*Pharmaceutical Research, Vol. 7, No. 7, 1990*

*Research Article*

# Chemical Pathways of Peptide Degradation. II. Kinetics of Deamidation of an Asparaginyl Residue in a Model Hexapeptide

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*Received October 12, 1989; accepted January 23, 1990*

Deamidation of Asn residues is a major chemical pathway of degradation of peptides and proteins. To understand better the external factors that influence deamidation, we studied the degradation of the hexapeptide Val–Tyr–Pro–Asn–Gly–Ala, a fragment of adrenocorticotropic hormone, by HPLC. The deamidation of this model peptide showed marked dependence on pH, temperature, and buffer composition. In the pH range 5 to 12, the peptide deamidated exclusively via a cyclic imide intermediate with the formation of both the Asp- and the isoAsp-hexapeptides. Buffer catalysis was also observed in the pH range of 7 to 11. However, at acidic pH's, the pathway of deamidation involved direct hydrolysis of the amide side chain of Asn residue to produce only the Asp-hexapeptide.

**KEY WORDS:** asparaginyl; isoaspartyl; aspartyl; deamidation; cyclic imide.

## INTRODUCTION

Over the past two decades many investigators have observed altered forms of protein which have been attributed to deamidation (1). In the deamidation reaction, the side-chain amide of a Gln[3] or Asn residue is hydrolyzed to form a free carboxylic acid. Earlier studies have suggested that the intermediate formation of a five-membered cyclic imide ring may be involved in the deamidation of Asn residues in proteins and peptides (2–9). In the deamidation of Asn residues, breakdown of the cyclic imide intermediate can produce both Asp and isoAsp residues (Scheme I) (2–9). The analyses of deamidated products have shown that isoAsp peptides generally predominate (2–9). The sequence Asn–Gly in peptides and proteins is particularly susceptible to deamidation and rearrangement to an isoAsp peptide bond (3–6,9). It has been shown that cyclic imides may also be prone to racemization and contribute to the formation of D-Asp residues in proteins (3,8). Similarly, Gln residues can also undergo deamidation via formation of a six-membered ring, but usually at a slower rate than for Asn residues (10).

Using synthetic pentapeptides, it has been shown that deamidation is favored by increased pH, temperature, and ionic strength (1). Similar results were obtained with cytochrome *c* (11) and human triosephosphate isomerase (12). However, these studies did not investigate the formation of either isoAsp or cyclic imide. Recently, using Boc–Asn–Gly–Gly–$NH_2$, Capasso *et al.* have shown that, from pH 5 to pH 10, deamidation occurs via a cyclic imide intermediate (7).

Our primary goal was to investigate the pathways of deamidation of Asn residues over a wide pH range, as well as the effect of external factors such as buffer concentration, buffer species, and temperature on the rate of deamidation and also on the ratio of isoAsp/Asp peptides. Initially, we chose porcine adrenocorticotropic hormone (ACTH) as a model peptide, because previous work had implied facile cyclic imide formation at the Asn residue in the intact hormone (13,14). However, we were not able to develop analytical methodology to separate isoAsp-ACTH from Asp-ACTH (15). Thus, we chose a hexapeptide having the sequence of residues 22–27 of adrenocorticotropic hormone (Val–Tyr–Pro–Asn–Gly–Ala, Asn-hexapeptide) for our studies. Geiger and Clarke have shown that at pH 7.4 this hexapeptide undergoes deamidation exclusively via a cyclic imide (L-Val–L-Tyr–L-Pro–L-Asu–Gly–L-Ala) intermediate (3). In this study, we have developed an HPLC assay for the separation of Asn-hexapeptide from the deamidated products, Asp-hexapeptide (L-Val–Tyr–L-Pro–L-Asp–Gly–L-Ala) and isoAsp-hexapeptide (L-Val–L-Tyr–L-Pro–L-isoAsp–Gly–L-Ala). We have investigated the pathways of deamidation of this model hexapeptide at different pH's, buffer concentrations, and temperatures and have shown that the pathway of deamidation is different at acidic and alka-line pH's.

## MATERIALS AND METHODS

### Materials

L-Val–L-Tyr–L-Pro–L-Asn–Gly–L-Ala and L-Val–

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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. Asu is used as an abbreviation of the cyclic imide form of Asn.



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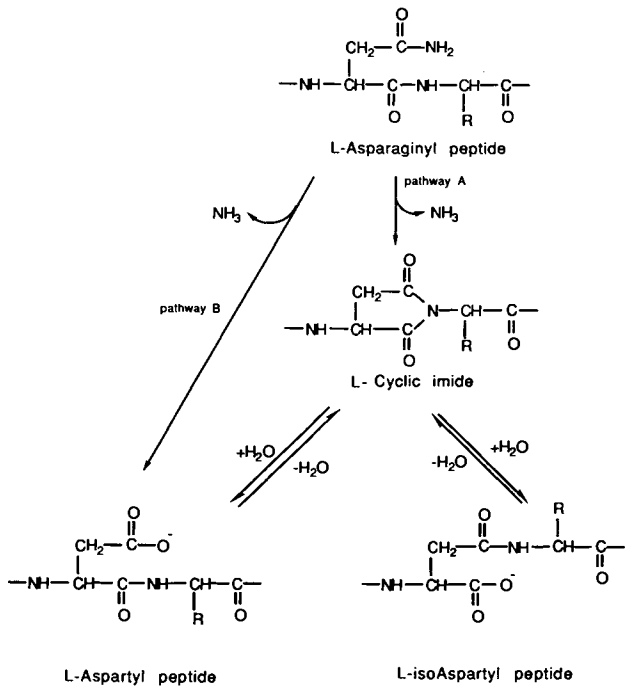



Scheme I

L-Tyr-L-Pro-L-Asp-Gly-L-Ala were synthesized by Dr. Thomas Lobl (Upjohn Company, Kalamazoo, MI). In the synthesis of peptides containing Asp residues, the major side reaction is the rearrangement of the Asp ester to the cyclic imide under acidic or alkaline conditions (16–21). Thus L-Val-L-Tyr-L-Pro-L-Asu-Gly-L-Ala was isolated during the purification of Asp-hexapeptide as described by Murray and Clarke (16). L-Val-L-Tyr-L-Pro-L-Asp (tetrapeptide) was manually synthesized following the standard Merrifield solid phase method (22). All t-Boc amino acid derivatives and Boc-Asp(OBzl)-*O*-resin were purchased from Peninsula Laboratories Inc, Belmont, CA. Gly-L-Ala and protease from *Staphylococcus aureus*, Type XVII-B, Strain V8 (P-2922), were purchased from Sigma Chemical Company, St. Louis, MO. Trifluoroacetic acid (HPLC grade) was purchased from Pierce, Rockford, IL. All the other chemicals were of analytical grade and used as received. All the peptides were purified by $C_{18}$ reversed-phase HPLC as described by Murray and Clarke using a semi-preparative Alltech Econosphere (10 × 250-mm, 10-μm resin) column (16).

The tetrapeptide and Asn- and Asp-hexapeptides were characterized by sequence analysis and amino acid analysis by the Biochemical Service Laboratory at The University of Kansas using standard techniques (23). The identification of the cyclic imide was confirmed by (i) the presence of stoichiometric amounts of each of the amino acids after strong acid hydrolysis and (ii) the generation of two hydrolysis products (Asp- and isoAsp-hexapeptides) upon mild alkaline treatment as described by Murray and Clarke (16). The FAB mass spectrum of the cyclic imide showed a molecular weight of 602, corresponding to the loss of a mole of water from Asp-hexapeptide.

### Buffer Solutions

The following buffers were used for all kinetic experiments: pH 1.00 to 2.00, HCl; pH 6.00 to 7.50, 11.10, sodium phosphate; pH 5.00, sodium acetate; pH 8.00, 9.00, Tris HCl; pH 10.00, sodium bicarbonate or sodium borate; and pH 12.00, NaOH. A constant ionic strength of 0.5 *M* was maintained for each buffer by adding an appropriate amount of sodium chloride. All the buffers were prepared at the experimental temperature. An Orion (701A) pH meter equipped with a Ross combination electrode was used to measure the pH (±0.05 pH unit) of the buffer solutions.

### Kinetic Measurements

All kinetic experiments were carried out in aqueous buffer solutions at 37 ± 1°C or otherwise indicated. For faster reactions, after the addition of peptide to give an initial concentration of about $2 \times 10^{-4}$ *M*, aliquots of 100 μl were removed from a single reaction vessel at appropriate intervals and frozen in dry ice–acetone mixture to quench the reaction. For the slower kinetic runs or those carried out at elevated temperatures, 250 μl of the reaction mixture was transferred to a 1-ml ampoule, flame-sealed, and temperature equilibrated. At known time intervals, ampoules were removed and stored at −70°C until analysis. All the reactions were carried out for three or more half-lives. Upon removal of the last sample, all of the stored samples were thawed and analyzed by HPLC as described below. The pH values of the reaction solutions were measured at the end of the experiment and no significant changes in pH were observed. Peptide concentration was based on the absorbance of a diluted aqueous solution at 274.5 nm using an extinction coefficient of 1413 $M^{-1}$ $cm^{-1}$ for Tyr (24).

### HPLC Analysis

Analysis of the degradation of the Asn-hexapeptide was performed by HPLC using a $C_{18}$ reversed-phase column. The HPLC system included a Shimadzu LC-6A pump, a SCL-6A controller, a SIL-6A autoinjector, a CR3A integrator, and a SPD-6A UV detector.

*Method A.* Separation of Asn-hexapeptide and its deamidated products was done on an Alltech Econospere $C_{18}$ column (5 μm resin, 4.6 × 250 mm) at room temperature, using an isocratic system consisting of 7% (v/v) acetonitrile, 0.1% trifluoroacetic acid in water at 1.0 ml/min and detection at 214 nm. Typical elution times (min) for peptides were as follows: Gly-Ala, 4.3; tetrapeptide, 21.19; isoAsp-hexapeptide, 26.8; Asn-hexapeptide, 32.9; Asp-hexapeptide, 40.3; and cyclic imide, 73.5.

*Method B.* For the cyclic imide hydrolysis experiments, an isocratic system consisting of 10% ACN, 0.1% (w/v) TFA in water at 1.5 ml/min was used. Typical elution times (min) were as follows: isoAsp-hexapeptide, 9.3; Asp-hexapeptide, 12.2; and cyclic imide, 20.3.

### Proteolytic Digestion of Asp- and isoAsp-Hexapeptides

Asp and isoAsp-hexapeptides were digested with protease from *S. aureus*, strain V8, as follows: approximately 20 nmol of peptide in 50 μl of 10 m*M* phosphate buffer, pH 7.8, was incubated with approximately 100 units of *S. aureus*

protease (615 units/mg) in 50 μl of distilled water for 12 hr at 37°C. Aliquots from the digestion mixture were applied directly to a $C_{18}$ reversed-phase HPLC column and eluted as described above in method A. Enzyme and peptide blank incubations were also performed.

## RESULTS

### Effect of pH and Buffer on Deamidation of Asn-Hexapeptide

Previous investigators have shown that at neutral to alkaline pH, an Asn–Gly sequence in a peptide can undergo deamidation to form both Asp–Gly and isoAsp–Gly sequences (3–7,9). Geiger and Clarke (3) have also detected the racemized Asp and isoAsp peptides at pH 7.4. In our studies we have addressed only the deamidation of Asn residues and no attempts were made to separate racemized products. Incubation of Asn-hexapeptide at pH 5 to 12 resulted in the appearance of two products as detected by elution from an HPLC column (Fig. 1a). The product which elutes after Asn-hexapeptide ($t$ = 40.3 min) was identified as Asp-hexapeptide by coinjecting an authentic sample. The product which elutes just ahead of Asn-hexapeptide ($t$ = 26.8 min) was assigned as the isoAsp-hexapeptide. The amino acid analysis of this peak gave a stoichiometric number for each of the amino acid residues Val, Tyr, Pro, Asp, Gly, and Ala. Proteolytic digestion was also used to verify the assignment of the isoAsp structure to this degradation product. It has been shown that the protease from *Staphylococcus aureus* cleaves peptide bonds specifically on the carboxyl terminal side of either Asp or Glu residues (25). Since we have only one Asp residue and no Glu residue in our deamidated products, we reasoned that such an enzymatic digestion of the Asp-hexapeptide should result in the formation of a tetrapeptide (Val–Tyr–Pro–Asp) and a dipeptide (Gly–Ala), while no such cleavage should occur with isoAsp-hexapeptide. When the synthetic Asp-hexapeptide was incubated with protease, formation of a tetrapeptide (Val–Tyr–Pro–Asp) was observed as expected (Figs. 2a and b). The tetrapeptide was identified by coinjecting an authentic sample. The corresponding dipeptide (Gly–Ala) was found to coelute very close to the solvent front. No such peptide bond cleavage (Figs. 2c and d) was observed with the HPLC peak eluting at 26.8 min (Fig. 1a). Thus, we were able to assign the peak at $t$ = 26.8 min (Fig. 1a) as isoAsp-hexapeptide.

At a fixed pH, the deamidation followed pseudo-first-order kinetics. The first-order rate constants were obtained from the plots of ln peak area *vs* time. The peak area was linearly related to the Asn-hexapeptide amount over the range of 0.1 to 4 nmol with a correlation coefficient of 1.00

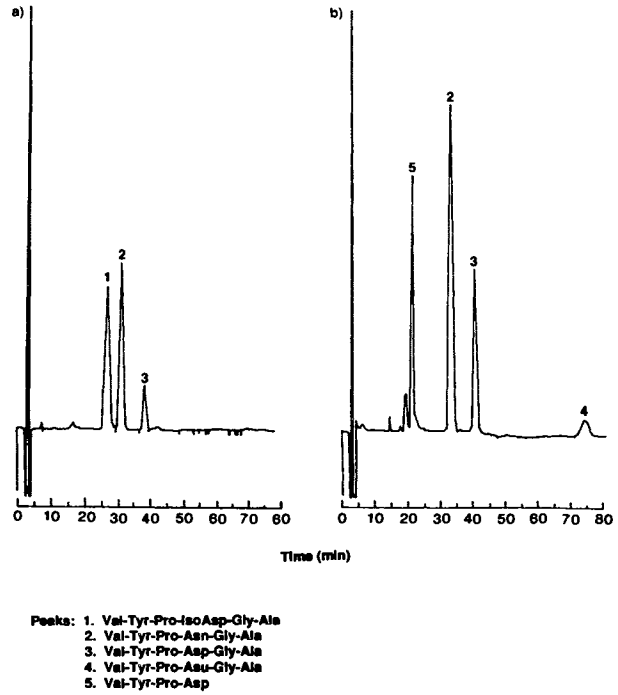



Fig. 1. Typical HPLC chromatograms for the deamidation of Asn-hexapeptide at (a) neutral to alkaline pH and (b) acidic pH.

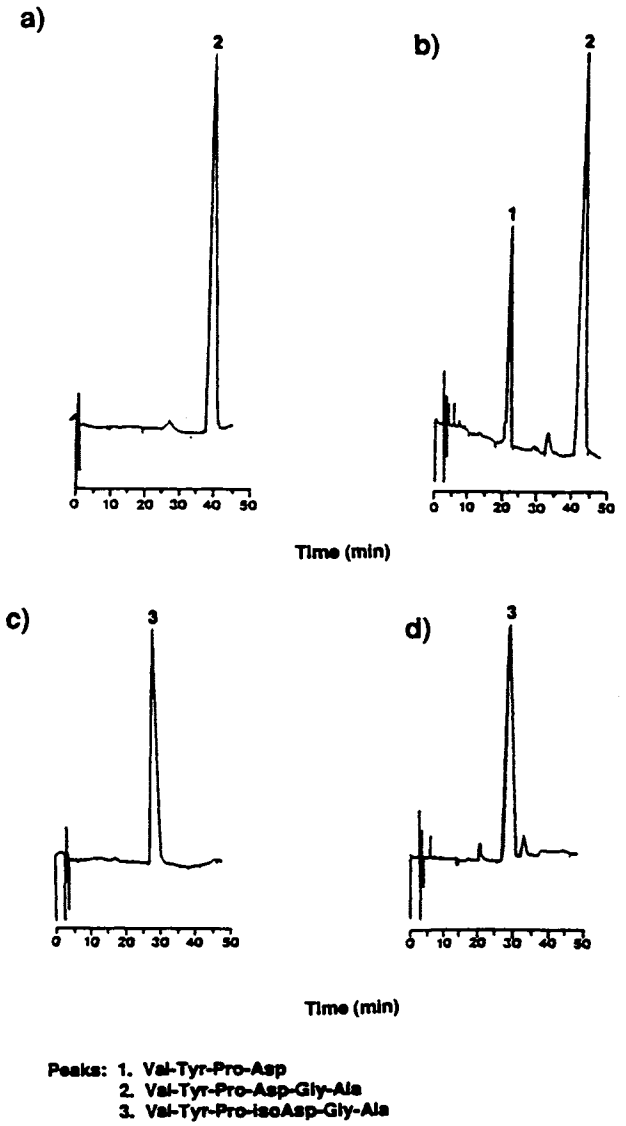



Fig. 2. HPLC chromatograms for the proteolytic mapping of deamidated products of Asn-hexapeptide using protease from *S. aureus*, V8. (a) Asp-hexapeptide (enzyme blank); (b) Asp-hexapeptide incubated with protease; (c) Peak 1 (Fig. 1a) (enzyme blank); (d) Peak 1 (Fig. 1a) incubated with protease.

(data not shown). No significant effect of the initial concentration was found in the range of (0.05 to 1.0) × $10^{-3}$ *M* of Asn-hexapeptide (pH 7.5, 0.05 *M* phosphate buffer, $I$ = 0.5, and 37°C). At pH 6–12, Asp- and isoAsp-hexapeptides were the only products detected. Figure 3 shows a typical time course of disappearance of Asn-hexapeptide and the appearance of the Asp- and isoAsp-hexapeptides at pH 7.0.

The catalytic effect of buffer was determined by measuring the rate of deamidation at constant pH, ionic strength, and temperature while varying only the buffer concentration. The catalytic effects of phosphate, Tris, and bicarbonate buffers on the rate of deamidation of Asn-hexapeptide are shown in Fig. 4. In the pH range 7 to 11, the deamidation of Asn-hexapeptide was subject to buffer catalysis. The rate constants at zero buffer concentration were obtained from the intercepts of plots of $k_{obs}$ *vs* total buffer concentration. No buffer catalysis was observed at pH 6 and 6.5 using phosphate buffer. At pH 5.0, using acetate buffer, the reaction was found to be very slow at 37°C. Thus the reaction was carried out at higher temperatures (i.e., 50, 70, 90°C) and using an Arrhenius plot (Fig. 5) and an activation energy of 22.2 kcal $mol^{-1}$, the rate constant at 37°C was obtained by extrapolation. At pH 5.0, using acetate buffer, no buffer catalysis was observed at 90°C, and thus the subsequent experiments at 50 and 70°C were carried out using only one buffer concentration, assuming no buffer catalysis would be observed at lower temperatures (i.e., 0.05 *M* acetate, $I$ = 0.5). For pH 5.0, at all temperatures, both Asp- and isoAsp-hexapeptides were detected including small amounts of the cyclic imide (<10% of total). The cyclic imide was identified by coinjecting an authentic sample of the cyclic imide. Table I summarizes the dependence of the rate constant on the buffer concentration for pH 1 to 12. At a fixed pH (6–12), the ratio of the two products (isoAsp/Asp) remained relatively constant (3.2–4.2) during the entire reaction (Table II). At any pH, no significant effect of buffer concentration on the ratio of these products was observed (Table II).

At acidic pH's (1 to 2), the deamidation of Asn-hexapeptide was much slower than at alkaline pH's and only the Asp-hexapeptide was produced upon deamidation of the Asn-hexapeptide (Fig. 1b). Formation of the isoAsp-hexapeptide was not detected. Interestingly, the cyclic imide was also detected, but the maximum concentration was only 10% of the total. At least one major new degradation product was observed by HPLC (Fig. 1b). This new degradation product was identified as a tetrapeptide by coinjecting the authentic sample. Since peptides with Asp residues are prone to undergo peptide backbone cleavage in acidic pH at Asp-X linkage (26), this tetrapeptide could have formed from the degradation of Asp-hexapeptide. This pathway was in fact confirmed by incubating purified Asp-hexapeptide at pH 1.0, $I$ = 0.5, and 37°C. Under these conditions, only the tetrapeptide and cyclic imide were detected and their rates of formation were slower than the deamidation of Asn-hexapeptide ($t_{1/2}$ = 388.6 hr). The reactions at pH 3.0 and 4.0, using formate and acetate buffer respectively, at 37°C were very slow, e.g., degradation of Asn-hexapeptide was not detected at 37°C up to 60 days.

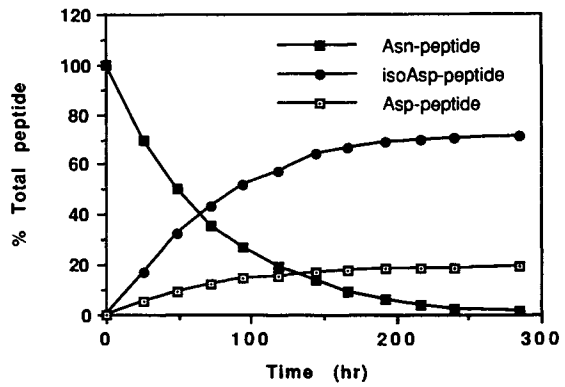



Fig. 3. Typical time profile for the disappearance of Asn-hexapeptide and for the appearance of deamidated products at pH 7.0, 0.1 *M* phosphate buffer ($I$ = 0.5) at 37°C.

### pH Dependence

The pH dependence of the overall rate of deamidation of the Asn-hexapeptide at 37°C and $I$ = 0.5 is shown in Fig. 6 and also in Table III. The rate constants used in the construction of the graph were obtained from the intercepts of the graphs of $k_{obs}$ *vs* total buffer concentrations at various pH values. Results from runs performed in dilute HCl (pH 1–2) and sodium hydroxide (pH 12.0) are also included. At 37°C, deamidation shows strong dependence on pH, with rate constants increasing with increasing pH in the region of pH 5 to 12. The pH of maximum stability is found to be about pH 3 to 4, since the rate of deamidation again increases at pH 1.

### Hydrolysis of Cyclic Imide

The hydrolysis of the cyclic imide was studied at pH 1.0 (0.1 *N* HCl), pH 5.0 (0.025 *M* acetate), pH 6.0 (0.05 *M* phosphate), pH 7.5 (0.1 *M* phosphate), and pH 10.0 (0.05 *M* borate), $I$ = 0.5, at 37°C. At pH 5 to 10, the overall loss of cyclic imide peptide followed pseudo-first-order kinetics, with the exclusive formation of Asp- and isoAsp-hexapeptides. At a given pH, the ratio of the Asp- and isoAsp-hexapeptides remained relatively constant (3.2 to 4.0) (Table IV). In contrast to these results, at pH 1.0 the cyclic imide was stable, with no significant degradation up to 38 days at 37°C.

### Effect of Temperature on Deamidation of Asn-Hexapeptide

Deamidation of Asn-hexapeptide was studied at pH 7.5 (0.1 *M* phosphate buffer, $I$ = 0.5) from 25 to 70°C (±1°C). Similar experiments were carried out at pH 5.0 (0.05 *M* acetate buffer, $I$ = 0.5) from 50 to 90°C (±1°C). For pH 7.5, at all temperatures, Asp- and isoAsp-hexapeptides were the only products formed upon deamidation, with no indication of side reactions. However, at pH 5.0, the tetrapeptide (Val–Tyr–Pro–Asp) was also observed after one half-life, probably because of further degradation of deamidated products. For both pH's, the rates of deamidation were found to increase with the temperature, obeying the Arrhenius equation (Fig. 5). From the Arrhenius plot, it was possible to calculate observed activation energies of 21.7 and 22.2 kcal $mol^{-1}$ for pH 7.5 and 5.0, respectively. The other observed activation parameters were as follows: log$A$ = 10.3 $sec^{-1}$, $\Delta H^{\neq}$ = 21.3 kcal $mol^{-1}$, and $\Delta S^{\neq}$ = −13.7 cal $mol^{-1}$ $K^{-1}$ for pH 7.5

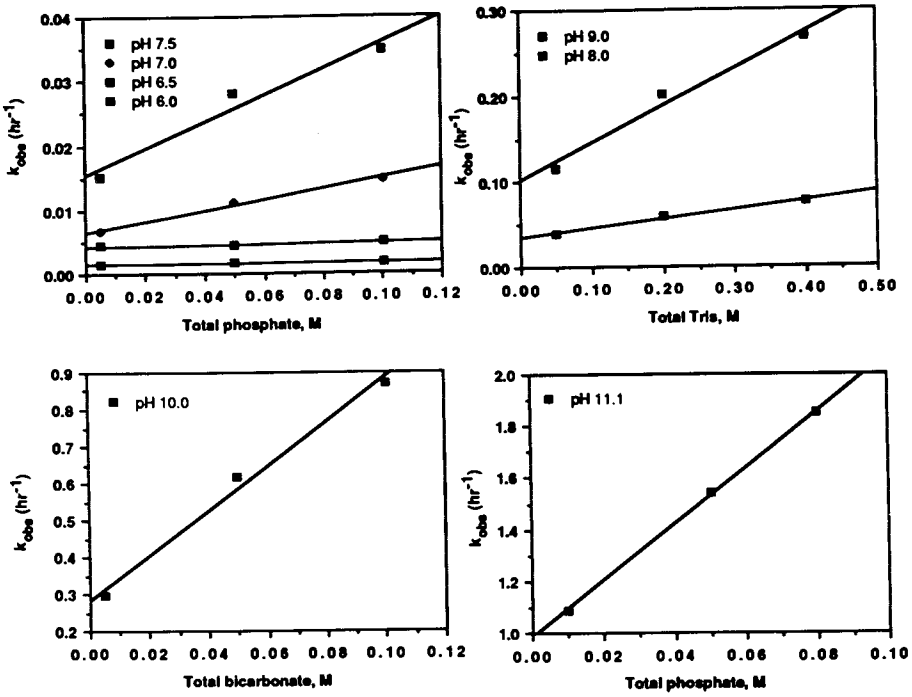



Fig. 4. Pseudo-first-order rate constants *versus* total buffer concentration for the deamidation of Asn-hexapeptide in aqueous solution at 37°C and $I = 0.5$.

and $\log A = 8.5\ \text{sec}^{-1}$, $\Delta H^{\neq} = 21.3$ kcal mol$^{-1}$, and $\Delta S^{\neq} = -21.8$ cal mol$^{-1}$ K$^{-1}$ for pH 5.0.

## DISCUSSION

### Degradation Pathways

The appearance of Asp- and isoAsp-hexapeptide in the pH range 5–12 suggests that the pathway of deamidation at neutral to alkaline pH's involves the formation of a cyclic imide intermediate. Since the rate of hydrolysis of the cyclic imide at pH 5 to 10 is substantially more rapid than the rate of hydrolysis of the Asn-hexapeptide, the rate-limiting step (slow step) in this pathway is the formation of the cyclic imide with subsequent rapid hydrolysis to the Asp-peptide and isoAsp-peptides (5).

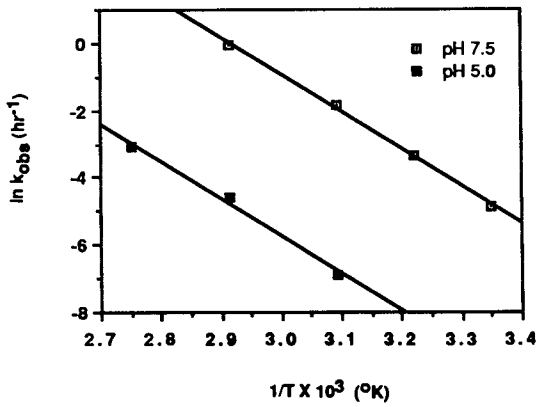



Fig. 5. Arrhenius plot for the deamidation of Asn-hexapeptide at pH 7.5, 0.1 *M* phosphate buffer ($I = 0.5$), and pH 5.0, 0.05 *M* acetate buffer ($I = 0.5$).

The deamidation of Asn residues can take place either via direct hydrolysis of the amide side chain or via formation of cyclic imide intermediate. At a given pH either one or both routes could be participating. Since the ratio of Asp and isoAsp hexapeptides in the deamidation reaction at neutral to alkaline pH coincides with the values obtained in the hydrolysis of the cyclic imide, we can propose that in the pH range 5–12, the deamidation reaction occurs exclusively through the cyclic imide rather than direct hydrolysis of the amide (pathway a, Scheme 1). If there were significant participation of direct hydrolysis of the amide linkage, an increase in the proportion of Asp-hexapeptide relative to isoAsp-hexapeptide would be expected.

These results are consistent with those reported by Geiger and Clarke at a single pH (pH 7.4) (3). Recently, using Boc–Asn–Gly–Gly–$NH_2$, Capasso *et al.* also showed that, in the pH range 5 to 10, the deamidation pathway is exclusively via cyclic imide intermediate (7). The observation that only the Asp-hexapeptide is generated from the Asn-hexapeptide under acidic conditions is consistent with the report that Asn–Gly and isoAsn–Gly dipeptides yield only the Asp–Gly and isoAsp–Gly dipeptides, respectively, when incubated with 1 *N* HCl at 37°C (5). However, the presence of low concentrations of the cyclic imide at acidic pH could mean that cyclic imide is formed from Asn-hexapeptide and it hydrolyzes exclusively to Asp-hexapeptide. However, this hypothesis was ruled out from the observation that the cyclic imide is stable at pH 1 up to 38 days, whereas the Asn-hexapeptide under identical conditions has $t_{1/2} = 301.3$ hr (Table IV). It seems that the cyclic imide is formed from Asn-hexapeptide very slowly compared to direct hydrolysis of the amide side chain and, once formed, it is very stable. On the other hand, the cyclic imide could also be formed

Table I. Observed Pseudo-First-Order Rate Constants for Deamidation of Asn-Hexapeptide in Aqueous Buffer Solutions of Various pH Values (37°C, $I$ = 0.5)

| pH (buffer) | Buffer concentration ($M$) | $k_{obs}$ ($hr^{-1}$) ± SD[a] |
|---|---|---|
| 1.0 (HCl) | 0.1 | $(2.33 \pm 0.06) \times 10^{-3}$ |
| 1.5 (HCl) | 0.032 | $(0.71 \pm 0.04) \times 10^{-3}$ |
| 2.0 (HCl) | 0.01 | $0.23 \times 10^{-3}$[b,c] |
| 5.0 (acetate)[d] | 0.01 | $(4.51 \pm 0.11) \times 10^{-2}$ |
| | 0.05 | $(4.54 \pm 0.03) \times 10^{-2}$ |
| | 0.1 | $(5.24 \pm 0.06) \times 10^{-2}$ |
| 6.0 (phosphate) | 0.005 | $(1.53 \pm 0.12) \times 10^{-3}$ |
| | 0.05 | $(1.67 \pm 0.06) \times 10^{-3}$ |
| | 0.1 | $(1.63 \pm 0.06) \times 10^{-3}$ |
| 6.5 (phosphate) | 0.005 | $(4.44 \pm 0.04) \times 10^{-3}$ |
| | 0.05 | $(4.49 \pm 0.31) \times 10^{-3}$ |
| | 0.1 | $5.0 \times 10^{-3}$[c] |
| 7.0 (phosphate) | 0.005 | $(0.63 \pm 0.04) \times 10^{-2}$ |
| | 0.05 | $(1.13 \pm 0.06) \times 10^{-2}$ |
| | 0.1 | $(1.47 \pm 0.05) \times 10^{-2}$ |
| 7.5 (phosphate) | 0.005 | $(1.51 \pm 0.08) \times 10^{-2}$ |
| | 0.05 | $(2.81 \pm 0.02) \times 10^{-2}$ |
| | 0.1 | $(3.48 \pm 0.13) \times 10^{-2}$ |
| 8.0 (Tris) | 0.05 | $(3.99 \pm 0.33) \times 10^{-2}$ |
| | 0.2 | $(5.91 \pm 0.05) \times 10^{-2}$ |
| | 0.4 | $(7.65 \pm 0.14) \times 10^{-2}$ |
| 9.0 (Tris) | 0.05 | 0.116 ± 0.016 |
| | 0.2 | 0.201 ± 0.006 |
| | 0.4 | 0.268 ± 0.009 |
| 10.0 (bicarbonate) | 0.005 | 0.294 ± 0.016 |
| | 0.05 | 0.617 ± 0.019 |
| | 0.1 | 0.871 ± 0.024 |
| 11.1 (phosphate) | 0.01 | 1.09 ± 0.2 |
| | 0.05 | 1.54 ± 0.04 |
| | 0.08 | 1.85 ± 0.09 |
| 12.0 (NaOH) | 0.01 | 1.85 ± 0.09 |

[a] $N$ = 3.
[b] Only 25% deamidation observed at pH 2.0, 37°C, in 80 days.
[c] $N$ = 1.
[d] Reactions carried out at 90°C.

Table II. Effect of pH and Buffer Concentration on the Ratio of isoAsp/Asp-Hexapeptides at 37°C, $I$ = 0.5

| pH | Buffer | Buffer concentration ($M$) | Average isoAsp/Asp |
|---|---|---|---|
| 12.0 | NaOH | 0.01 | 4.2 |
| 11.1 | Phosphate | 0.01 | 4.3 |
| | | 0.05 | 4.3 |
| | | 0.08 | 4.3 |
| 10.0 | Bicarbonate | 0.005 | 4.0 |
| | | 0.05 | 4.1 |
| | | 0.1 | 4.6 |
| 9.0 | Tris | 0.05 | 4.1 |
| | | 0.2 | 4.0 |
| | | 0.4 | 4.0 |
| 8 | Tris | 0.05 | 4.1 |
| | | 0.2 | 4.0 |
| | | 0.4 | 4.2 |
| 7.5 | Phosphate | 0.005 | 3.8 |
| | | 0.05 | 3.8 |
| | | 0.1 | 3.8 |
| 7.0 | Phosphate | 0.005 | 3.8 |
| | | 0.05 | 4.1 |
| | | 0.1 | 3.6 |
| 6.5 | Phosphate | 0.005 | 3.8 |
| | | 0.05 | 3.6 |
| | | 0.1 | 3.6 |
| 6 | Phosphate | 0.005 | 3.7 |
| | | 0.05 | 3.5 |
| | | 0.1 | 3.2 |

from Asp-hexapeptide in acidic media. Thus, we propose that, at acidic pH, the deamidation of Asn-hexapeptide involves mainly direct hydrolysis of the side chain rather than formation of the cyclic imide intermediate (Scheme II). It is not surprising that the rate of cyclic imide formation is very slow at acidic pH, because even for Asp-beta-ester peptides, it requires very strong acidic media (e.g., HF, HBr, etc.) to form cyclic imide, and furthermore, it requires alkali for further hydrolysis (17–21).

We have also shown that, at acidic pH, Asp-hexapeptide further degrades to form fragments, suggesting that Asp residues can also be potential sites for proteolysis in peptides and proteins. Significant irreversible thermoinactivation in lysozyme (27) and ribonuclease A (28) at 90–100°C and pH 4 was found to be due to peptide bond cleavage at Asp–X bonds.

Asp residues can also interconvert to isoAsp-peptide via cyclic imide intermediates in neutral to alkaline media [e.g., glucagon (29), calmodulin (30), daptomycin (31)]. However, this interconversion is very slow compared to deamidation of Asn residues at the pH's studied here. Racemization of the cyclic imide is another common reaction involving Asn/Asp residues (3,8). However, the presence of diastereomers was not detected with Asn-hexapeptide under the experimental conditions used in the HPLC analysis.

The results presented in this paper show that peptides deamidate at acidic and alkaline pH via different pathways. Determination of the pathway of deamidation in larger proteins is complicated by the difficulty in detection and sepa-

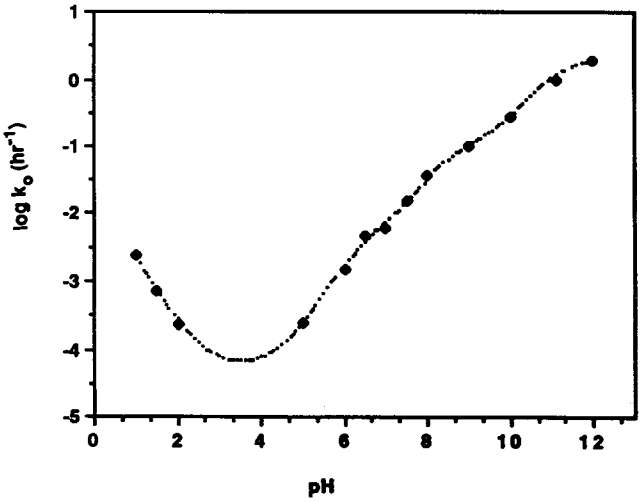



Fig. 6. pH-rate profile for the deamidation of Asn-hexapeptide in aqueous solution at 37°C and $I$ = 0.5.

Table III. The Rate Constants of Deamidation of Asn-Hexapeptide at Zero Buffer Concentration ($k_o$), 37°C, $I$ = 0.5

| pH | $k_o$ ($hr^{-1}$) ± SD |
|---|---|
| 1.0 | (2.33 ± 0.06) × $10^{-3}$ |
| 1.5 | (0.71 ± 0.04) × $10^{-3}$ |
| 2.0 | 0.23 × $10^{-3}$ |
| 5.0[a] | (0.25 ± 0.01) × $10^{-3}$ |
| 6.0 | (1.53 ± 0.12) × $10^{-3}$ |
| 6.5 | (4.57 ± 0.27) × $10^{-3}$ |
| 7.0 | (6.2 ± 0.5) × $10^{-3}$ |
| 7.5 | (1.53 ± 0.08) × $10^{-2}$ |
| 8.0 | (3.61 ± 0.32) × $10^{-2}$ |
| 9.0 | 0.102 ± 0.01 |
| 10.0 | 0.281 ± 0.008 |
| 11.1 | 0.982 ± 0.177 |
| 12.0 | 1.85 ± 0.09 |

[a] Rate constant calculated using Arrhenius plot.

ration of the deamidated and rearranged products. It has been reported that isoAsp residues are found upon incubation of peptides and proteins in neutral to alkaline media, suggesting the formation of cyclic imide intermediates, since cyclic imide formation is the only known mechanism by which isoAsp residues may form (14,32,33). Recently, using bovine protein carboxymethyltransferase, which selectively methylates the carboxyl group of isoAsp residues, the isoAsp–ACTH was detected at pH 7.0 and 9.6, but not at pH 1.9 (15).

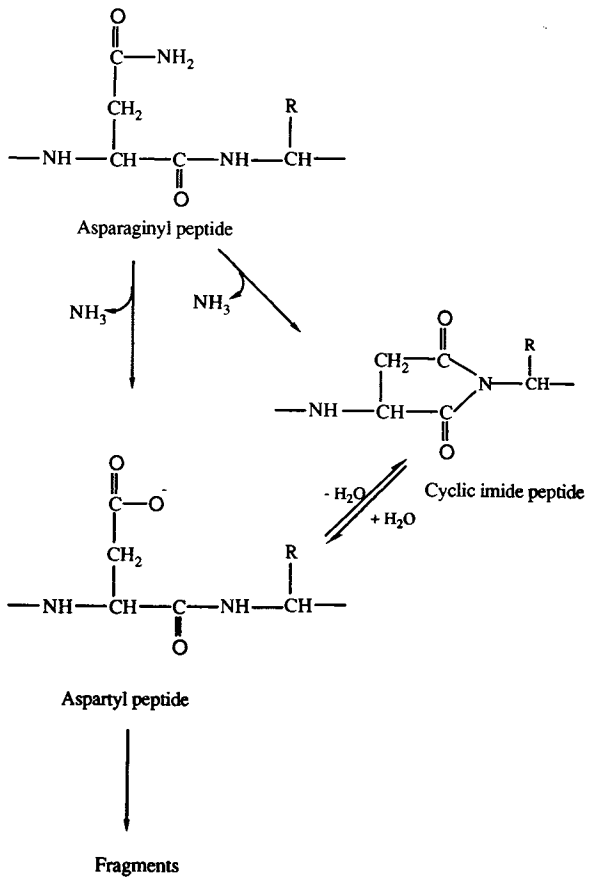
Because of conformational flexibility of short peptides, the rates of deamidation presented here probably represent the maximal rate of deamidation to be expected in proteins, assuming that side-chain participation (i.e., Ser) does not significantly accelerate the reaction. The cyclic imide formation and thus deamidation in proteins may be limited if the main-chain and side-chain groups are not already in the correct conformation or if they do not have the flexibility to assume the correct confirmation (34).

An alternative pathway for deamidation is via formation of an isoimide intermediate, where the peptide bond carbonyl oxygen is involved in nucleophilic attack on the carbonyl carbon of the side chain of the Asn residue. Several isoimides have been synthesized and have been shown to be readily hydrolyzed to the normal carboxylic acid (35,36). Although there is considerable evidence for cyclic imide intermediates in peptide Asp ester hydrolysis reactions (6,14,38–42), this evidence does not preclude the initial formation of an isoimide because it is known that rearrangement reactions may convert isoimide products to cyclic imide products (35,36).

Table IV. Rates of Hydrolysis of the Cyclic Imide Hexapeptide and the Distribution of the Deamidated Products at 37°C[a]

| pH | Buffer ($I$ = 0.5) | $k_{obs}$ ($hr^{-1}$) | isoAsp/Asp |
|---|---|---|---|
| 10.0 | 0.05 $M$ borate | 3.85 × $10^{1}$ | 3.9 |
| | | (4.15 × $10^{-1}$)[b] | (4.1)[b] |
| 7.5 | 0.1 $M$ phosphate | 3.94 × $10^{-1}$ | 3.6 |
| | | (3.48 × $10^{-2}$)[b] | (3.8)[b] |
| 6.0 | 0.05 $M$ phosphate | 2.65 × $10^{-2}$ | 3.2 |
| | | (1.67 × $10^{-3}$)[b] | (3.5)[b] |
| 5.0 | 0.025 $M$ acetate | 3.9 × $10^{-3}$ | 3.1 |
| | | (2.6 × $10^{-4}$)[b] | |
| 1.0 | 0.1 $M$ HCl | NR[c] | — |
| | | (2.33 × $10^{-3}$)[b] | (100% Asp)[b] |

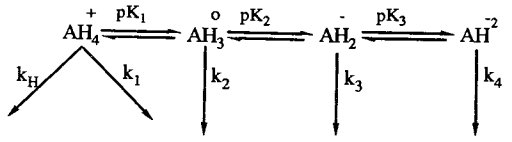
[a] $N$ = 2. Initial concentration was ~1 × $10^{-4}$ $M$.
[b] Data obtained for hydrolysis of Asn-hexapeptide under identical experimental conditions.
[c] No reaction up to 38 days.




Scheme II

## pH Rate Profile

The pH rate profile presented here has a unit negative slope on the acidic side (pH 1–2), indicating specific hydronium ion catalysis. From pH 5 to pH 12, the rate constant increases with hydroxide ion concentration but is not first-order in hydroxide ion (approximate slope = 0.54). This may be due to the complexity of the mechanism involving the cyclic imide. Several kinetic models could be proposed to describe this pH rate profile. We have presented here one particular kinetic model, which adequately describes the rate profile and is kinetically possible. Since in the pH range studied the Asn-hexapeptide has three ionizable groups (alpha carboxyl group of the C terminus, alpha amino group of the N terminus, and Tyr–OH), there are numerous ionic species, all of which may or may not have different propensities to deamidation (Scheme III). The shape of the pH rate pro-



Scheme III

file suggests that the overall velocity of the degradation of Asn-hexapeptide at a given pH can be expressed as

$$k_o = k_H[H_3O^+][AH_4/A_T] + k_1[AH_4/A_T] + k_2[AH_3/A_T] + k_3[AH_2/A_T] + k_4[AH/A_T] \quad (1)$$

where $[A]_T = [AH_4] + [AH_3[ + [AH_2] + [AH]$, $k_o$ is the pH-dependent first-order rate constant, $k_H$ is the second-order specific hydronium ion catalysis constant, and $k_1$, $k_2$, $k_3$, and $k_4$ are first-order constants for the reaction of $AH_4$, $AH_3$, $AH_2$, and $AH$ species, respectively.

Equation (1) can be rewritten as follows:

$$k_o = \frac{k_H[H_3O^+]^4 + k_1[H_3O^+]^3 + k_2[H_3O^+]^2K_1 + k_3[H_3O^+]K_1K_2 + k_4K_1K_2K_3}{[H_3O^+]^3 + [H_3O^+]^2K_1 + [H_3O^+]K_1K_2 + K_1K_2K_3} \quad (2)$$

where $K_1$, $K_2$, and $K_3$ are the ionization constants for Asn-hexapeptides. The experimental data were fitted to Eq. (2) using the RS1 nonlinear curve-fitting program. The data were weighted using reciprocal variance as the weight. The best fit was obtained by using $K_1 = 3.16 \times 10^{-7}$ ($pK_1 = 6.5$), $K_2 = 3.16 \times 10^{-9}$ ($pK_2 = 8.5$), and $K_3 = 1 \times 10^{-11}$ ($pK_3 = 11.0$). The individual values of rate constants are given in Table V. In Fig. 6, the line represents the theoretical curve calculated by substituting the rate constants from Table V into Eq. (2), while the points show the experimental results. Since no data are available between pH 2 and pH 5, we have assumed that Eq. (2) also obeys in this pH range. The good agreement observed shows that Eq. (1) can adequately describe the kinetics of the total degradation. Even though we have not determined individual ionization constants using standard techniques, it is not unreasonable to assign $pK_2 = 8.5$ and $pK_3 = 11$ to the N terminus (alpha-$NH_3$) and Tyr–OH, respectively (43). While $pK_1$ (6.5) seems reasonably high for the C terminus (alpha-COOH), it is not unusual for many amine bases and carboxylic acids in proteins to have an anomalously high or low $pK_a$ because of their microenvironment (44). At present we do not have more information to support the values of ionization constants. However, we speculate that either the $pK_a$ for alpha-COOH is unusually high due to some structural effects such as hydrogen bonding or this is a kinetic $pK_a$ representing change in the rate-determining step.

Table V. Calculated Rate Constants for Deamidation of Asn-Hexapeptide[a]

| | |
|---|---|
| $k_H$ | $(2.2 \pm 0.1) \times 10^{-2}\ M^{-1}\ hr^{-1}$ |
| $k_1$ | $(0.054 \pm 0.02) \times 10^{-3}\ hr^{-1}$ |
| $k_2$ | $(6.6 \pm 0.5) \times 10^{-3}\ hr^{-1}$ |
| $k_3$ | $(11.6 \pm 0.9) \times 10^{-2}\ hr^{-1}$ |
| $k_4$ | $(20.1 \pm 1.1) \times 10^{-1}\ hr^{-1}$ |

[a] The rate constants are for the reaction of numerous ionic species as shown in Scheme III and Eq. (1).

In conclusion, our results suggest that deamidation of the –Asn–Gly– sequence in peptides involves a cyclic imide intermediate at neutral to alkaline pH's with subsequent formation of isoAsp and Asp peptides. However, at acidic pH's, it involves mainly direct hydrolysis of the amide side chain to produce only Asp-containing peptides. The deamidation reaction is also subject to buffer catalysis in the pH range of 7 to 12. The pH of maximum stability toward deamidation appears to be about pH 3 to 4.

## ACKNOWLEDGMENTS

K. Patel acknowledges the support provided by the Parenteral Drug Association in the form of a Predoctoral Fellowship. The authors also acknowledge the many helpful comments provided by Professors Richard L. Schowen, Valentino J. Stella, and Mark C. Manning, The University of Kansas.

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