

Spontaneous degradation of polypeptides at aspartyl and asparaginyl residues: Effects of the solvent dielectric

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Abstract

We have investigated the spontaneous degradation of aspartate and asparagine residues via succinimide intermediates in model peptides in organic co-solvents. We find that the rate of deamidation at asparagine residues is markedly reduced in solvents of low dielectric strength. Theoretical considerations suggest that this decrease in rate is due to the destabilization of the deprotonated peptide bond nitrogen anion that is the postulated attacking species in succinimide formation. This result suggests that asparagine residues in regions with low dielectric constants, such as the interior of a protein or in a membrane bilayer, are protected from this type of degradation reaction. On the other hand, we found little or no effect on the rate of succinimide-mediated isomerization of aspartate residues when subjected to the same changes in dielectric constant. In this case, the destabilization of the attacking peptide bond nitrogen anion may be balanced by increased protonation of the aspartyl side chain carboxyl group, a reaction that results in a superior leaving group. Consequently, any protein structure or conformation that would increase the protonation of an aspartate side chain carboxyl group can be expected to render that residue more labile. These results may help explain why particular aspartate residues have been found to degrade in proteins at rates comparable to those of asparagine residues, even though aspartyl-containing peptides degrade more slowly than corresponding asparaginyl-containing peptides in aqueous solutions.

Keywords: asparagine; aspartate; deamidation; dielectric; isomerization; succinimide

The isomerization of aspartate residues to isoaspartate (β -aspartate) residues and the deamidation of asparagine residues to both normal and isoaspartate residues are widespread spontaneous reactions that can alter protein structure and function (Robinson & Rudd, 1974; Harding, 1985; Johnson et al., 1989; Stadtman, 1990; Wright, 1991). For example, the isomerization of Asp 101 in hen egg-white lysozyme results in reduced affinity for its chitin substrate (Yamada et al., 1985) and the isomerization of Asp 11 in human epidermal growth factor leads to a fivefold reduction in its mitogenic activity (Araki et al., 1989; George-Nascimento et al., 1990). In addition, the deamidation of an asparagine residue has been shown to facilitate the subunit dissociation of human triosephos-

phate isomerase (Yüksel & Gracy, 1986) as well as to cause a 25–500-fold reduction in the potency of human growth hormone releasing factor (Friedman et al., 1991).

Both the isomerization and the deamidation reactions proceed at physiological pH via an intermediate cyclic succinimide ring formed by the nucleophilic attack of the peptide bond nitrogen of the following residue on the carbonyl carbon atom of the side chain, releasing water and ammonia for aspartate and asparagine residues, respectively (Fig. 1). The succinimide then undergoes rapid hydrolysis at either carbonyl to open to the aspartyl or isoaspartyl forms (Geiger & Clarke, 1987; Patel & Borchardt, 1990a,b).

The rate-limiting step of the overall reactions shown in Figure 1 is the initial formation of the succinimide. Based on several lines of evidence, succinimide formation appears to be dependent upon the deprotonation of the attacking peptide bond nitrogen to form a more nucleo-

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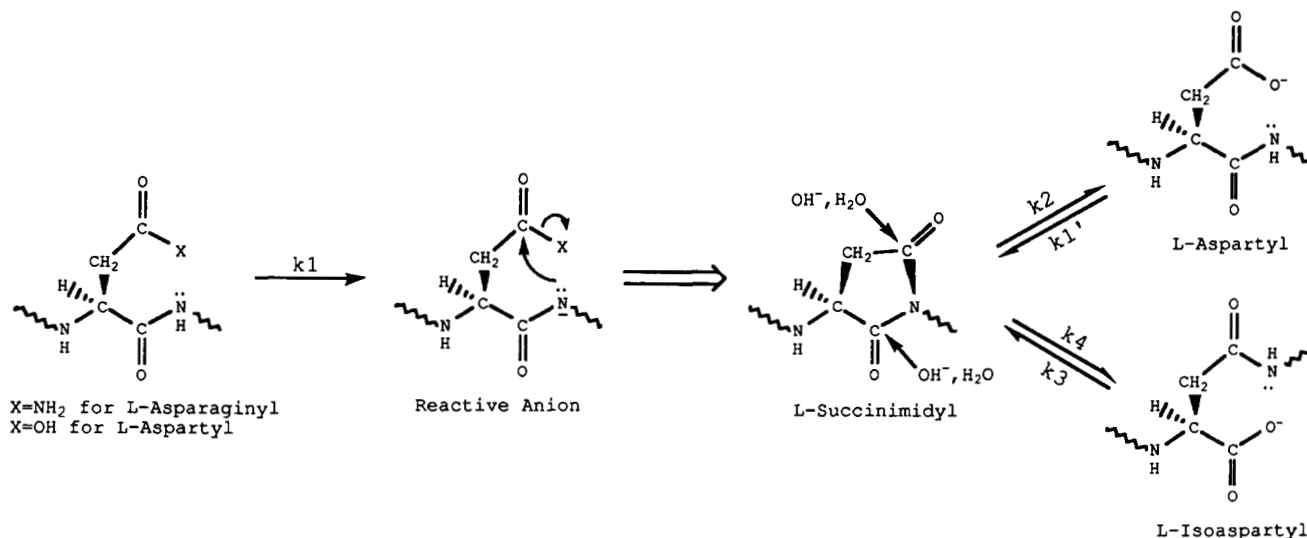


Fig. 1. Succinimide-mediated pathway for the nonenzymatic degradation of asparagine and aspartate residues leading to deamidation and isomerization products.

philic anion (Bernhard et al., 1962; Fölsch, 1966; Ondetti et al., 1968). First, the rate of succinimide formation increases when peptides are incubated in buffers of high pH and ionic strength (Lewis et al., 1970; Capasso et al., 1989; Patel & Borchardt, 1990a), conditions that enhance the stability of the nitrogen anion. Second, the presence of electron-withdrawing residues carboxyl to aspartyl or asparaginyl residues increases the rate of succinimide formation as compared to residues with electron-donating aliphatic side chains (Bernhard et al., 1962; Fölsch, 1966). Third, when the peptide bond nitrogen is incapable of deprotonation, as in the case when the carboxyl residue is proline, succinimide formation via the pathway outlined in Figure 1 does not occur (Piszkiwicz et al., 1970; Geiger & Clarke, 1987).

Following the hypothesis that the formation of the succinimide intermediate is dependent on the deprotonation of the peptide bond nitrogen, we have analyzed succinimide formation in model peptides under conditions that would affect the stability of this nitrogen anion. This was accomplished by measuring the rates of succinimide formation in buffers of constant pH and buffer concentration, but with varying dielectric constants attained through the use of organic co-solvents. The hypothesis is that solvents of differing dielectric constants will change the pK_a of the attacking peptide bond nitrogen (Greenstein & Winitz, 1961) and affect the rate of formation of the succinimide that leads to both isomerization and deamidation.

The elucidation of the solvent effects on succinimide formation may not only provide clues for predicting the protein regions most susceptible to spontaneous damage in vivo, but may also aid in choosing conditions for maximum stability of aspartate and asparagine residues during peptide synthesis and storage.

Results

Effects of solvent dielectric constant on the rates of deamidation of asparaginyl residues and isomerization of aspartyl residues

We found that the addition of increasing amounts of organic co-solvents, resulting in lowered solvent dielectric strengths, produces substantial decreases in the rate of spontaneous deamidation of the synthetic hexapeptide Val-Tyr-Pro-Asn-Gly-Ala (Table 1). This peptide, derived from a sequence in adrenocorticotrophic hormone, has been widely studied as a model for spontaneous degradation reactions (Geiger & Clarke, 1987; Stephenson & Clarke, 1989; Patel & Borchardt, 1990a,b). The effect of solvent dielectric seen here can be rationalized in terms of the pathway for succinimide-mediated deamidation reaction outlined in Figure 1. Here, a charged activated complex that results from the deprotonation of the attacking peptide bond nitrogen is the crucial step in succinimide formation. Because the stability of the anionic peptide bond nitrogen is decreased in media of low dielectric strength (Hughes & Ingold, 1935), reactions dependent on its formation proceed slower as the organic content of the solvents increase. Three different organic co-solvents that encompass a range of physical properties were used in this study to lower the dielectric strength. The fact that the results obtained are largely independent of the specific solvent utilized demonstrates the central importance of the dielectric effect itself (Table 1; Fig. 2). For example, incubation of this peptide in very different concentrations of the co-solvents glycerol (80%, $D_{70} = 39.1$), ethanol (50%, $D_{70} = 40.4$), and dioxane (30%, $D_{70} = 40.8$) all resulted in similar half-lives for the deamidation reaction (4.8, 4.5, and 4.7 h at 70 °C, respectively; Table 1). These

Table 1. Effects of organic co-solvents on succinimide formation and hydrolysis reactions of Val-Tyr-Pro-Asx-Gly-Ala at pH 7.4 in 0.10 M sodium phosphate buffer

Solvent (v/v)	Temperature (°C)	Dielectric constant ^a	Half-life of imide formation in asparagine peptide (h)	Half-life of imide formation in aspartate peptide (h) ^b	Half-life of imide hydrolysis in succinimidyl peptide (h)
100% Water	37	74.2	29		2.5
	70	63.5		25.1	
20% Glycerol	37	67.5	35		2.2
	70	57.5		18.1	
40% Glycerol	37	62.1	60		3.3
	70	52.7		17.8	
60% Glycerol	37	54.0	62		5.3
	70	45.7		17.6	
70% Glycerol	37	50.1	80 ^c		15.1
	70	42.5	2.7	19.4	
80% Glycerol	37	46.1	142 ^c		15.4
	70	39.1	4.8	17.1	
15% Ethanol	37	67.5	59 ^c		3.6
	70	57.5	2.0	38.3	
30% Ethanol	37	60.0	101 ^c		7.1
	70	50.9	3.4	34.1	
50% Ethanol	37	48.2	133 ^c		16.1
	70	40.4	4.5	27.0	
20% Dioxane	37	56.7	80 ^c		5.8
	70	47.8	2.7	33.3	
30% Dioxane	37	48.4	139 ^c		10.5
	70	40.8	4.7	31.7	
40% Dioxane	37	40.2	243 ^c		30.1
	70	33.5	8.2	24.6	

^a Dielectric values were obtained by interpolating (w/w) values and correcting for temperature using the relationship $D = ae^{-bT}$, where T is the temperature in °C and a and b are empirically determined constants (Akerlöf, 1932; Harned & Owen, 1958).

^b The formation of the succinimide is not a first-order reaction in the aspartyl peptide as it is in the asparaginyl peptide (Fig. 1). Consequently, it was necessary to model the kinetics of the isomerization reactions with the associated differential equation $[\text{Asp}]_t = [\text{Asp}]_{(t-\Delta t)} + k_2[\text{imide}]_{(t-\Delta t)} \Delta t - k_1[\text{Asp}]_{(t-\Delta t)} \Delta t$. The equation was solved using an iterative procedure to find k_1 at 70 °C as described by Geiger and Clarke (1987).

^c Extrapolated to 37 °C, for comparison with other data at this temperature, using the Arrhenius equation and an activation energy of 21.7 kcal/mol found for L-asparagine in the identical peptide at pH 7.5 and 0.10 M phosphate (Patel & Borchardt, 1990a).

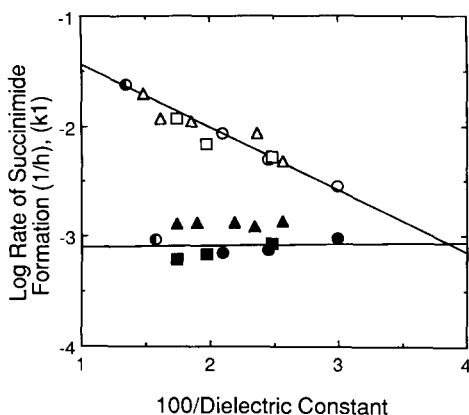


Fig. 2. Effect of solvent dielectric constant on the rate of degradation of the asparaginyl (open symbols)- and aspartyl (closed symbols)-containing peptides Val-Tyr-Pro-Asx-Gly-Ala in 0.10 M phosphate buffer (pH 7.4) at 37 °C. The co-solvents are: glycerol (Δ), ethanol (\square), dioxane (\circ), and 100% water (\bullet).

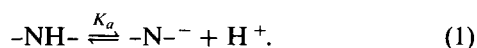
results also suggest that changes in the viscosity of the medium (especially with regard to the glycerol co-solvent) or in the concentration of water do not significantly affect the overall reaction rates.

Similar studies with the corresponding aspartyl-containing peptide (Val-Tyr-Pro-Asp-Gly-Ala) produced a different trend in the rate of succinimide formation with changes in solvent composition. An increase in the percentage of each of the organic co-solvents tested resulted in fact in a slightly increased rate of succinimide-mediated isomerization (Table 1; Fig. 2). This finding suggests that the decreased nucleophilicity of the peptide bond nitrogen may be countered by other changes in the reacting species. Additionally, we found that the rates of succinimide formation in the glycerol co-solvents are about 40–50% higher than those measured in the ethanol or dioxane co-solvents. The reason for this latter effect is not clear, although the presence of the three hydroxyl groups on

glycerol may accelerate the reaction by general acid/base catalysis. It is also unclear why this effect was not observed with the asparagine-containing peptide.

The only difference between the asparaginyl and aspartyl-containing peptides is the presence of a carboxamide versus a free carboxyl side chain, respectively. The aspartyl side chain carboxyl, with a pK_a value of about 4, exists almost entirely in the deprotonated charged form (COO^-) at pH 7.4 and presents a poor leaving group to nucleophilic attack (Stephenson & Clarke, 1989). In this case, as the dielectric constant of the media is lowered and the protonated uncharged state (COOH) is favored, a much better leaving group is formed. For example, Homandberg et al. (1978) found the pK_a of *N*-acetylglycine to increase in 80% glycerol by 0.9 units, in 80% ethanol by 1.4 units, and in 80% dioxane by 2.9 units. The side chain amide of the asparagine residue is uncharged at physiological pH values, and the characteristics of its potential leaving group would not be changed enough to offset the simultaneous changes in the nucleophilicity of the peptide bond nitrogen. Consequently, although the increased protonation of the aspartyl side chain can offset the changes in the rate of succinimide formation resulting from decreased nucleophilicity of the peptide bond nitrogen, no such balancing factor is present in asparagine residues.

Thus, by considering the effects of the dielectric constant on the stability of the nucleophilic species and the potential leaving group, the trends measured for deamidation of asparagine and isomerization of aspartate can be qualitatively understood. To attempt to explain the data more quantitatively, we made the assumption that the overall rate of succinimide formation in the asparagine peptide is proportional to the fraction of the attacking peptide bond nitrogen that occurs in the unprotonated state.



The electrostatic work necessary to separate the resulting charges will be inversely proportional to the dielectric constant as shown in the Born equation (N_A = Avogadro constant; e = electronic charge; D = dielectric constant; r = radii of respective atoms [Born, 1920]):

$$\Delta G_{\text{elect}} = \frac{N_A \cdot e^2}{2 \cdot D} \left(\frac{1}{r_{\text{N}^-}} + \frac{1}{r_{\text{H}^+}} \right). \quad (2)$$

Changes in the solvent dielectric constant will consequently affect the free energy difference ($\Delta\Delta G$) for the ionization of the peptide bond nitrogen (Eq. 1) (Amis & Hinton, 1973; Reichardt, 1979).

$$\begin{aligned} \Delta\Delta G &= (\Delta G_{\text{elect}})_{\text{solv. 1}} - (\Delta G_{\text{elect}})_{\text{solv. 2}} \\ &= 2.3 \cdot R \cdot T \cdot \log \frac{K_{a1}}{K_{a2}}. \end{aligned} \quad (3)$$

Figure 2 shows a plot of the rate of succinimide formation ($\log k_1$) versus the inverse dielectric constant for both the deamidation and isomerization reactions. The asparaginyl deamidation assays conform to the linearity predicted in Equation 3 and are evidence that an activated charged intermediate is central in the overall rate of formation of the cyclic succinimide. On the other hand, the nearly independent nature of the rate of isomerization in the aspartyl-containing peptide demonstrates that this rate is governed not only by the nature the peptide bond nucleophile but also by changes in the pK_a of the side chain leaving group.

Effect of solvent dielectric constant on the rate of succinimidyl hydrolysis

We also measured the rates of the hydrolysis of the peptide Val-Tyr-Pro-Asu-Gly-Ala (Asu = succinimidyl) under different solvent conditions (Table 1). The rates were found to be about 10-fold greater than the overall deamidation or isomerization rates in any of the solvents given in Table 1. This confirms that the rate-limiting step of deamidation and isomerization is the formation of the cyclic imide. We were interested, however, in the effect of solvent dielectric on the succinimide hydrolysis step itself. This hydrolysis reaction is dependent on the concentration of hydroxyl ion. Although all the assays were conducted at pH 7.4, the ion product of water (K_w) will vary with solvent dielectric strength and result in different pOH values. We estimated the ion product from graphical interpolation of K_w values in dioxane-water mixtures presented by Harned and Owen (1958) to approximate the effect of the altered hydroxyl ion concentration on the rate of succinimide hydrolysis. The results shown in Figure 3 are in fair agreement for first-order hy-

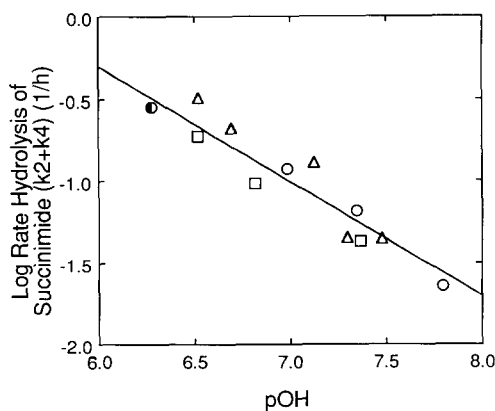


Fig. 3. Effects on the rates of hydrolysis of the succinimide-containing peptide Val-Tyr-Pro-Asu-Gly-Ala in 0.10 M phosphate buffer (pH 7.4) at 37 °C, as a function of the calculated pOH that results from the effect of solvent dielectric constant on the pK_a of water (K_w). The co-solvents are: glycerol (Δ), ethanol (\square), dioxane (\circ), and 100% water (\bullet).

droxyl ion concentration dependence. If these data are analyzed in terms of the pOH-independent component ($k_2 + k_4/[OH]$), a small negative dependence on dielectric is seen, similar to that observed for the hydrolysis of *N*-carbobenzyl-aspartylsuccinimide-serine amide (Bernhard et al., 1962) (data not shown).

Electron induction contributions to deamidation by amino acid residues carboxyl to asparagine

We tested the possibility of enhancing the rate of deamidation of the asparaginyl peptide by increasing the stability of the peptide bond anion through electron induction effects of neighboring groups. We thus synthesized a peptide corresponding to Val-Tyr-Pro-Asn-Gly-Ala where the glycine residue was replaced with a phenylglycine residue. The phenyl side chain on this amino acid has significant electron-withdrawing capabilities that would be expected to enhance the rate of succinimide formation (Fölsch, 1966). We compared the rate of succinimide formation in peptides with analogs containing glycine, serine, alanine, phenylglycine, and valine residues at this position (Table 2). Our results show that the rate was increased slightly over what is seen for a similarly sterically hindered residue such as valine at this position. The electron inductive effect is also illustrated by the faster rate of succinimide formation in the serine peptide derivative when compared to the alanine-containing derivative. By far the fastest rate of deamidation, however, occurred when a glycine residue was carboxyl to the asparagine residue. The lack of a side chain of glycine may provide for the backbone flexibility that allows for the interaction of the reactive groups with other atoms in the peptide (Stephenson & Clarke, 1989; Patel & Borchardt, 1990b). Further-

more, glycine's high Taft sigma constant (reflecting an absence of electron-releasing tendency) predicts that it would stabilize the peptide bond anion better than most naturally occurring amino acids.

Discussion

Because deamidation and isomerization are spontaneous reactions, their rates are dependent upon the local environment experienced by the labile residue. Although it is not yet possible to predict the reactivity of aspartate and asparagine residues under diverse conditions, approximations are available to estimate the effects of isotropic solvent interactions. One intrinsic property of a solvent is its ability to minimize the strong electrostatic attractions between oppositely charged ions and to thereby facilitate their solvation. This ability is expressed as a dielectric constant and is specific to a solvent at a given temperature.

Correlating changes in solvent dielectric constant to equivalent changes in pH

An alteration in solvent dielectric can impart a change on the rate of ionization of the peptide bond nitrogen, similar to that which can be obtained by actual variations in the solvent pH. Making the assumption that the rates of deamidation in the asparagine-containing peptide are proportional to the amount of deprotonated peptide bond nitrogen, we were able to calculate a change in pH that would give equivalent changes in the degree of protonation. Patel and Borchardt (1990a) measured the effect of varying the actual pH on the rate of deamidation using the same peptide and buffer utilized in this study. A comparison of their data with the calculated effective changes in pH caused by changes in solvent dielectric constant is shown in Figure 4. The conformity of these two sets of data implies that the observed rate changes are likely attributable to similar factors—the degree of deprotonation of the peptide bond nitrogen.

The independence of the aspartyl residue degradation rates to changes in solvent dielectric constant may explain why this reaction rate was found to be constant throughout this pH range in the 44-residue human growth hormone-releasing factor (Bongers et al., 1991) and in a 13-residue lipopeptide, daptomycin (Kirsch et al., 1989). In this pH range, a 10-fold increase in protonation of the peptide bond nitrogen is accompanied by an equivalent increase in protonation of the side chain carboxyl. In both of these examples, however, the rate of isomerization begins to decrease at approximately pH 3–4. This transition occurs in the region of the pK_a of the aspartyl side chain carboxyl, where a 10-fold increase in protonation of the peptide bond nitrogen is no longer accompanied by an equivalent increase in protonation of the side chain carboxyl group.

Table 2. Effect of the amino acid residue carboxyl to the asparaginyl residues in Val-Tyr-Pro-Asn-X-Ala on the rate of its deamidation in 0.10 M phosphate (pH 7.4) aqueous solutions

Amino acid	Corresponding side chain	Half-life for asparaginyl peptide at 37 °C in days ^a	Taft sigma* constant (aliphatic systems) ^b
Glycine	Hydrogen	1.21	0.49
Serine	Methanol	8.0 ^c	0.55
Alanine	Methyl	20.2 ^c	0.00
Phenylglycine	Phenyl	68.5	0.60
Valine	Isopropyl	106 ^d	-0.19

^a Except for the glycine peptide, all half-lives were extrapolated from data taken at 70 °C using the Arrhenius equation and an activation energy of 21.7 kcal/mol found for the L-asparagine-containing hexapeptide in 0.10 M phosphate (pH 7.5) buffer (Patel & Borchardt, 1990a).

^b Data from Taft (1956).

^c Data from Stephenson and Clarke (1989).

^d Data from Patel and Borchardt (1990b) at pH 7.5.

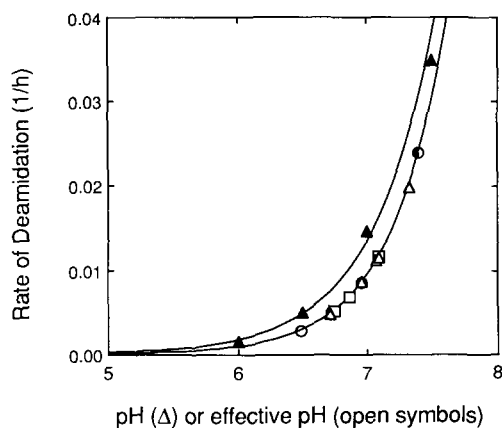


Fig. 4. Effect of the effective pH attributed to the changes in solvent dielectric constant on the rate of succinimide formation in the asparaginyl peptide (open symbols). These results are compared with the experimentally measured effect of pH on the rate of deamidation of the identical asparaginyl peptide in same buffer (0.10 M phosphate) and at the same temperature (37 °C) (▲) (data from Patel & Borchardt, 1990a). The co-solvents are: glycerol (Δ), ethanol (□), dioxane (○), and 100% water (●).

Implications for the in vivo degradation at asparagine and aspartate residues

The results of this study suggest that the lability of asparagine residues will increase for residues exposed at the surface of soluble proteins, where the environment is usually polar, and will decrease in the interior, where the environment is usually hydrophobic. Amino acids that are buried within proteins have been previously estimated to experience a dielectric constant on the order of 1–5 (Pethig, 1979; Rees, 1980). If the trends in rate caused by changes in solvent dielectric constant were extrapolated to these values, then the deamidation half-life of the asparagine residue in the model peptide utilized here would be on the order of 10^8 years at 37 °C. On the other hand the dielectric constant at the surface of a protein should approach that of the solvent. If the solvent is water, with a dielectric constant at 37 °C of around 74 (Akerlöf, 1932), then the half-life would be on the order of 1–2 days. Consequently, the environment to which an asparagine residue is exposed can greatly affect its stability in vitro or in vivo.

The fact that the aspartate residues are less affected by changes in the solvent dielectric suggests that their degradation to isoaspartate should proceed more or less independently of the polarity of the medium in their vicinity. Consequently, although a cell may be able to prevent spontaneous damage to asparagine residues by altering their local environment, it may be unable to similarly protect aspartate residues. These results may answer the question of why isoaspartyl residues in glucagon (Ota et al., 1987), calmodulin (Ota & Clarke, 1989), and epidermal growth factor (George-Nascimento et al., 1990)

can be preferentially formed from aspartate residues rather than from asparagine residues.

Pharmaceutical applications

The production of proteins through recombinant DNA technology has allowed for their widespread application as therapeutic pharmaceuticals (Pearlman & Nguyen, 1992). Unfortunately, the inherent instability of polypeptides has limited their use and has provided difficulties in their purification and storage (Manning et al., 1989; George-Nascimento et al., 1990). Because the deamidation of asparagine residues is a major pathway of protein degradation, peptide solvation in organic co-solvents may allow for product stabilization.

Materials and methods

Peptide synthesis and purification

The hexapeptides L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala and L-Val-L-Tyr-L-Pro-L-Asn-L-phenylglycine-L-Ala were synthesized by Dr. Janis Young at the UCLA Peptide Synthesis Facility using an Applied Biosystems model 430A instrument and trifluoroacetic acid (TFA) cleavage. L-Val-L-Tyr-L-Pro-L-Asu-Gly-L-Ala was synthesized by Vega Biotechnologies, Inc. L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala was prepared by incubating the analogous asparagine-containing peptide in 0.10 M sodium borate buffer at pH 10 and 37 °C for 24 h. This reaction yielded a mixture of the aspartyl- and isoaspartyl-containing peptide from which the aspartyl peptide was purified by high-performance liquid chromatography (HPLC) as described below.

Peptide purification and analysis was carried out on a Waters HPLC system composed of two model 510 pumps, a model 680 gradient controller, a model U6K injector, a model 441 UV detector set at 214 nm, and a Shimadzu C-R3A integrating recorder. Crude peptides, each less than 2.5 μmol , were purified at room temperature on an Econosphere C18 reverse-phase column (Alltech/Applied Science, 5- μm resin, 10-mm inside diameter, 190-mm length) at room temperature using a linear elution gradient from 0% to 30% solvent B in 30 min at a flow rate of 3.0 mL/min. The composition of solvent A is 0.1% TFA (w/v) in H_2O and solvent B is 0.1% TFA (w/v) in 90% (v/v) acetonitrile in H_2O . The concentration of peptides eluted from the HPLC purification was determined from their absorbance at 275 nm, using an extinction coefficient of $1,420 \text{ M}^{-1} \text{ cm}^{-1}$.

Analysis of peptide degradation

Aliquots of 10 or 20 nmol of purified peptide were placed in 500 μL polypropylene microcentrifuge tubes, and the

solvent was removed in a Savant Speedvac apparatus at room temperature and then stored at -20°C until used. Samples were dissolved in 200 μL of buffer—0.10 M monosodium phosphate and 0–80% glycerol (v/v), 15–50% ethanol (v/v), or 20–40% (v/v) dioxane in H_2O —and titrated to $\text{pH } 7.40 \pm 0.05$ at room temperature using NaOH. Samples were then incubated in sand baths pre-equilibrated at either 37°C or 70°C . The incubated samples were removed at various time intervals and frozen at -20°C to quench any further reaction. The samples were then thawed, vortexed, and centrifuged prior to analysis on the HPLC at room temperature using an Econosphere C18 reverse-phase column (Alltech/Applied Science, 5- μm resin, 4.6-mm inside diameter, 250-mm length). The peptides were eluted using a linear gradient from 0% to 40% solvent B in 40 min at a flow rate of 1.0 mL/min.

Typical elution times for the purified Val-Tyr-Pro-Asx-Gly-Ala peptides were: Asn and Asp, 23.0 min; IsoAsp, 20.7 min; and Asu, 26.8 min. Because the Asn and Asp peaks overlapped, it was necessary to determine the ratio of isoAsp:Asp formation using the succinimide-containing peptide. The amount of Asp could then be found by dividing the isoAsp peak by this ratio and then subtracting the resultant area from the Asn/Asp overlapping peak. The peptide containing phenylglycine (Val-Tyr-Pro-Asx-phenylglycine-Ala) had elution times of: Asp and Asn, 26.3 min; isoAsp, 25.4 min; Asu, 27.5 min.

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