Pharmaceutical Research, Vol. 7, No. 8, 1990

Research Article

Chemical Pathways of Peptide Degradation. III. Effect of Primary Sequence on the Pathways of Deamidation of Asparaginyl Residues in Hexapeptides

Kamlesh Patel[1] and Ronald T. Borchardt[1,2]

Received October 12, 1989; accepted January 23, 1990

Deamidation of Asn residues can occur either by direct hydrolysis of the Asn residue or via a cyclic imide intermediate. The effects of primary sequence on the pathways of deamidation of Asn residues were studied using Val-Tyr-X-Asn-Y-Ala hexapeptides with substitution on the C-terminal side (Y) and on the N-terminal side (X) of the Asn residue. In acidic media the peptides deamidate by direct hydrolysis of the Asn residue to yield only Asp peptides, whereas under neutral or alkaline conditions, the peptides deamidate by formation of the cyclic imide intermediates which hydrolyze to yield both isoAsp and Asp peptides. At neutral to alkaline pH's the rate of deamidation was significantly affected by the size of the amino acid on the C-terminal side of the Asn residue. The amino acid on the C-terminal side of the Asn residue has no effect on the rate of deamidation at acidic pH. Changes in the structure of the amino acid on the N-terminal side of the Asn residue had no significant effect on the rate of deamidation at all the pH's studied. For peptides that underwent deamidation slowly, a reaction involving the attack of the Asn side chain on the peptide carbonyl carbon resulting in peptide bond cleavage was also observed.

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

INTRODUCTION

Peptides and proteins can undergo degradation via a variety of chemical reactions, which are specific to certain amino acid residues (1). Deamidation of Asn[3] residues is one of the most common chemical pathways of peptide/protein degradation (2). It is now well recognized that at neutral to alkaline pH, the major pathway for deamidation of Asn residues is via a cyclic imide intermediate resulting in the formation of Asp- and isoAsp-containing peptides/proteins (3–7). Since cyclic imide formation involves the attack of a peptide bond nitrogen atom on the side chain carbonyl carbon atom of the Asn residue, the properties of the amino acid on the C-terminal side of the Asn residue play an important role in determining the rate of formation of the cyclic imide (2–5,8–11). Using small peptides, it has been shown that when Gly is present on the C-terminal side of Asn residues, cyclic imide formation is particularly rapid because of minimal steric interference presented by the Gly residue (3–5). In contrast, peptides having amino acids with bulky side chains (e.g., Val, Leu) on the C-terminal side of the Asn residue have slower rates of cyclic imide formation (3–5,8). In addition to steric factors influencing the rate of formation of the cyclic imide, other properties of the C-terminal side amino acid can influence the rate of deamidation of Asn residues. For example, amino acids such as His and Ser can enhance the rate of cyclic imide formation possibly through the catalytic effects of the imidazole and hydroxyl groups, respectively (8,10,11).

Recently we showed that at acidic pH's, the model peptide L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala hydrolyzed to yield exclusively L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala (12). The pathway of hydrolysis at acidic pH's involved direct hydrolysis of the amide side chain of the Asn residue.

From previous studies (2–12) it is clear that pH can influence the pathway by which an Asn residue undergoes deamidation and, thus, the distribution of the products (e.g., Asp versus isoAsp peptides). In addition, it is well recognized that the primary sequence, particularly the amino acid on the C-terminal side of the Asn residue, can influence the rate of formation of the cyclic imide and thus the overall rate of deamidation of a peptide at neutral to alkaline pH. However, it is unclear from these studies what effect, if any, the primary sequence has on the rate of deamidation of Asn residues at acidic pH's and whether the primary sequence can influence the pathway and thus the distribution of the products (Asp versus isoAsp) at acidic pH's. It is also unclear whether Asn residues that undergo deamidation slowly are prone to degrade by alternate pathways (e.g., peptide bond cleavage) at neutral to alkaline pH.

[1] Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66045.

[2] To whom correspondence should be addressed.

[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 and Asx is used to indicate uncertainty as to whether the residue was Asn or Asp prior to amino acid analysis.

0724-8741/90/0800-0787$06.00/0

To answer these questions we have studied the degradation of a series of hexapeptides (L-Val-L-Tyr-X-L-Asn-Y-L-Ala) with substitutions on the C-terminal side (Y) and on the N-terminal side (X) of the Asn residue.

MATERIALS AND METHODS

Materials

All t-Boc amino acid derivatives, Boc-Ala-O-resin, and Boc-Asp (OBzl)-O-resin were purchased from Peninsula Laboratories, Inc., Belmont, CA. Trifluoroacetic acid (HPLC grade) was purchased from Pierce, Rockford, IL. *o*-Phthalaldehyde, *N*-acetyl-L-cysteine, L-Asp, D-Asp, Gly-L-Ala, L-Ser-L-Ala, and L-Val-L-Ala and protease from *S. aureas*, type XVII-B, strain V8 (P-2922), were purchased from Sigma Chemical Company, St. Louis, MO. All other chemicals were of analytical grade and used as received. L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala (Asn-Gly hexapeptide), L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala (Asp-Gly hexapeptide), L-Val-L-Tyr-L-Pro-L-Asu-Gly-L-Ala (Asn-Gly cyclic imide), and L-Val-L-Tyr-L-Pro-L-Asp (tetrapeptide) were synthesized and characterized as described previously (12). L-Val-L-Tyr-L-Pro-L-Asn-L-Ser-L-Ala (Asn-Ser hexapeptide), L-Val-L-Tyr-L-Pro-L-Asn-L-Val-L-Ala (Asn-Val hexapeptide) and L-Asp-Gly-L-Ala were manually synthesized following the standard Merrifield solid-phase method (13). Asp-Gly-Ala peptide, Asn-Ser, and Asn-Val hexapeptides were characterized by sequence analysis and amino acid analysis by the Biochemical Service Laboratory at The University of Kansas using standard techniques (14). L-Val-L-Tyr-Gly-L-Asn-Gly-L-Ala (Gly-Asn hexapeptide) and L-Val-L-Tyr-Gly-L-Asp-Gly-L-Ala (Gly-Asp hexapeptide) were synthesized and characterized (amino acid analysis) by Dr. Alan Rawitch at The University of Kansas Medical Center, Kansas City, KS. All the peptides were purified by C_{18} reversed-phase HPLC as described by Murray and Clarke (15) using a semipreparative Alltech Econosphere column (10 × 250 mm, 10 μm resin).

Buffer Solutions

The buffers used were as follows: pH 1.0, HCl; pH 7.5, sodium phosphate; and pH 10.0, sodium borate. 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 37°C. An Orion (701A) pH meter equipped with a Ross combination electrode was used to measure the pH of the buffer solutions.

Kinetic Measurements

All kinetic experiments were carried out in aqueous buffer solutions at 37°C or otherwise indicated. After the addition of peptide to give an initial concentration of about 2×10^{-4} *M*, 250 μl of the reaction mixture was transferred to 1-ml ampoules, flame sealed and temperature equilibrated. At known time intervals, ampoules were removed and refrigerated. For faster reactions, aliquots were removed from a single reaction vessel and frozen to quench the reaction. Upon removal of the last sample, all of the stored samples were thawed and analyzed by HPLC as described below. 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 (16).

HPLC Analysis

Peptide analyses were performed on an Alltech Econosphere C_{18} column (4.6 × 250 mm, 5 μm resin) using the HPLC system described previously (12). The separation of Asn-Gly hexapeptide and its deamidated products was carried out as described previously using an isocratic system consisting of 7% acetonitrile, 0.1% (w/v) trifluoroacetic acid (TFA) in water at 1.0 ml/min and detection at 214 nm (12). The following modifications were necessary to separate deamidated products from Asn peptide for the other three peptides: Asn-Ser hexapeptide [mobile phase, 14% methanol, 0.1% (w/v) TFA; flow, 1.5 ml/min], Asn-Val hexapeptide [mobile phase, 20% methanol, 0.1% (w/v) TFA; flow, 1.0 ml/min], and Gly-Asn hexapeptide (mobile phase, 10% methanol, 0.1% TFA; flow, 1.0 ml/min).

Determination of D-Asp and L-Asp

Analysis of D- and L-Asp was carried out following the method described by Aswad (17). Briefly, lyophilized samples of HPLC-purified peptides were acid hydrolyzed *in vacuo* (in 6 *M* HCl for 24 hr at 110°C) and were derivatized with an *o*-phthalaldehyde/*N*-acetyl-L-cysteine reagent. The resulting fluorescent D- and L-Asp adducts were chromatographed and quantified as described by Johnson *et al.* (18). No correction was made for the racemization during hydrolysis in acid.

Proteolytic Digestion of Asp and IsoAsp Hexapeptides

Proteolytic digestion of deamidated products with *S. aureus* V8 protease was carried out as described previously to identify Asp and isoAsp peptides (12).

RESULTS

The deamidation reactions were carried out at pH 1.0 (0.1 *M* HCl), pH 7.5 (0.1 *M* phosphate), and pH 10.0 (0.05 *M* borate), $I = 0.5$, at 37°C. At a given pH, the deamidation followed pseudo-first-order kinetics for all peptides. The rate constants were obtained from the plots of ln peak area *vs* time. Table I lists the observed rate constants for deamidation of Asn hexapeptides. Like the parent peptide (Asn-Gly hexapeptide), both Asp and isoAsp hexapeptides were detected at pH 7.5 and 10.0 for all peptides studied (Figs. 1a, 2a, 3a, and 4a). Peaks for the deamidated products (Asp and isoAsp peptides) were identified based on the amino acid analysis and proteolytic mapping as described previously (data not shown) (12). Whenever possible, the identification was confirmed by comparing retention time with an authentic sample. The reaction for Asn-Val hexapeptide was very slow at pH 7.5, at 37°C. Therefore, experiments were carried out at higher temperatures (50–90°C) and the rate constant at 37°C was calculated using an Arrhenius equation and the activation energy of 20.7 kcal mol^{-1} (Fig. 5). The other activation parameters for deamidation of the Asn-Val hexapep-

Table I. Pseudo-First-Order Rate Constants for the Deamidation Reaction of Asn Peptides at 37°C and $I = 0.5$

Peptide	k_{obs} (hr^{-1}) × 10^{2a}		
	pH 1.0 (0.1 *M* HCl)	pH 7.5 (0.1 *M* phosphate)	pH 10.0 (0.05 *M* borate)
Val-Tyr-Pro-Asn-Gly-Ala	0.233 ± 0.006	3.47 ± 0.13	41.5 ± 4.3
Val-Tyr-Pro-Asn-Ser-Ala	0.196 ± 0.006	0.52 ± 0.01	2.31 ± 0.04
Val-Tyr-Pro-Asn-Val-Ala	0.199 ± 0.003	0.027 ± 0.002[b]	0.23 ± 0.06
Val-Tyr-Gly-Asn-Gly-Ala	0.245 ± 0.007	5.59 ± 0.11	48.7 ± 5.6

[a] $N = 3$; k_{obs} were calculated from the disappearance of the Asn peptides as determined by HPLC analysis as described under Materials and Methods.
[b] Calculated using the Arrhenius equation and an activation energy of 20.7 kcal/mol.

tide were as follows: $\log A = 7.44\ sec^{-1}$, $\Delta H^{\neq} = 19.9$ kcal mol^{-1}, and $\Delta S^{\neq} = -26.6$ cal $mol^{-1}\ K^{-1}$. Interestingly, a tetrapeptide with a composition of Val, Tyr, Pro, Asx (after amino acid analysis) was also detected for Asn-Ser ($t = 14$ min) and Asn-Val ($t = 8.7$ min) hexapeptides (Figs. 3a and 4a, respectively). This peptide may represent the product of the attack of the side-chain amide nitrogen on the peptide bond carbonyl to release a dipeptide (Ser-Ala or Val-Ala, respectively) and form a COOH-terminal cyclic imide (Scheme I). This cyclic imide can then be further hydrolyzed to form a tetrapeptide with a C-terminal Asn or Asp amide residue (Scheme I). Generally, the preferential hydrolysis of this cyclic imide occurs to form a product with a C-terminal Asn (19). The corresponding dipeptides (Ser-Ala and Val-Ala) were found to elute very close to the solvent front, making identification difficult. The cleavage product from the Asn-Ser hexapeptide appears to be identical in composition to the cleavage product found for the Asn-Val hexapeptide, i.e., Val-Tyr-Pro-Asx. The extent of the cleavage reaction was not determined. Similar cleavage products were also observed with Asn-Leu and Asn-Pro hexapeptides by Geiger and Clarke at pH 7.4 and 37°C (3).

For Asn-Val hexapeptide, at pH 7.5 and 10.0, we detected another product which elutes at 30.9 min (Fig. 4a). This peak had an identical amino acid composition (Val_2, Tyr, Pro, Asp, Ala) and gave an identical proteolytic mapping profile as the peak at 34.3 min in Fig. 4a (data not shown). Since the peak at 44.4 min was identified as Asp-Val hexapeptide, the peaks at 30.9 and 34.3 min appear to be the diastereomers of isoAsp-Val hexapeptide. These diastereomers probably arise from the racemization of the cyclic imide and subsequent hydrolysis of the cyclic imides as shown for Asn-Gly hexapeptide by Geiger and Clarke (3). When these two peaks were analyzed for the presence of

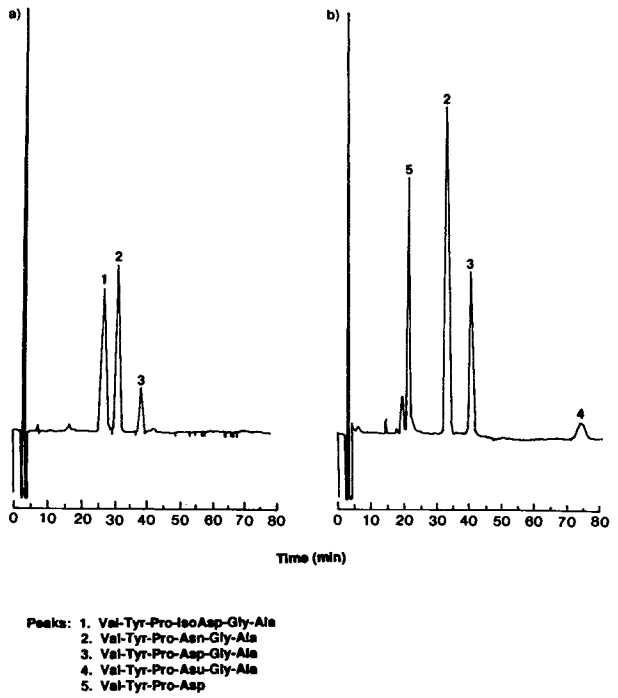


Fig. 1. HPLC chromatograms for the deamidation of Val-Tyr-Pro-Asn-Gly-Ala hexapeptide at (a) neutral to alkaline pH and (b) acidic pH.

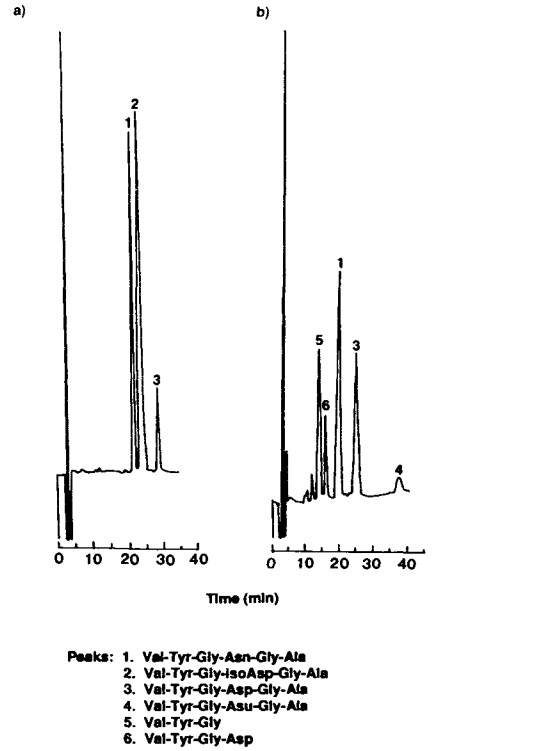
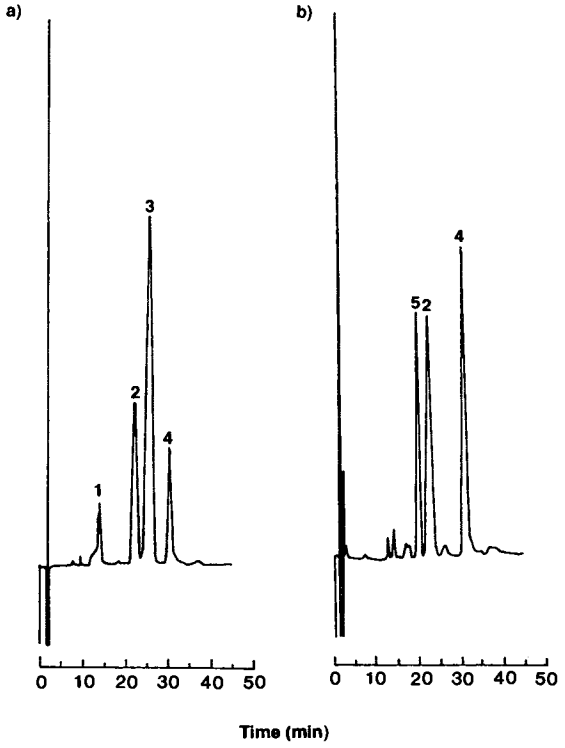
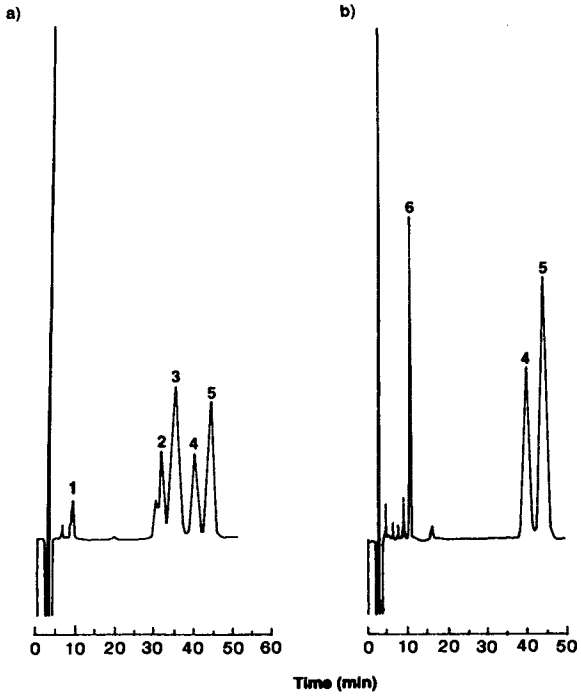


Fig. 2. HPLC chromatograms for the deamidation of Val-Tyr-Gly-Asn-Gly-Ala hexapeptide at (a) neutral to alkaline pH and (b) acidic pH.



Peaks: 1. Val-Tyr-Pro-Asx
2. Val-Tyr-Pro-Asn-Ser-Ala
3. Val-Tyr-Pro-isoAsp-Ser-Ala
4. Val-Tyr-Pro-Asp-Ser-Ala
5. Val-Tyr-Pro-Asp

Fig. 3. HPLC chromatograms for the deamidation of Val-Tyr-Pro-Asn-Ser-Ala hexapeptide at (a) neutral to alkaline pH and (b) acidic pH.



Peaks: 1. Val-Tyr-Pro-Asx
2. Val-Tyr-Pro-D-IsoAsp-Val-Ala
3. Val-Tyr-Pro-L-IsoAsp-Val-Ala
4. Val-Tyr-Pro-Asn-Val-Ala
5. Val-Tyr-Pro-Asp-Val-Ala
6. Val-Tyr-Pro-Asp

Fig. 4. HPLC chromatograms for the deamidation of Val-Tyr-Pro-Asn-Val-Ala hexapeptide at (a) neutral to alkaline pH and (b) acidic pH.

L-Asp and D-Asp, the peak at 30.9 min was shown to contain 93% of D-Asp and the peak at 34.3 min contained 95% L-Asp. Based on these results, we assigned the peak at 30.9 min as D-isoAsp-Val hexapeptide and the peak at 34.3 min as L-isoAsp-Val hexapeptide. However, the peak at 30.9 min could also be D-Asp-Val hexapeptide, since both D-Asp- and D-isoAsp-containing hexapeptides would give an identical amino acid composition and also be resistant to proteolytic cleavage. For the deamidated products of other peptides, separation of diastereomers was not observed under the HPLC conditions used.

For all the peptides, at both pH 7.5 and pH 10.0, the ratio of isoAsp and Asp hexapeptides (Table II) remained constant during the entire reaction. The ratio of IsoAsp and Asp hexapeptides for Asn-Ser and Gly-Asn hexapeptides is similar to that for the parent peptide (Asn-Gly hexapeptide) at pH 7.5 and 10.0, while for Asn-Val hexapeptide the ratio is lower than for the Asn-Gly hexapeptide.

Similar to the results obtained with the Asn-Gly hexapeptide (12), at pH 1.0 all three peptides studied here produce only Asp hexapeptides upon deamidation (Figs. 1b, 2b, 3b, and 4b). Formation of isoAsp hexapeptides was not observed. However, the Asp hexapeptides were found to degrade further at pH 1 to form tetra- and dipeptides because of cleavage of the Asp-X peptide bonds (Figs. 1b, 2b, 3b, and 4b) (20). The corresponding dipeptides for all hexapeptides studied were found to elute very close to the solvent front. Interestingly, hydrolysis of the Gly-Asp hexapeptide also formed the tripeptides (Val-Tyr-Gly) and (Asp-Gly-Ala) (Fig. 2b). These fragments probably form because of the peptide bond cleavage on the N-terminal side of the Asp residue (Gly-Asp), as described by Inglis (20). Such cleavage on the N-terminal side of the Asp residue was not observed for any of the other peptides. For the Gly-Asn hexapeptide at pH 1.0, there was also one late-eluting peak at 36.9 min

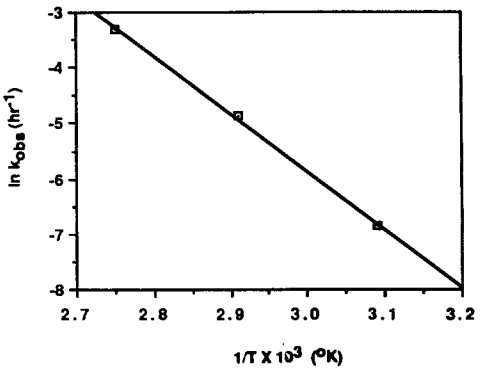
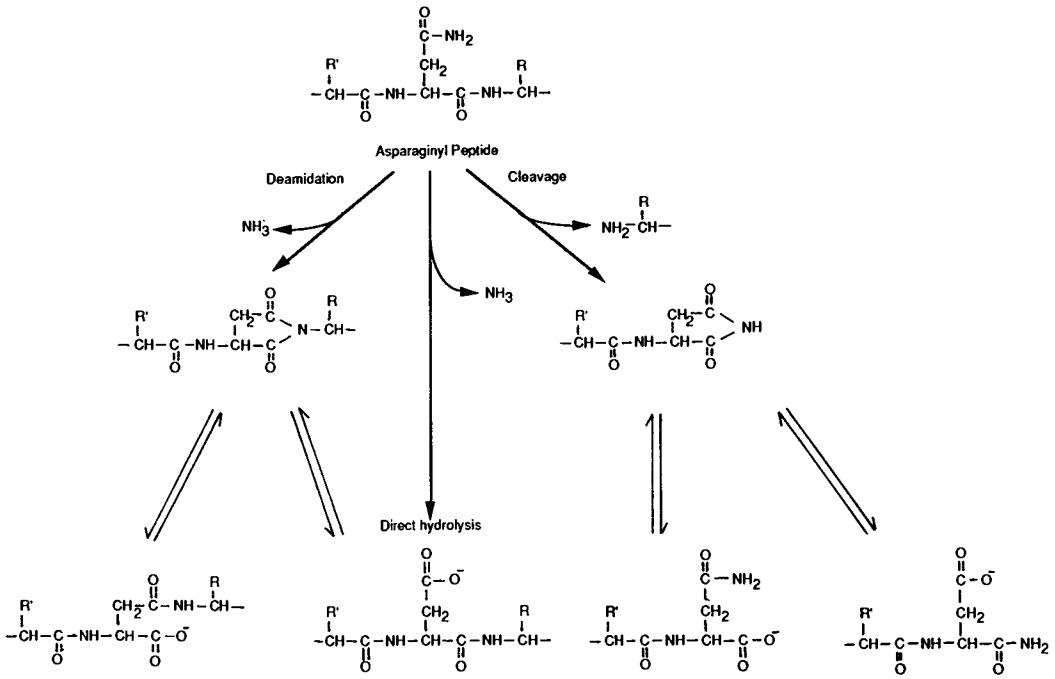


Fig. 5. Arrhenius plot for the deamidation of Val-Tyr-Pro-Asn-Val-Ala hexapeptide at pH 7.5, 0.1 *M* phosphate buffer, I = 0.5.



Scheme I

(Fig. 2b). As for the Asn-Gly hexapeptide, this peak was identified as the corresponding cyclic imide peptide (12). The maximum concentration of the cyclic imide was less than 10% of the total peptide. No such cyclic imide was detected for Asn-Val and Asn-Ser hexapeptides.

DISCUSSION

Upon deamidation under acidic conditions, each of the peptides used in this study degraded to give only the corresponding Asp hexapeptides, with no isoAsp hexapeptides being detected. If cyclic imide intermediates were involved in the deamidation of these hexapeptides at acidic pH, we would predict an order of reactivity of Asn-Val < Asn-Ser < Asn-Gly (4). This prediction is based on the steric bulk of the amino acid on the C-terminal side of the Asn residue (4). However, our results show that all the hexapeptides degrade with identical rates (Table I). This result clearly indicates that the pathway for deamidation at acidic pH is via direct hydrolysis of the amide side chain of Asn residues (Scheme I). With this pathway, we would not expect any effect of substitution on the N-terminal side of the Asn residue on the rate of deamidation and distribution of products. Indeed, no change occurred when Pro was replaced with Gly in the parent hexapeptide (Tables I and II). The deamidation pathway via cyclic imide intermediate in acidic media can be ruled out from our previous observation that the Asn-Gly cyclic imide was stable at pH 1 up to 912 hr, whereas the Asn-Gly hexapeptide under identical conditions had $t_{1/2}$ = 301.3 hr (12). The detection of small amounts of cyclic imide for the Asn-Gly and Gly-Asn hexapeptides indicates that it represents a very minor pathway for the deamidation of the Asn side chain at pH 1.0. The absence of cyclic imide for Asn-Val and Asn-Ser hexapeptides at pH 1 is not surprising because the added steric hindrance from the side chains of Ser and Val would further slow the formation of cyclic imides.

The peptide bond cleavage (Asp-Y) for all Asp-hexapeptides at pH 1.0 further confirms that Asp residues may also be hot spots for degradation of peptides and proteins (21,22). Asp residues in peptides can either undergo proteolysis (21,22) or interconvert to isoAsp residues via a cyclic imide intermediate (3,4). Interestingly, for the Gly-Asp hexapeptide, peptide bond cleavage was also observed

Table II. The Ratio of IsoAsp and Asp Hexapeptides at 37°C and I = 0.5

Peptide	pH 1.0 (0.1 *M* HCl)	pH 7.5 (0.1 *M* phosphate)	pH 10.0 (0.05 *M* borate)
Val-Tyr-Pro-Asn-Gly-Ala	100% Asp	3.8	4.1
Val-Tyr-Pro-Asn-Ser-Ala	100% Asp	4.3	4.2
Val-Tyr-Pro-Asn-Val-Ala[a]	100% Asp	2.0–2.6[b]	2.1
Val-Tyr-Gly-Asn-Gly-Ala	100% Asp	4.0	4.2

[a] The isoAsp hexapeptide was calculated combining both L-isoAsp and D-isoAsp hexapeptide peak areas.
[b] Reactions were carried out at 50 to 90°C and the ratio varied between 2.0 to 2.6 in the temperature range studied.

on the N-terminal side of the Asp residue. For the other peptides such cleavage was not observed. This indicates the influence of primary sequence on the proteolysis of X-Asp-Y bonds in acidic media. This is not surprising because these reactions also involve an intramolecular formation of either five- or six-membered anhydride intermediates (20). For Gly-Asn hexapeptide, the Gly residue offers minimal steric interference and allows flexibility to adopt conformations which favor such reactions. With the other three peptides, the presence of a Pro residue on the N-terminal side of the Asp residue may hinder such an intramolecular reaction by conformational restriction of the peptide backbone (23).

In contrast to the results at pH 1.0, each of the peptides tested here showed the formation of both isoAsp and Asp hexapeptides at pH 7.5 and 10.0. This suggests the formation of a cyclic imide intermediate at neutral to alkaline pH, similar to that observed with the Asn-Gly hexapeptide (12). We have found that the most rapid deamidation occurred when a Gly residue is present on the C-terminal side of the Asn residue. For example, the rate of deamidation with the Asn-Gly hexapeptide was 6 to 20 times that of the Asn-Ser hexapeptide and 120 to 180 times that of the Asn-Val hexapeptide. These results are not unexpected considering that the Gly residue provides minimal steric interference to attack of the peptide bond nitrogen atom on the side-chain carbonyl carbon atom. In Asp-beta benzyl ester-containing peptides with Thr and Ser residues following the Asp ester, cyclic imide formation in alkaline media was 3 to 6 times faster than when the ester was followed by a Gly residue (8). These studies suggested that a hydroxyl group on the Ser and Thr residues may catalyze the cyclic imide formation. However, in our studies, the Asn-Gly hexapeptide was still more reactive than the Asn-Ser hexapeptide. Similar results were also obtained by Stephenson and Clarke (4) and Capasso *et al.* (5). Stephenson and Clarke have proposed that Gly-containing peptides could adopt more conformations favorable for reaction compared to peptides where the presence of a beta side-chain carbon restricts the range of motion (4). Another factor may involve the electron-inductive effect of the side chain on the deprotonation of the peptide bond nitrogen that has been postulated to be necessary for the nucleophilic attack that forms the cyclic imide (11). The absence of electron-donating substituents on the Gly side chain can thus also contribute to the reactivity of Asx-Gly peptides (24).

Clarke and co-workers have reported deamidation rates for Asn-Ala, Asn-Leu, and Asn-Pro hexapeptides at pH 7.4, 37°C (Table III) (3,4). The difference in the rate of degradation among the Asn-Val, Asn-Pro, and Asn-Leu hexapeptides is very small. In fact, the Asn-Val hexapeptide is slightly more stable than the Asn-Leu hexapeptide even though Leu is bulkier than Val (R = isobutyl *vs* isopropyl). It is not clear why the Asn-Val hexapeptide is more stable than the Asn-Leu hexapeptide, but similar results were also obtained in a study with an Asp-beta ester peptide in alkaline media by Bodanzky and Kwei (8).

The cyclic imide can also be formed by nucleophilic attack of the side-chain amide nitrogen on the peptide bond carbonyl group, with concomitant cleavage of a peptide bond. With Asn-Val and Asn-Ser hexapeptides, such cleavage products were observed at pH 7.5 and 10. Similar cleavage products were also observed for the Asn-Leu and Asn-Pro hexapeptides at pH 7.4 and 37°C (3). However, we do not detect such cleavage products with the Asn-Gly and Gly-Asn hexapeptides, probably because the deamidation reaction resulting in Asp and isoAsp hexapeptides is much more rapid and results in the loss of the side-chain amide nitrogen which would participate in such a cleavage reaction. These results suggest that although the rate of deamidation can be slowed down by replacing an amino acid with a bulky side chain on the C-terminal side of the Asn residue, the overall degradation can give a mixture of products at neutral to alkaline pH (e.g., Asp and isoAsp peptides, cleavage products). This was recently observed with α-crystallin at pH 7.4 and 70°C, where both deamidation and cleavage products were formed at an Asn^{101}-Glu^{102} sequence (19).

Table III. Effect of Amino Acid Sequence on Rates of Deamidation of Asn Peptides

Peptide	$t_{1/2}$ (days)
Val-Tyr-Pro-Asn-Gly-Ala	1.89[a]
Val-Tyr-Pro-Asn-Ser-Ala	5.55[a]
Val-Tyr-Pro-Asn-Ala-Ala	20.2[b]
Val-Tyr-Pro-Asn-Val-Ala	106[a]
Val-Tyr-Pro-Asn-Leu-Ala	70[b]
Val-Tyr-Pro-Asn-Pro-Ala	106[b]

[a] pH 7.5 (0.1 M phosphate buffer, I = 0.5), 37°C. Values of $t_{1/2}$ were calculated from the data given in Table I.
[b] pH 7.4 (0.1 M phosphate buffer), 37°C. Data from Refs. 3 and 4.

Modification on the N-terminal side of the Asn residue does not appear to have any significant effect, at pH 7.5 and 10.0, on the rate of deamidation and the ratio of the deamidated products. Since this amino acid is not directly involved in the formation of cyclic imide, these results are not surprising. A similar observation was made by Kossiakoff (25) with trypsin, where a side chain of the amino acid on the C-terminal side of the Asn residue was found to play a more important role in deamidation than the side chain of the amino acid on the N-terminal side.

Caution should be exercised when extrapolating these results to proteins, because in proteins, secondary and tertiary structures may also play an important role in determining the site and the rate of deamidation [e.g., trypsin (25), glucagone (26), calmodulin (27)].

In conclusion, these results suggest that the primary sequence does not influence the overall pathway of deamidation. In acidic media, the peptides deamidate by direct hydrolysis (Scheme I) of the Asn residue to yield only the Asp peptides, whereas under neutral or alkaline conditions, the peptides deamidate via the formation of cyclic imide intermediates, which hydrolyze to yield the isoAsp and Asp peptides (Scheme I). At neutral to alkaline pH, the primary sequence can affect the rates of deamidation, the distribution of the deamidated products, and the tendency of the peptides to undergo side reactions (e.g., attack of the Asn side chain on the peptide bond carbonyl resulting in peptide bond cleavage). Under acidic conditions, the sequence does not appear to alter the rate of deamidation nor the exclusive formation of the Asp peptides as the product of deamidation. However, the sequence can influence the tendency of the

peptide to undergo side reactions (e.g., cyclic imide formation).

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 and Mark C. Manning, The University of Kansas.

REFERENCES

1. M. C. Manning, K. Patel, and R. T. Borchardt. *Pharm. Res.* 6:903–918 (1989).
2. A. B. Robinson and C. J. Rudd. In B. L. Horecker and E. R. Stadtman (eds.), *Current Topics in Cellular Regulations, Vol. 8,* Academic Press, New York, 1974, pp. 247–295.
3. T. Geiger and S. Clarke. *J. Biol. Chem.* 262:785–794 (1987).
4. R. C. Stephenson and S. Clarke. *J. Biol. Chem.* 264:6164–6170 (1989).
5. S. Capasso, L. Mazzarella, F. Sica, and A. Zagari. *Pept. Res.* 2:195–200 (1989).
6. R. Lura and V. Schirch. *Biochemistry* 27:7671–7677 (1988).
7. Y. C. Meinwald, E. R. Stimson, and H. A. Scheraga. *Int. J. Peptide Protein Res.* 28:79–84 (1986).
8. M. Bodanszky and J. Z. Kwei. *Int. J. Peptide Protein Res.* 12:69–74 (1978).
9. I. Schon and L. Kisfaludy. *Int. J. Peptide Protein Res.* 14:485–494 (1979).
10. T. Baba, H. Sugiyama, and S. Seto. *Chem. Pharm. Bull.* 21:207–209 (1973).
11. S. A. Bernhard, A. Berger, J. H. Carter, E. Katchalski, M. Sela, and Y. Shelitiny. *J. Am. Chem. Soc.* 84:2421–2434 (1962).
12. K. Patel and R. T. Borchardt. *Pharm. Res.* 7:703–711 (1990).
13. J. Stewart and J. S. Young. In *Solid Phase Peptide Synthesis,* 2nd ed., Pierce, Rockford, IL, 1984, pp. 71–95.
14. G. Allen. In T. S. Work and R. H. Burdon (eds.), *Sequencing of Proteins and Peptides,* North-Holland, Amsterdam, 1981.
15. E. D. Murray, Jr., and S. Clarke. *J. Biol. Chem.* 259:10722–10732 (1984).
16. I. H. Segel. *Biochemical Calculations,* John Wiley and Sons, New York, 1968.
17. D. W. Aswad. *Anal. Biochem.* 137:405–409 (1984).
18. B. A. Johnson, E. D. Murray, Jr., S. Clarke, D. B. Glass, and D. W. Aswad. *J. Biol. Chem.* 262:5622–5629 (1987).
19. C. E. M. Voorter, W. A. de Haard-Hoekman, P. J. M. van den Oetelaar, H. Bloemendal, and W. W. de Jong. *J. Biol. Chem.* 263:19020–19023 (1988).
20. A. S. Inglis. *Methods. Enzymol.* 91:324–332 (1983).
21. T. J. Ahern and A. M. Klibanov. *Science* 228:1280–1284 (1985).
22. S. E. Zale and A. M. Klibanov. *Biochemistry* 25:5432–5444 (1986).
23. K. Patel, C. Oliyai, R. T. Borchardt, and M. C. Manning. *J. Cell. Biochem.* 13A:A318 (1989).
24. M. A. Ondetti, A. Deer, J. T. Sheehan, J. Pluscec, and O. Kocy. *Biochemistry* 7:4069–4075 (1968).
25. A. A. Kossiakoff. *Science* 240:191–194 (1988).
26. I. M. Ota, L. Ding, and S. Clarke. *J. Biol. Chem.* 262:8522–8531 (1987).
27. I. M. Ota and S. Clarke. *Biochemistry* 28:4020–4027 (1989).