

Effects of Amino Acid Sequence, Buffers, and Ionic Strength on the Rate and Mechanism of Deamidation of Asparagine Residues in Small Peptides*

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The nonenzymatic rates of deamidation of Asn residues in a series of pentapeptides with the sequences VSNXV and VXNSV, where X is one of 10 different amino acids, were determined at neutral, alkaline, and acid pH values. The results demonstrate that in neutral and alkaline solutions the amino acid residue on the amino side of the Asn had little or no effect on the rate of deamidation regardless of its charge or size. The group on the carboxyl side of Asn affected the rate of deamidation significantly. Increasing size and branching in the side chain of this residue decreased the rate of deamidation by as much as 70-fold compared to glycine in the N-G sequence, which had the greatest rate of deamidation. In acidic solution, the rate of deamidation of the Asn residue was not affected by the amino acid sequence of the peptide. The products for each deamidation reaction were tested for the formation of isoAsp residues. In neutral and alkaline solutions, all products showed that the isoAsp:Asp peptide products were formed in about a 3:1 ratio. In acidic solution, the Asp peptide was the only deamidation product formed. All peptides in which a Ser residue follows the Asn residue were found to undergo a peptide cleavage reaction in neutral and alkaline solutions, yielding a tripeptide and a dipeptide. The rate of the cleavage reaction was about 10% of the rate of the deamidation pathway at neutral and alkaline pH values. The rates of deamidation of Asn residues in the peptides studied were not affected by ionic strength, and were not specific base catalyzed. General base catalysis was observed for small bases like ammonia. A model for the deamidation reaction is proposed to account for the observed effects.

The nonenzymatic deamidation of Asn to Asp is known to occur in proteins and peptides (Clarke, 1985; Wright, 1991a, 1991b). Mechanistic studies have suggested that the reaction at neutral to basic pH is intramolecular and proceeds by way of a five-membered succinimide intermediate (Meinwald *et al.*, 1986; Geiger and Clarke, 1987; Capasso *et al.*, 1989a; Stephenson and Clarke, 1989) (Scheme I, path a). This intermediate is formed via a nucleophilic attack on the side chain carbonyl carbon of Asn by the backbone nitrogen of the ensuing amino acid residue, releasing NH₃. It has been pro-

posed that the peptide backbone amide is deprotonated prior to or concomitant with the nucleophilic attack, as this would greatly increase its nucleophilicity (Bernhard *et al.*, 1962; Clarke, 1987). The succinimide ring is labile to base and readily opens in two possible ways, yielding either aspartic acid in an isoAsp¹ linkage or aspartic acid in a normal peptide linkage. The isoAsp product predominates at a ratio of about 3:1 (Meinwald *et al.*, 1986; Geiger and Clarke, 1987; Lura and Schirch, 1989; Patel and Borchardt, 1990a, 1990b). Peptide cleavage at Asn residues has also been observed in peptides and proteins (Geiger and Clarke, 1987; Voorter *et al.*, 1988; Violand *et al.*, 1990). These cleavage reactions are assumed to proceed through a succinimide intermediate by attack of the side chain amide of Asn on the backbone peptide carbonyl of Asn as shown in path b of Scheme I.

The characterization of deamidation of Asn residues has been primarily elucidated from studies of short Asn-containing peptides. Several studies have shown that the structures of the amino acids which flank Asn have a marked effect on the rate of deamidation. For example, a study by Robinson *et al.* (1973) suggests that when the sequence of Asn-Ala is preceded by an amino acid with a large, bulky side chain (I, L, V, P) the deamidation rate is as much as 6-fold slower than when the preceding residue is Gly. When Arg precedes Asn it increases the rate of deamidation 2-fold over that of peptides with the Gly-Asn-Ala sequence (Robinson *et al.*, 1973; Robinson, 1974). Other reports have shown that, in general, when the residue following Asn is varied, the rate of deamidation decreases as the size and steric bulk of the residue increases, such that G > S > A > L > P when ranked with respect to reaction rate (Robinson and Rudd, 1974; Geiger and Clarke, 1987; Capasso *et al.*, 1989; Stephenson and Clarke, 1989). These sequence effects may be important in proteins as well. There is evidence that in proteins the identity of the nearest neighbors of Asn is non-random, suggesting that their selection is subject to evolutionary constraints (Clarke, 1985; Wright, 1991a, 1991b). In addition, some investigators have reported that the rate of deamidation is enhanced by both high pH (Scotchler and Robinson, 1974; Capasso *et al.*, 1989a; Patel and Borchardt, 1990a), and ionic strength (McKerrow and Robinson, 1971; Scotchler and Robinson, 1974) and that certain buffers tend to increase the reaction rate, especially

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¹ The abbreviations used are: isoAsp, a peptide linkage in which the amide bond between Asp and the next amino acid is through the 4-carboxyl group of Asp; BES, *N,N*-bis(2-hydroxyethyl)2-aminoethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, *N*-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; CAPS, cyclohexylaminopropanesulfonic acid; PIMT, protein isoaspartyl methyltransferase; *n*+1, the amino acid connected to the carboxyl group of Asn; *n*-1, the amino acid connected to the amino group of Asn; HPLC, high performance liquid chromatography; CHES, 2-(cyclohexylamino)ethanesulfonic acid; *t*-Boc, *t*-butoxycarbonyl.

at alkaline pH (McKerrow and Robinson, 1971; Scotchler and Robinson, 1974; Bhatt, *et al.*, 1990; Patel and Borchardt, 1990a). Others, however, have not observed buffer catalysis of these reactions in the alkaline pH range (Lura and Schirch, 1989; Capasso *et al.*, 1989).

In previous investigations, model peptide sequences were usually selected on the basis of amino acid sequences in proteins which were known to deamidate (Geiger and Clarke, 1987; Lura and Schirch, 1988; Stephenson and Clarke, 1989; Patel and Borchardt, 1990a, 1990b; Robinson *et al.*, 1973). No systematic approach with respect to the effects of charge and size of the amino acids adjacent to the Asn residue has been undertaken. In this study, we have investigated the rate of deamidation of Asn residues in two series of pentapeptides, VSNXV and VXNSV, where X is one of 10 different amino acids whose side chains vary in charge and size. The effects of buffer, pH, and ionic strength on the rate of deamidation of these peptides have been investigated. The results correct some misconceptions about the effect of these factors on the rate of deamidation of Asn residues in small peptides. We also describe the characterization of a peptide bond cleavage reaction which accompanies deamidation in some of the peptides.

EXPERIMENTAL PROCEDURES

Materials—*t*-Boc-amino acids and *t*-Boc-amino acid resins were obtained from Peninsula Laboratories, Inc. Sulfonic acid buffers, *S*-adenosyl-L-methionine, LiCl, and the SerGly dipeptide were purchased from Sigma. [³H]Methyl-*S*-adenosyl-L-methionine was from NEN Research Products. Guanidine hydrochloride was obtained from Schwarz/Mann. HPLC grade triethylamine was purchased from Fisher, and ammonium hydroxide (58%) was from Mallinckrodt. All organic solvents and inorganic compounds were of ACS reagent grade or better.

Peptide Synthesis—Peptides were synthesized manually (Stewart and Young, 1969) by solid-phase peptide synthesis using *t*-Boc-amino acids. Peptide purity was checked by HPLC, thin layer chromatography, and amino acid analysis. Most peptides were pure as synthesized. If further purification was required, it was done on a C-18 reverse-phase semipreparative HPLC column from Column Resolution, Inc.

Deamidation Reaction Conditions—Peptides at a concentration of 0.5, 1, or 2 mg/ml were reacted at 60 °C in a volume of 0.4 ml in stoppered polypropylene vials. At appropriate time points, a vial was removed from the incubator, and stored at 4 °C until the time of

analysis. Low pH reactions were carried out in 0.1 N HCl.

Buffer and Ionic Strength Studies—The effect of sulfonic acid buffer concentration on the rate of deamidation of the peptide VSNGV was determined in 20, 50, 100, and 200 mM solutions of the following buffers: BES, pH 7.3; TAPS, pH 8.0; CHES, pH 9.0. The pH profile of the rate of deamidation of VANSV and VSNGV was performed using a series of 20 mM sulfonic acid buffers as follows: pH 6.0, MES; pH 7.3, BES; pH 8.0, TAPS; pH 9.0, CHES; and pH 10.0, CAPS. Ionic strength and buffer catalysis studies were performed under the following conditions: 20 mM BES or TAPS buffer, pH 8.0, with 0, 20, 100, and 200 mM sodium phosphate; 20 mM TAPS, pH 8.0, plus 20 mM imidazole; 20 mM BES, pH 8.0, with 0, 0.1, 0.2, 0.4, and 1.0 M guanidine hydrochloride; 20 mM TAPS, pH 8.0, with 0, 0.05, 0.5, and 1.0 M LiCl; 20 mM TAPS, pH 8.0, with 0, 0.5, and 1.0 M NaCl; 20 mM CAPS brought to pH 10.0 with NaOH, and 20 mM CAPS brought to pH 10.0 with NH₄OH; 20 mM CAPS, pH 10.0, plus 50 mM NH₄OH, 50 mM glycine, and 250 mM triethylamine; 20 mM CAPS plus 50 mM NH₄OH at pH 8.5, 8.75, 9.0, 9.25, and 9.5.

HPLC Analysis of Deamidation Reaction Products—Deamidation reactions were analyzed on either a C-18 reverse-phase column from Column Resolutions, Inc., or on a C-18 reverse-phase column from Phenomenex. The solvent system was solvent A = 20 mM ammonium acetate, pH 5.0, and solvent B = acetonitrile. The peptides were eluted isocratically. The % B which gave optimal separation of the parent peptide and reaction products varied according to the sequence of the peptide, but was in the range of 0–8% B.

Reaction products formed during the pH profile studies of VANSV and the deamidation rates of GANSV, GSNAG, and GSNVG were analyzed on a Polyhydroxyethyl aspartamide (Poly-C) column from the Nest Group, Southboro, MA. The reaction products were eluted isocratically at 70–80% B, depending on the peptide, where A = 10 mM triethylamine phosphate, pH 5.0, and B = acetonitrile.

Thin Layer Chromatography—Chromatography was carried out on Baker Si250 silica plates with the solvent phenol/water/NH₄OH, 75:25:4. Peptides were identified by spraying the plates with an acetone solution of fluorescamine.

Kinetic Analysis—The *t*_{1/2} value for deamidation of each peptide was determined by measuring its time-dependent disappearance during the course of a deamidation reaction. For each peptide, the area under the HPLC absorbance curve at 5 time points (from 1 to 100 h) was determined in duplicate and plotted on semilog paper. Reactions with *t*_{1/2} values less than 30 h gave linear log plots for more than three half-lives. Slower reactions, some of which were followed for less than one half-life, were assumed to be first-order in peptide concentration. Each peptide was studied at least twice and several peptides were reacted three or more times.

Amino Acid Analysis—Peptides were hydrolyzed overnight at 110 °C in 6 N hydrochloric acid in sealed, evacuated tubes as previously described (Martini *et al.*, 1987). Amino acids were identified by post-column derivitization with *o*-phthalaldehyde.

Methylation Reactions—Methylation reactions were performed by the vapor diffusion method as described by Johnson and Aswad (1991). Incubations were carried out in 30–40 μl of citrate buffer, pH 6.2, for 1 h at 37 °C.

RESULTS

Deamidation Rates for VSNXV Peptides—The rates of deamidation of 10 peptides were investigated in 20 mM potassium phosphate, pH 7.3, at 60 °C. The high temperature increased the rate of deamidation and decreased the probability of bacterial growth during the long incubation periods. Most of the peptides were shown to deamidate by first-order kinetics through at least three half-lives. The peptides which deamidated slowly were assumed to deamidate by first-order kinetics. Column 1 of Table I shows the *t*_{1/2} values for deamidation of the 10 peptides with the sequence VSNXV where X = A, D, G, H, I, L, R, S, T, and V. The deamidation rates of the peptides fall into three groups with respect to reaction time: those which deamidate very rapidly (*t*_{1/2} < 10 h), those which deamidate very slowly (*t*_{1/2} > 200 h), and those which deamidate at an intermediate rate (*t*_{1/2} = 25–75 h).

The rapid deamidation rate of VSNGV (*t*_{1/2} = 5.8 h) is not surprising. The N-G sequence has previously been shown to be especially labile to deamidation both in proteins and pep-

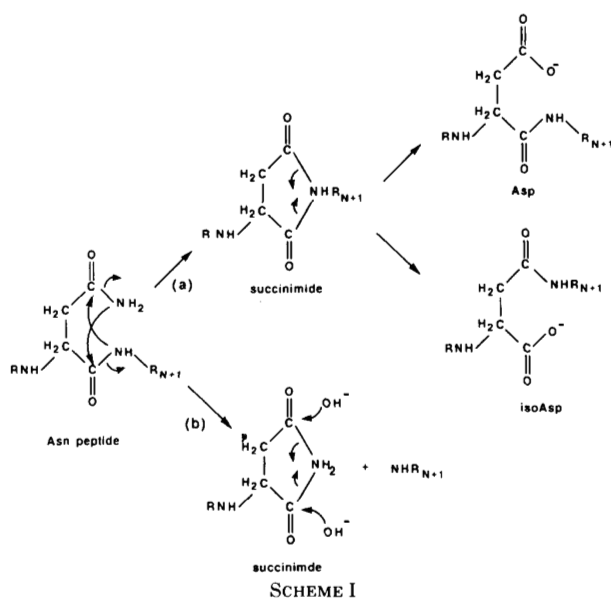


TABLE I
Rates of deamidation of pentapeptides in 20 mM phosphate, pH 7.3

Peptide	$t_{1/2}$	Peptide	$t_{1/2}$
	h		h
VSNGV	5.8	VGNSV	34 ± 1.7
VSNHV	26	VHNSV	45
VSNSV	45	VSNSV	45
VSNDV	65		
VSNVAV	66	VANSV	42 ± 1.7
VSNRV	76	VRNSV	40
VSNLV	240	VLNSV	46
VSNTV	240	VTNSV	52
VSNIV	330		
VSNVV	420 ± 54	VVNSV	45

tides (Haley *et al.*, 1966; DiDonato *et al.*, 1986; Clarke, 1987). This is usually attributed to the absence of steric hindrance in attaining the correct geometry for reaction. Our half-life for deamidation of VSNGV is in reasonable accord with other published values for small peptides with the N-G sequence (Lura and Schirch, 1988; Stephenson and Clarke, 1989; Patel and Borchardt, 1990b).

The peptides which deamidate with rates in the intermediate range have $X = A, D, H, R$, and S . The side chains of these amino acids are structurally diverse. At pH 7.3, D has a formal negative charge, H and R each have a formal positive charge and A and S are neutral. Nevertheless, their half-lives for deamidation are comparable. When the rate of deamidation of the peptide VSNGV is taken as 1, the reaction rates of this intermediate group are from 5 to 13 times slower. Within the intermediate group itself, however, there are some reproducible differences in rates. The peptide with the fastest rate of deamidation was VSNHV ($t_{1/2} = 26$ h) followed by VSNSV ($t_{1/2} = 45$ h).

The peptides in the third group, where $X = I, L, T$, and V , are the slowest to undergo deamidation (Table I). Their $t_{1/2}$ values are all greater than 200 h; they deamidate 40–70 times slower than VSNGV. Three of these residues (I, T , and V) contain β -branched methyl groups and L is γ -branched.

Deamidation Rates for the VXNSV Peptides—Column 2 of Table I shows the deamidation half-lives for the series of peptides VXNSV. One notes immediately that all of the rates of deamidation are very similar within this group. When compared to VSNGV, this series of peptides deamidates from six to nine times more slowly. Of special interest is the absence of a difference between peptides with and without β -branched residues. For example, substituting V for G or A has virtually no impact on the deamidation rate of the peptide. This finding is in marked contrast to that reported by Robinson *et al.* (1973) who found a range of deamidation rates of nearly 14-fold for peptides having the sequence GXNAG.

One possible explanation for the lack of amino acid sequence effects in the $n-1$ position is that Ser in the $n+1$ position is unique. Possibly, other amino acids in the $n+1$ position would be sensitive to the nature of the $n-1$ residue even though Ser is not. To examine this possibility, we tested whether having a β -branched residue in the $n+1$ position would enhance sensitivity to the identity of the residue in the $n-1$ position. Prior to initiating the experiments, we compared the sequences VSNVAV, VSNTV and VSNVV with $t_{1/2}$ values for deamidation of 66, 240, and 420 h, respectively (Table II). These values show that replacing Val with Thr in the $n+1$ position doubles the rate of deamidation, suggesting that the presence of a hydroxyl function on the β -carbon of the $n+1$ residue accelerates the deamidation reaction. The same effect is observed when the Asn-Ser sequence is compared to Asn-Ala (Table I). Because Val residues decrease the

rate of deamidation so severely, we decided to use the Asn-Thr sequence to investigate the effect of β -branching on residues in the $n-1$ position. We synthesized several peptides with β -branching in both the $n+1$ and $n-1$ position and compared their deamidation rates. The sequences VANTV, VTNTV, and VTNAV exhibited deamidation $t_{1/2}$ values of 120, 94, and 99 h, respectively. These values are essentially identical and suggest that β -branching in the $n+1$ position does not result in sensitivity to β -branching in the $n-1$ position. The peptide VSNTV appears to be an anomaly in this respect. It deamidates at one-half the rate of VANTV.

Effect of Amino Acids Two Residues Removed from Asn—Pentapeptides having G rather than V at their carboxyl and amino termini were studied in order to confirm that amino acids 2 residues removed from Asn do not affect the rate of deamidation. The results are shown in Table II. No significant differences in deamidation rates were observed between the two groups. Apparently, in these short peptides sequence effects are confined to those residues immediately adjacent to Asn. This is in accord with the finding of Capasso *et al.* (1989a) that there is no difference in the half-lives of two short peptides which contain the sequences N-G-G and N-G-A.

Methylation by PIMT—The deamidation of peptides containing the sequence Asn-Gly have been shown to occur via a succinimide intermediate, resulting in the formation of a 3:1 ratio of isoAsp-Gly to Asp-Gly sequences in the product peptides (Meinwald *et al.*, 1986; Clarke, 1987; Lura and Schirch, 1988). The enzyme PIMT specifically methylates isoAsp residues by transferring a methyl group from S -adenosylmethionine to the free α -carboxyl of isoAsp residues (Aswad, 1984; Murray and Clarke, 1984; DiDonato *et al.*, 1986; Aswad and Johnson, 1987). PIMT can therefore be used to identify isoAsp-containing peptides. The absorbance profiles from our HPLC studies suggested that each of the peptides in the series VSNXV and VXNSV were reacting to give isoAsp and Asp products in about a 3:1 ratio. We confirmed this by assaying for isoAsp residues in the products using the PIMT methylation assay. For each peptide recorded in Table I, equimolar quantities were incubated at pH 7.3 in phosphate buffer for periods which allowed for at least 80% completion of deamidation. These reaction mixtures were then assayed using PIMT and [C^3H_3]S-adenosyl-L-methionine. With only two exceptions, isoAsp residues were present in the samples after incubation at 60 °C, but not before incubation. The peptide VSNDV gave no evidence for the presence of isoAsp residues. This, however, is probably the result of the specificity of PIMT. Lowenson and Clarke (1990) have recently reported that peptides in which the first, second, or third

TABLE II
Effect of β -branched amino acids, which are 1 and 2 residues removed from Asn, on the rate of deamidation in 20 mM phosphate, pH 7.3

Peptide	$t_{1/2}$
	h
VSNVAV	66
VSNTV	240
VSNVV	420
VTNAV	99
VANTV	120
VTNTV	94
VANSV	42
GANSV	43
GANSV	330
GSNAG	72

residue on the carboxyl side of L-isoaspartate is negatively charged are very poor substrates for PIMT. Also, the peptides GSNAG and GANSNG did not show evidence of isoAsp residues by this method. We are confident that isoAsp is the major product of the deamidation of these peptides, however, for two reasons. First, two products are produced in approximately a 3:1 ratio. Second, when the major product of the GANSNG reaction was isolated by thin layer chromatography and analyzed by amino acid sequencing, the result showed the sequence G, A, D for the first three cycles, but the yield of the third cycle was less than 20% that of the second cycle. This pattern is characteristic of peptides which contain an isoAsp residue.

The levels of methylation for the deamidation products of VSNXV peptides were not as high as for the substrate *N*-acetyl-VisoDGA which is used as a control to determine the specific activity of the [^3H]-S-adenosylmethionine. Peptides in the VSNXV and VXNSV series which were more than 80% deamidated incorporated only 20–30% of the radioactivity that an equimolar quantity of *N*-acetyl-VisoDGA incorporated. This probably reflects the decreased specificity of PIMT for peptides containing sequences other than isoAsp-Gly. Previous studies have suggested that neighboring residues of the isoAsp moiety in peptides affect the K_m and V_{max} values for this enzyme (Lowenson and Clarke, 1990; Johnson and Aswad, 1991). To further confirm that the sequence of the peptide was not affecting the mechanism of deamidation, seven peptides which had base-line HPLC resolution of the major and minor products were collected and assayed for isoAsp residues with PIMT. For these seven peptides, only the major product contained the isoAsp residue.

Peptide Bond Cleavage—While investigating the rate of deamidation of peptides with the sequence VXNSV by thin layer chromatography, an increase in the number of reaction products was detected (Fig. 1). The peptides were reacted at pH 7.3 in phosphate buffer, and at pH 9.0 in borate buffer. Fluorescamine reactive spots were recovered from the thin layer plate and subjected to amino acid analysis which showed,

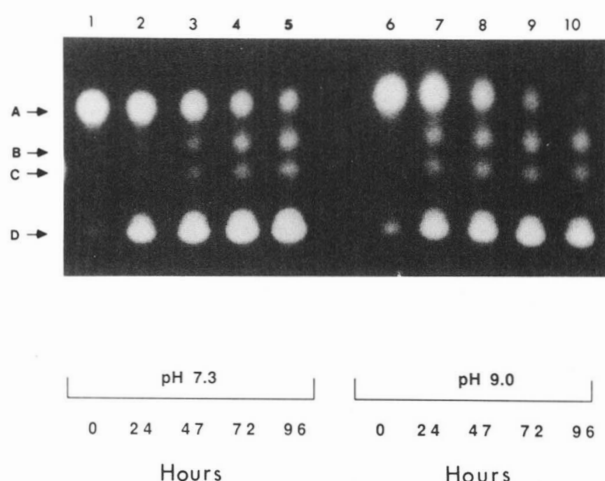


FIG. 1. Thin layer chromatography of reaction solutions of VANSV. The peptide was reacted at pH 7.3 in phosphate buffer (lanes 1–5) and at pH 9.0 in borate buffer (lanes 6–10) for 0, 24, 48, 72, and 96 h. Chromatography was carried out as described under "Experimental Procedures." The starting material migrates at position A. Amino acid analysis of the material recovered from positions B and C showed their compositions to be V/A/D, 1:1:1 and S/V, 1:1, respectively. Position D is a double spot and gives the amino acid analysis of the deamidated peptide. We assume the double spot corresponds to the isoAsp- and Asp-containing peptides.

in addition to the deamidated peptide, an unexpected dipeptide, S-V, and a tripeptide, V-X-D. A detailed analysis of the deamidation of the peptide VANSV by HPLC showed peaks which, according to amino acid analysis, represent the dipeptide SV and the tripeptide VAD (Fig. 2). The dipeptide Ser-Val was synthesized and shown to coelute with the reaction product containing S and V (data not shown). As anticipated, peaks representing VADSV and VAisoDSV (as determined by amino acid analysis and the PIMT reaction) were also detected. The extent of cleavage of VANSV was about 10%, relative to the amount of the deamidated pentapeptide during the entire course of the reaction, as determined by the areas from the HPLC elution profiles. In all, 14 peptides of the sequence VXNSV (where X = G, H, L, R, S, and V) or GXNSG (where X = A, D, E, K, S, P, R, and V) were shown by thin layer chromatography and/or HPLC analysis to undergo the peptide bond cleavage and deamidation reaction.

The pH and buffer dependence of the cleavage reaction were further investigated using the peptide GANSNG. The peptide was reacted at pH 7.3 in phosphate buffer and BES buffer, and at pH 9.0 in borate buffer and CHES buffer. Aliquots were removed and the formation of products determined by HPLC. Increasing pH values and buffer catalysis by phosphate or borate resulted in the same 9:1 ratio of deamidation without cleavage, to deamidation with cleavage (data not shown).

Effect of pH, Buffers, and Ionic Strength on the Rate of Deamidation—Previous reports have indicated that the deamidation reaction is sensitive to pH, temperature, and ionic strength (Capasso *et al.*, 1989b; Scotchler and Robinson, 1974) and to buffer type and concentration (Bhatt *et al.*, 1990; Patel and Borchardt, 1990a, 1990b). The majority of the experiments described in the following sections were carried out in

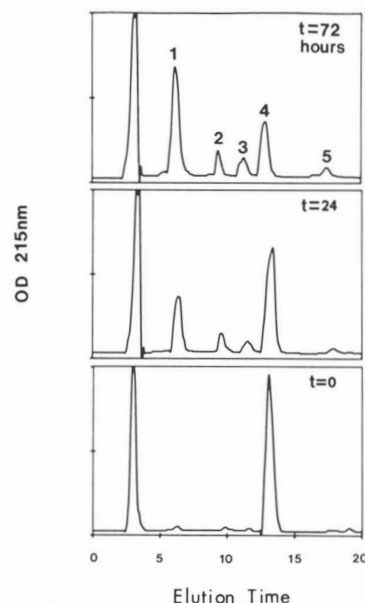


FIG. 2. HPLC analysis of reaction solutions of VANSV. The peptide was reacted at pH 10.0 in 20 mM CAPS buffer for 0, 24, and 72 h and analyzed on a polyhydroxyethyl aspartamide column as described under "Experimental Procedures." Fraction 4 contains the starting material. Amino acid analyses of fractions 3 and 5 showed their compositions to be S/V, 1:1 and V/A/D, 1:1:1, respectively. Fractions 1 and 2 have identical amino acid compositions, V/A/D/S, 2:1:1:1. The peptide in fraction 1 was a substrate for protein isoaspartyl methyltransferase, but the peptide in fraction 2 was not a substrate.

sulfonic acid buffers because they proved to have little effect on the rate of the deamidation reaction. The deamidation rate of VSNGV was determined at pH 7.3, 8.0, and 9.0 in BES, TAPS, and CHES buffers, respectively, at buffer concentrations of 20, 50, 100, and 200 mM. At all three pH values, a 10-fold increase in buffer concentration (from 20 to 200 mM) caused approximately a 2-fold increase in the rate of deamidation (data not shown). By extrapolating from 20 mM back to 0 mM buffer, one can show that the presence of 20 mM sulfonate buffer in the reaction solution has little effect on the intrinsic rate of deamidation (k_{hyd}) of the peptides under study. A concentration of 20 mM sodium sulfonate buffer (MES, BES, TAPS, CHAPS, CAPS) was used to buffer the reaction solutions for most of the following studies.

In order to establish more clearly the relationship between the rate of deamidation and hydroxide ion concentration, we determined the pH profile of the peptide VANSV. The peptide was reacted in 20 mM sulfonate buffers at pH 6.0, 7.3, 8.0, 9.0, and 10.0. A plot of $\log k_{\text{obs}}$ versus pH was linear with a slope of 0.2, indicating that the rate of deamidation is not proportional to hydroxide ion concentration and, therefore, is not subject to specific base catalysis. Essentially the same results were obtained with the peptide VSNGV.

McKerrow and Robinson (1971) and Scotchler and Robinson (1974) have reported that an increase of one unit in ionic strength decreases the half-time of deamidation by a factor of about two. In those experiments, the ionic strength was increased by the addition of NaCl and it has become a common practice to hold ionic strength constant in deamidation experiments by the addition of this salt. We investigated the ability of two other salts, guanidine hydrochloride and LiCl, to accelerate the rate of deamidation. The peptide VSNGV was reacted in 20 mM sulfonic acid buffer, pH 8.0, with guanidine hydrochloride at concentrations of 0, 0.1, 0.2, 0.4, and 1.0 M, and with LiCl at concentrations of 0.0, 0.5, and 1.0 M. The half-life of deamidation was measured at each salt concentration. We found no significant difference in the deamidation rate of VSNGV over the entire range of guanidine hydrochloride and LiCl concentrations (Table III). The experiment was then carried out with the same peptide under identical conditions but with 0.0, 0.5, and 1.0 M NaCl. Again, no increase in the deamidation rate was observed by the addition of up to 1.0 M salt (Table III). We conclude that in our system, the rate of deamidation of the peptide VSNGV is not accelerated by an increase in ionic strength.

Because of the dissimilar findings of Lura and Schirch (1988), Capasso *et al.* (1989b), and Patel and Borchardt (1990a) with respect to buffer catalysis by phosphate ion in the alkaline pH range, we investigated the ability of phosphate to catalyze the deamidation reaction. We have determined the rate of deamidation of the peptide VSNGV at pH 8.0 in 20 mM sulfonic acid buffer plus 0, 20, 50, and 100 mM phosphate (Table III). The presence of as little as 20 mM phosphate accelerates the deamidation reaction 3.9-fold over the rate in sulfonic acid buffer. However, a plot of $k_{\text{obs}} - k_{\text{hyd}}$ versus phosphate concentration was not linear. The rate of deamidation at 100 mM phosphate was only 53% of the value predicted from a linear response to phosphate catalysis. Phosphate catalysis was also investigated as a function of pH. Catalysis increased with pH and was proportional to the concentration of the dianionic form of the buffer (data not shown). These results are in accord with the findings of Patel and Borchardt (1990a) who have reported that increasing the concentration of phosphate increased the rate of deamidation of the adrenocorticotrophic hormone-derived hexapeptide VYPNGA. In contrast, the peptide sequence examined by

TABLE III
Effect of buffers and ionic strength on the rate of deamidation of the peptides VSNGV and VSNLV

Peptide	pH ^a	Buffer or ion	Conc.	$t_{1/2}$
			M	h
VSNGV	8.0	None		17
		Guanidine HCl	1	15
		LiCl	1	16
		NaCl	1	17
		Phosphate	0.02	4.2
		Phosphate	0.05	2.7
		Phosphate	0.1	2.0
	10.0	None		6.5
		Glycine	0.02	1.5
		NH ₄ OH	0.05	1.1
		Triethylamine	0.25	6.0
	9.0	None		9.0
		NH ₄ OH	0.05	5.0
				3.0
				2.8
				1.7
				1.3
VSNLV	10.0	None		6.5
		NH ₄ OH	0.006	1.0
				33
			0.006	30

^a Each reaction was performed in 20 mM buffer at 60 °C as follows: pH 8.0, TAPS; pH 10.0, CAPS; pH 9.0 to 9.5, CHES.

Capasso *et al.* (1989b), Boc-Asn-Gly-Gly-NH₂, was reported not to be susceptible to buffer catalysis by phosphate ion in the alkaline pH range, as were the tetrapeptides VNGA and acetyl-VNGA which were investigated by Lura and Schirch (1988).

The ability of imidazole to catalyze the deamidation of VSNGV was also tested. The results showed that at pH 8.0, in 20 mM TAPS buffer, the addition of 20 mM imidazole increased the deamidation rate ~2-fold. At pH 8.0, phosphate ($pK_{a2} = 7.2$) and imidazole ($pK_a = 7.0$) should be deprotonated to about the same extent. Therefore, we conclude that the dianionic form of phosphate is a better catalyst for the deamidation reaction than is imidazole by a factor of 2.

We also investigated the ability of several other ions to participate in the deamidation of VSNGV. The first of these was the potential acid-base catalyst ammonia. The peptide VSNGV was reacted in 20 mM CAPS buffer with and without 6 mM ammonia at pH 10.0. The rate of deamidation was 6.5-fold faster in the solution which contained ammonia, showing that the presence of ammonia, rather than the high pH, was the crucial factor in catalyzing the reaction. However, when the experiment was repeated with VSNLV as the peptide, addition of ammonia did not increase the rate of deamidation.

The ability of ammonia to catalyze the reaction at high pH was compared to that of two other potential general acid-base catalysts, glycine and triethylamine. The pK_a values of NH₄⁺, glycine (pK_{a2}) and triethylamine are 9.3, 9.6, and 10.6, respectively. At pH 10.0, solutions of 50 mM NH₄OH, 50 mM glycine, and 250 mM triethylamine contain 42, 36, and 37 mM of their deprotonated forms, respectively. The $t_{1/2}$ values for deamidation of VSNGV with these three amines were 1.5, 1.1, and 6 h, respectively (Table III). Ammonia is apparently a slightly better catalyst than glycine, but triethylamine is not a catalyst.

In order to determine which form of ammonia (protonated or unprotonated) catalyzes the reaction, a detailed study was carried out in 50 mM NH₄OH at several different pH values.

The peptide VSNGV was reacted in 20 mM CHES buffer plus 50 mM NH_4OH which had been adjusted to pH 8.5, 8.75, 9.0, 9.25, and 9.5. The value of k_{cat} ($k_{\text{obs}} - k_{\text{hyd}}$) was found to be proportional to the concentration of the unprotonated amine (data not shown). These results suggest that ammonia is an effective general base catalyst of deamidation of Asn residues in peptides.

Rate and Mechanism of Deamidation at Low pH—The mechanism of deamidation at alkaline pH has been shown to occur via the formation of a succinimide intermediate. The hydrolysis of the intermediate at either the α -carboxamide or the β -carboxamide explains the observation that two different reaction products (isoAsp and Asp) are formed. When deamidation is carried out at acidic pH values, only one reaction product is detected. Meinwald *et al.* (1986) have reported that the peptide acetyl-Asn-Gly-NHMe yields only the normal Asp peptide upon incubation in 1 N HCl. Patel and Borchardt (1990a, 1990b) report that only the normal Asp peptide is formed when the hexapeptide VYPNGA is reacted at pH 1 or 2. These findings imply that at acidic pH, deamidation of peptides with N-G sequences occur by a direct hydrolytic mechanism which does not involve an intramolecular reaction and subsequent rearrangement. If this is so, the sequence of the peptide might not play a role in the rate of deamidation at acid pH. Factors such as steric hindrance, which are important at alkaline pH might not affect the rate of direct hydrolysis which depends only on the accessibility of the side chain of Asn to solvent.

To test this possibility, we compared the rates of deamidation at acidic pH of three peptides (VSNGV, VSNAV, and VSNIV) which have very different half-lives for deamidation at pH 7.3 (5.8, 62, and 330 h, respectively). In 0.1 N HCl, we found that their half-lives (39, 44, and 40 h, respectively), were essentially identical within experimental error.

We observed a single deamidation product for each peptide at acidic pH. In HPLC traces, the product of acidic deamidation eluted at the position of the minor product of alkaline deamidation, the normal D peptide (data not shown). We also observed that, with time, the amount of deamidated product formed was less than the amount of Asn peptide lost. Analysis of the reactions of VSNAV and VSNIV by thin layer chromatography showed additional minor products that are probably the result of peptide bond cleavage. This is consistent with the results of Patel and Borchardt (1990a, 1990b) who also observed cleavage products when reacting the hexapeptide VYPNGA at acidic pH. This result is probably due to the well-known tendency of Asp containing peptides to cleave at acidic pH values (Schultz, 1967).

DISCUSSION

Nonenzymatic deamidation of Asn residues in peptides and proteins is well documented (Clarke, 1985; Wright, 1991b). Several laboratories have studied the rates and conditions related to deamidation and, in some cases, general conclusions have been drawn and perpetuated on the basis of data which were actually very specific in nature. Among these generalizations are those relating to the effects of amino acid sequence, pH, buffers, and ionic strength on the rate of deamidation of Asn residues in peptides. By determining the rate of deamidation of a series of closely related Asn-containing peptides, we hoped to test the validity of some of these previous conclusions and to further define the requirements and mechanism for the nonenzymatic deamidation of Asn residues in peptides and proteins.

All previous studies, relating deamidation rates to sequence, observed that the $n+1$ residue in Asn peptides affects the rate

of deamidation (Stephenson and Clarke, 1989; Wright, 1991b). This conclusion is substantiated by our results (Table I) which further suggests that the most critical factor of the $n+1$ residue is its degree of side chain branching. The charge on the residue does not seem to greatly affect the rate of deamidation. The three exceptions to this rule are when $n+1$ is Ser, Thr, or His. These residues result in deamidation of Asn at a faster rate than predicted from the size of their side chains.

Another generalization which is often repeated in the literature is that the $n-1$ residue also affects the rate of deamidation of Asn residues (Robinson, 1974; Robinson and Rudd, 1974). When the $n+1$ residue is Ser this is clearly not true (Table I). In a limited study which tested residues other than Ser in the $n+1$ position, we looked at peptides in which the $n+1$ residue was Ala, Thr, or Val, and either Ser, Ala, or Thr was in the $n-1$ position. We found that most peptides in this group had very similar half-times; a single exception, VSNTV, had a deamidation half-time approximately 2-fold longer than the other peptides. Thus, our results show that the $n-1$ residue, even when β -branched, does not substantially affect the rate of deamidation. Similar conclusions were proposed by Patel and Borchardt (1990b) on the basis of comparison of deamidation rates of Gly-Asn and Pro-Asn peptides.

The previously reported observation by Robinson and Rudd (1974) that bulky residues in the $n-1$ position decrease the rate of deamidation led Kossiakoff (1988) to conclude that the deamidation of three Asn-Ser sequences in trypsin were not related to sequence, but rather only to conformation. The three $n-1$ residues for these three Asn residues were Ile, Leu, and Tyr, all of which were predicted from peptide studies to deamidate slowly. Our results suggest, however, that these Asn-Ser sequences should be susceptible to deamidation. It is of interest that the products of the 3 deamidated Asn residues in trypsin were all Asp-Ser sequences with no evidence of them being isoAsp-Ser. Either these residues did not deamidate by the succinimide mechanism or the succinimide intermediate was hydrolyzed by a mechanism which favored the Asp-Ser sequence.

The rate of deamidation of Asn residues has been reported to have a positive dependence on ionic strength (Scotchler and Robinson, 1974; McKerrow and Robinson, 1974). However, these studies were very limited in scope, did not show dramatic changes in deamidation rates with increasing ionic strength (2-fold for each 1 unit of increase in ionic strength), and were probably never intended to serve as a definitive measure of ionic strength effects on nonenzymatic rates of deamidation. Using three different ions, up to ionic strengths of 1.0, we find that there is no effect on the rate of deamidation by increasing ionic strength for the peptide VSNGV (Table III).

Previous studies have resulted in conflicting reports with respect to the effect of buffers and pH on the rate of deamidation. Usually it is stated that pH has a marked effect on deamidation rates, while buffer effects have been reported to be both important and not important (Capasso, 1989b; Patel and Borchardt, 1990a; Lura and Schirch, 1988). Our results show that in the pH range of 6 to 9 a 1000-fold increase in hydroxide ion concentration results in only an 8-fold increase in the rate of deamidation. The failure to observe a proportional increase in rate (1000-fold) with hydroxide ion concentration suggests that nonenzymatic deamidation of Asn residues, in peptides at neutral to slightly alkaline solution, are not specific base catalyzed. However, we do find that buffer catalysis is important, and several of the previous studies which attribute rate increases to pH effects are probably the

result of increased general base catalysis by the buffer. Carbonate has been shown to be a catalyst of deamidation reactions and unless care is taken to remove carbonate from buffers increasing pH may result in both increased concentration of carbonate and its increased rate of catalysis. A careful study to remove all carbonate from the reaction solution and extrapolation of the rates of deamidation to zero peptide concentration may well further reduce the effect of pH on the rate of deamidation. It is clear from our studies that to increase the rate of deamidation of Asn residues in a peptide or protein one should use ammonia as a catalyst. As one example, calmodulin is often deamidated *in vitro* by incubating the enzyme at pH 10. However, the pH is usually adjusted to 10 with NH_4OH , and it is probably the ammonia which is the most important factor in determining the rate of deamidation of calmodulin, and not the increased pH (Johnson *et al.*, 1985).

We previously reported that the deamidation of the peptides VNGA and acetylVNGA was not catalyzed by phosphate or imidazole (Lura and Schirch, 1988). This would be in agreement with the results of Capasso (1989) who observed that phosphate did not catalyze the deamidation of *t*-Boc-Asn-Gly-Gly- NH_2 at neutral to alkaline pH. However, in this study we find that the peptides VSNGV and VSNAV show catalysis by phosphate, ammonia, and imidazole but not by triethylamine (Table III). These differences in effects of base catalysis can be explained in part by the observations that catalysis is not proportional to the concentration of the base, and catalysis is dependent on both the nature of the base and the structure of the peptide. In our previous studies we had checked phosphate catalysis by comparing the rates of deamidation of 50 and 100 mM phosphate solutions to our standard conditions of 20 mM phosphate. We saw little or no increase in the rate of deamidation, and thus concluded that phosphate was not a catalyst. However, in this study we have determined the rate of deamidation in the absence of any buffer (*i.e.* k_{hyd}) and find that 20 mM phosphate accelerates the rate of deamidation severalfold. However, above 20 mM phosphate, increasing concentrations of phosphate have less than the predicted effect as a catalyst. We have also observed that ammonia is an effective catalyst of peptides and gives linear increases in rate with concentration when the sequence is Asn-Gly. However, the secondary amine, imidazole, was a less effective catalyst than either phosphate or ammonia, and the tertiary amine triethylamine was not a catalyst at all. Furthermore, with the peptide VSNLV even ammonia was not a catalyst. In conclusion, caution needs to be taken in determining whether a particular peptide is subject to base-catalyzed deamidation. The mechanistic implications of buffer catalysis are discussed below.

Another statement encountered in the literature is that the deamidation of Asn residues in acid is slower than at neutral pH (Meinwald *et al.*, 1986; Patel and Borchardt, 1990a). While this is true if one is comparing the deamidation rates of Asn-Gly sequences, it may not be a generally valid precept. For example, the rate of deamidation of the Asn-Val sequence at pH 1.0 is 8-fold faster than its rate of deamidation at pH 7.3. Our results, as well as the recent work of Patel and Borchardt (1990b), suggest that in acid the rate of deamidation of Asn residues in peptides may not be dependent on sequence. We are in agreement with Patel and Borchardt (1990b) that in acid solution the only mechanism of deamidation that appears to operate is the direct hydrolysis of the side chain amide group of the Asn residue.

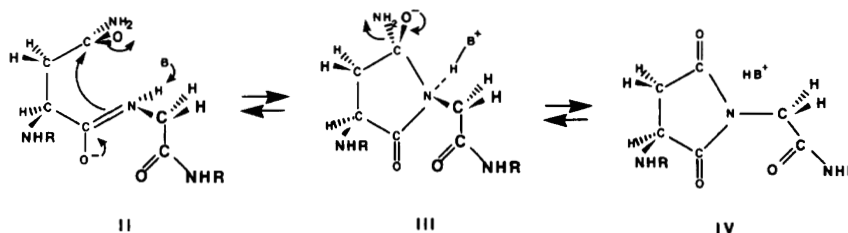
The principle mechanism for deamidation of Asn residues in short peptides in neutral or alkaline solution is through a

succinimide intermediate, regardless of the sequence of the peptide and buffer (Patel and Borchardt, 1990a, 1990b; Geiger and Clarke, 1987). This conclusion is further confirmed by the observation that every peptide we studied resulted in the formation of both Asp and isoAsp-containing peptides. Only the succinimide intermediate pathway gives rise to isoAsp residues. Since the ratio of isoAsp to Asp residues in the deamidated products was about 3:1 for each peptide, we conclude that the sequence of the peptide does not significantly affect the mechanism of hydrolysis of the succinimide intermediate (Patel and Borchardt (1990b) observed small changes in the ratio of Asp:isoAsp with sequence). Also, except for the isoAsp-Asp sequence and the GXNXXG peptides, the enzyme PIMT was able to methylate all of the product isoAsp-X peptides, showing that this enzyme has a broad substrate specificity for the $n+1$ residue. Lowenson and Clarke (1990) have previously shown that the enzyme does not methylate isoAsp residues when a negatively charged residue is in the $n+1$ position.

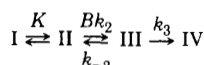
A previously proposed mechanism for deamidation of Asn residues involves orientation of the peptide so that the χ_1 and ψ bonds of the Asn are $+120^\circ$ and -120° , respectively. This aligns the $n+1$ amide nitrogen for attack on the side chain amide of Asn (Clarke, 1987). Hydroxide then removes the proton from the attacking amide to generate the amide anion. This mechanism would predict specific base catalysis, but we observe only general base catalysis. In Scheme II we propose a variation of the previous mechanism to account for the difference in base catalysis. As previously described, the peptide can be considered to be a series of interconverting rotational conformers. These are grouped as structure I, and are not shown in Scheme II. There is now considerable evidence that small peptides assume favored conformational states in solution (Imperiali and Shannon, 1991; Dyson *et al.*, 1990; Lura and Schirch, 1988; Capasso *et al.*, 1984; Capasso *et al.*, 1987). Only one of these conformers has the correct orientation to proceed to the transition state which may then form the succinimide intermediate (structure II in Scheme II). In this orientation the χ_1 and ψ angles for the Asn residue are well outside the limits predicted to be allowable for stable conformers in Ramachandran calculations (Ramachandran *et al.*, 1963). The first intermediate formed from the optimal conformer (II) would be a tetrahedral adduct after attack of the Gly amide on the side chain amide of Asn (structure III in Scheme II). The transition state in forming this intermediate requires the removal of the Gly amide proton by a base. Collapse of the tetrahedral intermediate III by expulsion of the side chain amide group would give the succinimide intermediate (structure IV). Previous studies have shown that the formation of the succinimide intermediate is the rate-determining step in the deamidation of Asn residues (Geiger and Clarke, 1987). The succinimide intermediate can hydrolyze by breaking either of the two imide bonds to form the Asp and isoAsp residues.

Models of the tetrahedral intermediate (structure III in Scheme II) show that there is shielding of the $n+1$ amide proton by its own side chain. This could explain why a bulky base, such as triethylamine, was not a good catalyst for the Asn-Ser peptides in these deamidation reactions. It is less clear why the values of k_{obs} versus phosphate concentration were not linear. One possibility is that as the concentration of base is increased, there is a change in rate-determining step to one which is not base-catalyzed. The minimal reaction path for formation of the succinimide intermediate is shown in Scheme III. In this scheme, structure I represents all rotational conformers of the peptide which are not geometri-

SCHEME II



cally competent to form the tetrahedral intermediate, and structure II represents the conformer which is "geometrically correct".



SCHEME III

Assuming that the rate-determining step is the formation of the tetrahedral intermediate the rate law for the base catalyzed reaction will be

$$v = k_2 [II] [B] \quad (1)$$

Since the correct conformation (structure II) is known to be a high energy species, the solution to the rate equation can be simplified by letting the concentration of structure I equal the total concentration of peptide *i.e.*, the value of K is a very small number. The rate law for the mechanism of the reaction in Scheme III would be

$$v = Kk_2 [I] [B] \quad (2)$$

Equation 2 predicts that the rate will be first order in both peptide and the base B . However, it is likely that the formation of the high energy conformer (structure II) will also be slow and that its formation may become rate determining at high concentrations of the base catalyst. Since the conversion of structure I to structure II is not subject to base catalysis, k_{cat} would become independent of the concentration of B .

The degree to which the structure of the $n+1$ residue effects the value of K (formation of the correct conformer) or k_2 (the rate of formation of the tetrahedral intermediate) is left in doubt by the mechanism shown in Scheme III. The value of k_2 will also reflect the pK of the amide proton of the $n+1$ residue. This pK has been shown to vary with the structure of the side chain of this residue adding yet another factor in the effect of the structure of the $n+1$ residue on the rate of deamidation (Molday and Kallen, 1972).

We have found that when the sequence in the peptide is Asn-Ser a cleavage reaction takes place at the Asn-Ser bond at about 10% of the rate of deamidation without cleavage. This cleavage reaction shows the same sensitivity to pH and buffer as the mechanism for the side chain deamidation of Asn. This suggests that the cleavage reaction is occurring by a mechanism that is very similar to the succinimide mechanism reported for Asn peptides. A similar tetrahedral intermediate to the one shown in Scheme II can also be constructed as the result of the side chain amide attacking the main chain peptide carbonyl of Asn. A model of this tetrahedral inter-

mediate shows that the Ser hydroxyl can come within hydrogen bonding distance of the α -carbonyl of Asn. As noted by others the Ser hydroxyl can stabilize the developing negative charge in the formation of the tetrahedral intermediate. This may account for the sequence specificity of the cleavage reaction since collapse of this intermediate would form a succinimide intermediate with expulsion of the Ser residue. The similarity between the mechanisms for side chain deamidation and cleavage could account for why cleavage and deamidation product ratios remain constant with changing pH and buffer concentration.

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