Effect of N-1 and N-2 Residues on Peptide Deamidation Rate in Solution and Solid State

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ABSTRACT

The deamidation kinetics of 7 model peptides (VYPNGA, VYGNGA, VFGNGA, VIGNGA, VGGNGA, VGPNGA, and VGYNGA) were studied at 70°C in pH 10 buffer solutions and at 70°C and 50% relative humidity in lyophilized solid formulations containing polyvinyl pyrrolidone (PVP). The disappearance of the model peptides from solution and solid-state formulations followed apparent first-order kinetics, proceeding to completion in solution. In the solid state, the reactions showed plateaus with ~10% to 30% of the model peptides remaining; this was thought to be due to reversible complexation of the peptides and the PVP followed by slow dissociation of the complexes. The residues immediately N-terminal to asparagine (N-1, N-2) influenced the rate of deamidation significantly in the solid state but had minimal effect in solution. Increases in the volume and hydrophobicity of the N-1 and N-2 residues decreased the rate of deamidation in the solid state, but neither parameter alone adequately accounted for the observed effects. An empirical model using a linear combination of volume and hydrophobicity was developed; it showed that the influences of the volume and the hydrophobicity of the residues in the N-1 and N-2 positions are approximately equally important for the N-1 and N-2 residues.

KEYWORDS: Peptides, deamidation, PVP, solid-state stability, N-1 position, N-2 position

INTRODUCTION

The advent of recombinant DNA technology and modern synthetic chemistry has enabled the production of large quantities of highly pure peptides and proteins for possible pharmaceutical applications. However, because of the susceptibility of peptides and proteins to physical and chemical instability, the development of successful formulations has been a major challenge confronting pharmaceutical scientists.¹⁻³ Deamidation of asparagine (Asn) residues in peptides and proteins is one of the major pathways of chemical degra-

dation.^{2,4} This chemical pathway of degradation can occur under physiological conditions^{5,6} as well as in formulations intended for use in humans.⁷⁻⁹ Deamidation of a protein affects its structure and function and may play a role in the regulation of cell apoptosis, protein folding, aging, and immunogenicity.¹⁰⁻¹⁵

At pH values above 5 in aqueous solution,^{16,17} Asn deamidation proceeds via an intramolecular mechanism involving attack of the C-terminal peptide-bond nitrogen atom on the side-chain carbonyl carbon atom, resulting in the formation of a 5-membered succinimide ring (Asu) (Figure 1). The succinimide then undergoes rapid hydrolysis at either the α - or β -carbonyl group to generate isoaspartate (iso-Asp) and aspartate (Asp) residues in a ratio of ~3:1, respectively. The succinimide is also prone to racemization at the α -carbon to generate a mixture of D- and L-Asu, which then hydrolyzes to a mixture of D- and L-Asp and D- and L-iso-Asp. Recently, the formation of D-Asn has been observed under deamidation conditions, suggesting that racemization can occur in the tetrahedral intermediate (TI) before the expulsion of ammonia.¹⁸

The effects of exogenous and endogenous factors on the deamidation of Asn residues in proteins and peptides in solution at a pH ≥ 5 have been extensively studied using small model peptides.¹⁹⁻²² Under these conditions, the rate of Asn deamidation increases with increases in temperature, pH, and solvent dielectric constant.¹⁹ Amino acids in close proximity to the Asn residue can also play a major role in determining its rate of deamidation.²³⁻²⁷ Most studies have focused on the effect of the structure of the amino acid residue immediately C-terminal to the Asn residue (N+1 position) on deamidation. Structural variations in the N+1 residue can result in up to a 100-fold change in the deamidation rate of an Asn residue.¹⁹ A few studies have examined how the structure of the amino acid residue immediately N-terminal to the Asn residue (N-1 position) affects its deamidation.^{20,23} In general, the amino acid residue in the N-1 position has little effect on Asn deamidation. In proteins, residues distant from the Asn residue in the primary sequence can also affect its rate of deamidation because of the proximity achieved through secondary or tertiary structure.^{26,27} The extensive literature on Asn deamidation in peptides and proteins in solution has enabled investigators to develop models to predict the rate of this reaction with

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Figure 1. Pathways of deamidation of Asn residues in proteins and peptides at pH > 5. Asn indicates asparagine; Asp, aspartate; Asu, 5-membered succinimide ring; iso-Asp, iso-aspartate.

reasonable accuracy from a known primary sequence and 3-dimensional structure.²⁸⁻³¹

In an attempt to stabilize therapeutic proteins and peptides to chemical degradation, solid-state formulation strategies, often including excipients, have been developed.^{32,33} Unfortunately, chemical stability of proteins and peptides in the solid state is not well understood. Recently, studies in our laboratories have examined the influence of some exogenous factors, including effective pH, moisture content, and physical state of the matrix, on the rate and mechanism of peptide and protein deamidation.³⁴⁻³⁶ These studies suggest that, in general, one cannot use solution stability data to predict the effect of exogenous factors on the propensity of peptides and proteins to undergo deamidation in the solid state. Here, we investigate the possibility of a correlation between the effects of an endogenous factor (eg, primary sequence) on solution stability and the solid-state stability of Asn-containing peptides. Specifically, we describe the effect of the amino acid residues in the N-1 and N-2 positions on the rate of deamidation of Asn-containing peptides in polyvinyl pyrrolidone (PVP) matrices.

METHODS AND MATERIALS

Materials

Seven hexapeptides, Val-Tyr-Pro-Asn-Gly-Ala (VYPNGA), Val-Tyr-Gly-Asn-Gly-Ala (VYGNGA), Val-Phe-Gly-Asn-Gly-Ala (VFGNGA), Val-Ile-Gly-Asn-Gly-Ala (VIGNGA), Val-Gly-Gly-Asn-Gly-Ala (VGGNGA), Val-Gly-Pro-Asn-Gly-Ala (VGPNGA), and Val-Gly-Tyr-Asn-Gly-Ala (VGYNGA), were synthesized by California Peptide Research, Inc (Napa, CA). All had free N- and C-termini, and

all the residues in the peptides, except Gly, were in the L- configuration. The purity of all the peptides was >95%. PVP (Kollidon 17PF, average molecular weight [MW] 10 000) was obtained from BASF Corporation (Parsippany, NJ). All chemicals and solvents used to make buffer solutions and mobile phases were purchased from Fisher Scientific (Fair Lawn, NJ). Water used in the study was purified through a Millipore MILLI-Q system (Billerica, MA).

Purification of PVP

To remove low-MW impurities, PVP was purified before being used in the preparation of peptide formulations. PVP was dissolved in water and dialyzed for 48 hours using Spectra/Pro MW cutoff (MWCO) cellulose membranes (MWCO = 3500 Da; Spectrum Medical Industries, Houston, TX). The polymer was then lyophilized in a VirTis Advantage lyophilizer (Gardiner, NY) using the cycle as described under "Preparation of Solid Samples."

Buffers

Buffer solutions of pH 10 were prepared with sodium carbonate and sodium bicarbonate at 70°C. The buffer concentration was 0.1M, and the ionic strength was adjusted to 0.5 with potassium chloride. A quench buffer of 0.5M of formic acid/ammonium hydroxide, pH 3.5, was prepared at room temperature.

Preparation of Solid Samples

The peptides were dissolved in the pH 10, 0.1M sodium carbonate/bicarbonate buffer containing 5% (wt/vol) purified PVP at a concentration of ~200 µg/mL. Aliquots of 50 µL were transferred to 250 µL polypropylene high performance liquid chromatography (HPLC) vials and lyophilized using a VirTis Advantage lyophilizer (Gardiner, NY). The following lyophilization cycle was applied: (1) freezing at a shelf temperature of -35°C for 2 hours; (2) primary drying at -35°C for 10 hours; and (3) secondary drying at -15, -5, 5, 15, 20, and 25°C for 10, 8, 6, 6, 12, and 10 hours, respectively.

Formulation Stability Studies

For the solution stability studies, appropriate buffer solutions containing 5% (wt/vol) purified PVP were equilibrated in a 70°C water bath for 30 minutes, and the peptides were added to yield an initial concentration of ~200 µg/mL. At time zero and at predetermined time intervals, 3 aliquots (50 µL each) were removed and placed in three 250-µL polypropylene HPLC vials prefilled with 50 µL of pH 3.5

quench buffer in each vial. The vials were vortexed immediately to mix the solutions well. The samples were stored at -20°C until they were analyzed by HPLC.

For the solid-state stability studies, the samples were stored in a ESPEC LHH-112 Humidity Cabinet (Hudsonville, MI) at 70°C and 50% relative humidity. At time zero and at pre-determined time intervals, 3 vials were removed and 100 μL of the quench buffer was added to each vial. The vials were vortexed until the solid was completely dissolved. The samples were stored at -20°C until analyzed by HPLC.

HPLC Analysis

The model peptides and their degradation products were analyzed using a Shimadzu LC-10A system (Shimadzu, Tokyo, Japan) with an on-line column-switching system. The system consisted of a Shimadzu SCL 10A system controller, a Sil-10A auto injector, 2 LC-10AS pumps, an SPD-10A UV spectrophotometric detector, and an FCV-1AH high-pressure switching valve controlled by an SCL-6A controller. The mobile phase for pump A (mobile phase A) consisted of 0.05% formic acid, 0.03% ammonium hydroxide, and 5% to 12% acetonitrile. The percentage of acetonitrile was varied with each peptide to provide optimum separation. The mobile phase for pump B (mobile phase B) was 95% methanol. The flow rate of the mobile phase was 1 mL/min for both pumps. The peptides and their degradation products were detected by UV at 210 nm. Two columns were employed: a Keystone Hypersil ODS guard column (5 μm , 120 $\text{\AA}$, 20×4.6 mm) (column 1) and a Keystone Hypersil ODS analytical column (5 μm , 120 $\text{\AA}$, 250×4.6 mm) (column 2). The system was set up so that column 2 was exposed to only mobile phase A. Column 1 was exposed to mobile phase A for the first 2 minutes, then washed with mobile phase B for at least 25 minutes, and then washed again with mobile phase A for 6 minutes.

Kinetic Analysis

The disappearance of the parent peptides from solution and solid-state formulations followed apparent first-order kinetics. The observed first-order rate constants (k_{obs}) were obtained through nonlinear least-squares regression using the software package Origin (Microcal Software, Inc, Northampton, MA). The following equation was used for the fitting:

$$A = (A_0 - A_{\infty})\exp(-k_{\text{obs}}t) + A_{\infty} \quad (1)$$

where A was the concentration of a model peptide at time t , A_0 was the initial concentration of the model peptide, and A_{∞} was the concentration of the model peptide at infinity ($t \rightarrow \infty$). For the solution stability studies, A_{∞} was set at 0 because the deamidation of the model peptides went to completion.

Correlation of N-1 and N-2 Amino Acid Structural Features and Their Effects on Asn Deamidation

Attempts were made to correlate the rates of deamidation of the model peptides with the volume, hydrophobicity, and flexibility of the amino acid residues in the N-1 and N-2 positions. The volumes of the residues were taken from Chothia³⁷ and normalized for equal weighting of this parameter and hydrophobicity, as described by Ragone et al.³⁸ The lowest value in the scale (X_{min}) was taken to be 0, and the highest value (X_{max}) was 100. The normalized value (X_{norm}) was calculated from the original value (X) using the following equation:

$$X_{\text{norm}} = 100 \left(\frac{(X - X_{\text{min}})}{(X_{\text{max}} - X)} \right) \quad (2)$$

The hydrophobicity parameters were taken from the calculations by Manavalan and Ponnuswamy³⁹ and normalized by the same procedure.

To evaluate the relative importance of the volume and hydrophobicity of the residues in the N-1 and N-2 positions in determining the rate of Asn deamidation in the solid state, the following empirical equation based on a linear combination of the 2 factors was employed:

$$\log k_{\text{obs}} = a + bV_{\text{norm}} + cH_{\text{norm}} \quad (3)$$

where V_{norm} and H_{norm} are the sum of the normalized volumes of the N-1 and N-2 residues and the sum of the normalized hydrophobicity of the N-1 and N-2 residues, respectively, and a , b , and c are constants. The experimental data were fitted to Equation 3 using multiple linear regression. The t values and corresponding P values for the null hypothesis $x = 0$ (where $x = a$, b , or c) were calculated by the software. The product of the normalized volume and normalized hydrophobicity of each residue was used as an indicator of its contribution to the flexibility of the peptide.

RESULTS AND DISCUSSION

HPLC Method

Our laboratory had studied the stability of VYPNGA, one of the model peptides, in solution and solid state in the presence of PVP.^{35,36} In these studies, it was noted that repeated injections of samples of VYPNGA and its deamidation products containing PVP on an ODS analytical column resulted in decreases in retention times and loss of resolution of the degradation products. In addition, for some peptides used in the current study, significant decreases in peptide recovery were also observed when repeated injections of samples containing PVP were made to the analytical column.

In an attempt to eliminate the interference of the polymer in the analysis of the peptides used in the current studies, an on-line column-switching system was developed. In

developing this analytical method, we had the following goals: (1) baseline separation of the model peptides and their deamidation products in the absence of PVP with mobile phase A; (2) elution of the model peptides and their deamidation products from the guard column in <1.5 minutes, with retention of PVP on the guard column for >30 minutes with mobile phase A; and (3) elution of PVP from the guard column by mobile phase B within 25 minutes. These goals were met using the column-switching system developed.

Column switching is a well-established strategy for on-line sample cleanup. It is labor- and time-saving and has been applied frequently for the direct injection of biological samples.^{40,41} The results described in this article show that column switching can also be used in the analysis of formulations that contain large amounts of excipients that interfere with the analysis of the components of interest. For this method to be used, the interfering excipient should have chromatographic behavior markedly different from that of the solutes of interest.

Deamidation Mechanism and Kinetics

Under the experimental conditions employed in these studies, all of the model peptides degraded exclusively to the corresponding iso-Asp- and Asp-containing peptides in ratios of ~4:1 (data not shown). No other products were observed, and the mass balance was higher than 85%. These results suggest that the degradation of the Asn residues in the model peptides in the solid state and in solution occurred via the succinimide intermediate shown in Figure 1.

The disappearance of the model peptides from solution and solid-state formulations followed apparent first-order kinetics. In solution, the deamidation of the model peptides went to completion. However, in the solid state, the reactions showed plateaus with ~10% to 30% of the model peptides remaining. The unusual kinetic profiles in the solid state can be attributed to the reversible formation of a non-covalent complex (A·B) between the peptide (A) and PVP (B) and the irreversible formation of deamidation products (C) (Figure 2). The formation of the complex could make the peptide inaccessible to deamidation. If the rate of dissociation (k_{-1}) of the complex (A·B) is much slower than the rate of deamidation (k_2) of the peptide, a kinetic plateau can

be reached in the disappearance of the model peptide. In this case, it can be approximated that, in the solid formulation, there is no conversion between the free peptide (A) and the complex (A·B) during deamidation (ie, $k_1 = k_{-1} \approx 0$). If it is also assumed that the complex (A·B) dissociates on analysis, the observed kinetic profile of the disappearance of the peptide can be described by Equation 1, with $k_{\text{obs}} = k_2$. In the solid state, the rate of dissociation of the complex A·B (k_{-1}) is likely to be much slower than the rate of deamidation (k_2) because the dissociation of the complex requires translational mobility of the peptide and PVP molecules, whereas deamidation is an intramolecular process that does not require translational mobility of the molecule. How the formation of the complex is influenced by the properties of the peptide is being investigated further.

Effects of N-1 and N-2 Residues on the Rate of Deamidation of Asn-Containing Peptides in Solution

The deamidation rate constants (k_{obs}) of the 7 model peptides in solution are shown in Table 1. The k_{obs} values of the peptides VYGNGA and VYPNGA are very similar, differing by only 16%. A similar observation was made with the peptides VGYNGA and VGGNGA. These results suggest that the N-1 residue does not have a major influence on the rate of Asn deamidation in solution. These results are consistent with a previous report from our group using VYGNGA and VYPNGA but at a different temperature and pH.²⁰ The lack of effect of the N-1 residue on the deamidation rate of peptides/proteins in solution has also been observed by others. Tyler-Cross and Schirch,²³ in studies of peptides with the sequence VXNSV (X = G, H, S, A, R, L, T, V), showed deamidation half-lives at pH 7.3 and 60°C within a factor of 1.5. Robinson and Robinson⁴² have studied an extensive peptide library in an attempt to determine how primary sequence affects Asn deamidation.

Table 1. Rate Constants of Deamidation of the Model Hexapeptides in pH 10 Sodium Carbonate/Bicarbonate Buffer Solutions*

Peptides	k_{obs} in Solution (h^{-1})	$10^2 k_{\text{obs}}$ in Solid (h^{-1})
VYGNGA	9.77 ± 0.11	10.2 ± 1.4
VYPNGA	8.19 ± 0.15	11.7 ± 1.5
VGPNGA	18.9 ± 0.43	138 ± 22
VGYNGA	5.54 ± 0.09	8.63 ± 0.65
VGGNGA	6.33 ± 0.12	232 ± 44
VIGNGA	5.91 ± 0.16	5.07 ± 0.91
VFGNGA	5.32 ± 0.14	8.92 ± 0.99

*For solutions (buffer = 0.1M, I = 0.5) containing 5% polyvinyl pyrrolidone at 70°C and in the solid-state formulation lyophilized from the same solutions at 70°C and 50% relative humidity.

Figure 2. Reversible binding of peptide (A) and polyvinyl pyrrolidone (B) with parallel deamidation to products (C).

Many (306) of the peptides in this library had the sequence Gly-Xxx-Asn-Yyy-Gly, where Xxx included all of the 20 common amino acids except Asn and Gln, and Yyy included all except Asn, Gln, and Pro. The half-lives of deamidation were measured at pH 7.4 and 37.0°C in 0.15M Tris buffer.⁴² For peptides with the same Yyy residue, the deamidation half-lives of peptides with different Xxx residues were within a factor of 2.

Effects of N-1 and N-2 Residues on the Rate of Deamidation of Asn-Containing Peptides in the Solid State

The deamidation rate constants of the 7 model peptides in the solid state are also shown in Table 1. All peptides deaminate significantly more slowly in the solid state than in solution. For example, the differences between the deamidation rate constants in solution and in the solid state for VGGNGA and VGPNGA are ~3-fold and 10-fold, respectively. For all the other peptides, the differences in the rates of deamidation of Asn in solution and solid state are ~100-fold. These differences are reasonable, considering that in the solid state, molecular motion is markedly restricted. In addition, in the solid state there is less water, and could serve as either a reactant or a catalyst of the deamidation reaction.³⁴

In the solid state, the amino acids in the N-1 and N-2 positions of these peptides appear to have a significant influence on their deamidation rates (Table 1). For example, VGPNGA and VGGNGA both deaminate more than 20 times faster than VGYNGA (Table 1). Perhaps the bulkier structure of the side chain of tyrosine in the N-1 position, compared with glycine and proline, can explain these differences. However, a similar phenomenon was observed when bulky amino acids (eg, tyrosine, phenylalanine, isoleucine) were incorporated into the N-2 position. For example, VYGNGA, VFGNGA, and VIGNGA all have deamidation rates that are more than 20-fold slower than that of VGGNGA (Table 1). Furthermore, the magnitude of the effect of a bulky amino acid on the rate of deamidation does not seem to change with its location in the primary sequence (N-1 vs N-2 position) (eg, VYGNGA and VGYNGA have very similar deamidation rate constants).

A bulky amino acid residue in close proximity to an Asn residue could decrease rate of deamidation by hindering the access of water to the reaction center; as stated above, this lack of water can serve as a catalyst or reactant in the deamidation reaction.³⁴ A bulky amino acid residue may also restrict the molecular motion of a peptide and, thus, decrease the rate of deamidation. For deamidation to occur, intramolecular motion of the peptide molecule is required to bring the nucleophilic backbone nitrogen into proximity with the side-chain amide carbonyl group (Figure 1). A bulky residue in close proximity to these functional groups could restrict their mobility, thereby reducing the likelihood of a

reaction. Finally, a bulky residue close to the reaction center could hinder the release of ammonia from L-TI (Figure 1). The size of the residues in the N-1 and N-2 positions alone cannot account for the effects observed in the solid state (Figure 3), however. Although there is clearly a trend of deamidation rate constants decreasing with an increase in the volume of the residues in the N-1 and N-2 positions, the correlation is poor ($R^2 = 0.63$) (Figure 3).

Another characteristic of the amino acid residues that could contribute to these differences is their hydrophobicity. The hydrophobicity of the residues in the N-1 and N-2 positions could modulate the rate of deamidation in the solid state by reducing the hydration of the peptide, thus reducing the availability of water for the reaction. Alternatively, increases in the hydrophobicity of the residues in the N-1 and N-2 positions could decrease the dielectric constant in the local environment around the reaction center, thus stabilizing the reactant rather than the charged intermediate. In solution, it has been shown that deamidation of Asn in model peptides slows down with decreasing dielectric constant of the solvent.^{43,44} In the solid state, because of the removal of water molecules, neighboring amino acid residues could contribute more significantly than they can in the solution state to the creation of the local dielectric constant. The relationship between the deamidation rate constants of the model peptides in the solid state and the hydrophobicity of the residues in the N-1 and N-2 positions is shown in Figure 4. These data show that the deamidation rate constant decreases with increasing hydrophobicity of the residues in the N-1

Figure 3. The relationship between the observed first-order rate constants (k_{obs}) of deamidation in the solid state and the normalized volume of the residues (V_{norm}) in the N-1 and N-2 position residues. The x-axis is the sum of the V_{norm} of the residues in the N-1 and N-2 positions. The solid line is fitted to the data by linear regression. The sequences of the peptides are VGGNGA (1), VGPNGA (2), VYPNGA (3), VYGNGA (4), VGYNGA (5), VFGNGA (6), and VIGNGA (7).

Figure 4. The relationship between the observed first-order rate constants (k_{obs}) of deamidation in the solid state and the normalized hydrophobicity (H_{norm}) of the residues in the N-1 and N-2 positions for all the 7 peptides (A) and the 5 peptides containing tyrosine, phenylalanine, or isoleucine (B). The x-axis is the sum of the H_{norm} of the residues in the N-1 and N-2 positions. The solid lines are fitted to the data by linear regression. The sequences of the peptides are VGGNGA (1), VGPNGA (2), VYPNGA (3), VYGNGA (4), VGYNGA (5), VFGNGA (6), and VIGNGA (7).

and N-2 positions. As with residue size, the linear correlation of the deamidation rate constants and the hydrophobicities of the residues in the N-1 and N-2 positions was poor ($R^2 = 0.56$) when all 7 peptides were included in the analysis (Figure 4A). Interestingly, there is a much better correlation ($R^2 = 0.93$) when only those peptides with bulky residues (eg, tyrosine, phenylalanine, isoleucine) in the N-1 or N-2 positions are considered (Figure 4B). Those peptides containing the bulky residues have deamidation rates that do not correlate well with the volume of residues in the N-1 and N-2 positions (Figure 3). This suggests that the 2 parameters, volume and hydrophobicity, might lead to a better prediction of deamidation propensity if considered together.

A plot of volume versus hydrophobicity of the residues in the N-1 and N-2 positions (Figure 5) shows that the 2 param-

Figure 5. The relationship between the sum of the normalized hydrophobicity (H_{norm}) versus the sum of the normalized volume (V_{norm}) of the residues in the N-1 and N-2 positions. The sequences of the peptides are VGGNGA (1), VGPNGA (2), VYPNGA (3), VYGNGA (4), VGYNGA (5), VFGNGA (6), and VIGNGA (7).

eters appear to be independent of each other ($R^2 = 0.043$). The relative importance of the 2 parameters in determining deamidation rate was evaluated using an empirical model (Equation 3) in which volume and hydrophobicity are considered to be linearly independent. The resulting values of the coefficients (a, b, and c) in the model obtained from fitting the data to the model are shown in Table 2, together with the standard errors and the t and P values. The data are well described by the model, as indicated by a coefficient of determination near unity ($r^2 = 0.98$) and regression coefficients significantly different from 0 (Table 1). As volume and hydrophobicity were equally weighted through normalization, the similar values of b and c (Table 2) indicate that the influences of the volume and the hydrophobicity of the residues in the N-1 and N-2 positions on the rate of Asn deamidation in the solid state are approximately equally important.

We also explored the possibility of a correlation between the deamidation rate constant and the product of the volume and hydrophobicity ($V_{norm} \times H_{norm}$) of the residues in the N-1 and N-2 positions; a linear relationship was obtained ($R^2 = 0.93$) (Figure 6). The product $V_{norm} \times H_{norm}$ was chosen because it takes into account the effects of both the volume and the hydrophobicity and has been used previously as an

Table 2. Regression Coefficients in Equation 3 Obtained Through Multiple Linear Regression Using Origin

Parameter	Value	Standard Error	t Value	P Value
a	3.21282	0.1428	22.49946	<0.0001
b	-0.0117	0.00121	-9.70449	0.0006
c	-0.01401	0.00159	-8.8265	0.0009

Figure 6. The relationship between the observed first-order rate constants (k_{obs}) of deamidation in the solid state and the sum of the products of the normalized volume and hydrophobicity ($V_{\text{norm}} \times H_{\text{norm}}$) of the residues in the N-1 and N-2 positions. The solid line is fitted to the data by linear regression. The sequences of the peptides are VGGNGA (1), VGPNGA (2), VYPNGA (3), VYGNGA (4), VGYNGA (5), VFGNGA (6), and VIGNGA (7).

index of the local flexibility of proteins.³⁸ If the product $V_{\text{norm}} \times H_{\text{norm}}$ is truly an indicator of its contribution to the local flexibility of a peptide, the correlation observed between this parameter and the rate of deamidation shown in Figure 6 would suggest that the flexibility of the peptide might be a key factor in determining the rate of this reaction in the solid state. This correlation is reasonable because the ring-closure step in the deamidation mechanism (Figure 1) requires the intramolecular motion of the peptide molecules. Studies in solution have shown that if the flexibility of an Asn residue is restricted by the conformation of the protein, the deamidation rate decreases markedly.^{26,45} For example, upon denaturation, 2 Asn residues in lymphotoxin showed 31- and 9-fold increases in the deamidation rate, which could be due to the increase in the flexibility of the residues in the denatured state compared with the native state in which the 2 residues are located in β -turn structures.⁴⁵ Lai et al³⁵ have proposed that in the solid state the rigidity of the matrix can be used to predict the reaction rate. However, the flexibility of the peptide molecule itself needs to be considered as well. The current study shows that $V_{\text{norm}} \times H_{\text{norm}}$ might be a useful indicator of a residue's contribution to the local flexibility of a peptide. However, as discussed above, modulating the flexibility of the peptide may not be the only way through which the volume and hydrophobicity of the residues in the N-1 and N-2 positions affect the deamidation rate.

CONCLUSIONS

The solid-state stability of Asn-containing peptides/proteins cannot be readily predicted from their solution stability. Residues in the N-1 and N-2 positions can significantly influence the rate of deamidation of Asn in the solid state,

even though these residues have minimal effects in solution. Increases in the volume and the hydrophobicity of the residues in the N-1 and N-2 positions decrease the rate of deamidation in the solid state. However, neither the volume nor the hydrophobicity of the residues alone can adequately account for the observed effects. The effects of the residues in the N-1 and N-2 positions on the rate of deamidation of Asn appear to result from a combination of the 2 factors, as demonstrated by an empirical model developed using a linear combination. This model shows that the influences of the volume and the hydrophobicity of the residues in the N-1 and N-2 positions are approximately equal. The volume and the hydrophobicity of the residues in the N-1 and N-2 positions could influence the rate of deamidation in a variety of ways, including modulating the flexibility of the peptide molecules, controlling the availability of water to the reaction center, and changing the dielectric constant of the local environment.

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