Pharmaceutical Research, Vol. 6, No. 11, 1989

Review

Stability of Protein Pharmaceuticals

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Recombinant DNA technology has now made it possible to produce proteins for pharmaceutical applications. Consequently, proteins produced via biotechnology now comprise a significant portion of the drugs currently under development. Isolation, purification, formulation, and delivery of proteins represent significant challenges to pharmaceutical scientists, as proteins possess unique chemical and physical properties. These properties pose difficult stability problems. A summary of both chemical and physical decomposition pathways for proteins is given. Chemical instability can include proteolysis, deamidation, oxidation, racemization, and β-elimination. Physical instability refers to processes such as aggregation, precipitation, denaturation, and adsorption to surfaces. Current methodology to stabilize proteins is presented, including additives, excipients, chemical modification, and the use of site-directed mutagenesis to produce a more stable protein species.

KEY WORDS: protein stability; biotechnology; mutagenesis; denaturation.

INTRODUCTION

With the recent advances in recombinant DNA technology, the commercial production of proteins for pharmaceutical purposes has become feasible (1,2). As a result, the preparation of proteins as medicinal agents has become an integral part of the pharmaceutical industry. Currently, there are more than 150 recombinant proteins in Phase I clinical trials or beyond, and almost a dozen have received FDA approval. Unfortunately, proteins possess chemical and physical properties which present unique difficulties in the purification, separation, storage, and delivery of these materials. Therefore, formulation of proteins differ greatly from that of rigid small organic molecules. Future pharmaceutical scientists will need to be properly trained to address the various aspects of protein instability. An introduction to these concepts is presented below, with the view that understanding protein stability at a molecular level is essential to solving many of their formulation problems.

Degradation pathways for proteins can be separated into two distinct classes, involving chemical instability and physical instability. First, chemical instability can be defined as any process which involves modification of the protein via bond formation or cleavage, yielding a new chemical entity. Second, physical instability does not involve covalent modification of the protein. Rather, it refers to changes in the higher order structure (secondary and above). These include denaturation, adsorption to surfaces, aggregation, and precipitation. A summary of the current understanding of each of these processes is presented and illustrated by well-characterized systems. Finally, approaches for retarding or inhibiting these processes and, thereby, increasing protein stability is presented.

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[3] Unless otherwise noted, all amino acids listed are L-enantiomers of the 20 common amino acids and are referred to by their three-letter abbreviations.

CHEMICAL INSTABILITY

A variety of chemical reactions is known to affect proteins (Fig. 1). These reactions can involve hydrolysis, including both cleavage of peptide bonds as well as deamidation of Asn and Gln side chains.[3] Hydrolysis at Asp-X sites is particularly accelerated. Oxidation of Cys can lead to disulfide bond formation and exchange, whereas oxidation of Met and other amino acids may inactivate or alter the activity of a protein. Other decomposition reactions include beta-elimination and racemization.

Deamidation

In the deamidation reaction, the side chain amide linkage in a Gln or Asn residue is hydrolyzed to form a free carboxylic acid. Over the past two decades many investigators have observed altered forms of proteins which have been attributed to deamidation. Such a list contains lysozyme (3), bovine growth hormone (bGH) (growth hormone is also known as somatotropin) (4), human growth hormone (hGH) (5,6), insulin (7,8), α-crystallin (9), cytochrome c (10), γ-immunoglobulin (11), epidermal growth factor (EGF) (12), hemoglobin (13), triosephosphate isomerase (TIM) (14,15), neocarzinostatin (16), prolactin (17), gastrin releasing peptide (18), and adrenocorticotropic hormone (ACTH) (19,20), suggesting that *in vitro* deamidation is a common phenomenon.

The hydrolysis of Asn and Gln residues for many proteins and peptides has been observed under a variety of

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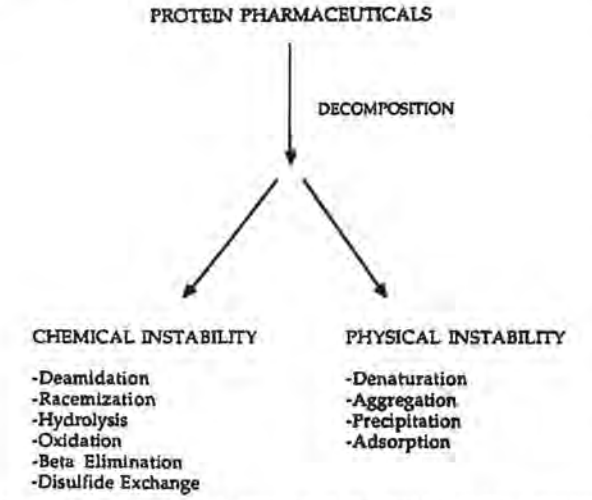

Fig. 1. Summary of the chemical and physical instability processes observed in protein pharmaceuticals.

chemical conditions and has been reviewed by Robinson and Rudd (21). Interestingly, it was realized that the deamidation of Asn residues, which occurs most often at the sequence Asn-Gly, was accelerated at neutral or alkaline conditions (22–24). The rates were also higher relative to the hydrolysis of the amino acid Asn itself (21). An explanation is that deamidation is believed to proceed through a five-membered cyclic imide intermediate formed by intramolecular attack of the succeeding peptide nitrogen at the side chain carbonyl carbon of the Asn residue (see Fig. 2) (25). Subsequently, the cyclic imide spontaneously hydrolyzes to give a mixture of peptides in which the polypeptide backbone is attached via an α-carboxyl linkage (Asp) or is attached via a β-carboxyl linkage (iso-Asp) (23). Similarly, Gln can also undergo deamidation via formation of a six membered ring (23). Most of the information on the mechanism and rate of deamidation of

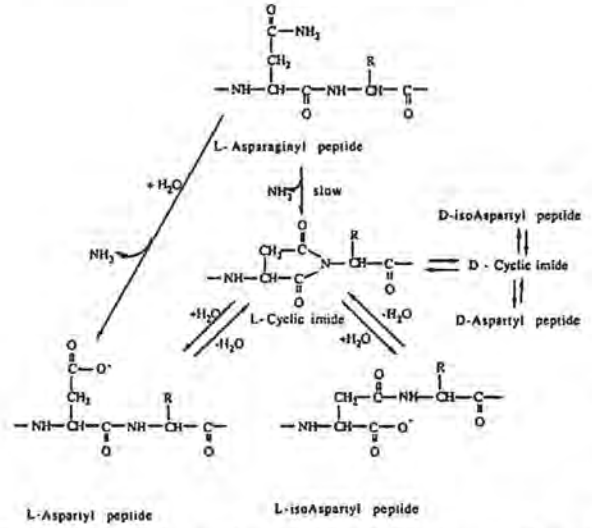

Fig. 2. Pathways for spontaneous deamidation, isomerization, and racemization for aspartyl and asparaginyl peptides.

Asn residues has been obtained from studies on short model peptides (26,27). Clear evidence for a deamidation mechanism involving the cyclic imide intermediate has been obtained by Geiger and Clarke (26). In their study, deamidation of a hexapeptide sequence based on residues 22–27 of ACTH (Val-Tyr-Pro-Asn-Gly-Ala) was studied at 37°C and pH 7.4. Evidence from these studies supporting cyclic imide formation include the appearance of iso-Asp, Asp, and cyclic imide peptides upon deamidation and a ratio of the iso-Asp to Asp peptide formed in the deamidation of this hexapeptide (2.8:1) is the same as that found when purified cyclic imide is hydrolyzed (3.1:1). If there is a significant amount of direct solvent hydrolysis of the amide linkage occurring, the proportion of Asp peptide relative to iso-Asp peptide in the deamidation of a hexapeptide would have increased, which is not the case. The presence of iso-Asp products from the incubations of proteins and peptides implies cyclic imide formation as an intermediate in deamidation reaction (28–32). The Fourier transform infrared photoacoustic spectroscopic measurements (FTIR-PAS) have also provided direct evidence for the formation of a cyclic imide in peptides with Asn-Gly sequences induced by heating in the dry state (33).

Recently, we have shown that both Asp- and iso-Asp-hexapeptides are formed upon deamidation of Val-Tyr-Pro-Asn-Gly-Ala, $ACTH^{22-27}$ (Asn-hexapeptide), in the pH range of 5 to 12 at 37°C (34,35). This further confirms the formation of a cyclic imide intermediate in the deamidation process at neutral and alkaline pH's. In the pH range 7 to 12, buffer concentration had significant effect on the rate of deamidation, indicating general acid-base catalysis. No buffer catalysis was observed at pH 5, 6, and 6.5. The ratio of iso-Asp- and Asp-hexapeptides was independent of buffer concentration at all pH's and was approximately 4:1. At acidic pH's (pH 1–2), the deamidation was much slower than at alkaline pH, and only Asp-hexapeptide was produced upon deamidation. Although iso-Asp-hexapeptide was not detected at acidic pH, one new product (Val-Tyr-Pro-Asp) was observed by HPLC. These results suggest that at acidic pH, the probable mechanism of deamidation is direct hydrolysis of the amide side chain of Asn, to form the Asp-hexapeptide, which further degrades in acidic media via peptide bond cleavage at the Asp-Gly bond. Reactions at pH 3 and 4 were very slow at 37°C (degradation of Asn-hexapeptide was not detected for 60 days).

By comparison, when deamidation experiments were carried out with ACTH (1–39), the separation of Asp- and iso-Asp products could not be achieved by either isoelectric focusing or cation-exchange HPLC (20). However, these techniques did separate native ACTH from the deamidated ACTHs (Asp- and iso-Asp-ACTH). The rate constants for the deamidation of both ACTH and Asn-hexapeptide ($ACTH^{22-27}$) at pH 2.0, 7.0, and 9.6 at 37°C were similar. Formation of the iso-Asp product upon deamidation of ACTH at pH 7.0 and 9.6 was verified by the protein carboxymethyltransferase (PCM)-catalyzed methylation of deamidated ACTH. No such methylation was observed when ACTH was incubated at pH 2.0, 37°C. These data indicate the involvement of cyclic imide intermediate at neutral and alkaline pH but not at pH 2.0.

Since the formation of a cyclic imide involves participation of the succeeding amino acid, the size and physico-

chemical properties of neighboring amino acid side chain is expected to play an important role in the rate of formation of cyclic imide. Evidence in support of this conclusion comes from studying the rates of cyclic imide formation in peptides containing Asn (26) or Asp β-benzyl esters (36–45). For example, the rate of cyclic imide formation at pH 7.4 was approximately 50 times slower in the Asn-Leu-hexapeptide than the Asn-Gly-hexapeptide due to steric hinderance by the Leu side chain (26).

Robinson and co-workers (46–49) investigated the nonenzymatic deamidation of Asn residues in synthetic pentapeptides, and the effects of amino acid sequence, pH, temperature, buffer species, and ionic strength. Using synthetic pentapeptides, it has been shown that deamidation is favored by increased pH, temperature, and ionic strength (46,47). These studies showed the importance of primary sequence around the Asn residue, but did not investigate the formation of either iso-Asp or cyclic imide. Similar results are obtained for cytochrome c (50). Similarly, the rate of deamidation of human TIM was facilitated by high temperatures, and was also found to be dependent on the presence of substrate and specific buffers (14). Unlike the hydrolysis of peptides containing esters of Asp, where the cyclic imide intermediate can be trapped (22,24), cyclic imide formation during the deamidation of Asn peptides is the rate determining step (26,27).

The rates of deamidation of Asn residues in proteins are influenced by the secondary and tertiary structures of proteins. Clarke has shown that Asp and Asn residues in native proteins generally exist in conformations where the peptide bond nitrogen atom cannot approach the side chain carbonyl carbon without large scale conformational changes (51). Therefore, certain proteins will not undergo deamidation unless they have been denatured. Cyclic imide could only be formed *in vitro* at Asn^{67} of bovine pancreatic ribonuclease in the unfolded state (52). While in the native structure, this residue is poorly positioned for cyclic imide formation. Similarly, it has been shown that urea (a strong denaturant) accelerates the deamidation of bGH, hGH, and prolactin, presumably by unfolding the protein (5). Tertiary structure appears to be the principle determinant for the deamidation of trypsin (53). The study also showed that adjacent Ser residues aid in the formation of the cyclic imide intermediate, consistent with earlier studies on small peptide systems (36,37). Recently, Lura and Schirch (54) have shown that the mechanism of deamidation of Val-Asn-Gly-Ala and N-acetyl-Val-Asn-Gly-Ala varies according to the conformation of the peptide backbone. Above pH 9.0, both peptides have similar conformations and thus deamidate by the same mechanism to give mixture of Asp and iso-Asp peptides. However, at pH 7.0, while the N-acetyl peptide yielded a mixture of Asp and iso-Asp peptides, the non-acetylated peptide gave no detectable amounts of these products, but rather yielded a cyclic peptide believed to be formed by nucleophilic attack of the amide of the Asn residue by the terminal amino group.

It is well known that peptides of Asp esters undergo intramolecular cyclization, under both acidic and basic conditions, leading to a cyclic imide derivative (39,55). However, no reports are available showing the formation of cyclic imide from Asn peptides in acidic media. There are few examples in the literature which at least indicate that cyclic imide is not involved in the deamidation reaction under acidic conditions. For example, insulin (7,8), neocarzinostatin (16), and ribonuclease A (56), when incubated in acidic media, yield only Asp-containing products from the deamidation of Asn residues. Similar results were obtained by Meinwald and co-workers (27), where Ac-Asn-Gly-NHMe produced only Ac-Asp-Gly-NHMe and the analogous iso-Asn produced only the iso-Asp-containing peptide after 1 day in 1 *M* HCl.

It has been postulated that deamidation may play a central role as a timer in protein turnover and in aging (21). However, for pharmaceutical preparations, the major concern is the change in protein function upon deamidation. In a few cases, the deamidation of specific Asn residues has been linked to the changes in the protein function, for example, deamidation at two Asn-Gly sequences in TIM resulted in subunit dissociation (15). Deamidation at an Asn-Gly site in a hemoglobin mutant (Hb providence) changed its oxygen affinity (57), and deamidation at an Asn-Asp site in hGH altered its proteolytic cleavage properties (58). Recently, deamidation was shown as one of the major chemical processes responsible for irreversible enzyme inactivation of lysozyme (59) and ribonuclease (60) at 100°C. Deamidation was also responsible for the decrease in biological activity for porcine ACTH (62) and slower rate of refolding after deamidation for ribonuclease (63,64).

With small peptides, the iso-Asp and Asp peptides are separable by chromatographic or electrophoretic methods (65–67). However, with larger proteins similar methodology has not been successful. Chromatofocusing (68) and HPLC (20,29) have been used for separating the native protein from the product mixture, but these techniques do not separate the iso-Asp-peptide from the Asp-peptide. However, there are several indirect ways of showing the presence of iso-Asp residues in proteins. These include (i) NMR methods to distinguish Asp and iso-Asp (27,54,69); (ii) Leu aminopeptidase digestion, since this enzyme will not cleave an iso-Asp peptide bond (67,70); (iii) tryptic peptide mapping and amino acid sequencing (71); and (iv) use of PCM, which is known to methylate selectively the free α-carboxy group of iso-Asp peptides (72). Recently, Johnson and co-workers have shown the use of this enzyme as a powerful analytical tool for estimating minimum levels of protein deamidation (73). In their work, they monitored the increase in methylation for aldolase, bovine serum albumin, cytochrome c, lysozyme, ovalbumin, ribonuclease A, and TIM upon incubation at pH 11, finding evidence that iso-Asp is formed upon deamidation.

Oxidation

The side chains of His, Met, Cys, Trp, and Tyr residues in proteins are potential oxidation sites. Even atmospheric oxygen can oxidize Met residues. Oxidation has been observed in many peptide hormones during their isolation (74–77), synthesis (78), and storage (79). Since the thioether group of Met is a weak nucleophile and is not protonated at low pH, it can be selectively oxidized by certain reagents under acidic conditions (80). For example, hydrogen peroxide can modify indole, sulfhydryl, disulfide, imidazole, phe-

nol and thioether groups of proteins at neutral or slightly alkaline conditions, but under acidic conditions the primary reaction is the oxidation of Met to Met sulfoxide (81). In addition to hydrogen peroxide, a variety of other reagents have been used to oxidize Met to Met sulfoxide. These include periodate, iodine, dimethylsulfoxide, a dye-sensitized photooxidation, chloramine-T, and *N*-chlorosuccinamide (82,83). To oxidize Met to Met sulfone, more drastic conditions and reagents are needed, e.g., 95% performic acid. The structures of the oxidation products of Met, i.e. Met sulfoxide and Met sulfone, are shown in Fig. 3.

Oxidation of Met residues to their corresponding sulfoxides is associated with loss of biological activity for many peptide hormones [e.g., corticotropin (84), α- and β-melanotropins (85), parathyroid hormone (86), gastrin (87), calcitonin (88), and corticotropin releasing factor (77)] as well as nonhormonal peptides and proteins (81). It has been shown that *E. coli* ribosomal protein L12 loses activity after oxidation of Met residues to Met sulfoxide and that the activity can be restored by incubating the protein with high concentrations of β-mercaptoethanol (89). Restoration of biological activity was found to coincide with the reduction of Met sulfoxide to Met (89). Alpha-1-proteinase inhibitor protein, which is a major serum inhibitor of elastase activity, loses its ability to inactivate elastase when chemically oxidized (90,91). Oxidation by hydrogen peroxide of a single Met residue in subtilisin at pH 8.8 occurs concurrently with changes in kinetic parameters of the enzyme, although it does not abolish enzymatic activity (92). Similar results were obtained with a disulfoxide derivative of α-chymotrypsin (93,94), and trypsin (95). In many cases, such as parathyroid hormone (86), ribonuclease S-peptide (96), ribonuclease (97), and lysozyme (98), reduction of Met sulfoxide by thiols results in the recovery of nearly full biological activity.

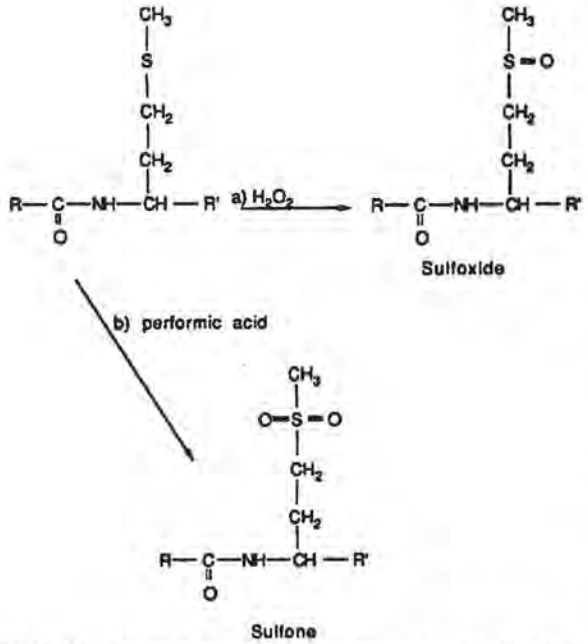

Fig. 3. Mechanism of oxidation of Met-containing peptide under (a) mild and (b) strong conditions.

There are also examples where protein functions are not affected upon Met oxidation. Active monosulfoxide derivatives of pancreatic ribonuclease (99), α-chymotrypsin (100), and Kunitz trypsin inhibitor (101) have been prepared using mild hydrogen peroxide treatment at low pH (pH 1 to 3). Similarly, EGF (102,103) and glucagon (104) are biologically active when chemically oxidized.

It is also shown that within a given protein, the reactivity of Met residues towards oxidation may be different depending upon their position. For example, in hGH, Met^{170} was found to be completely resistant to oxidation by hydrogen peroxide (105). In addition, it was shown that when biosynthetic hGH is chemically oxidized at Met^{14}, it exhibits full biological activity and has immunoreactivity identical to that of authentic hGH (6). In human chorionic somatomammotropin (hCS), Met^{64}, Met^{16}, and Met^{179} have markedly different reaction rates (105). The oxidation of Met^{64} and/or Met^{179} markedly reduced both its affinity for lactogenic receptors and its *in vitro* biological potency (105).

Determination of oxidized Met in proteins is generally a problem, because during conventional amino acid analysis Met sulfoxide is converted to Met during acid hydrolysis. Therefore, Met is commonly determined by using its specific reactions with alkyl halides (106) or cyanogen bromide (107), to which the sulfoxide is resistant. After alkylating the Met residues of the peptide, its Met sulfoxide is oxidized with performic acid to the acid stable sulfones; the sulfone content, determined by amino acid analysis, is then used to correct the Met estimate obtained by conventional amino acid analysis (99). Alternatively, Met containing peptides have been separated from peptides containing oxidized Met residues by ion-exchange chromatography (108), countercurrent distribution (109), HPLC (103,110), or affinity chromatography (111). A radioassay for non oxidized Met in peptide hormones based on its specific reaction with iodo[2-^{14}C]acetic acid is also developed (112).

The thiol group of Cys (RSH) can be oxidized in steps, successively, to a sulfenic acid (RSOH), a disulfide (RSSH), a sulfinic acid (RSO_2H), and, finally, a sulfonic acid (RSO_3H), depending upon reaction conditions. The factors which influence the rate of oxidation include the temperature, pH, and buffer medium used, the type of catalyst (e.g., traces of metal ions), and the oxygen tension (113). An important factor is the spatial positioning of the thiol groups in the proteins. In those cases where contact between thiol groups within the molecule of the protein is hindered, or when the protein contains only a single thiol group, intramolecular disulfide bonds are not formed, but sometimes, under favorable steric conditions, intermolecular disulfide bonds arise, and the protein aggregates (114). Thiol groups are oxidized not only when oxidizing agents (e.g., iodine, ferricyanide, tetrathionate, *O*-iodosobenzoate, and hydrogen peroxide) are added, but also "spontaneously," by oxygen from the air (autooxidation). The oxidation of thiol groups by molecular oxygen takes place at an appreciable rate in the presence of catalytic quantities of metal ions, such as iron and copper ions (115,116). The speed of oxidation of thiol groups is also greatly influenced by the nature of neighboring

groups. This was clearly demonstrated by Barron *et al.* (117) and also by Ovaberger and Ferraro (118). From their findings it appears that the rate of oxidation of dithiols is diminished on increasing the distance between the thiol groups in the molecule and also under the influence of neighboring electronegative groups such as carboxyl group (i.e., groups that raise the pK_a of the thiol group). This fact indicates that the mercaptide ion is oxidized more easily than the undissociated thiol group. Thus, it is shown that usually the oxidation rate increases with increasing pH (119). At 90°C and pH 8.0, α-amylase from *Bacillus* was shown to undergo irreversible thermoinactivation due to air oxidation of the Cys residues along with formation of incorrect or "misfolded" structures (120). Inactivation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by hydrogen peroxide has been shown to result from sulfhydryl group modification to sulfenic acid (114). Various methods for quantitative determination of thiol and disulfide groups in proteins are described by Torchinskii (121).

The side chains of His, Tyr, Met, Cys, and Trp residues can also be oxidized by visible light in the presence of dyes, i.e., via photooxidation. The specificity for the various amino acid side chains is particularly determined by pH. Oxidation of His is a rapid reaction at neutral pH but is quite slow at low pH. At higher pH, Tyr is most reactive (122–124), while Trp and Met are the only amino acids readily oxidized below pH 4. More information on photooxidation is available in a few review articles (125–127). In many cases, loss of enzymatic activity following photooxidation has been attributed to the destruction of critical His residues. For example, the inactivation of rabbit muscle aldolase (128), pig heart aspartate aminotransferase (129,130), cytochrome c (131), renin, and yeast enolase (132) has been attributed to photodegradation of His residues.

Proteolysis

It has been established that peptide bonds of Asp residues are cleaved in dilute acid at a rate at least 100 times faster than other peptide bonds (133). Selective hydrolysis is usually achieved by heating for 5–18 hr at 110°C in either 0.03 *N* HCl or 0.25 *N* acetic acid (134). The mechanism of hydrolysis undoubtedly involves intramolecular catalysis by a carboxyl group of the Asp residue. Hydrolysis can take place at either the N-terminal and/or C-terminal peptide bonds adjacent to the Asp residue. Inglis (135) has described the mechanism for such hydrolysis as shown in Fig. 4, where cleavage of the N-terminal peptide bond would proceed via an intermediate containing a six membered ring rather than via a five-membered ring as proposed for C-terminal peptide fission. Such peptide bond cleavage can contribute to the inactivation of proteins. Significant irreversible thermoinactivation in lysozyme (59) and ribonuclease A (60) at 90–100°C and pH 4 was found to be due to peptide bond cleavage at Asp-X bonds.

It is now well established that Asp-Pro peptide bonds are particularly labile and are hydrolyzed under conditions where other Asp peptide bonds are stable (136). For example, when rabbit antibody light chain was subjected to 10% acetic acid-pyridine (pH 2.5) in 7 *M* guanidinium hydrochloride (GnHCl) for 24–90 hr, selective cleavage was observed at Asp^{109}-Pro^{110} (137). Piszkiewicz *et al.* have suggested that the hydrolytic reaction proceeds via intramolecular catalysis by carboxylate anion displacement of the protonated nitrogen of the peptide bond and the rate enhancement occurs due to the greater basicity of the Pro nitrogen (136). Marcus has compared the lability of the Asp-Pro bonds to the lability of other peptide bonds, in particular to those of Asp residues (138). In his study, a variety of dipeptides was heated at 110°C in 0.015 *M* HCl. The concentration of amino acid released during the heat treatment was determined by amino acid analysis. The results indicated that Asp-Pro bonds were 8- to 20-fold more labile than other Asp-X or X-Asp peptide bonds. Other peptide bonds that do not involve Asp were found to be stable to hydrolysis under these conditions.

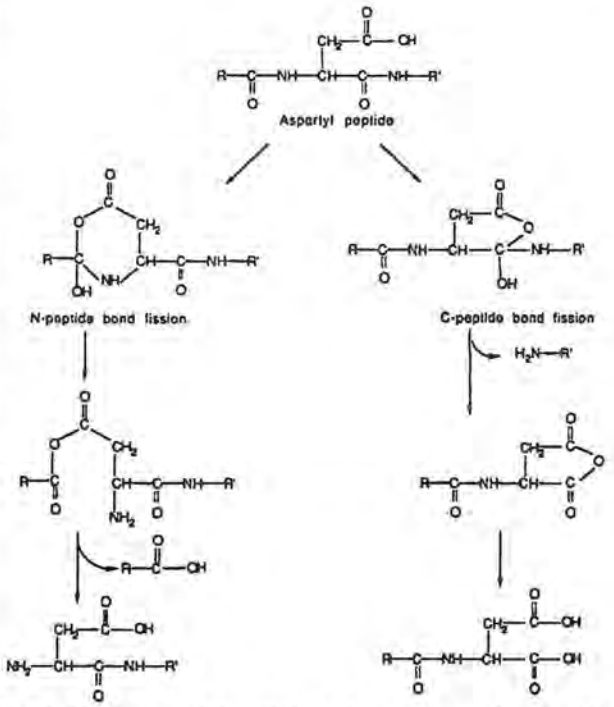

Fig. 4. Mechanism of degradation of aspartyl peptides in acidic media.

Asp-X peptide bonds also undergo a reversible isomerization between the Asp and the iso-Asp forms via the cyclic imide intermediate as shown in Fig. 3 (139,140). This reaction was first noted by Swallow and Abraham with Asp-Lys derived from hydrolyzates of bacitracin (139). Similar interconversion was also shown for Val-Tyr-Pro-Asp-Gly-Ala ($ACTH^{22-27}$), displaying a half-life of 53 days at pH 7.4 and 37°C (26). Even storage of aqueous solutions of an Asp-containing peptide can result in the formation of cyclic imide derivatives (141). The ring closure is particularly fast when an Asp residue is followed by Gly in the sequence (142). Peptide bonds formed by X-Ser and X-Thr are also labile, but require strong acidic conditions (e.g., 11.6 *M* HCl) (143). The mechanism involves N–O acyl rearrangement (144).

The time course of hydrolysis of amide peptide bonds

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