Chemical Pathways of Peptide Degradation. IV. Pathways, Kinetics, and Mechanism of Degradation of an Aspartyl Residue in a Model Hexapeptide

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In this study the hexapeptide Val-Tyr-Pro-Asp-Gly-Ala (Asp-hexapeptide) was used as a model to investigate the kinetics of aspartate degradation in aqueous solution. The apparent rate of degradation of the Asp-hexapeptide was determined as a function of pH, buffer concentration, and temperature. At very acidic pH levels (0.3, 1.1, 1.5, 2.0, and 3.0), the apparent rate of degradation followed pseudo-first-order kinetics. In this pH region, the Asp-hexapeptide predominantly underwent specific acid-catalyzed hydrolysis of the Asp-Gly amide bond (Asp-X hydrolysis) to form a tetrapeptide (Val-Tyr-Pro-Asp) and a dipeptide (Gly-Ala). In addition, parallel formation of a cyclic imide intermediate could be observed, although no isoAsp-hexapeptide was detected. At pH 4.0 and 5.0, the Asp-hexapeptide simultaneously isomerized via the cyclic imide to form the iso-Asp-hexapeptide and underwent Asp-X hydrolysis to produce the cleavage products. The pH–rate profiles (pH 0.3–5.0) for the Asp-X hydrolysis and the formation of cyclic imide revealed that the degree of ionization of the carboxylic acid side chain of Asp residue significantly altered the rate of reaction, with the ionized form being more reactive than the unionized form. Little or no buffer catalysis was observed for either pathway. Solvent isotope experiments were used to probe the mechanism of the Asp-X hydrolysis reaction. At pH values above 6.0, the apparent rate of degradation of the Asp-hexapeptide followed pseudo-first-order reversible kinetics, with the isoAsp-hexapeptide being the only observed product (isomerization). Above pH 8.0, the isomerization kinetics were found to be independent of pH and buffer concentration. The kinetics of degradation of Asp-hexapeptide (Val-Tyr-Pro-Asp-Gly-Ala) and Asn-hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala) were compared to determine the relative instability of the Asp and Asn residues and to understand the mechanism of formation of cyclic imide at near neutral to basic pH.

KEY WORDS: Asp-hexapeptide; isoAsp-hexapeptide; Asp-X hydrolysis; conversion of Asp to isoAsp; aspartyl; asparaginyl; cyclic imide; degradation pathways.

INTRODUCTION

With the emergence of interest in proteins and peptides as pharmacotherapeutic entities (1,2), a comprehensive understanding of enzymatic and nonenzymatic chemical stability of these agents is necessary in order to design rational formulation strategies (3–6). Earlier studies in this series (7–9) using adrenocorticotropic hormone and a model hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala; Asn-hexapeptide) have focused on the instability of Asn[3] residues, which, depending upon pH, have been shown to degrade to Asp and isoAsp residues. However, it is also well documented in the literature that Asp residues in peptides and proteins are potential sites of chemical instability (3–6). Although it is known that chemical degradation involving conversion of Asp to isoAsp and Asp-X hydrolysis can alter or diminish the biological activity of Asp-containing proteins and peptides (10–17), the kinetics and mechanism of these degradation routes have not been fully characterized.

Therefore, in the present study a hexapeptide (Val-Tyr-Pro-Asp-Gly-Ala; Asp-hexapeptide) has been selected as a model peptide for studying the kinetics and mechanism of the degradation of a peptidic Asp residue. The rates of decomposition of the Asp-hexapeptide were studied in aqueous solution as a function of pH, buffer concentration, and temperature. In addition, the kinetics of degradation of the Asp-hexapeptide was compared to that of the corresponding Asn-hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala) in order to determine the relative instability of these residues and to elucidate the mechanism of the formation of the Asu-hexapeptide (Val-Tyr-Pro-Asu-Gly-Ala).

MATERIALS AND METHODS

Materials

The Asp-hexapeptide (Val-Tyr-Pro-Asp-Gly-Ala) was synthesized by Dr. Thomas Lobl (The Upjohn Company, Kalamazoo, MI). The Asu-hexapeptide (Val-Tyr-Pro-Asu-Gly-Ala), a major side product in the synthesis of the Asp-hexapeptide, was isolated during reversed-phase preparative purification of the Asp-hexapeptide. The tetrapeptide (Val-Tyr-Pro-Asp) was manually synthesized by Dr. Kamlesh Patel using the standard Merrifield solid phase method (8,18).

All chemicals were analytical grade and were used as received from the commercial suppliers. Trifluoroacetic acid (TFA; HPLC grade) was purchased from Pierce Chemicals (Rockford, IL). Deuterium chloride (20 wt% solution in D_2O) and deuterium oxide (99.9 atom% D) were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Cambridge Isotope Laboratories (Woburn, MA), respectively. HPLC-grade acetonitrile was supplied by Fisher Chemical (Fair Lawn, NJ). The water used in all studies was from a Millipore MILLI-Q water system.

Apparatus

High-performance liquid chromatography (HPLC) was done with a system consisting of a Shimadzu LC-6A pump, a SCL-6B system controller, a SPD-6A variable-wavelength UV detector, a Rheodyne manual injector equipped with 20- and 200-μl loops, and a C-R4A Chromatopac integrator. The pH readings were recorded using a POPE Model 1501 pH/ion meter.

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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 Asp.

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Characterization of Peptides

The Asp-hexapeptide, tetrapeptide, and Asu-hexapeptide were characterized by sequence analysis and amino acid analysis by The Biochemical Service Laboratory at The University of Kansas using manual Edman degradation and postcolumn derivatization with ninhydrin techniques, respectively (19). The identities of these peptides were further confirmed by FAB-mass spectrometry and, in the case of Asu-hexapeptide, generation of two hydrolysis products (Asp- and isoAsp-hexapeptides) upon alkaline treatment (20). In addition to sequence analysis, amino acid analysis, and FAB-mass spectrometry, proteolytic digestion by a protease from *Staphylococcus aureus,* Type XVII-B, Strain V8 (P-2922), was used to verify the assignment of the isoAsp-hexapeptide (Val-Tyr-Pro-isoAsp-Gly-Ala) (see Ref. 8 for methods).

Kinetic Measurements

The decomposition of the Asp-hexapeptide was studied in aqueous solution at 37°C. The following buffers were used: pH 0.3, 1.1, 1.5, and 2.0 HCl; pH 3.0 (0.005, 0.05, and 0.1 *M*) formate; pH 4.0 and 5.0 (0.005, 0.05, and 0.1 *M*) acetate; pH 6.1 and 7.4 (0.005, 0.05, and 0.1 *M*) phosphate; pH 8.0 (0.005, 0.05, and 0.1 *M*) Tris; and pH 9.0 and 10.0 (0.02, 0.05, and 0.1 *M*) borate. A constant ionic strength of 0.5 *M* was maintained for each buffer by adding an appropriate amount of NaCl. The pH of the buffer solutions was adjusted at the experimental temperature (37°C).

The purified Asp-hexapeptide (0.81 μmol) was dissolved in a sufficient volume of buffer solution to make 5.0 ml of bulk solution with the resulting concentration of 1.6×10^{-4} *M*. Aliquots (100 μl) of the bulk solution were added to ampoules, which were then sealed and stored in a 37°C oven. At various times, ampoules were removed, cooled, and refrigerated at 4°C prior to being analyzed by HPLC.

Solvent Isotope Study

The kinetic study in D_2O was carried out at pD 1.1 and 37°C using DCl buffer in which the ionic strength was adjusted to 0.5 *M* by adding a calculated amount of NaCl. The pD was determined from the pH meter reading, applying the appropriate corrections (21). The kinetic solvent isotope effect (KSIE) is expressed as k_{H_2O}/k_{D_2O}, where k_{H_2O} and k_{D_2O} are the rate constants in H_2O and D_2O, respectively.

HPLC Analysis

Analysis of the Asp-hexapeptide and its degradation products was performed on an Alltech ODS Hypersil C_{18} column (5 μm resin, 4.6 × 250 mm) at room temperature, using an isocratic method consisting of 11% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid in water at 1.0 ml/min and detection at 214 nm. Typical elution times (min) for peptides were as follows: Asp-hexapeptide, 21.3; isoAsp-hexapeptide, 15.1; tetrapeptide, 13.5; and Asu-hexapeptide, 35.1. The dipeptide eluted at the solvent front.

For pH 0.3 to 3.0, the observed rate constants for the loss of the Asp-hexapeptide (k_{obs}) were determined from the slopes of linear plots of the logarithm of peptide concentration vs time. Pseudo-first-order rate constants of the parallel formations of Asu-hexapeptide (k_{ab}) and tetrapeptide (k_{ad}) were calculated using the following equation: $k_{obs} = k_{ab} + k_{ad}$ and $k_{ab}/k_{ad} = [\text{Asu-hexapeptide}]_f/[\text{tetrapeptide}]_f$. At pH 4.0, all rate constants were generated from fitting the data to LaPlace MicroMath (Salt Lake City, UT). For pH 5.0 to 10.0, the rate constants were obtained by curve-fitting the data using nonlinear least-squares regression (MINSQ, MicroMath, Salt Lake City, UT). The differential equations used to describe the kinetics scheme at pH 5.0 were successfully solved by Dr. Jeffrey Fox (The University of Utah, Salt Lake City). At pH 6.1 to 10.0, the decomposition of the Asp-hexapeptide was characterized by pseudo-first-order reversible kinetics behavior whereby the apparent rate constant of the loss of the starting peptide (k_{obs}) was calculated using the equation $k_{obs} = k_r$ (apparent rate constant of the reverse reaction) + k_f (apparent rate constant of the forward reaction), where k_r and k_f were generated from the best fit obtained.

RESULTS AND DISCUSSION

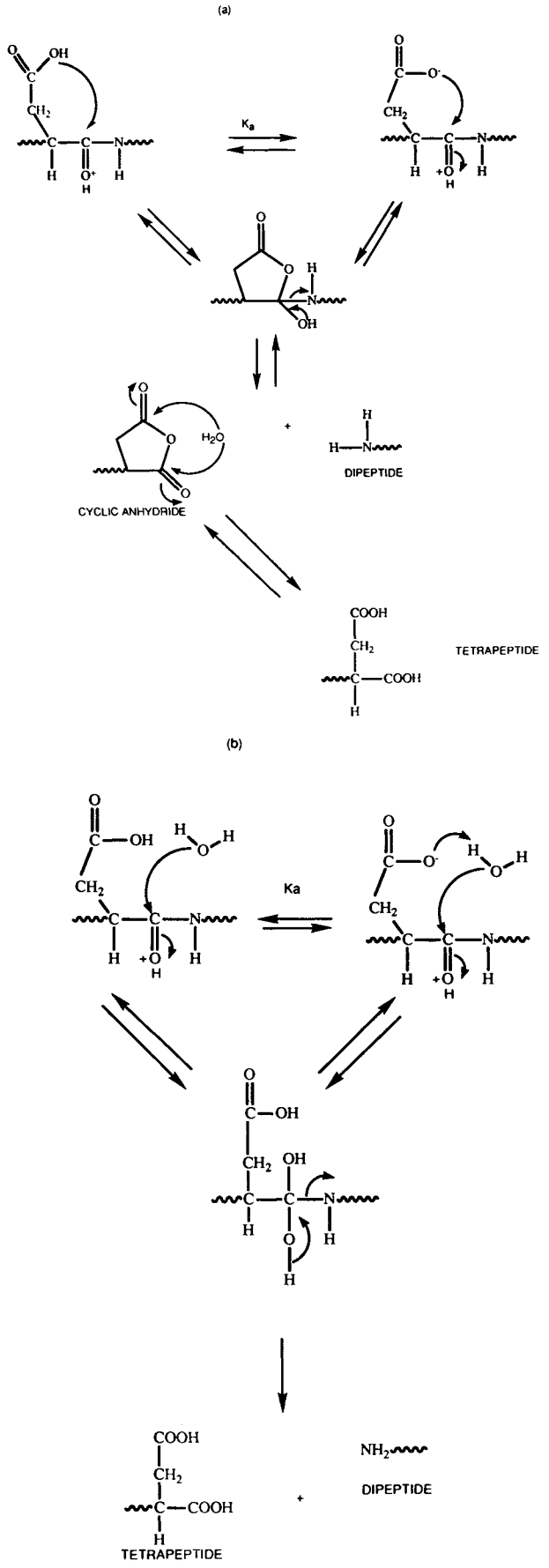
Degradation Pathways

The major degradation pathways for the Asp-hexapeptide consisted of the cleavage at the Asp-Gly amide bond (formation of tetrapeptide) and the isomerization of Asp to isoAsp via cyclic imide intermediate. The extent and routes of degradation were found to be pH dependent.

Highly Acidic

Under highly acidic conditions (pH 0.3–3.0), the Asp-hexapeptide decomposed predominantly via intramolecular cleavage of the Asp-Gly amide bond, forming a tetrapeptide, Val-Tyr-Pro-Asp, and a dipeptide, Gly-Ala (Fig. 1). In addition, the starting peptide could also cyclize to generate a Asu-hexapeptide, which remained stable up to 1400 hr and constituted approximately 7% of the total degradation products (Fig. 1). It has been established that peptide bonds of aspartyl residues are readily hydrolyzed in dilute acid compared to other peptide bonds (16,22). This is corroborated by the findings of A. S. Inglis, who reported that the majority of aspartyl peptide bonds in cytochrome *c,* wool proteins, and egg yolk apovitellenins is hydrolyzed after only 2 hr in dilute HCl (17). Similarly, Tsuda *et al.* found that under storage at 60°C and at pH values lower than 4.0, the intestinal hormone secretin, which contains two Asp residues at positions 3 and 15, degraded to a relatively larger amount of fragment peptides as a result of the cleavage reactions that occurred at Asp^3-Gly and Asp^{15}-Gly and to a lesser amount of aspartoyl secretin, the cyclic imide form of secretin (14). Furthermore, it was noted in an independent study of the hydrolysis of aspartoyl secretin that this compound was much more easily degraded in basic solution (pH 8.0) than in the pH 4.0 solution (14). High acid stability of the aspartimidyl residue was also confirmed by Patel and Borchardt in a similar study on the cyclic imide form of the Asn-hexapeptide (8).

The pH dependency of the hydrolysis of the Asp-Gly amide bond at 37°C as shown in Fig. 2 can be adequately described by Eq.(1), where k_{ad} is the pseudo-first-order rate constant for hydrolysis of the Asp-Gly amide bond, K_a is the



Scheme I. Plausible mechanisms of Asp-Gly amide bond hydrolysis: (a) intramolecular nucleophilic attack; (b) intramolecular general base-catalyzed water attack.

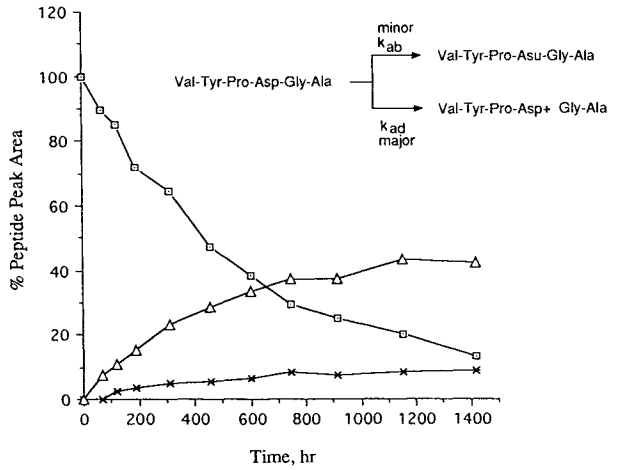


Fig. 1. Time course for the disappearance of Asp-hexapeptide (⊡) and appearance of tetrapeptide (△) and cyclic imide (⁎) at pH 1.1 (37°C and μ = 0.5). The inset shows the major and minor pathways of degradation of the Asp-hexapeptide at pH 0.3–3.0.

apparent dissociation constant for the side-chain carboxylic acid of the Asp residue, and k_H and k'_H are the microscopic second-order rate constants for specific acid-catalyzed hydrolysis of the Asp-Gly amide bond of the protonated and deprotonated species, respectively.

$$k_{ad} = \frac{k_H [H^+]^2 + k'_H K_a [H^+]}{K_a + [H^+]} \quad (1)$$

The break in the partial pH–rate profile (Fig. 2) most reasonably represents the ionization of the carboxylic acid side chain of the Asp residue. The kinetically generated pK_a was estimated to be 3.5 ± 0.2 by curve-fitting the pH–rate

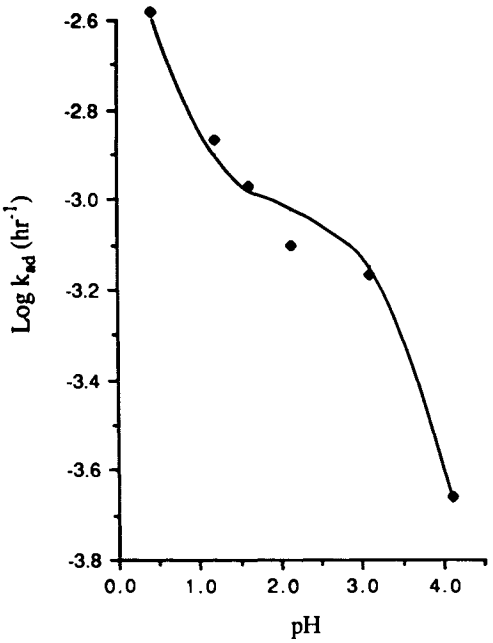


Fig. 2. Partial pH–rate profile for the Asp-Gly hydrolysis at 37°C (μ = 0.5). The solid represents the theoretical profile based on Eq.(1) and values of 3.4 (±0.3) × 10^{-3} M^{-1} hr^{-1} for k_H, 2.7 (±1.2) M^{-1} hr^{-1} for k'_H, and 3.3 (±1.6) × 10^{-4} for K_a.

profile. The plot is linear, with a negative slope approximately equal to unity at pH values above and below the pK_a, indicating apparent hydrogen ion catalysis. It was noted that the deprotonated species appears to be inherently more reactive with acid catalysis than the protonated forms as evidenced by the following calculated rate constants: $k_H = 3.4 \pm (0.3) \times 10^{-3}\ M^{-1}\ hr^{-1}$ and $k'_H = 2.7 \pm (1.2)\ M^{-1}\ hr^{-1}$. However, at pH values above the pK_a of the carboxylic acid side chain, the rate of Asp-Gly amide bond hydrolysis decreases in spite of the fact that the equilibrium concentration of the deprotonated species increases (Fig. 2). This is consistent with the view that the formation of tetrapeptide is specific acid-catalyzed, with the result that the rate is governed by the rapidly diminishing hydronium ion concentration at higher pH values. Further support for specific acid catalysis derives from the pseudo-first-order rate constants (k_{ad}) for the hydrolysis of the Asp-Gly amide bond as determined at different formate buffer concentrations at pH 3.0 (μ = 0.5, 37°C). No significant buffer catalysis was observed [0.005 *M* formate, 5.9 (±0.8) × 10^{-4} hr^{-1}; 0.05 *M* formate, 6.7 (±0.3) × 10^{-4} hr^{-1}; and 0.1 *M* formate, 6.9 (±0.6) × 10^{-4} hr^{-1}]. The substantial difference in the chemical reactivities of the protonated and deprotonated forms of the carboxylic acid side chain of the Asp residue and the absence of a significant catalytic effect by the buffers employed are not inconsistent with the formation of the tetrahedral intermediate being the rate-limiting step in the hydrolysis of the Asp-Gly amide bond.

Apparent enthalpies and entropies of activation for the Asp-Gly amide bond hydrolysis and the formation of Asu-hexapeptide at pH 1.1 were estimated from Eyring plots (Figs. 3a and b) of the logarithms of the respective rate constants (k_H and k_{ab}) versus the reciprocal of the temperatures of 37, 50, and 70°C. The specific acid-catalysis rate constant, k_H, for the hydrolysis of the Asp-Gly amide bond was used instead of the observed rate constant of tetrapeptide formation, k_{ad}, choosing the standard state of hydrogen ion concentration to be 1 *M*. Although the formations of Asu-hexapeptide (k_{ab}) and the Asp-Gly amide bond hydrolysis were enthalpically similar, the hydrolytic pathway was entropically more favorable ($\Delta S^{\neq} = -13.4 \pm 0.9$ eu, $\Delta H^{\neq} = 21.8 \pm 0.7$ kcal/mol) (Fig. 3b) than the formation of the Asu-hexapeptide ($\Delta S^{\neq} = -32.8 \pm 3.7$ eu, $\Delta H^{\neq} = 20.9 \pm 0.5$ kcal/mol) (Fig. 3a). The more positive value of entropy of activation for the acid-catalyzed hydrolysis reaction is consistent with an increase in entropy at the transition state due to expulsion of water molecules from highly solvated hydrogen ions.

Two plausible mechanisms of the Asp-Gly hydrolysis that are consistent with the data are illustrated in Scheme I. The first mechanism (Scheme Ia) involves hydrogen ion-catalyzed intramolecular nucleophilic attack by the Asp carboxylic acid side chain on the electrophilic carbonyl center of the amide backbone, leading to the expulsion of a dipeptide fragment and the formation of a cyclic anhydride intermediate which, in turn, can be hydrolyzed at either carbonyl center to generate a tetrapeptide. Both protonated and deprotonated species could undergo the same degradation mechanism (Scheme Ia) except that the carboxylate anion of the latter is a much better nucleophile than the carboxylic acid of the former accounting for the substantially higher

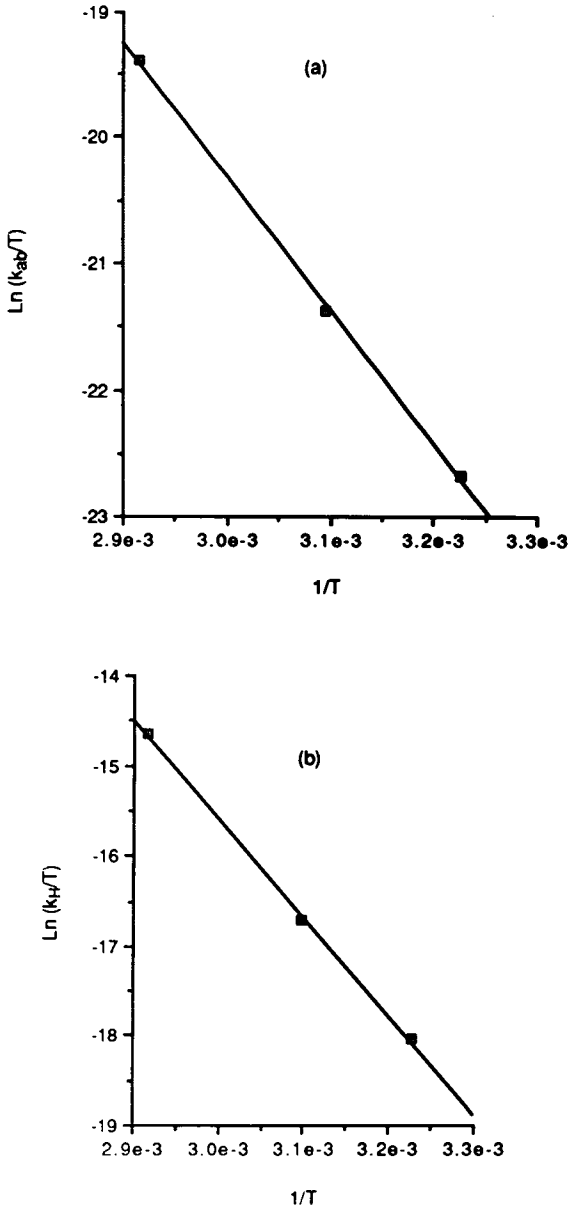


Fig. 3. Eyring plots for the temperature dependence of (a) formation of cyclic imide and (b) Asp-Gly hydrolysis at pH 1.1 and μ = 0.5.

reaction rate constant for the deprotonated species. The second mechanism (Scheme Ib) involves acid-catalyzed amide hydrolysis in which the deprotonated species can act as an intramolecular general base to catalyze the water attack on the electrophilic carbonyl center of the Asp-Gly amide backbone, generating the tetrahedral intermediate, which can be further degraded to form the same tetrapeptide and dipeptide fragments. However, the protonated species are less capable of such catalysis and, hence, react much more slowly than the deprotonated form.

Since both of these mechanisms are kinetically indistinguishable, it is impossible to differentiate them based on the pH–rate profile. To elucidate the governing mechanism, solvent isotope experiments were carried out at pD 1.1 and 37°C. The rationale is that if the first mechanism (Scheme Ia) is the operating mechanism, there should be no solvent iso-

tope effect (k_{H_2O}/k_{D_2O} = 1.0) following protonation of the substrate because solvent molecules are not involved in the rate-determining step. However, the solvent isotope effect following protonation should be considerably greater than one (k_{H_2O}/k_{D_2O} = 2–3) if the second mechanism (Scheme Ib) is operative, since solvent molecules participate in the rate-determining step in which significant breakage of the O-D or O-L bond occurs. The experimentally determined solvent isotope effect (k_{H_2O}/k_{D_2O}) obtained for the apparent hydrolysis of Asp-Gly amide bond was found to be 0.81. This effect includes both prior protonation and any effect following protonation. The inverse solvent isotope effect is consistent with either a prior equilibrium proton transfer to the carbonyl followed by rate-limiting nucleophilic attack by the side-chain carboxyl group or a concerted proton transfer and nucleophilic attack. Since H_3O^+ is a weaker acid than D_3O^+, an inverse solvent isotope effect is expected for the protonation step in the reaction (21,23). This inverse effect could be as small as 0.5, and the solvent isotope effect for the nucleophilic attack would then be approximately 1.6. This means that we cannot unambiguously rule out one or the other mechanism since this is a small but, nevertheless, possibly significant solvent isotope effect.

Mildly Acidic

As the pH of the reaction mixture was increased, the formation of Asu-hexapeptide became progressively predominant. This is illustrated by the results obtained at pH 4.0 (Fig. 4), where, initially, approximately 60% of the Asp-hexapeptide favored the pathway yielding the Asu-hexapeptide and only 10% of the Asp-hexapeptide underwent the Asp-Gly amide bond hydrolysis reaction to generate the tetrapeptide and dipeptide fragments. In addition, the chemical stability of the Asu-hexapeptide progressively diminished with increasing pH. The Asu-hexapeptide, which was extremely stable at pH 1.1 (Fig. 1), degraded at pH 4.0 to generate the isoAsp-hexapeptide and to regenerate the starting Asp-hexapeptide. The overall degradation pathways at pH 4.0 are illustrated in Fig. 4. The same kinetics scheme was observed for the degradation of secretin at pH 4.0 and 60°C (14).

Figure 5 illustrates the partial pH–rate profile for the formation of Asu-hexapeptide at 37°C. The profile was fitted to Eq.(2), where K_{a_1} and K_{a_2} are apparent dissociations constants, k_H is the second-order hydrogen ion catalysis rate constant for the reaction of the positively charged fraction, k_o is the first-order spontaneous rate constant for the reaction of the positively charged fraction, and k'_o is the first-order rate constant for the reaction of the zwitterionic species.

$$k_{ab} = \frac{k_H [H^+]^3 + k_0 [H^+]^2 + k'_0 K_{a_1} [H^+]}{[H^+]^2 + K_{a_1} [H^+] + K_{a_1} K_{a_2}} \qquad (2)$$

The kinetic rate constants and dissociation constant parameters used to generate the theoretical pH–rate profile (Fig. 5) are the following: k_H = 4.4 (±0.8) × 10^{-4} M^{-1} hr^{-1}, k_o = 8.9 (±2.3) × 10^{-5} hr^{-1}, k'_o = 2.0 (±0.05) × 10^{-3} hr^{-1}, K_{a_1} = 9.0 (±0.9) × 10^{-4} (pK_{a_1} = 3.1), and K_{a_2} = 6.5 (±0.7) × 10^{-6} (pK_{a_2} = 5.2). The second-order hydro-

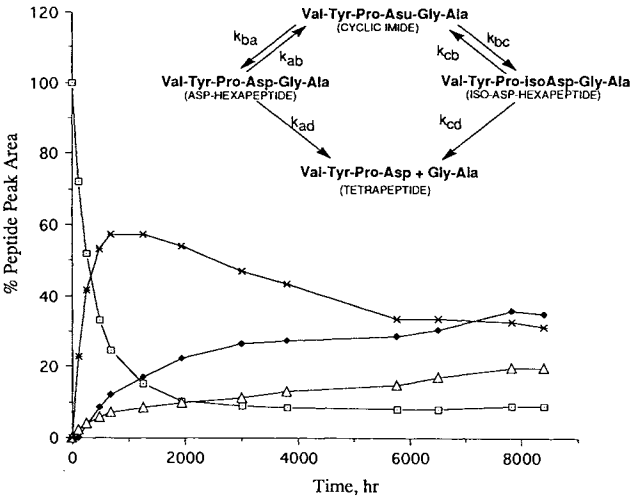


Fig. 4. Time course for the disappearance of Asp-hexapeptide (□) and appearance of cyclic imide (*) tetrapeptide (△) and isoAsp-hexapeptide (◆) at pH 4.0 (37°C and μ = 0.5). The inset shows the pathways of degradation of the Asp-hexapeptide at pH 4.0–5.0.

nium ion-catalyzed reaction was estimated to be five times faster than the spontaneous, water-catalyzed formation of the Asu-hexapeptide for the positively charged species. Also, the zwitterionic species appeared to be two orders of magnitude more reactive than the positively charged species in the first-order water-catalyzed formation of the Asu-hexapeptide. In comparison, the first-order rate constant for the deamidation reaction of the zwitterionic forms of the Asn-hexapeptide was three times faster than that for the zwitterionic species of the Asp-hexapeptide (8).

The pK_a of 3.1 was assigned to the ionization of the carboxylic acid side chain of the Asp residue. Although the

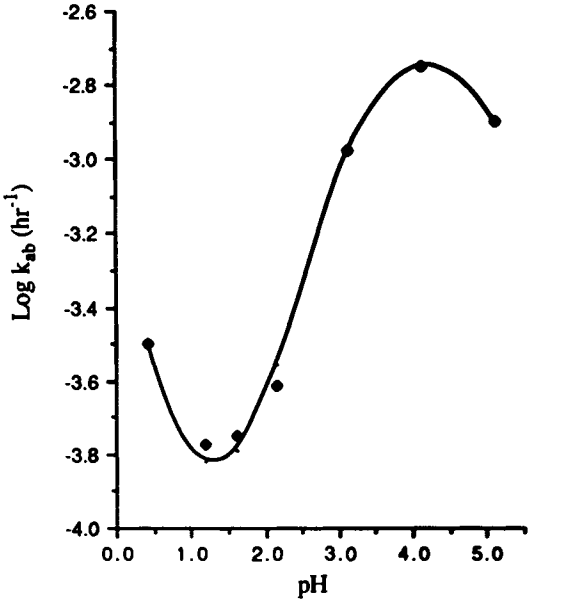


Fig. 5. Partial pH–rate profile for the formation of cyclic imide at 37°C (μ = 0.5). The solid line represents the theoretical profile based on Eq.(2) and values of 4.4 (±0.8) × 10^{-4} M^{-1} hr^{-1} for k_H, 8.9 (±2.3) × 10^{-5} hr^{-1} for k_o, 2.0 (±0.05) × 10^{-3} hr^{-1} for k'_o, 9.0 (±0.9) × 10^{-4} for K_{a_1}, and 6.5 (±0.7) × 10^{-6} for K_{a_2}.

pK_a assignment for the Asp side chain is reasonable, the kinetically generated pK_a of 5.2 is slightly higher than the typically reported pK_a values (pK_a = 3.0 – 4.7) for C-terminus carboxylic acids in small peptides (24). Similarly, Patel and Borchardt acquired an unusually high apparent pK_a value of 6.5 for the same C terminus from the theoretical fit of the pH–rate profile for deamidation of the Asn-hexapeptide (8). If this pK_a value does represent the dissociation of the C-terminus carboxylic acid, it is not clear why the pK_a value itself is elevated and, more importantly, how the dissociation of the C-terminus carboxylic acid mechanistically alters the rate. Another possible explanation would be that the inflection in the pH–rate profile at a pH value of approximately 5.2 is, in reality, a kinetic pK_a and corresponds to a change in the rate-determining step in the reaction sequence rather than the second dissociation of the reactant.

Near-Neutral to Basic

As previously mentioned, the Asu-hexapeptide readily degrades under slightly acidic, neutral, and basic conditions, affording the isoAsp-hexapeptide and the starting Asp-hexapeptide. Thus, at pH values above 6.1, the concentration of this intermediate was sufficiently low that it was no longer detectable since its rate of degradation is much faster than its rate of formation. In addition, the contribution of the Asp-Gly amide bond hydrolysis reaction to the overall degradation is insignificant at these pH values. Therefore, the complicated kinetics scheme that was observed at pH 4.0 collapsed to a simple pseudo-first-order reversible Asp-to-isoAsp interconversion under near neutral-to-basic conditions (Fig. 6). Figure 6 shows a typical time course for this interconversion at pH 10.0 and 37°C. The results from our study are comparable to those reported by Geiger and Clarke, who observed that the formation of isoAsp-hexapeptide via the Asu-hexapeptide was the major degradation pathway in the isomerization of the Asp-hexapeptide at physiological conditions (pH 7.4, 37°C) (25). Similarly, Tsuda *et al.* reported that, above pH 4.0, secretin reversibly converted to the rearranged peptide, β-aspartyl secretin (isoAsp), via hydrolysis of the aspartoyl secretin (Asu) at the Asp[3]-Gly position (14). This interconversion was thought to be responsible for the diminished potency of secretin in aqueous solutions. A previous study by Kirsch *et al.* demonstrated that with increasing pH, this reversible transpeptidation of Asp residues became the predominant degradation pathway for Daptomycin, a lipopeptide antibiotic which also contains an Asp-Gly sequence found in the Asp-hexapeptide (11).

Buffer catalysis did not occur to any significant extent for the forward (k_f) and backward (k_r) reactions, which were also essentially pH independent at pH 8.0 and above (Table I). The mechanistic implications of these observations are discussed in the subsequent section.

Effect of Asn Substitution

Replacing the Asp residue with an Asn residue effected a substantial impact on the overall stability of the hexapeptide. The observed rate constants for the degradations of the Asn-hexapeptide (see Fig. 6 in Ref. 8) and Asp-hexapeptide (Table I) under similar experimental conditions revealed the following observation. Although the Asn-hexapeptide experienced maximum stability near the pH range of 3 to 5, the Asp-Gly hexapeptide underwent the most rapid degradation at these pH values. Also, the Asn-hexapeptide was most unstable in neutral to alkaline conditions, whereas the Asp-hexapeptide exhibited highest chemical stability at these pH values.

More importantly, the substitution of Asp for Asn exerted a significant change in the kinetics of the degradation of the hexapeptide in the neutral-to-basic pH region, where the formation of isoAsp-hexapeptide via the Asu-hexapeptide intermediate predominantly occurs. The key

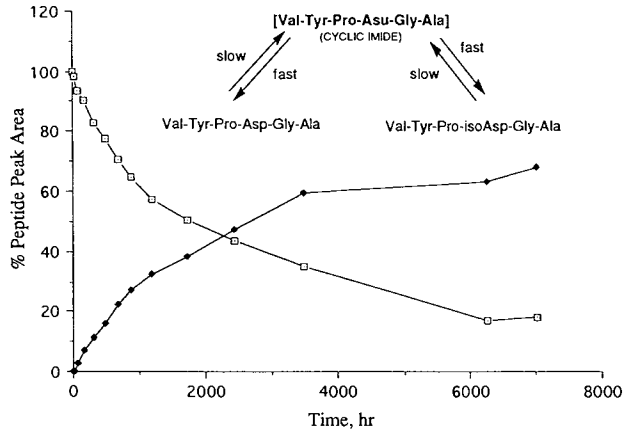
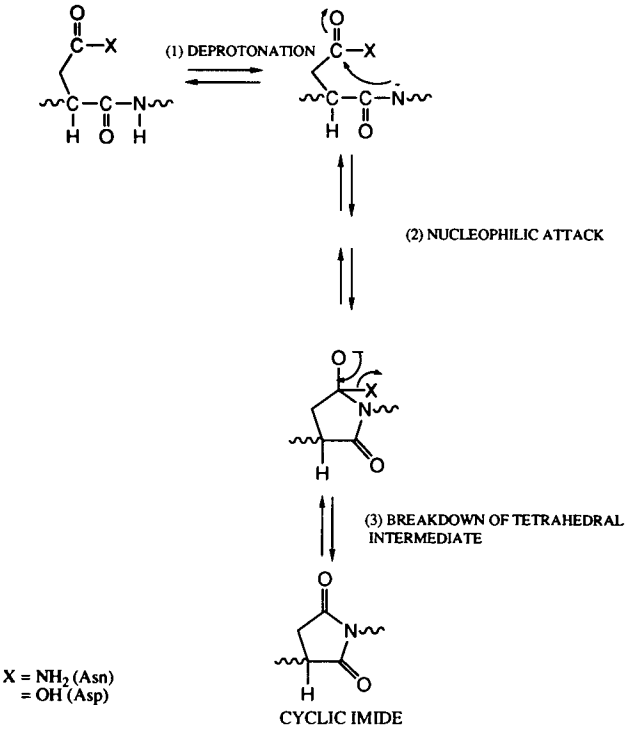


Fig. 6. Time course for the disappearance of Asp-hexapeptide (□) and appearance of isoAsp-hexapeptide (◆) at pH 10.0 (37°C and μ = 0.5). The inset shows the pathways of degradation of the Asp-hexapeptide at pH 6.1–10.0.



Scheme II. Proposed steps in the formation of cyclic imide.

Table I. Summary of Rate Constants for the Degradation of Asp-Hexapeptide in Buffer Solutions at Different pH Values (37°C, μ = 0.5)

pH	Buff. conc. (*M*)	Rate constant × 10^4 (hr^{-1}) ± SD								
		k_{obs}	k_{ab}[a]	k_{ad}[b]	k_r[c]	k_f[d]	k_{ba}[e]	k_{bc}[f]	k_{cb}[g]	k_{cd}[h]
0.3	—	28.4 ± 1.4	3.5 ± 0.2	25.0 ± 1.2	—	—	—	—	—	—
1.1	—	14.4 ± .4	1.40 ± .05	13.0 ± 0.4	—	—	—	—	—	—
1.5	—	11.8 ± 0.4	1.59 ± 0.06	10.2 ± 0.4	—	—	—	—	—	—
2.0	—	9.9 ± 0.4	2.3 ± 0.1	7.6 ± 0.3	—	—	—	—	—	—
3.0	0.005	17.0 ± 1.5	11.0 ± 1.4	5.9 ± 0.8	—	—	—	—	—	—
	0.05	17.0 ± 0.8	11.0 ± 0.5	6.7 ± 0.3	—	—	—	—	—	—
	0.1	16.0 ± 1.3	8.8 ± 0.7	6.9 ± 0.6	—	—	—	—	—	—
4.0	0.005	23.1	20.4	2.7	—	—	3.8	3.3	4.1	0.44
	0.05	28.1	25.1	3.1	—	—	5.5	2.4	2.4	0.012
	0.1	29.5	24.3	5.2	—	—	1.2	5.8	40.9	1.9
5.0	0.01	12.0 ± 1.8	11.7 ± 1.8	0.09 ± .06	—	—	51.9 ± 13.9	37.9 ± 3.5	2.5 ± 0.6	—
	0.05	13.0 ± 0.8	12.2 ± 0.8	0.45 ± 0.1	—	—	9.7 ± 1.5	20.0 ± 2.2	7.4 ± 1.4	—
	0.1	17.0 ± 1.5	16.5 ± 1.5	1.1 ± .2	—	—	6.9 ± 1.3	13.4 ± 2.6	8.0 ± 1.7	—
6.1	0.005[i]	7.0	—	—	1.6	5.4	—	—	—	—
	0.05	5.0 ± .7	—	—	0.6 ± .3	4.4 ± .4	—	—	—	—
	0.1	6.7 ± 1.4	—	—	0.9 ± .5	5.8 ± .9	—	—	—	—
7.4	0.005[i]	5.9	—	—	1.1	4.8	—	—	—	—
	0.05[i]	7.3	—	—	1.7	5.6	—	—	—	—
	0.1	5.2 ± 1.0	—	—	1.0 ± .5	4.2 ± .5	—	—	—	—
8.0	0.005	5.4 ± .5	—	—	1.3 ± .2	4.1 ± .3	—	—	—	—
	0.05	4.0 ± .4	—	—	0.8 ± .2	3.2 ± .2	—	—	—	—
	0.1	4.2 ± .7	—	—	0.8 ± .3	3.4 ± .4	—	—	—	—
9.0	0.02	5.3 ± .4	—	—	1.3 ± .2	4.0 ± .2	—	—	—	—
	0.05	3.6 ± .4	—	—	0.6 ± .2	3.0 ± .2	—	—	—	—
	0.1	4.1 ± .7	—	—	0.7 ± .3	3.4 ± .4	—	—	—	—
10.0	0.02	5.6 ± .6	—	—	1.4 ± .3	4.2 ± .3	—	—	—	—
	0.05	4.1 ± .5	—	—	0.7 ± .2	3.4 ± .3	—	—	—	—
	0.1	4.1 ± .9	—	—	0.5 ± .4	3.6 ± .5	—	—	—	—

[a] Rate constant of the appearance of the Asu-hexapeptide.
[b] Rate constant of the formation of the tetrapeptide from the Asp-hexapeptide.
[c] Apparent rate constant of the regeneration of the Asp-hexapeptide.
[d] Apparent rate constant of the formation of the isoAsp-hexapeptide.
[e] Rate constant of the formation of the Asp-hexapeptide from the Asu-hexapeptide.
[f] Rate constant of the formation of the isoAsp-hexapeptide from the Asu-hexapeptide.
[g] Rate constant of the back generation of the cyclic imide from the isoAsp-hexapeptide.
[h] Rate constant of the formation of the tetrapeptide from the isoAsp-hexapeptide.
[i] Rate constants at 37°C were extrapolated from accelerated temperature studies.

difference was that the degradation of the Asn-hexapeptide was subjected to both specific hydroxide ion (Table II) and general buffer catalysis (see Fig. 4 in Ref. 8) at pH values above 7.4, while the kinetics of the Asp-hexapeptide lacked pH and buffer dependency under identical conditions (Tables I and II). These observations provide some fundamental insights regarding the governing mechanism of the Asu-hexapeptide formation. Scheme II delineates three major steps that may be involved in the formation of Asu-hexapeptide from either the Asn-hexapeptide or the Asp-hexapeptide. These steps consist of the deprotonation of amide nitrogen, nucleophilic attack by the amide ion resulting in formation of the tetrahedral intermediate, and finally, the breakdown of the tetrahedral intermediate.

Based on NMR evidence, the amide protons of both Asn-hexapeptide and Asp-hexapeptide were subjected to rapid exchange in deuterium oxide in the absence of chemical degradation (unpublished data). This observation mechanistically implies that the deprotonation of the amide proton is relatively fast and can be ruled out as the rate-determining step in the overall degradation of either hexapeptide.

To probe the mechanistic nature of the rate-determining step in the formation of Asu-hexapeptide, we examine two

Table II. Comparison of the Effect of pH on Rate Constants for the Degradation of Asp-Hexapeptide and Asn-Hexapeptide Under Neutral to Basic Conditions (37°C, μm = 0.5)

pH	k_{obs} (hr^{-1})[a] ± SD (Asp)	pH	k_o (hr^{-1})[b] ± SD (Asn)
7.4	(6.1 ± 1.0) × 10^{-4}	7.5	(1.53 ± 0.08) × 10^{-2}
8.0	(4.5 ± 0.7) × 10^{-4}	8.0	(3.61 ± 0.32) × 10^{-2}
9.0	(4.3 ± 0.9) × 10^{-4}	9.0	0.102 ± 0.01
10.0	(4.6 ± 0.8) × 10^{-4}	10.0	0.281 ± 0.008

[a] Average of the observed rate constants (k_{obs}) of the degradation of Asp-hexapeptide at three buffer concentrations at a given pH.
[b] Rate constants of deamidation of Asn-hexapeptide at zero buffer concentration. Data taken from Ref. 8.

principal differences between the Asn and Asp residues. First, under basic conditions, it is recognized that hydroxide or alkoxide possesses greater ability as a leaving group, compared to amine ion (26). Thus, changing the leaving group from an amine ($-NH_2$) to a hydroxyl moiety (–OH) by replacing the Asn residue with an Asp residue equips the reactant with a better leaving group. Second, the carboxylic acid of the Asp side chain exists mainly as the carboxylate anion whose negative charge increases the electron density surrounding the carbonyl carbon center via resonance, rendering the carbonyl center less electrophilic and, hence, less reactive to the nucleophilic attack by the backbone amide nitrogen.

Taken these two factors together, it may be reasonable to suggest that the formation of Asu-hexapeptide from the Asp-hexapeptide involves the nucleophilic attack by the deprotonated amide nitrogen on the free carboxylic acid species. Thus, as the pH of the solution is increased, the equilibrium concentration of the nucleophilic amide ion increases but the fraction of protonated species decreases. These two effects exactly offset each other and the observed rate becomes independent of pH.

The presence of both general and specific base catalyses, as is the case for the Asn-hexapeptide, and lack of pH effect and general buffer catalysis, in the case of the Asp-hexapeptide, indicate a drastic change in the kinetic behavior upon structural substitution such as a change in the rate-determining step. However, there is no concrete evidence to assign unambiguously the rate-determining step in the formation of Asu-hexapeptide from either Asp- or Asn-hexapeptide.

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