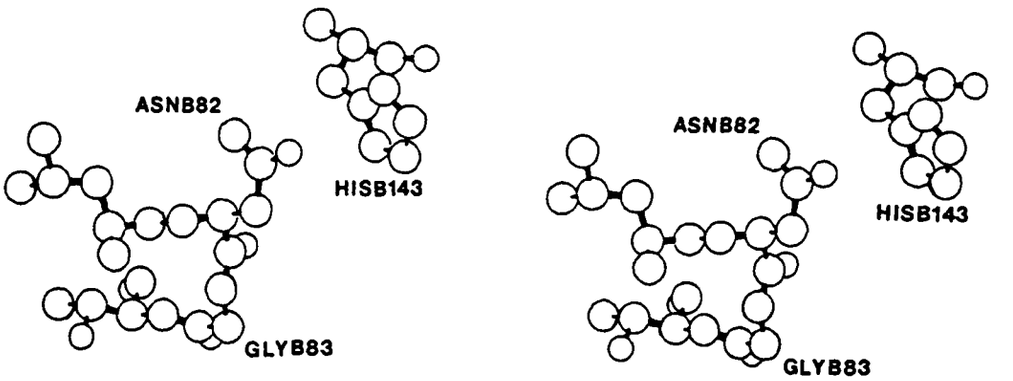

FIGURE 13. Environment of Asn(β82) in hemoglobin Providence. (From Wright, H. T., *Protein Engineer*, 4, 283. With permission.)

essary to bring these residues into contact distance with Asn(α78).

3. Hemoglobin Wayne

Another mutant hemoglobin with a variant α chain, called hemoglobin Wayne, has been found to exist in two forms of low abundance in the individual carrying the mutation.[44] Characterization of these forms clearly established that the mutation is the result of a frameshift mutation near the carboxyl terminus at residue Ser(α138)-Lys(α139), which eliminates the stop codon at residue 142, resulting in an extension of the chain. The native sequence is Ser-Lys-Tyr-Arg. The mutant sequence in this region is Ser-Asn-Thr-Val-Lys-Leu-Glu-Pro-Arg. The Asn residue in this sequence deamidates and accounts for the two variant forms *in vivo*. The flanking Ser and Thr residues probably assist in the deamidation reaction, and the location of the labile Asn near the carboxyl terminus may provide flexibility in the chain, which makes deamidating conformations accessible. Hemoglobin Wayne and its deamidated form account for only about 5% of the total hemoglobin in this patient. The low abundance of the deamidated and undeamidated forms may be due to rapid degradation of the deamidated form, though other explanations are possible.

N. Insulin

The stability of insulin is important in its pharmaceutical production, distribution, and delivery. Human insulin has been found to deamidate slowly at Asn(A21)[45] and Asn(B3),[49] the former being the carboxyl-terminal residue of the A-chain. The rate of deamidation depends on the pH and on additives in the different formulations.[50] Neutral conditions diminish the rate of deamidation relative to acid ones, consistent with the known pH dependence of deamidation.

Asn(A21) at the carboxyl terminus may deamidate through a cyclic anhydride, as discussed above, or at acid pH a protonated terminal carboxylate group may function as a general acid in catalyzing the deamidation of Asn(A21). Lys(B29) and Arg(B22) could both stabilize an anhydride or tetrahedral intermediate, and Thr(B27) could function as a general acid. Asn(B3) can be brought into contact distance with the general acid, Ser(A12) (Figure 14).

Insulin also undergoes a dimerization reaction in which intramolecular deamidation occurs. This dimerization involves the attack of an amino-terminal amino group on the amide side chain of an Asn or Gln residue in the A-chain of another molecule. It presumably resembles the intramolecular deamidation that occurs in the tetrapeptide Val-Asn-Gly-Ala, in which the neutral form of the amino-terminal amino group functions as a

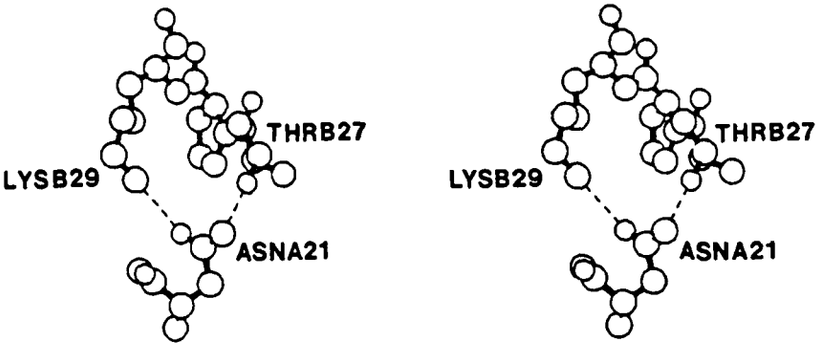

FIGURE 14. Stereo view of environment of Asn(A21) of insulin.

nucleophile in deamidating the Asn residue.[162] Site-directed mutagenesis has been used to replace Asn(A21) with several other amino acids.[51] All substitutions decreased the biological potency of the insulin 27 to 80%, but the stability of the mutant insulins to deamidation was increased from 10- to 100-fold.

O. Interleukin-1α

Human interleukin-1α synthesized by *E. coli* has been found to occur in two forms, differing as a result of a single deamidation at Asn(36).[145] Similar charge heterogeneity occurs in interleukin-1α from human monocytes. The labile Asn(36) occurs in the sequence Ala-Asn-Asp. Replacement of the Asn(36) by a serine yields a protein that is stable against deamidation, clearly establishing the identity of the deamidating residue in the original sequence.[146] The physical, chemical, and biological properties of the two forms were identical, except for charge.

P. Lysozyme

It has been known for a long time that hen egg-white lysozyme is heterogeneous,[52-54] and the original two determinations of the sequence differed at four positions in the assignment of Asp and Asn.[55,57] The identities of residues 46 and 65 in the respective sequences Arg-Asn-Thr and Cys-Asn-Asp have been established from the DNA sequence.[60] Residue 103, which was identified as an Asp in both amino acid sequence determinations has now been shown to be an Asn in the sequence Gly-Asn-Gly.

It cannot be established whether the variability at these positions occurred as a result of treatment during sequencing or preexists in the native protein. However, examination of the structure of hen egg-white lysozyme supports the deamidation of Asn(46) in the native protein, while it is less convincing for Asn(65). The side chain of Asn(46) is within 3.6 Å of the side chain carboxylate of Asp(52) and 4.4 Å of the side chain –OH of Ser(50). Small changes in side chain position could bring these groups close enough to the side chain of Asn(46) to catalyze deamidation in the native enzyme, and thus have given rise to heterogeneity at this position. Asn(65) is 4.8 Å from the side chain of Ser(72), but is otherwise not in proximity to potential intramolecular catalytic groups. Its original misassignment may have been a switch with residue 66, which is an Asp. Asn(103) is most likely to have deamidated by the β-aspartyl shift mechanism, though whether that occurs in the native protein or during sequencing is not known.

Robinson and Tedro (1973)[8] synthesized and measured the deamidation rates of 11 model peptides with X-Asn-Y and X-Gln-Y sequences corresponding to 11 of the 16 amide-containing sequences in chicken egg-white lysozyme. The estimated half-life for the protein derived from deamidation half-lives of these model peptides was similar to that of rat kidney lysozyme,[58] whose sequence at the time was not known. However, the sequence of rat lysozyme[59] has since been determined, and only 3 of the 11 peptides synthesized by Robinson and Tedro[8] occur in its

sequence. Failure to take into account the possible rapid deamidation of Asn(103) in the calculation of lysozyme half-life from peptide deamidation rates makes this result further suspect. Finally, as pointed out above, the groups that are most likely to catalyze the deamidation of Asn in the native enzyme are not in residues that are its immediate neighbors. This argues against nearest-neighbor catalyzed deamidation and suggests that the agreement between the peptide-derived deamidation rate and the *in vivo* deamidation rate of the rat kidney enzyme is fortuitous.

Q. Prolactin

Prolactin isolated from either stored tissue homogenates or from secreted pituitary organ culture medium is heterogeneous.[153] Ovine prolactin has been shown to exist as one major form and four minor ones, all of which were active in the mammary gland receptor assay.[158] Ovine prolactin converts to more acidic forms on incubation at high pH, and the correlation of ammonia release with these changes indicates that deamidation is occurring.[155] In these experiments, the dependence of the rate of conversion on temperature, pH, and ionic strength was also consistent with studies of these parameters on model peptides.[1]

By incubating samples of secreted mouse prolactin and stored ovine prolactin at several pHs (pH 4, 8, 10) and monitoring ammonia release and electrophoretic homogeneity on alkaline polyacrylamide gels, the mobility of the more electrophoretically positive forms changed in a way consistent with a net increase in negative charge.[159,160] It is not clear whether the more negative forms generated by alkaline incubation are identical to the more negative forms occurring in the original sample. Of the three deamidated forms of prolactin, the most electrophoretically negative is the least abundant in both mouse and ovine. *In vitro*, the deamidated forms appear 20 times faster at alkaline pH than at acid pH. Each of the forms was electrophoretically isolated and rerun in electrophoresis, and none showed any signs of break down to more electrophoretically negative forms during this procedure. Test of the three deamidated forms for binding to isolated membrane preparations containing prolactin receptor showed progressively reduced binding with increased deamidation. Antigenicity also decreased stepwise with deamidation of ovine prolactin, but the first deamidation of mouse prolactin had no effect on antigenicity measured by radioimmune assay.

The sequence of ovine prolactin[154,156,157] contains a number of potential deamidating Asns. Asn(6), which has been shown to deamidate,[154] occurs in the sequence: Cys-Pro-Asn-Gly and the carboxyl terminus has the sequence Tyr-Asn-Asn-Asn-Cys-COO. Both horse-heart cytochrome C and rabbit muscle aldolase have rapidly deamidating Asns near their carboxyl termini, possibly a result of unconstrained conformational flexibility at the terminus.

Rat and pig prolactin have two Asn residues at the carboxyl terminus, but lack the Asn-Gly sequence near the amino terminus. Comparison of the breakdown products of the prolactins from these different species might help to identify the deamidating residues. The Asn at the amino terminus and the ones at the carboxyl terminus of ovine prolactin both occur in short disulfide-linked loops.

R. Ribonuclease

1. Bovine Ribonuclease

Bornstein and Balian[62] first showed that Asn(67) in ribonuclease A deamidates when the protein is in the denatured but not in the native form. Later, ribonuclease modified in all of its eight half-cysteine residues to form octa-S-ribonuclease, which is enzymatically inactive and conformationally disordered, was also shown to undergo deamidation at Asn(67) under mild, physiological conditions.[188]

A number of studies under harsh conditions (e.g., 0.5 N HCl, 30°, 10 to 20 h) have yielded several distinct forms of RNAse deamidated in the region of residues 67 to 85.[64-68] Residues Asn(67) and Asn(94) have been shown to be deamidated in this work, and are probably also deamidated during sequencing of African porcupine and Casiragua RNAses.[67]

The conformation at Asn(67)-Gly(68)[57] does

not favor the formation of the succinimide intermediate in native ribonuclease A, and the requirement for some local or global denaturation of the molecule in order for this deamidation to occur is consistent with this. The other two amide residues, beside Asn(67), in this segment of the sequence are in positions where minor structural changes could bring them into proximity with functional groups that could catalyze their deamidation. It is possible that Asn(67) is the first to deamidate by a β-aspartyl shift mechanism and that this change contributes to the deamidation of the other nearby residues. These amides form a nest pointed toward His(119), and side chain rotations alone could bring the imidazole side chain of this histidine to within 3.3 to 5.5 Å of the other amides, where it could function as a general acid or nucleophile. The side chain of Glu(111) is near enough to attack Asn(71) and possibly also Gln(69), and Gln(74) is very close to His(105). There are no potential nucleophiles near to Asn(94). The fact that RNAse deamidates only when unfolded or exposed to harsh conditions suggests that the native conformation is resistant to deamidation. The difference in deamidation rates between the native and completely unfolded form was recently quantitated.[69] At pH 8 and 37° the unfolded form deamidated 30 times faster at Asn(67) than the native form.

2. Bovine Seminal Ribonuclease

Bovine seminal ribonuclease is a dimer of identical monomers that are highly similar in sequence to bovine ribonuclease-A. The monomers are linked by two disulfide bridges. Three forms of the dimeric enzyme are separable on CMC chromatography.[70] These forms correspond to homo- and heterodimers — AA, AB, BB — where B is the undeamidated form and A a monodeamidated form. The ratios of the three subforms are constant and, in contrast to RNAse A, the deamidation appears to occur *in vivo* and under physiological conditions *in vitro*. There is no difference in catalytic activity among the three forms. As in RNAse-A, Asn(67) is the labile residue, probably undergoing deamidation by a β-aspartyl shift reaction with Gly(68).[71] Because of the high degree of sequence identity between bovine ribonuclease A and bovine seminal RNAse, the relative ease with which the bovine seminal enzyme undergoes deamidation must be due either to one of the few differences in sequence between the two enzymes or to interactions that arise from the linkage of the two monomers by disulfide bridges.

It is interesting that the isolated tryptic peptide containing the deamidated isoAsp-Gly sequence is a substrate for methylation by methyltransferase II, while the intact, deamidated bovine seminal RNAse is not.[71,73,74] However, denatured, monomeric, deamidated bovine seminal ribonuclease is methylatable by this enzyme, implying that the tertiary and/or quaternary structure prevents methylation of the isoAsp formed as a result of deamidation.

S. Somatotropin

Two components of human chorionic somatomammotropin (somatotropin), differing in charge in electrophoresis, have been separated on DEAE.[164] The kinetics of their interconversion have been studied and the conversion found to depend on temperature, but only slightly on pH. Under denaturing conditions, the conversion is much more rapid, suggesting that tertiary structure retards the rate of deamidation of the labile residue(s) and that neighboring groups are probably involved in deamidation of the unfolded form. Although the two forms are very similar by a number of criteria, there are differences between them in some assays, one of which showed the deamidated form to be the more active. The DNA sequence of human somatotropin shows some similarity with human growth hormone, including the rapidly deamidating region, Thr-Asn-Ser-His-Asn-His. Both Asns are good candidates for deamidation, though the reference cited above showed evidence for only one modified form. It is possible that both Asns deamidate, but that they are mutually exclusive, the deamidation of one precluding the deamidation of the other.

Crystallization of human somatotropin that has been isolated under harsh conditions yields crystals that are extensively disordered.[165] However, avoidance of extremes of pH, temperature,

and organic solvent in the preparation gave well-ordered crystals.[166]

T. Triosephosphate Isomerase

Two residues have been identified as sites of deamidation in human erythrocyte triosephosphate isomerase (TIM): Asn(15) and Asn(71).[77,80] Both of the labile Asns are succeeded by Glys in the amino acid sequence of the human enzyme and presumably deamidate by a β-aspartyl shift mechanism. The rate of deamidation of Asn(15) and Asn(71) *in vitro* increases with temperature and pH, and depends on the nature of the buffer ions, in a way consistent with general base catalysis. The deamidation half-time at pH 7.0, 37°C, is 22 or 38 d in 0.05 *M* triethylamine and 0.05 *M* sodium phosphate buffers, respectively.[83] The four deamidations appear to be part of an initial step in the degradation of this enzyme *in vivo*.[84]

There is an obligatory order of deamidation in human TIM in which Asn(71) deamidates before Asn(15). In the chicken enzyme, residue 71 is a Lys and no deamidation of Asn(15) occurs, supporting deamidation of the former as prerequisite for deamidation of the latter in the human enzyme. Furthermore, the conformations around residue 71 in the chicken enzyme are closer to optimal for the β-aspartyl shift mechanism than those around residue 15. In the chicken enzyme,[75] residues 15 and 71 from opposite subunits are juxtaposed across the subunit interface (Figure 15), so that an initial deamidation of Asn(71) in the human enzyme by the β-aspartyl shift mechanism introduces a new carboxylate group immediately adjacent to Asn(15) of the opposite subunit. The appearance of this new nucleophile may be the basis for the order of deamidation of the two residues.

The *in vitro* deamidation rate of human erythrocyte TIM is higher when the concentration of TIM decreases, and it has been suggested that this favors dissociation of the subunits, which is necessary to allow solvent to enter. However, the order of deamidation and the proximity of Asn(15) and Asn(71) from different protomers of the dimer suggest that subunit contacts are important for the cooperative deamidation. Dissociation of the dimers would abolish these close contacts and break any linkage between the deamidations of the distinct residues in different subunits. Furthermore, the crystal structure of the chicken TIM shows residues 15 and 71 to be exposed to solvent. The deamidated dimer is likely to be destabilized as a result of the close contact of the two new negative charges,[77] but there is no clear structural reason why subunit dissociation should favor deamidation. Bound substrate increases the rate of deamidation, possibly as a result of structural changes in the active site, which are transmitted to the subunit interface. This may be the reason why TIM deamidates more rapidly *in vivo* than *in vitro*.

Confirmation of the effect of deamidation on TIM stability comes from site-directed mutagenesis experiments on the yeast enzyme (see below). Replacement of Asn(78) [corresponding to Asn(71)], which is in the subunit interface, by Asp reduced the stability of the enzyme to denaturation and increased its susceptibility to protease degradation.[85,86] It also lowered the k_{cat} of the enzyme-catalyzed reaction.

U. Trypsin

From a neutron diffraction study of deuterated and undeuterated crystals of bovine trypsin,[87] Kossiakoff discovered that three Asn residues are deamidated. Asn(48), Asn(95), and Asn(115) show no density for deuterium at their side chain termini, and it is inferred from this that they have deamidated to Asps. All three Asns are followed in sequence by serine residues and show very similar local main chain torsion angles. Each is located just before a tight β-bend (see below) and makes a hydrogen bond from its side chain amide oxygen to the peptide –NH– of the n + 2 residue.

Kossiakoff has proposed that the deamidation reaction goes by way of a β-aspartyl shift mechanism that depends on the main-chain configuration. However, since all three Asps in the crystal structure appear in the normal α configuration, while the predominant product of this reaction is usually the β configuration, it is possible that the reaction is simply catalyzed by the neighboring Ser residue acting as a general acid. If a normal

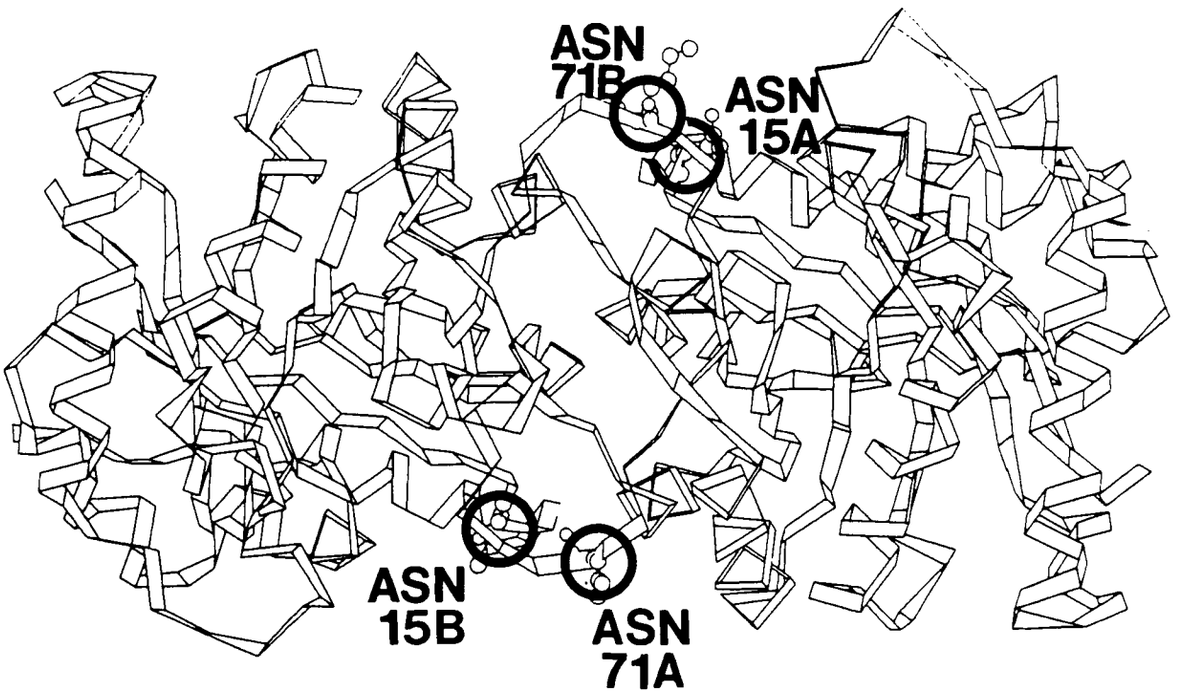

FIGURE 15. Backbone diagram of the dimer of chicken triosephosphate isomerase showing the positions of the labile Asn(15) and Asn(71) on each of the chains (A and B). (From Wright, H. T., *Protein Engineer*, 4, 283. With permission.)

β-aspartyl shift mechanism did occur, we must assume that crystallization selected out the α form or that constraints on the structure in the crystal changed the usual distribution of products in favor of the α-form. No solution measurements have been made of the deamidation of trypsin, but the exceptionally long time for crystallization and the high ionic strength used in these experiments may be necessary to observe these deamidations.

V. TWO TYPES OF DEAMIDATION

We can distinguish two types of Asn or Gln residues that deamidate in proteins. The first type is controlled by the sequence immediately adjacent to the Asn or Gln. These neighboring residues catalyze the deamidation reaction by the mechanisms described above. The second type of deamidating Asn or Gln is that which lies close to potential catalytic side chains of amino acids that are not all sequence proximal, but that are brought into spatial proximity as a result of the tertiary structure of the protein. The same residues can catalyze both types of deamidation, and the mechanisms of the deamidation reactions may be the same or similar (with the exception of the β-aspartyl shift mechanism).

A. Relationship between Protein Sequence and Deamidation

The data of Table 1 and Figure 1 and the earlier rate studies by Robinson and co-workers on peptide deamidation[1] confirm the important role of residues immediately adjacent to Asn and Gln. In peptides, the relatively low barriers between different conformations increase the frequency with which the peptide adopts a deamidating conformation. In larger peptides and proteins, secondary, tertiary, and quaternary structure effects will influence the conformational space to which various residues have access, and so determine whether residues that are potential catalysts of deamidation will be able to function in that role. Comparison of the deami-

dation rate of an Asn or Gln in a protein with that of a model peptide of the same sequence can indicate whether the neighboring sequence in the protein is the principal influence on the reaction. Similar rates for the peptide and protein deamidation reaction imply that this segment of the protein can enter the same conformational state(s) as the peptide and that the same steps in the deamidation are probably rate-limiting for the peptide and the protein.

Robinson used these comparisons to conclude that the first deamidation of cytochrome C and the deamidation of aldolase are under sequence control.[1,11] Differences in deamidation rate for the model peptide and the protein are evidence for an influence of higher order structure on the reaction in the protein. In proteins where the neighboring residues of a labile Asn or Gln provide no functional groups that could catalyze the reaction, it can be inferred that the protein conformation is essential to bring functional groups from elsewhere in the sequence into proximity with the labile amide.

B. Sequence Biases Around Asn and Gln

If the sequence and structure-dependent mechanisms of Asn and Gln deamidation discussed above occur, then we expect selection against them in favor of protein stability and for them in favor of protein lability. For sequence-dependent deamidation, such selection should show up as biases against or in favor of particular amino acids occurring adjacent to Asn and Gln.

To search for positive and negative biases in protein sequences around Asn and Gln residues, a database consisting of 1076 nonredundant sequences with a total of 399,153 amino acids was assembled and the frequencies of triplets of amino acids with either Asn or Gln as the middle amino acid were scored.[179] Expectation values (f_e) for the frequency of occurrence of pairs of amino acids surrounding Asn and Gln were based on the frequencies of the amino acids in the database. The fractional deviation of the observed frequencies (f_o) from the expected frequencies (f_e):

$$\frac{f_o - f_e}{f_e}$$

were tabulated. The distributions of these fractional deviations are approximately normal. Histogram plots of the standard normal deviate (expressed as multiples of sigma, the calculated standard deviation) for the positive and negative tails of the distributions of all X-Asn-Y and X-Gln-Y triplets are shown in Figures 16 and 17. The amino acids are ordered according to the polarity and bulk scales of Jones.[215] It should be noted that the properties of side chain bulk, hydrophobicity, and polarity follow similar rank order for the 20 naturally occurring amino acids, and therefore any biases in this distribution may reflect the effects of one or a combination of these properties upon the amino acid sequences of the database.

The positive tail of the X-Asn-Y histogram shows clear selection in favor of nonpolar residues both before and after Asn. Exceptions to this generalization are Asn-Asn-Asn and Cys-Asn-Cys triplets, which show the largest deviations from expected values, and their maxima have been truncated in the figure so that the other triplet values can be displayed on scale. No distinction is made in the database between thiol-cysteine or cysteine in disulfide linkage, so the significance of the high frequency of Cys-Asn-Cys cannot be fully assessed. The Asn-Asn-Asn triplet is somewhat exaggerated in frequency due to the bias in favor of all amino acids occurring next to themselves, but reasonable corrections to the observed high frequency for Asn-Asn-Asn triplets do not eliminate the significance of this higher frequency. The general bias in favor of nonpolar bulky neighbors of Asn is even more significant than these figures indicate, since Asn itself is a polar amino acid and would be expected to occur with higher frequency next to itself and other polar side chains.

The negative tail of the distribution for X-Asn-Y tripeptides is roughly complementary

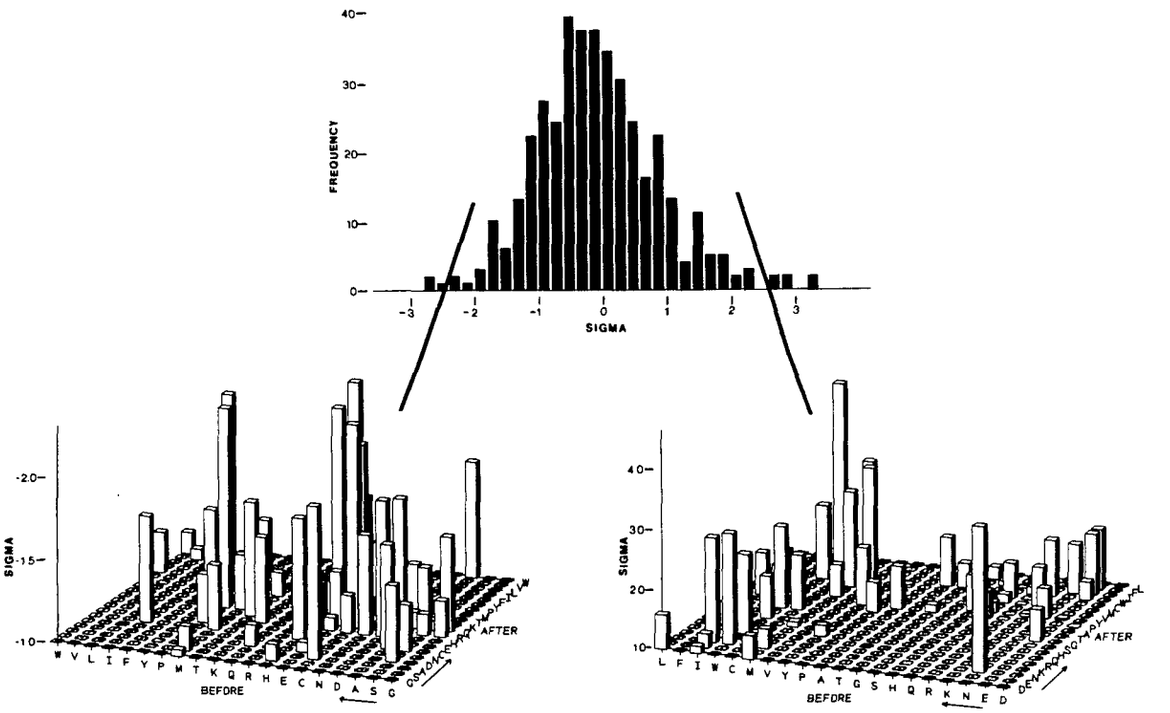

FIGURE 16. Frequency distribution of all pairs of amino acids surrounding asparagine tabulated from a selected, nonredundant database of 1076 proteins composed of 399,153 amino acids. The abscissa is measured in standard deviations from the mean for the positive (right) and negative (left) tails of the distribution. Frequency histograms for all amino acid pairs around asparagine that occur more than one standard deviation from the mean in the negative (left histogram) and positive (right histogram) tails of the distribution. The length of the bars is a measure of the deviation of these frequencies from the mean. For the negative tail of the distribution, the longer the bar, the higher the probability that this low frequency is nonrandom. For the positive tail of the distribution, longer bars measure increased probability that this high frequency is nonrandom. (From Wright, H. T., *Protein Engineer*, 4 283. With permission.)

to the positive tail. It shows a clear discrimination against certain polar residues as neighbors of Asn. In particular, there is discrimination against histidine, both before and after Asn: of the 20 most rejected X-Asn-Y triplets, histidine occurs 10 times in 9 of them.

It appears that there is a stronger consistency in the selection for bulky, nonpolar residues around Asn than there is against polar residues, which could catalyze deamidation. This difference between positive and negative selection may be reflecting other factors that influence deamidation rates in proteins. In addition to mutual constraints on sequence and secondary and tertiary structure of proteins, positive selection for nonpolar residues adjacent to Asn may reflect selection against potential deamidating configurations through the exclusion of water from thei vicinity.

For Gln, strong selection for Gln-Gln-Gl triplets, similar to the Asn-Asn-Asn triplets, oc curs, and also for Trp next to Gln. Both of thes may arise, in part, from the similarity in prop erties of Gln and Trp to the central Gln. Ther is also selection in favor of nonpolar, bulk neighbors of Gln, similar to that for Asn. Asp i strongly selected against as a neighbor of Gln and a few other polar amino acids (Ser, His) ar less strongly selected against. There is not th widespread selection against polar residues aroun Gln, as is observed for the neighbors of Asn. Th weaker negative bias for nearest neighbors of Gl may be related to the intrinsically slower rate o Gln deamidation (see above). It is interesting tha

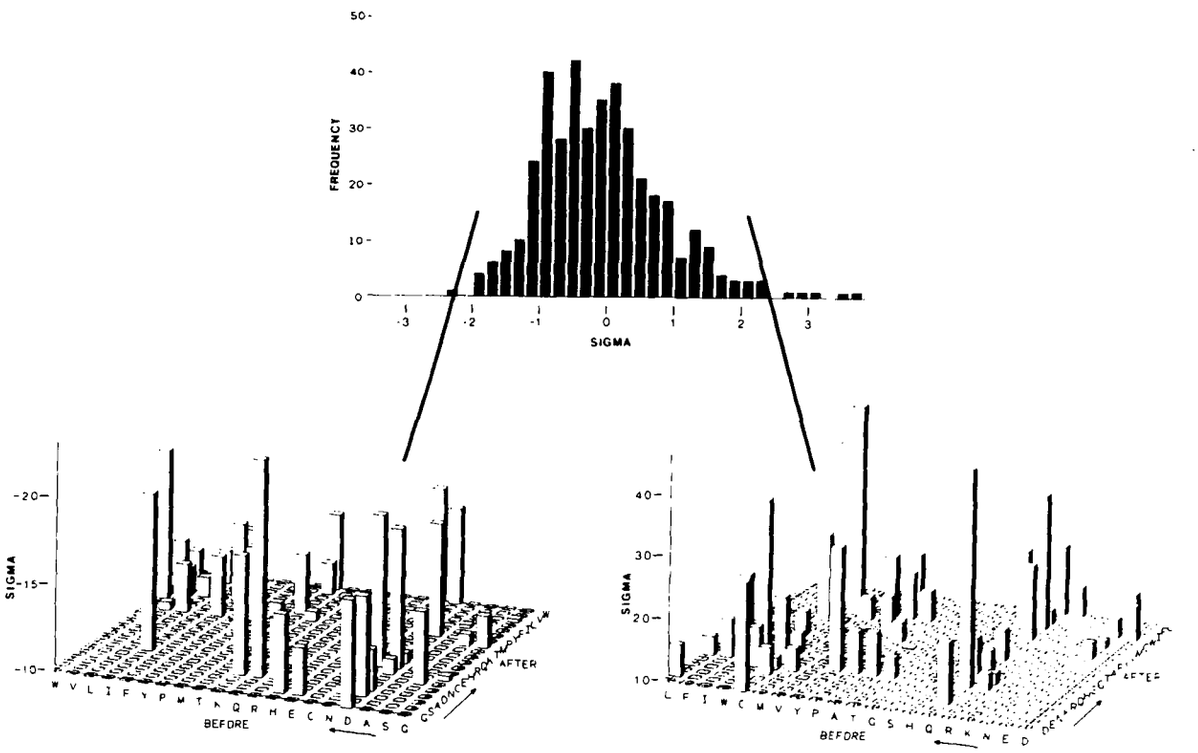

FIGURE 17. Same as Figure 13 but for amino acids on either side of Gln in the same database. (From Wright, H. T., *Protein Engineer*, 4, 283. With permission.)

the Gln-Gln-Asp triplet is very strongly selected against, while the Gln-Gln-Gln triplet is most strongly selected for. Clearly, the presence of the Asp and not the polar or bulk properties of the side chain is responsible for this discrimination.

Taken together, these preferences and rejections of neighboring amino acids of Asn and Gln suggest that there has been evolutionary selection in favor of large, functionally inert residues adjacent to Asn and Gln, and some selection against polar residues, particularly histidine adjacent to Asn and against Asp adjacent to Gln. These biases in sequence reflect requirements of secondary or tertiary structure, but the fact that peptide deamidation rates are frequently determined by sequence is consistent with selection that minimizes nearest-neighbor catalyzed deamidation of Asn.

VI. POLYPEPTIDE AND PROTEIN CONFORMATION EFFECTS ON DEAMIDATION

A. Local Conformation Effects on Sequence-Controlled Deamidation

There are currently more examples of nearest-neighbor, sequence-controlled deamidation than of deamidation catalyzed by residues remote in sequence from the labile amide. In the first class, the requirement that Asn or Gln occur adjacent in sequence to potential catalytic groups is a necessary but not sufficient condition for this type of deamidation. In order for the potential catalytic groups to function in the deamidation reaction, the polypeptide chain conformation and

the side chain orientations must permit close approach of the functional groups to the amide side chain.

Clarke[216] and Kossiakoff[87] have examined the local conformations around Asn residues in selected proteins of known structure. In the former work, the stereochemistry inferred for the formation of the succinimide intermediate in the β-aspartyl shift mechanism was used as a criterion for examining the conformations around Asn residues in a small number of known crystal structures. Using the criterion of minimizing the distance from the side chain amide carbonyl carbon to the amido –NH– of the next residue, Clarke found an optimal ψ angle of $-120°$ for the labile Asn. This is an unfavorable conformation, occurring only rarely as a type II′ β-hairpin turn.[217] The type II′ β turn in proteins occurs almost exclusively with a Gly in the second position of the turn. Theoretical and physical studies of small peptides having a succinimide at position 1 of this turn confirm this conformation, even for peptides where the amino acid following the succinimide ring at position 2 of the turn is as large as a Phe.[218-224] In a set of 10 known protein structures, Clarke[216] found no cases in which Asn or Asp occur in this type of turn. A survey of the Asn-Gly sequences in 9 proteins of known structure also revealed that none (or at most one) of the 11 Asn-Gly sequences approached the presumed optimal conformation for nucleophilic attack from the amido –NH– group of the Gly. A survey of all Asn and Asp residues in this same set of proteins also showed none of them in conformations with a $\psi = 120°$ in which the following amido –NH– group would be at minimal distance from the side chain amido carbonyl carbon. Clarke[216] concluded that it is a general restriction on conformation that excludes Asn and Asp, and all other residues, from the range of ψ values in which the succinimide intermediate could form.

Succinimide intermediate formation in the β-aspartyl shift mechanism also requires side chain dihedral angles of $\chi_1 = 120°$ and $\chi_2 = +/-90°$. Clarke[216] found that these values did not occur in the 11 protein structures examined. This requirement may not be as restrictive as that on the main chain conformation, because Asn (and Gln) occur predominantly on the surface of proteins of known structure,[225,229] where constraints on side chain rotation are likely to be lower than in the interior of proteins. This exposed location also guarantees access of solvent water to any succinimide intermediate, which will consequently be unstable and break down to the α- and β-products.

The conformation that Clarke has postulated to be the most favored for succinimide intermediate formation is the one presumed to occur in the case of base-catalyzed nucleophilic attack, where the peptide –NH– group has been transiently deprotonated. Abstraction of the proton from the peptide –NH– group by a general base might be sterically obstructed in the conformation suggested by Clarke. The deprotonation of the attacking peptide nitrogen prior to attack on the amide side chain could occur in a different conformation from that which is optimal for the bond breaking and bond making around the transition state of the deamidation reaction. Then, changes in the local peptide chain structure or the change in hybridization of the peptide nitrogen could orient it for attack on the C_γ of the Asn side chain. Since a general base-catalyzed reaction seems the most probable mechanism for this reaction, any consistencies in the observed structures of proteins that undergo the β-aspartyl shift mechanism at Asn-Gly sequences may reflect susceptibility to proton abstraction rather than to succinimide formation.

It is also possible that a peptide nitrogen still carrying its proton could attack the side-chain carbonyl carbon. Although this is unlikely, it might occur if there were an increase in positive charge at the side chain amide carbon as a result of interaction of the amide group with electron-withdrawing constituents. In this case, the most favorable orientation of the attacking peptide nitrogen would be with the plane of the peptide bond parallel to that of the side chain amide group of the Asn. In this orientation, the lone pair orbitals of both the peptide nitrogen and the side chain C_γ atom will be perpendicular to their trigonal planes and have maximum overlap.

Kossiakoff[87] examined the conformation around three Asn residues in trypsin that were found by neutron diffraction to have deamidated. The three Asn residues [Asn(48), Asn(95), and Asn(115)] are all followed by a Ser. Kossiakoff

noted that these three Asn residues have similar main chain and side chain conformations, each occurring in the turn noted by Richardson[226] in which the Asn side chain Oδ1 oxygen hydrogen bonds to the amido –NH– of the n + 2 residue. The main chain conformations around these residues have values of ϕ/ψ: Asn(48) −171/180, Asn(95) −118/108, and Asn(115) −153/168, which differ significantly from the ψ = −120° postulated by Clarke. The hydrogen-bond interaction of the side chain would have to be disrupted and the side chains rotated by about 80° about their Cα-Cβ bonds (χ1) to be brought into position for attack by the succeeding peptide nitrogen. In this position, all three Asn-Ser sequences have Ser side chain conformations that permit hydrogen bonding to the side chain of the potential labile amide group. Kossiakoff[87] suggests that this Ser residue could also be oriented to form a hydrogen bond to its own amido –NH– and thereby assist in its deprotonation, which is presumed necessary for the nucleophilic attack on the side chain amide group. This implies an important role for the hydrogen bonding of potential functional groups in determining whether a residue will deamidate by this mechanism.

There is no experimental evidence that these three Asn residues in trypsin deamidate through a succinimide intermediate. As mentioned above, all three are in the usual α-configuration, the less favored product of the β-aspartyl shift mechanism. Given the requirement for disruption of hydrogen bonds and a change in main chain conformation in order for these residues to deamidate through the succinimide intermediate, it is more parsimonious to conclude that the deamidation may simply occur through general acid and/or base catalysis by the Ser residues following the labile Asns.

Richardson[226] has noted several properties of Asn that distinguish it from the other amino acids in protein structures. Asn is more likely than any other residue, except glycine, to have ϕ,ψ angles outside the normally allowed regions. Also, Asn often forms hydrogen bonds different from those of Gln or Asp. Wright[179] has cataloged the main chain conformations (ϕ/ψ plots) around all Asn and Gln residues that occur in proteins of known structure (Table 1) that undergo deamidation (Figure 18). The conformational angles for the labile amide residue and the two amino acids on either side of it are tabulated. The angle values for those residues that deamidate are noted. With this restricted database, there is no evidence that Asn occurs more frequently outside the range of allowed values for main chain torsion angles. There does not seem to be any strong selection for conformation at the other positions examined, with the exception of the residue following the labile amide group. The higher frequency of conformational angles in the usually unfavorable region of positive ϕ values is due to the predominance of Asn-Gly sequences in this population. Since these conformations are accessible primarily to Gly residues, it is possible, as has been suggested elsewhere,[199] that the tendency of some Asn-Gly (or Asp-Gly) sequences to undergo the β-aspartyl shift mechanism may depend on local conformation.

The higher frequency with which deamidating Asn residues are followed by a residue with positive ϕ values, usually accessible only to glycine, may be related to the orientation of the n + 2 residue.[179] For example, values of +80° and +40° for the residue n + 1 to Asn direct the orientation of the n + 2 residue. If the n + 2 residue has main chain conformational angles of an α helix, then the peptide –NH– of the n + 2 residue can form a hydrogen bond to the Asn side chain oxygen (Figure 19a). This could stabilize the tetrahedral, oxyanion transition state of the Asn during the deamidation reaction. This stabilizing hydrogen bond cannot form if the n + 2 residue has conformational angles in the range of those of a β-sheet (Figure 19b). In both the α-helical and β-sheet conformations for the n + 2 residue, for n + 1 in the range of ϕ = +80° and ψ = +40°, the peptide nitrogen of n + 1 can be well oriented for side on attack of the amide side chain of the Asn. However, only in the α-helix conformation for the n + 2 residue can the stabilizing hydrogen bond form. The frequency with which residues n + 2 to deamidating Asn occur in the α-helix conformation is higher than that for β-sheet, but the database is still too small to draw firm conclusions on this point.

Although the question is still open, the preference for Gly, and to a lesser extent Ser and Ala, after labile Asn in the β-aspartyl shift mechanism may be due less to obstruction by the bulk

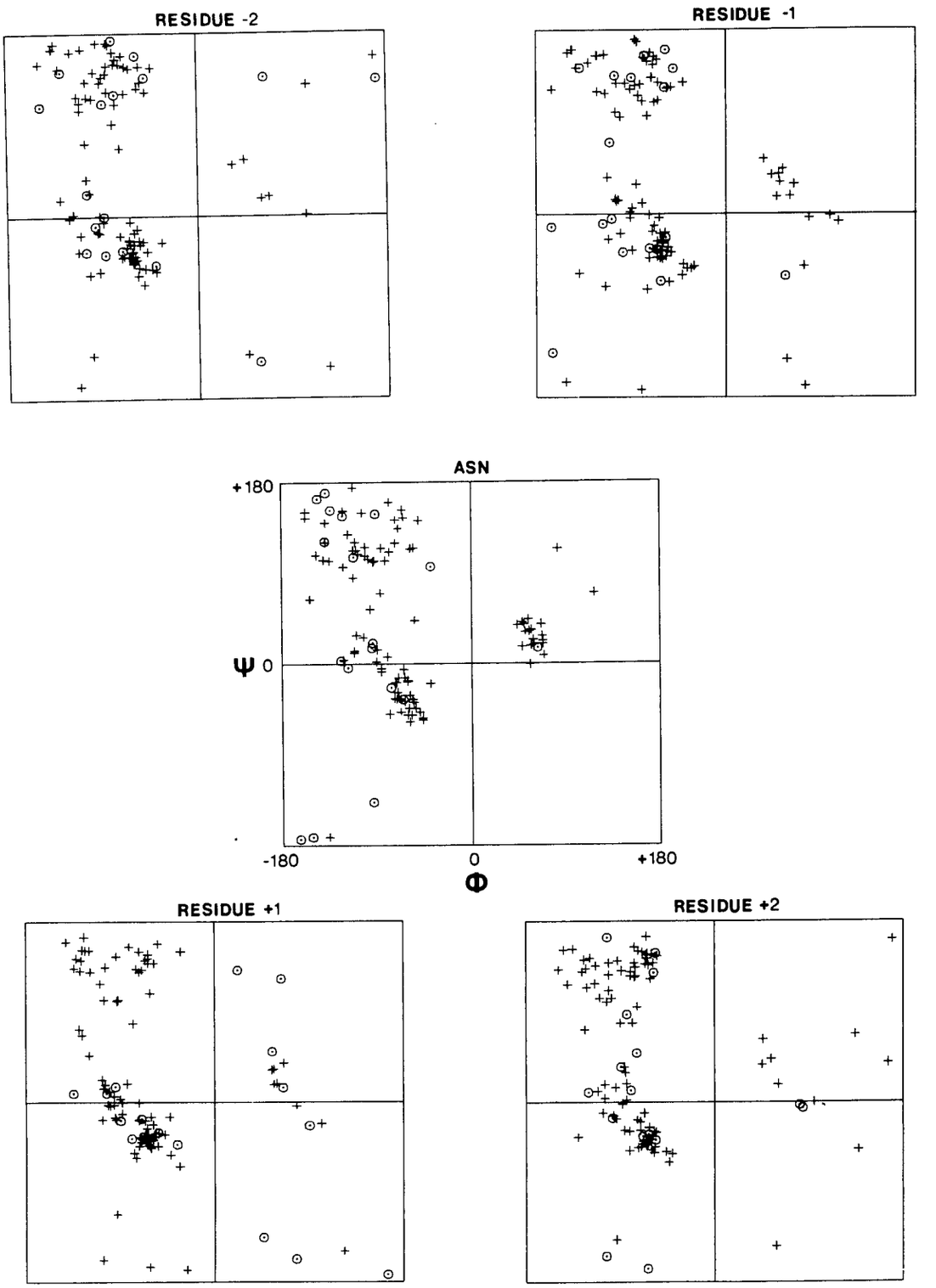

FIGURE 18. ϕ/ψ plots for the five residues centered on each Asn that occur in the proteins of known structure in Table 1. Circles denote values for those residues that deamidate. (From Wright, H. T., *Protein Engineer*, 4, 283. With permission.)

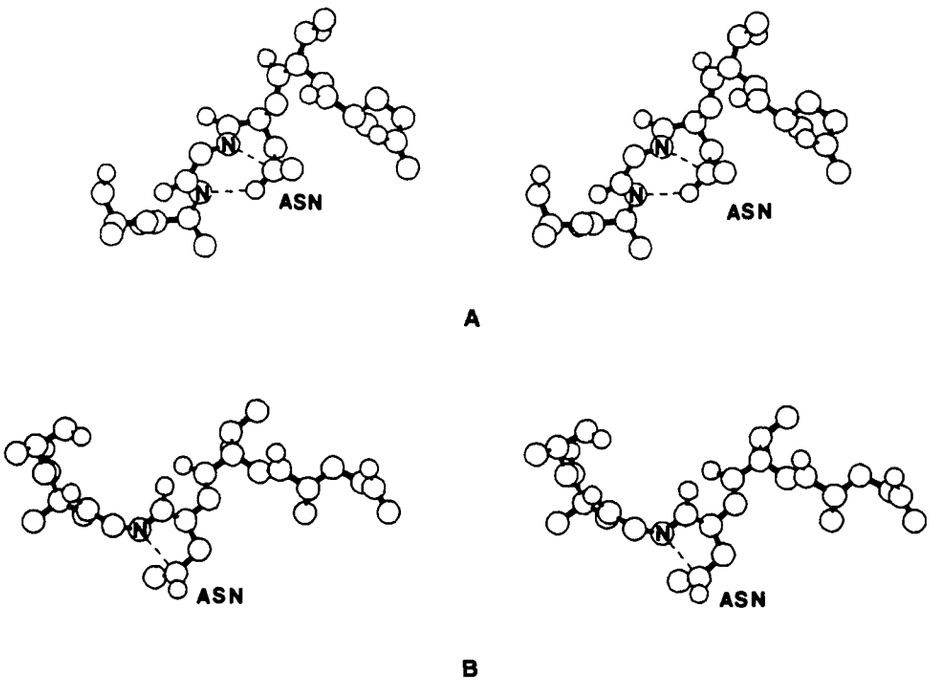

FIGURE 19. Stereo views of Asn residues in Asn-Gly sequences showing potential hydrogen bonds for (a) α helical conformation, (b) β-sheet conformation.

of the following side chain than to the main chain conformation of that residue, though these two effects are difficult to separate.

The structure of chicken triosephosphate isomerase suggests how local conformation may impose the obligatory order in which Asn(15) and Asn(71) deamidate in the human TIM. The chicken enzyme has Lys at position 71 and does not undergo deamidation like that of the human enzyme. Examination of the main chain ϕ and ψ angles for the chicken enzyme structure shows that the conformation around residue 71 is slightly more favorable to the β-aspartyl shift mechanism than that around residue 15. These differences are only significant if the human enzyme has the same conformation as the chicken enzyme used in this analysis, but if true it may explain the order of deamidation in the human enzyme.

The tabulation by Robinson and co-workers[1] of deamidation rates for Asn and Gln peptides provides a catalog in which sequence is the dominant influence on the rates. Unfortunately, none of the peptides studied had an Asn-Gly sequence, and there are only a few controlled measurements of the deamidation rates of Asn-Gly peptides.[180,199] These latter measurements show the Asn-Gly deamidation rate in hexapeptides to be more rapid than any of the peptides measured by Robinson and co-workers. Asn-Gly sequences may be the most rapidly deamidating sequences in proteins, but this sequence effect, and all others, are clearly dependent on secondary and tertiary structure, since the majority of such sequences in proteins do not undergo the deamidation reaction.

Asn, Asp, Gln, and Glu show some differences in their segregation into different types of secondary structure,[227,228] which makes it likely that destabilization of some of these structures could occur as a result of deamidation. Asp and Glu are preferred over Asn and Gln in the amino-terminal segment of α-helices, although all four amino acids are favored in this region. More recently,[214] it was noted in a large set of helices that Asn occurs with a significantly higher frequency than other amino acids at the amino-terminal residue initiating the helix. No strong preferences for amidated or carboxylate forms of these amino acids occur within α-helices or at their carboxyl termini. Both Asp and Glu are selected

against at the amino terminus of β-sheet regions relative to Asn and Gln. All four of these residues are strongly selected against at internal β-sheet positions, but there is strong selection against Glu and for Gln at the carboxyl termini of β-sheets. No distinction between Asn and Asp at flanking, turn regions of the α-helix and β-sheet were found, and only minor selection in favor of Gln over Glu at the amino terminus and Glu over Gln at the carboxyl terminus of β-sheets. Asn occurs frequently just before type I tight turns with its side chain amide oxygen hydrogen bonded to the main chain amido –NH– of the n + 2 residue (Figure 20).

Asn and Gln occur most frequently on the surface of proteins. In a selected set of proteins, 88% of Asn and 93% of Gln residues were found on the surface,[229] where they are exposed to bulk solvent, which is necessary for deamidation. Although there is only limited information, differences in the interaction of Asn and Gln with water molecules may distinguish them from each other and from their deamidated Asp and Glu products. Abraham and Leo[230] have noted the frequent hydration of Gln residues in deoxy-hemoglobin A,[231] in which a water molecule is immobilized through hydrogen bonding to the Gln side chain and to main chain proton donors or acceptors. Similar stereochemistry cannot occur with Asn or Asp, because of the shorter extension of its side chain, and the Glu side chain will be restricted to only one of the two types of conformation that Gln can adopt in sequestering water. This tendency of Gln to trap and immobilize water could also contribute to the reduced rate at which it is deamidated relative to Asn.

B. Tertiary and Quaternary Structure Effects on Deamidation

Although their number is still small, the class of labile Asn and Gln residues that appear to deamidate as a result of catalysis by residues brought into their proximity by higher order structure may be more interesting than those under the control of adjacent sequence. In particular, those that deamidate more rapidly than would be predicted from the peptides of the same sequence constitute a group where the tertiary structure provides a conformation that accelerates the deamidation reaction. As discussed above, the rate of deamidation of Gln is intrinsically slower than that of Asn in peptides and proteins. Several factors were cited as probable causes of this difference: diminished stabilization of the tetrahedral oxyanion intermediate, unfavorable stability of the intermediate in the β-aspartyl shift-type mechanism, and possible exclusion of free water. Therefore, anomalously rapid deamidation of Gln is likely to be the result of tertiary or quaternary conformations that catalyze the deamidation.

There are only a few good examples of this second type of structure-controlled deamidation: cytochrome C, triosephosphate isomerase, insulin, and hemoglobin Providence. However, a number of other cases of this type of deamidation almost certainly appear in Table 1, particularly where the sequence immediately around the labile residue provides no functional groups to catalyze the deamidation.

The second (and probably the third) deamidation of cytochrome C described above are un-

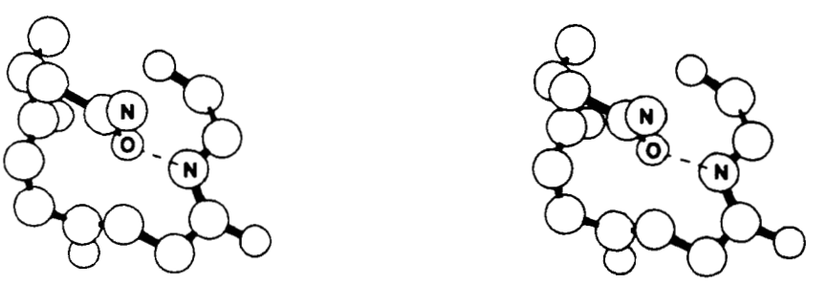

FIGURE 20. Stereo view of hydrogen-bond interaction of Asn side chain with main-chain amide –NH– in β turns. Proton of hydrogen bond is omitted for clarity.

usual in that their rates are slow in the native protein but anomalously rapid, relative to pentapeptides of the same sequence, in cytochrome C that has undergone prior deamidation of Asn(103) near the carboxyl terminus. The deamidation of Asn(103) appears to be controlled by neighboring sequence, as judged by the similarity of its rate to that of the free tetrapeptide of the same sequence. In contrast, the second labile amide, Asn(54),[33] deamidates slowly in the native protein, but more rapidly than a model pentapeptide containing its same tripeptide sequence, after the deamidation of Asn(103).[11] It has been proposed that the first deamidation results in a change of tertiary structure that opens a new conformation in which the second amide is rapidly hydrolyzed.[1]

Inspection of the cytochrome C structure shows that the side chains of Asp(50) and Lys(53) are close to the Asn(54) side chain and could function as a nucleophile and as a transition-state stabilizer, respectively, if local constraints on their orientation were relaxed. This appears to be a clear care where the native structure inhibits deamidation, and changes in it result in configurations that can catalyze the deamidation reaction. The identity of the third deamidating residue in cytochrome C is not known, but Asn(31) (see above) is a likely candidate.

Human triosephosphate isomerase is an interesting example of a dimeric enzyme of identical subunits undergoing two deamidations on each subunit. The obligatory order of deamidation described above suggests that changes resulting from the first deamidation are necessary to permit the second deamidation.

In the structure of the native, dimeric chicken triosephosphate isomerase, the residues corresponding to the two pairs of Asn-Gly that occur in the human enzyme lie at the interface of the subunits. The deamidation of Asn(71) on each monomer results in two negative charges, which lie close to the side chains of the other labile Asn(15). These new carboxylate groups could participate directly in the deamidation as general bases, or they could induce changes in the conformation or interaction of the subunits that promote the second deamidation. The failure of Asn(15) to deamidate significantly in the chicken enzyme, which lacks an Asn at position 71, is further proof of the requirement for either the newly deamidated Asp group and/or changes in conformation or quaternary interactions resulting from the prior deamidation of Asn(71).

Deamidation in hemoglobin Providence occurs at the site of the point mutation Lys(β82) ⇒ Asn. The mutant Asn residue may deamidate by a β-aspartyl shift mechanism involving Gly(β83), though the conformation in wild-type human hemoglobin at this site is unfavorable. It seems more likely that His(β143) catalyzes this deamidation, either as a nucleophile or as a general acid and/or base. His(β143) is bound to diphosphoglycerate in the deoxy form of hemoglobin, but in the oxy form it is free and presumably solvated.

C. Deamidation and Protein Folding

It has been shown that the irreversible thermal denaturation of ribonuclease, lysozyme, and α-amylase is controlled largely by the deamidation of Asn and Gln at acid and neutral pH,[232-235] and that a single deamidation in ribonuclease can affect folding kinetics.[236] These studies have resolved the longstanding question of the principal mechanism by which thermally, irreversibly denatured proteins lose their activity. At acid pH, ribonuclease also undergoes peptide bond cleavage with heating to 90°C. These cleavages occur almost exclusively at Asp residues (carboxyl-terminal side), which raises the possibility that deamidation could yield new potential sites of cleavage in a degradation process mechanistically similar to the thermal denaturation reaction.

The observation that deamidation of Asn residues is a principal cause for the irreversible denaturation of proteins has important implications for the role of Asn and Gln in the folding of proteins, their assembly into biologically active complexes, and their breakdown. The rate and extent of deamidation during thermal denaturation are increased by the increase in temperature.[2,3] A factor of equal if not greater importance is the loss of constraints on secondary and tertiary structure that occurs when the protein is heated. It has been shown from arguments based on the rates of deamidation of Asn and Gln in peptides

vs. their inferred rates as free amino acids[12] that incorporation of Asn and Gln into polypeptides increases their rates of deamidation. Although the extent of deamidation in native proteins is almost certainly underestimated, the fact that most amides in proteins deamidate only slowly, if at all, suggests that deamidation is slow in proteins. This failure of deamidation rates in peptides to extrapolate to proteins implies that the intrinsically rapid rates of deamidation of Asn and Gln in short peptides are suppressed by the higher order folding and aggregation states of native proteins. This is also supported by observations made during sequencing of proteins, in which isolated peptides undergo deamidation at sites that are stable in the intact protein. Some of the entries in Table 1 fall in this class and are more pertinent to a discussion of deamidation in peptides than in proteins. The harsh conditions necessary to observe deamidation in certain proteins (e.g., ribonuclease) imply that the native protein is relatively resistant to deamidation.

While we think of conformations such as the β-turn as being stabilized by the side chain hydrogen bond of an Asn residue (Figure 20), it may be equally correct in this case to think of the protein conformation as stabilizing the amide side chain of Asn against deamidation. Stabilization against deamidation of Asn and Gln may also be conferred by the sequestration of potential catalytic side chain groups through their hydrogen bonding or interaction with cofactors or ligands.

Kossiakoff[87] has proposed a protective effect of hydrogen bonding against deamidation of certain residues in trypsin. He documented the hydrogen bonds in trypsin that appear to inhibit deamidation of Asn(34) as one example of why this Asn does not deamidate. Three other Asns followed by a Ser do deamidate, but Asn(34), which is in a similar conformation and followed by a Ser, does not deamidate. Other nondeamidating Asn residues in trypsin are hydrogen bonded to main chain peptide nitrogens or are in conformationally constrained regions of the peptide chain. Kossiakoff infers that the constraints imposed by hydrogen bonding on the main chain in which an Asn lies may be more important in inhibiting deamidation than whether the side chain of the Asn is hydrogen bonded.

Ligand binding can also block deamidation by complexing with potential catalytic side chains. Hemoglobin Providence deamidates at Asn(β83) in the oxy form but not in the deoxy form with diphosphoglycerate bound. The ligand hydrogen bonds to the side chain of His(β143) and may prevent its participation in the deamidation reaction in the deoxy form of the protein. The histidines that complex with the Zn atom of carbonic anhydrase are potential catalysts of the deamidation of Asn residues, if the Zn is removed, and deamidation of calmodulin is diminished at increased calcium concentrations (see above). Kossiakoff[87] notes that one of the constrained, nondeamidating Asns in trypsin lies in a loop that is stabilized by the binding of calcium. As suggested above, these apo forms of the proteins would then be more susceptible to deamidation, and this would be a means of selectively removing them from the pool of holoproteins.

D. Deamidation and Protein Crystallization

Although it is peripheral to the structural basis and possible biological consequences of deamidation, the influence of deamidation of proteins on their crystallizability has been clearly demonstrated in several instances. Proteins often fail to crystallize under a wide variety of conditions or crystallize in forms that are too small or so poorly ordered that the limit of resolution is too low to yield useful information. There are an increasing number of examples of proteins that crystallize in forms that are inadequate for X-ray diffraction analysis, but that are improved to usability by careful fractionation or by the avoidance of purification conditions that promote deamidation.[120,166,237] This improvement in crystal quality with elimination of microheterogeneity in protein samples may arise from removal of bad contacts in the crystal lattice where a residue is variable (Asn and Asp or Gln and Glu) in the population of molecules composing the crystal. Destabilization of tertiary structure as a result of microheterogeneity induced by deamidation could also have the effect of raising the thermal or statistical temperature factor of segments of molecules in the crystal lattice, and so diminish lat-

tice order, with consequent effects on the resolution of the diffraction pattern.

E. Point Mutations in Proteins

1. Natural Point Mutations

The occurrence of point mutations leading to replacement of one amino acid by another has been known since the discovery of the molecular basis for the difference between normal and sickle-cell hemoglobin S.[238] The ability to clone and sequence regions of DNA in which point mutations are known to occur has drastically increased the number of cases in which the amino acid replacement resulting from a point mutation is characterized (see below).

Attempts to relate naturally occurring sequence changes to phenotypes usually involve rationalizations of changes in the properties or functions of proteins in which the single amino acid change occurs. Instances where an amino acid residue is shown to be mutated to an Asn or Gln require particular care in interpretation, because of the possibility that the mutant residue deamidates to Asp or Glu or forms a mixture of the mutant and deamidated forms. These deamidations are somatic mutations leading to post-translational products that are also natural amino acids and will not be detected by amino acid sequence analysis alone. On the other hand, a DNA sequence reveals the Asn or Gln residue coded for, but provides no information on the abundance of these residues or their deamidated forms in the translated protein. This latter must be provided by amino acid sequence analysis. The cases of the mutant hemoglobins described above illustrate the possible pitfalls in relying exclusively on either the DNA or the amino acid sequence in determining the nature of point mutations to Asn and Gln, and in understanding the mechanisms by which they determine phenotype.

2. Deamidation in Engineered Proteins

The effects of sequence and tertiary structure on the rate of Asn and Gln deamidation have implications for the design of proteins to be produced by recombinant DNA methods in conjunction with site-directed mutagenesis. Particular sequences or configurations containing Asn or Gln residues will be more susceptible to deamidation than others and should be taken into account in considering the properties and stability of the protein to be synthesized. Furthermore, the final steps of biosynthesis in which the newly synthesized polypeptide chain folds into its native conformation can be affected by intentionally introduced mutations.

One such example is that of dihydrofolate reductase, which was used to test mutant suppressor tRNA function.[36] Site-directed mutagenesis of the cloned dihydrofolate reductase structural gene introduced Stop-Asn codons in place of Val-Asp codons at amino acid positions 10 and 11. Translation of the mutant sequence using a serine-inserting amber suppressor tRNA at the Stop codon yielded full-length dihydrofolate reductase with a Ser-Asn replacing the Val-Asp sequence. Edman analysis of the dihydrofolate reductase synthesized with Ser-Asn at positions 10 and 11 showed significant deamidation of Asn(11). This is consistent with the high frequency with which serine occurs next to deamidating Asns in proteins (see Figure 1).

An example of the application of site-directed mutagenesis to the study of the role of deamidation in proteins is that of triosephosphate isomerase (TIM). The double deamidation of the monomeric subunits in dimeric human TIM was described above. These deamidations are both at Asn-Gly sequences and presumably occur by way of the β-aspartyl shift mechanism. The tertiary and quaternary structures are also important in their effects on the order of residue deamidation.

To assess the influence of Asn residues on the stability of the dimer, Asn residues in the intersubunit interface were replaced.[86] The substitution of either of the two Asn by threonine or isoleucine led to an increase of 25% in the stability of the resulting mutant enzyme to thermal denaturation. The double replacement of Asn(14) and Asn(78) by threonine and isoleucine, respectively, led to a doubling of the enzyme stability to thermal denaturation. Conversely, the substitution of an Asp residue for Asn(78) [corresponding to Asn(71) in human TIM] led to a decrease in enzyme stability to thermal denatur-

ation and produced an enzyme that was unstable at room temperature. The effect of this latter replacement is proposed to be on the stability of the subunit interaction, as was suggested by earlier work on the deamidation of this enzyme.[77,83] These experiments further strengthen the earlier conclusion that irreversible thermal denaturation of proteins is due to the deamidation of Asn residues. The low frequency of Asn in triosephosphate isomerase from *B. stearothermophilus* (2 Asn) relative to the same enzyme from *E. coli* (10 Asn) is consistent with selection against deamidation, which would be more rapid at higher temperature.

With a larger catalog of half-lives for peptides containing Asn and Gln, we expect that some rules will emerge that will permit investigators to anticipate the effects of substituting different amino acids at various positions in proteins of known sequence, and in the best cases, known tertiary structure. Experiments using site-directed mutagenesis to introduce Asn and Gln residues into proteins of known tertiary structure will be valuable in testing the emerging rules for predicting the deamidation rates.

A survey of site-directed mutagenesis experiments in which Asn or Gln are introduced in place of another amino acid shows that experimenters seldom check the integrity of the mutated Asn or Gln in the expressed protein by amino acid analysis. This runs the risk that effects observed, both *in vitro* and *in vivo*, are the result of an Asp or Glu resulting from the posttranslational deamidation of Asn or Gln. Furthermore, amino acid replacements other than Asn and Gln may precipitate deamidation of preexisting Asn and Gln through the introduction of catalytic groups or through changes induced in the structure.

VII. POSSIBLE PHYSIOLOGICAL ROLES FOR DEAMIDATION

Robinson et al.[6] first suggested that deamidation may be a molecular timer that determines the *in vivo* turnover time of proteins. Although there is little direct evidence to support this hypothesis, it is consistent with several observations. A few proteins show increased proteinase susceptibility in their deamidated forms relative to their native forms (see next section). Recent site-directed mutagenesis experiments, and the demonstration that thermal denaturation of certain proteins is irreversible as a result of deamidation, also support decreased conformational stability for deamidated forms, and by implication, increased susceptibility to proteolytic degradation. Since the deamidation of Asn or Gln introduces a new charged amino acid into a protein, it is comparable to the incorporation of amino acid analogs, which has been shown to increase sensitivity to proteases.[239,240]

Any hypothetical role for deamidation in timing the degradation of proteins *in vivo* would have evolved closely with the sequence and structure of the proteins. The discrimination among amino acids as immediate neighbors of Asn and Gln described above may be a manifestation of selection pressures, driven in part by the tendency of Asn and Gln to deamidate. Selection pressures on tertiary structure that discriminate for and against deamidating configurations would influence the evolution of protein conformation through sequence changes as well. Fixation of point mutations that introduce or remove Asn or Gln residues or their neighbors from environments where deamidation is favored would be a mechanism by which the lifetime of certain proteins could be modulated by adjustments in sequence and/or conformation.

If it is true, as proposed here, that the teritary structure of proteins suppresses the rate of Asn and Gln deamidation, then the continued existence of rapidly deamidating residues, particularly those in configurations determined by the tertiary structure, suggests that the retention of their lability is an aspect of their function as timers. The apparent requirement for protection against the deamidation of Asn and Gln, and the possible role of these residues as timers of protein turnover, may have conferred an important role on these two amino acids in the evolution of protein sequence and structures.

A. Deamidation as a Regulator of Protein Integrity

1. Deamidation and Protein Breakdown

Although some proteins can undergo deamidation reactions with little or no loss of biological activity, and presumably, little loss of tertiary structure, others do lose activity. In some of these cases, the loss of activity is due to specific effects, such as the destabilization of bound cofactor (alcohol dehydrogenase from *Drosophila*) or subunit interactions (triosephosphate isomerase). The effect of introducing a new negatively charged group into a protein will depend on its location in the structure and on the magnitude of the sum of the stabilizing forces that maintain the native structure. Urry[241] has proposed that apolar-polar interactions can modulate protein folding and subunit assembly through effects on ordered solvent. The cross-linked polymer of the repeating peptide of elastin (L-Val-L-Pro-Gly-L-Val-Gly)$_n$ ($n > 120$) exists in a β-spiral structure with temperature-dependent contractile properties. Introduction of 20% Glu residues at position 4 results in an assembly that undergoes contraction and relaxation as a function of pH[242,243] due to folding and unfolding of the β-spiral. Deamidation of Gln or Asn in mono- or oligomeric proteins could produce similar effects, leading to unfolded states that are more susceptible to further deamidation and to proteolytic attack, with subsequent accelerated degradation of the protein.

The deamidation of cytochrome C, rabbit muscle aldolase, and human triosephosphate isomerase may be linked to proteolytic processes that are responsible for regulating their concentrations and degradation *in vivo*. As mentioned in an earlier section, the large excess of undeamidated over deamidated forms of cytochrome C (8:1) *in vivo* can be best accounted for by increased susceptibility of the deamidated forms to degradation. The first deamidation near the carboxyl terminus, which is sequence controlled, appears to control the half-life of cytochrome C *in vivo*.[11] Formation of this singly deamidated form increases the rate of the second deamidation, possibly through changes in structure induced by the first deamidation. Whether the singly or more highly deamidated forms are more susceptible to proteolytic degradation is not known, but their low abundance *in vivo* suggests that they are more rapidly degraded than the native form.

The *in vitro* degradation of rabbit muscle aldolase is initiated at the carboxyl terminus,[108,244,245] where a single rapid deamidation occurs. Cathepsins B and L have been shown to release carboxyl terminal dipeptides sequentially from this protein. Cytochrome C and rabbit muscle aldolase are similar in that their initial deamidations occur near the carboxyl terminus of the protein. These deamidations are probably controlled by a neighboring sequence and require the reduced constraints on structure that a chain terminus may have. Insulin and epidermal growth factor (Table 1) also deamidate near a chain terminus.

The mutant hemoglobins in Table 1 differ in the abundances of the several mutant forms and wild-type chains. These data are only suggestive of possible relative rates of degradation, since the amounts of each form initially synthesized are not known. Hemoglobin Providence shows a ratio of about 7:4 of the deamidated to the undeamidated mutant form[39] and a measurable increase in the abundance of the deamidated form over the lifetime of the erythrocyte.[42] This suggests that there is not a preferential degradation of the deamidated form or that the degradative process is saturated. Hemoglobin Singapore has about a 3:1 ratio of normal A chain to mutant A chain, which is consistent with an increased rate of degradation of the mutant form. Hemoglobin Wayne shows very low levels *in vivo*, and may be more rapidly degraded than the wild type, though other explanations are possible.[44]

Human triosephosphate isomerase undergoes two deamidations on each of its identical monomeric subunits. The resulting negatively charged Asp residues lie in the interface between the two subunits and may destabilize their association. This deamidation could show cooperative properties if deamidated subunits exchange with undeamidated ones, which are then more rapidly deamidated as a result of the Asp residues in the subunit interface. Dimeric TIM is resistant to enzymatic proteolysis, but dissociation of subunits leads to rapid degradation by trypsin.[77] Deamidation of Asn or Gln residues lying in the

interface of multisubunit proteins could be a general mode of initiating degradation processes by favoring dissociation to protease-susceptible monomers.

Rogers and Rechsteiner[246] have correlated the properties of 35 well-characterized proteins with their metabolic half-lives in HeLa cells. Of the many properties included in these correlations, only Asn and Gln content showed a significant (inverse) correlation with *in vivo* half-life. Other indirect observations are consistent with a role for deamidation in regulating the rate of protein breakdown. Dice and Goldberg[247,248] found a positive correlation between low isoelectric point and shorter half-life for a small collection of proteins. Since deamidation results in a decrease in isoelectric point, its occurrence could shorten the half-life of a protein, if the above correlation reflects some aspect of protein degradation processes. Possibly related to this is the observation of Momany et al.,[249] who found an inverse correlation between *in vivo* half-life and excess surface density of Asx and Glx residues in a small sample of proteins.

The above examples are possible instances where deamidation(s) initiate the process of degradation of the protein. The rapidly deamidating Asns are embedded in sequences and structures that promote the deamidation and that may increase the susceptibility of the protein to other degradative processes. It is proposed here that there is a second, more general influence of Asn and Gln deamidation on the breakdown of proteins *in vivo*. It arises from a fundamental relationship between protein structure and the lability of Asn and Gln side chains. The enhanced rates of deamidation of Asn and Gln in peptides relative to the free amino acids[12] are suppressed in proteins by sequence selection and higher order structure. Intrinsically flexible regions of a protein or changes in the integrity of protein structure that destabilize secondary or tertiary structure are likely to lead to increased rates of deamidation. Further deamidations increase the stability of unfolded forms relative to native, which in turn promotes more deamidations. Such a cooperative process could serve as a switch that rapidly shunts incorrectly folded or damaged proteins into protease-susceptible unfolded conformations for degradation. Inhibition of refolding of proteins to their native state by deamidation has the effect of making unfolding processes irreversible. Deamidation is thus unique among the many posttranslational modifications of proteins.[250] Any of these reactions, including deamidation itself, that destabilize protein structure will lead to increasing rates of deamidation among other Asn and Gln residues and drive unfolding processes in native proteins.

Asn and Gln are uniquely suited to such a role as a resident regulator of protein breakdown. They are natural amino acids and their deamidation is a simple hydrolytic reaction, requiring no other reactants. Also, it depends only on protein sequence and conformation, and not on exogenous agents. The reaction is irreversible, even to potential repair enzymes, since the reamidation of Asn and Gln cannot occur without amidation of Asp and Glu residues that are correctly coded for. Any such reamidation repair pathway would inhibit refolding to native conformation, because of these incorrect amidations.

2. Deamidation and Protein Biosynthesis

Deamidation may regulate folding of newly synthesized proteins, as well as the unfolding of older ones. Newly or partially synthesized proteins that are incorrectly or incompletely folded will be at least transiently more susceptible to deamidation than their native, completely folded forms. Studies have revealed that protein conformation is an important determinant in the export of secretory and integral membrane proteins from the endoplasmic reticulum.[251] Through the use of antibodies specific for unfolded forms of influenza virus hemagglutinin, it was shown that non-native forms of the protein are trapped in the endoplasmic reticulum and not secreted.[251] Furthermore, protein(s) endogenous to the endoplasmic reticulum appear to bind to newly synthesized but misfolded proteins within that compartment and to prevent them from being further processed.[251-254] I suggest that some of the forms of proteins trapped in the endoplasmic reticulum may have undergone deamidation and that the helper proteins have the function of stabilizing incompletely folded or incompletely synthesized proteins against deamidation.

The *N*-glycosylation of Asn residues is initiated in the endoplasmic reticulum, in contrast to most other glycosylations, which are carried out in the Golgi apparatus.[255] The integrity of the Asn receptor for the glycosyl group is an essential determinant of the overall glycosylation reaction, and prior deamidation would render it dysfunctional. It may be significant that the first step of *N*-glycosylation occurs in the endoplasmic reticulum, since glycosylation results in relative protection of the amide group to deamidation by transforming a primary amide group to a more stable secondary amide. In addition to its pleiotropic functions in cellular metabolism,[256] glycosylation of Asn in the endoplasmic reticulum may serve a protective function in preventing deamidation before completion of the synthesis, folding, and processing of new proteins.

B. Deamidation and Aging

The possibility that protein and peptide nonenzymatic deamidation could be a timing mechanism regulating aging was first proposed by Robinson et al.[6,257,258] Deamidation has been shown to occur with aging of stored proteins and in proteins that turn over little or not at all during the lifetime of the cell or organ in which they occur. The accumulation of deamidated forms of proteins *in vivo* with aging could result from either or both of two general changes in the organism: increasing rate of Asn and Gln deamidation with age, or decrease in rate at which deamidated protein molecules are degraded with age.

The latter possibility is known to be true[250] and has been ascribed, in part, to a decrease in activity of cellular proteases responsible for the degradation of aberrant protein forms. While it may seem unlikely that the rate of deamidation of Asn and Gln residues increases with age, the hypotheses of the previous section provide a basis for understanding how this might occur. If, as proposed, the higher order structure of proteins suppresses Asn and Gln deamidation, any aberrant forms of proteins in the population are likely to have higher rates of deamidation than the thermodynamically most stable form. If the degradation machinery is saturated, accumulation of these forms would be expected to occur. The recent studies mentioned above on the processing of secretory proteins in the endoplasmic reticulum and Golgi apparatus imply that checking mechanisms on the aggregation state and integrity of the folding of protein molecules exist. Deterioration of this checking mechanism with age could lead to the accumulation of slightly misfolded species of protein structures, particularly in the endoplasmic reticulum. Some studies have found evidence for the accumulation of modified forms of proteins with increasing cellular and organismic age.[109-111,259-268] Holliday has noted[269] that enzymes in which age-related changes have been detected are frequently polymeric. This may reflect the stabilizing influence of subunit interactions, which diminish the rate of misfolding and other predegradative events. If, as proposed here, the higher order structure of proteins is important in suppressing deamidation, these misfolded forms will undergo further deamidation and unfolding with increased susceptibility to proteolytic degradation. If there is no compensating increase in the degradative mechanisms for removing these forms, and/or if these mechanisms are saturated, then deamidated species will accumulate.

There are a few studies of protein deamidation that have been correlated with age. The lens crystallins are a useful family of proteins for study of the long-term damage to proteins, since they turn over little or not at all during the lifetime of the organism. Aged human αa_x-crystalline deamidates at Asn(101) in a His-Asn-Glu sequence.[126] Chicken αA-crystallin progressively deamidates at Ser-Asn(149)-Met from 15% deamidated form at 4 months of age to 50% at 1 year and 70% at 10 years.[270] McFadden and Clarke[271] have shown that the susceptibility to methylation of lens crystallins by type II methyltransferases increases with age, consistent with an increase in the abundance of β-isoaspartyl linkages as a result of deamidation. However, it was noted in that work that there is a decrease in endogenous methyltransferase enzymes in lens with age, which could lead to higher values of methyl acceptor available. The same observation of age-dependent increase of methyl acceptor has been made earlier on human erythrocyte methylation substrate sites.[272,273] and on cerebral, membrand-bound proteins.[274] The physiological

function of the methyl transferase enzymes is not known, but it has been frequently proposed that it is a repair enzyme for aberrant isoAsp linkages, for which deamidation is one cause.[200,207,275-278]

An increase in abundance of the deamidated forms of TIM with age has also been observed in human fibroblasts, both from old donors[79] and from cells in culture,[78] from patients with Werner's syndrome and progeria,[78] from erythrocytes,[76] and in the eye lens.[279]

An interesting connection between deamidation and the tendency of τ-protein in the brain to associate in the neurofibrillary tangles characteristic of Alzheimer's disease has been made.[280] τ-Protein that has been denatured in urea or treated with glutaminase enters into neurofibrillary tangles *in vitro*, whereas control samples do not. It is not known whether this Gln deamidation occurs *in vivo*, and, if so, whether it is enzymatic or nonenzymatic, but its occurrence under denaturing conditions *in vitro* demonstrates a connection between the stability of that Gln and the integrity of the structure of τ-protein.

There are some general observations in the literature that could implicate deamidation in the aging process.[281,282] Deamidated forms of proteins could elicit immune responses to newly exposed antigenic determinants, which could lead to autoimmune effects.[283] Lukash et al.[284] have found that deamidated serum albumin has altered antigenic properties, making it immunogenic in the body, and has suggested that these changes could induce autoimmune reactions during aging. The appearance of a new class of molecules with new properties would also lead to many side reactions that do not occur with the native, undeamidated molecules. For instance, deamidation of amide groups in membrane-bound proteins could change the association of the protein with the membrane and alter the many critical functions of membrane proteins in controlling cellular exchange with the environment.

The possible release of Asn-linked carbohydrate as a result of deamidation,[12] though unlikely, could have widespread metabolic effects in an organism. There are a variety of functions for protein-linked carbohydrates, including those of cell surface receptors and signals for catabolism. Disruption of these and other cellular processes as a result of carbohydrate loss due to deamidation would likely lead to complicated sequelae.

In view of the still primitive state of understanding of the determinants and structural and functional consequences of deamidation in proteins, these speculations on the possible role in aging of Asn and Gln deamidation in proteins are premature. However, the mechanisms suggested here for the role of deamidation in protein degradation and biosynthesis may provide a starting point for testing its involvement in organismic processes.

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