Controlling Deamidation Rates in a Model Peptide: Effects of Temperature, Peptide Concentration, and Additives

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ABSTRACT: The rate of deamidation of the Asn residue in Val-Tyr-Pro-Asn-Gly-Ala (VYPNGA), a model peptide, was determined at pH 9 (400 mM Tris buffer) as a function of temperature and peptide concentration. Over the temperature range 5–65°C, deamidation followed Arrhenius behavior, with an apparent activation energy of 13.3 kcal/mol. Furthermore, increasing the peptide concentration slows the rate of deamidation. Self-stabilization with respect to deamidation has not been reported previously. The rate of deamidation was also determined in the presence of sucrose and poloxamer 407 (Pluronic F127). In both cases, the rate of deamidation was retarded by up to 40% at 35°C. In aqueous solutions containing poloxamer 407, the degree of stabilization is independent of formation of a reversible thermosetting gel. With sucrose, maximum reduction in the deamidation rate was attained with as little as 5% (w/v). Addition of sucrose results in a greater conformational preference for a type II β-turn structure, which presumably is less prone to intramolecular cyclization and subsequent deamidation. J Pharm Sci 90:2141–2148, 2001

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INTRODUCTION

Deamidation of asparagine (Asn) residues is probably the most common pathway for chemical inactivation of protein pharmaceuticals[1,2] The reaction rate for deamidation in aqueous solution is dependent on a number of extrinsic factors, such as pH,[3–6] solvent dielectric,[7] buffer concentration,[5] and temperature,[5] as well as intrinsic factors, such as the primary sequence[8–10] and the presence of secondary[2,11,12] and tertiary structure.[13] Despite numerous studies, little has been reported on the ability of additives to affect the rate of deamidation in peptides and proteins. In the case of deamidation of human epidermal growth factor (hEGF), a wide variety of excipients were reported to have an effect, but with no explanation as to why some were effective and others were not.[14]

It is now well established that the conformation of the peptide backbone (i.e., the secondary structure), as well as the side chain dihedral angles, can affect the rate of deamidation.[2,3] Therefore, one approach for slowing deamidation might be to control peptide conformation. One way this control might be accomplished is through the use of preferentially excluded solutes. These solutes are additives that are selectively excluded from the hydration sphere of the protein. In other words, there will be a relatively higher concentration of

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the excluded solute in the bulk relative to near the surface of the protein. The mechanism by which these solutes stabilize proteins has been described in detail by Timasheff and co-workers.[15–17]

It has also been demonstrated that preferentially excluded solutes can lead to compaction of the native structure of a globular protein.[15,16] The same additives may be able to alter the conformational distribution of a flexible peptide in aqueous solution.[15] In short, one might be able to use any number of excluded solutes (e.g., polymers, sugars, salts, or amino acids) to affect the solution conformation of the peptide, thereby having an effect on the deamidation rate. If this effect can be demonstrated, it would provide a new strategy for stabilizing hydrolytically sensitive peptides in aqueous formulations. It is important to note that preferentially excluded solutes are maximally effective at relatively high concentrations of solute (as much as 1 M).

Herein we describe the deamidation of a previously studied model peptide, Val-Tyr-Pro-Asn-Gly-Ala (VYPNGA),[4–7,10] as a function of temperature, peptide concentration, and the addition of solutes. We chose to examine two different excipients, sucrose and poloxamer 407, both of which are known to act as preferentially excluded solutes. Sucrose is well known to alter the conformation and flexibility of proteins through a preferential exclusion mechanism.[17] As has been shown for interleukin-1 receptor antagonist, addition of sucrose produces a more compact protein structure,[15,17] which is less prone to aggregation as well as deamidation.[18] Similar effects have been observed for sucrose interacting with interferon-γ.[16] However, the effect of excluded solutes on the solution conformation of a peptide has not been assessed.

The possible effects of poloxamer 407 (Pluronic F127), a triblock copolymer of poly(oxyethylene) and poly(oxypropylene), on deamidation of a peptide is less clear. Within a certain concentration range, aqueous solutions of poloxamer 407 will undergo a cooperative phase transition from a sol state to a gel with an increase in temperature. This transitition property has made it an attractive vehicle for controlled drug delivery.[19,20] It was hypothesized that poloxamer 407 might retard deamidation as well because it has been shown to salt out native proteins presumably due to preferential exclusion,[19] but that the stabilization may also be related to the sol–gel transition rather than solely due to poloxamer 407 effects on peptide conformation.

MATERIALS AND METHODS

Materials

Poloxamer 407 (Pluronic F127) was obtained from BASF and used as received. Sucrose was purchased from Pfannstiehl. All solvents were obtained from Aldrich.

Peptide Synthesis

Amino acid derivatives and resin were purchased from Applied Biosystems Incorporated (Foster City, CA). All other solvents and reagents were purchased from Fisher Scientific (Pittsburgh, PA). The peptide was assembled on Boc-Ala-PAM resin (0.5 meq) using an ABI 430A automated peptide synthesizer. Boc groups were removed with 33% trifluoroacetic acid (TFA) in dichloromethane. Subsequent Boc amino acid derivatives were coupled to the resin in fourfold excess using diisopropylcarbodiimide and hydroxybenzotriazole. Coupling reactions were monitored by the quantitative ninhydrin test.[1] All yields were $>99\%$ after a single coupling. Once the desired sequence was assembled, the peptide was simultaneously deprotected and cleaved from the resin using a mixture of TFA, trifluoromethanesulphonic acid, ethanedithiol and thioanisole (80/8/4/8, v/v/v/v). The crude peptide product was loaded onto a Vydac 218TP101550 preparative C18 column (250×50 mm, 300, 10–15 m) from The Separations Group (Hesperia, CA) that was previously equilibrated with triethylammonium phosphate buffer (100 mM, pH 2.5). The concentration of acetonitrile in the eluent was raised to 11% over a period of 50 min. The eluent was continuously monitored at 230 nm and collected in 50 mL fractions. Fractions containing only the peptide were pooled, diluted twofold with water and desalted on the same column now equilibrated with 0.1% TFA. The concentration of acetonitrile was increased to 30% over a period of 30 min. Fractions containing the product were pooled and lyophilized to yield 150 mg of VYPNGA as a fluffy white powder (48% overall yield based on the initial resin substitution). FAB-MS $[M+H]+$ found 620.4, theoretical 620.3.

HPLC Protocol

The degradation of VYPNGA was monitored using a LKB Bromma high-performance liquid chromatography (HPLC) system with a Applied

Biosystems variable wavelength detector set at 214 nm. Separation was achieved using a Vydac reverse phase C-18 column (4.6 × 25 mm length, 5 μm bead diameter and 80 Å pore size). The mobile phase was a 24/76 mixture of water and 20% acetonitrile in 0.1% aqueous TFA. The flow rate was 1.5 mL/min. This protocol is similar to those reported previously for deamidation studies of VYPNGA.[5,10]

Deamidation Studies

Samples were prepared by dissolving a weighed amount of VYPNGA in 1 mL of 400 mM aqueous Tris buffer (pH 9), with or without poloxamer 407 present. The sample was placed in a water bath to control the temperature. Periodically, samples were removed to assay for the extent of deamidation using the HPLC protocol already described.

Circular Dichroism Studies

Samples were prepared by dissolving 1 mg of VYPNGA in 1 mL of 400 mM Tris buffer containing various amounts of sucrose or poloxamer 407. Samples were then placed in a 0.1 mm quartz cuvette. Far ultraviolet (UV) spectra ($\lambda \approx 250$–180 nm) were collected using an AVIV 62-DS circular dichroism (CD) spectrometer with a thermoelectric temperature control unit regulated to $\pm 0.1^{\circ}$C. Data were taken every 0.25 nm, using an averaging time of 4 s and a bandwidth of 1.5 nm.

RESULTS AND DISCUSSION

Deamidation of a polypeptide proceeds through a cyclic imide intermediate formed by intramolecular attack of the succeeding peptide nitrogen on the carbonyl of the Asn side chain above pH 6[3,4] (see Figure 1). This intermediate can hydrolyze, via nucleophilic attack of water, at either of the carbonyl groups; one attack will form a product containing normal aspartic acid (Asp) in place of the Asn residue, and one leads to a rearranged form of aspartic acid referred to as isoaspartic acid (isoAsp). As a model of a peptide prone to deamidation, we chose the model hexapeptide, VYPNGA, which has been used widely in the past.[4–7,10] This peptide represents the

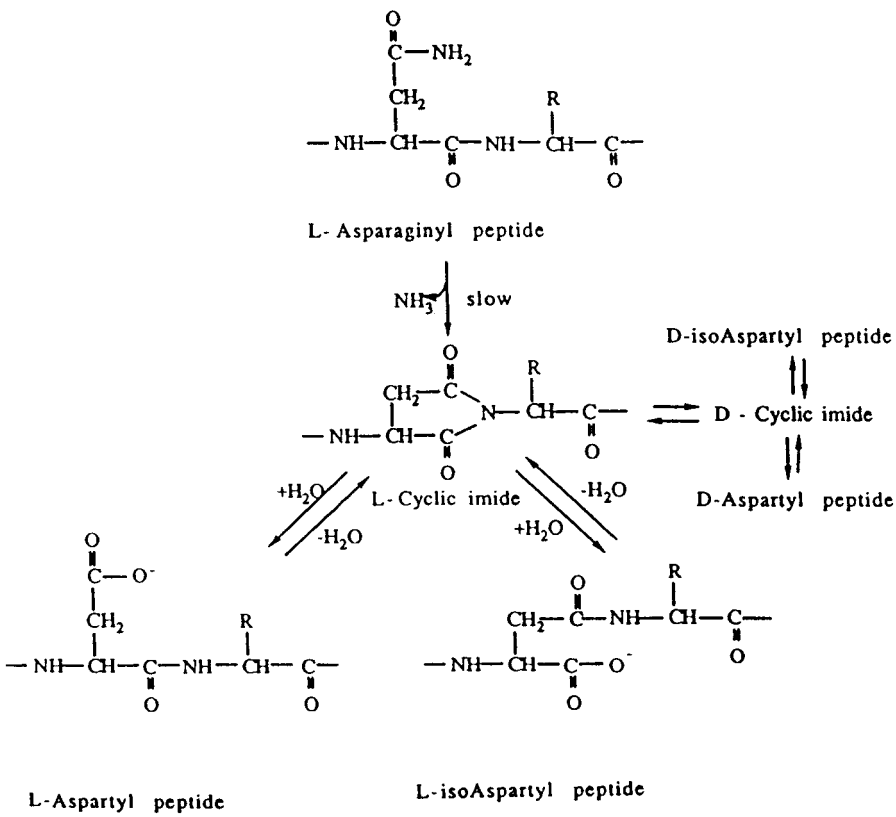

Figure 1. General mechanism of deamidation of Asn residues under basic conditions.

deamidation site in the peptide hormone, ACTH. The chromatographic properties of this peptide and its degradation products are well established,[4–7,10] making determination of the reaction kinetics straightforward. To have the reaction proceed quickly, we used conditions known to accelerate deamidation; namely, high pH and high buffer concentration. For this study, 400 mM Tris buffer at pH 9 was employed throughout. Under these conditions, a 1 mg/mL sample of VYPNGA will deamidate almost completely at 45 °C within 6 h.

The first studies were conducted to determine whether the mechanism of deamidation was altered by the use of high Tris concentrations at pH 9, conditions that have not been reported previously for this peptide. Therefore, the temperature dependence of the reaction was followed over a range spanning 5 to 65°C. It should be noted that there is the possibility that Tris will decompose at elevated temperatures,[21] producing reactive species, such as formaldehyde. However, there was no chromatographic evidence for the formation of species other than the deamidated forms of VYPNGA.

Within experimental error, the reaction follows Arrhenius behavior (Figure 2). The activation energy was 13.3 kcal/mol, with an A-value of $10^{14.9}$ ($r^2 = 0.98$), which is significantly less than the ~20 kcal/mol reported for this reaction.[4,5] However, those activation energies were for reactions extrapolated to zero buffer concentration. At high buffer concentrations, buffer catalysis becomes significant, and the activation energy could be affected. The ratio of isoAsp to Asp was similar to that reported in the literature for VYPNGA,[4,5,10] averaging ~3.0–3.5 to 1.0 at all temperatures tested, as determined by HPLC.

Concentration Effects

One aspect of peptide deamidation that has not been reported is whether peptide concentration has any effect. Presumably, increased concentration could lead to aggregates that might be more stable or increased concentration may favor a solution conformation that is more or less reactive, depending on the relative spatial arrangement of the peptide backbone and Asn side chain.[8] On increasing the peptide concentration from 0.1 to 100 mg/mL, an approximate threefold decrease in reaction rate was observed (Figure 3). A number of possible explanation exist for such an observation. First, it is possible that the peptide is interacting with itself, assembling to form a structure that is less reactive than the free monomer in solution. Second, small changes in pH (< 1 pH unit) could cause a modest decrease in rate, and high concentrations of the peptide may be able to shift the pH to this degree.[22] However, no significant change in overall pH was measured for the concentrated solutions. Third, a peptide concentration of 100 mg/mL will cause increases in the viscosity of the solution. It has been reported that viscosity increase of <5 cP can slow the reaction to this extent.[23] Whereas this most plausible explanation may be self-assembly, these other mechanisms cannot be excluded.

Additive Effects-Sucrose

Sucrose is well known to alter both the conformation and flexibility of proteins through a preferential exclusion mechanism.[15–17] Briefly, an exclusion of solute, such as sucrose, means that the solute would rather exist in the bulk solution rather than at the surface of the protein. Such

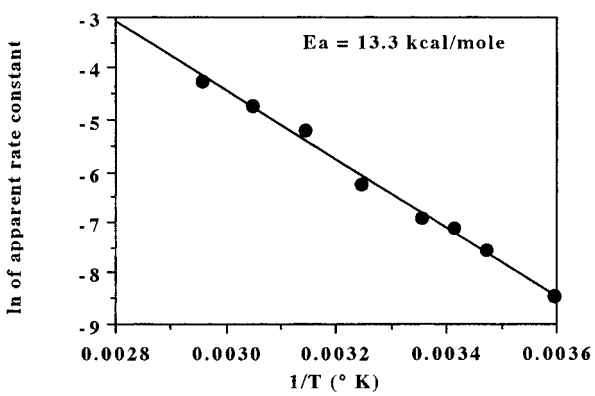

Figure 2. Arrhenius plot for deamidation of VYPNGA (1 mg/mL) at pH 9 (400 mM Tris buffer).

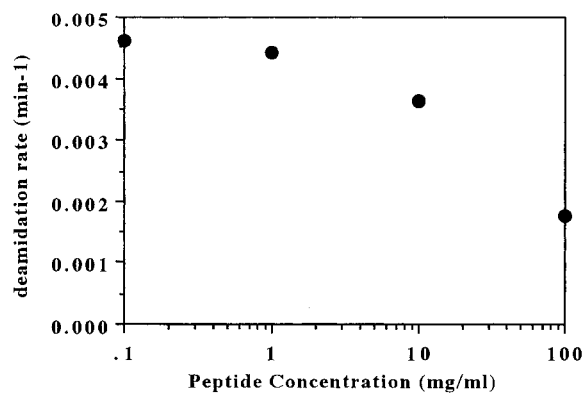

Figure 3. Concentration effects on the rate of deamidation of VYPNGA (pH 9, 400 mM Tris buffer, 35°C).

'negative' binding is thermodynamically destabilizing. The same exclusion exists for the unfolded state, as well as the native state. Because the unfolded state is typically larger in surface area than the folded state, the effect is larger in magnitude, meaning that the denatured or unfolded state is destabilized more than the native state. Together, these effects result in an increase in the free energy required to go from the folded to unfolded state; that is, there is a net stabilization. Moreover, for most proteins, excluded solutes also induce the protein to adopt a more compact native state.[15,16] For interleukin-1 receptor antagonist, the more compact protein structure is less prone to aggregation[15] as well as deamidation.[18] However, the effects of sucrose on peptide stability have not been well characterized. On addition of increasing amounts of sucrose, the reaction rate for deamidation decreased, even with as little as 2% sucrose present. The maximum effect could be achieved with as little as 5% sucrose (Figure 4). With 5% sucrose present, the relative deamidation rate was 77%. With 10% sucrose present, the relative rate was 78% of comparable solutions with no sucrose present.

At these concentrations, lower rates due solely to increased viscosity is unlikely, especially because the rate-limiting step is intramolecular cyclization.[6] At a sucrose concentration of 2%, there should be little change in the viscosity of an aqueous solution (< 5 cP). Although the spectroscopic evidence given later suggests that rate retardation is due to a conformational change, the effects of the increased viscosity, although modest, cannot be excluded as having some modulating effect on deamidation rates, especially in light of the work of Li et al.[23]

As just hypothesized, retardation of deamidation rates by an excluded solute suggests that the peptide has changed conformation, making intramolecular cyclization to the succinimide intermediate more difficult. The effect of secondary structure on deamidation has been attributed to this type of effect, where the peptide and the asparagine side chain are in less favorable positions for intramolecular attack when in an ordered secondary structure.[2,3,8] In this case, the deamidation site is not within a fixed secondary structure, but the distribution within the native state ensemble is being shifted.

CD spectra were obtained for VYPNGA both in the absence and presence of sucrose at pH 9. The spectra were taken at 25°C and within 10 min of sample preparation to minimize the extent of deamidation. A difference spectrum for a sample in 5% sucrose versus no sucrose reveals that a certain secondary structure is induced by the addition of sucrose (Figure 5). The induced structure is easier to detect using difference spectroscopy because the original CD spectra are dominated by contributions from the Tyr side chain. The difference CD spectrum shown a negative band near 225 nm and a positive band near 200 nm, indicative of a type II β-turn structure,[24,25] presumably with Pro at position $i+1$. This structure would place the Asn residue at the corner of a type II turn. It is known that there is a subset of conformations that favors deamidation by positioning the side chain for attack by the succeeding nitrogen atom in the polypeptide chain.[3] Such a structure would be

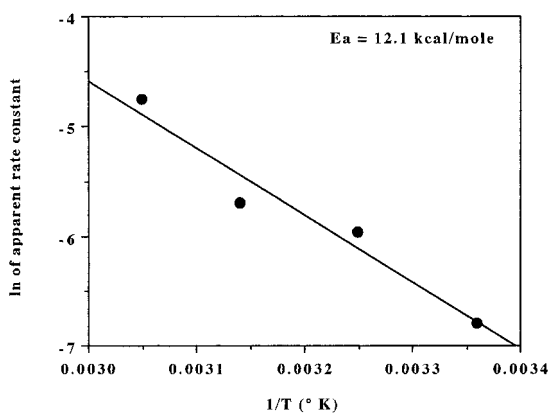

Figure 4. Effect of added sucrose on the deamidation rate of VYPNGA (1 mg/mL) at pH 9, 400 mM Tris buffer) at 35°C.

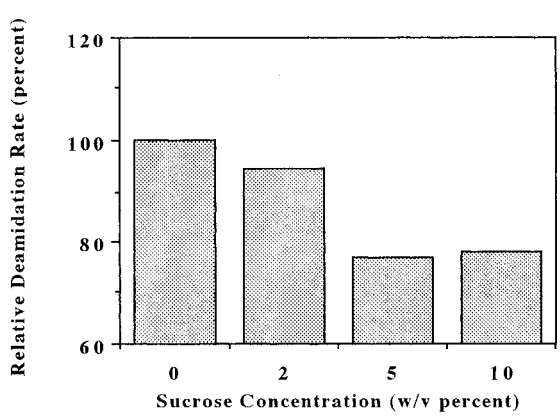

Figure 5. Difference CD spectrum of VYPNGA in the (A) presence and (B) absence of 5% (w/v) sucrose (pH 9, 400 mM Tris buffer, 25°C).

predicted to slow deamidation for two reasons. First, the orientation of the carbonyl group of Asn relative to the succeeding amide nitrogen of the Gly residue is not favorable for nucleophilic attack. Second, the amide NH is presumably involved in stabilizing the β-turn, making its deprotonation more difficult. The deprotonation must occur to generate a species sufficiently nucleophilic to form the cyclic imide intermediate. Other researchers have observed that residues located in β-turns do deamidate more slowly than residues in unordered or random coil structure.[2,26,27] Analysis of the sequence preferences for certain amino acids in various positions of β-turns indicates that the combination of Pro-Asn is highly favored for occupying positions $i+1$ and $i+2$ in a type II β-turn.[28,29]

Although these data do not constitute definitive proof for rate retardation arising from an altered solution conformation of a peptide, the magnitude of the rate changes and the substantial increase in the β-turn population present a compelling observation. The ability of additives to modulate peptide conformation and thereby affect chemical degradation rates appears to be one possible formulation strategy. Recently, a similar finding was observed for active site oxidation retardation by the addition of sucrose.[30]

Additive Effects- Poloxamer 407

Few data have been reported on the ability of excipients to slow chemical degradation in proteins, especially for deamidation. Son and Kwon reported that numerous polymers were effective at retarding deamidation in hEGF.[14] However, no mechanistic explanation was given and the additives were very different in chemical and physical properties (various polymers, surfactants, etc.), making generalization difficult.

Poloxamer 407, when dissolved in water at high concentrations, has the ability to form either a fluid solution (at low temperatures) or a stiff gel (at higher temperatures). It was of interest to see if formation of an aqueous gel would retard deamidation in a peptide, particularly as a function of physical state. Relatively little is known about the detailed interaction of poloxamers and proteins. Recently, we demonstrated that poloxamer 407 is a preferentially excluded solute that is able to salt-out proteins when dissolved in aqueous buffer at high concentrations.[19] Therefore, as an excluded solute, it should be possible for poloxamer 407 to affect the solution conformation of VYPNGA in the same fashion as sucrose, and thereby alter the rate of cyclic imide-mediated deamidation.

At concentrations $>17\%$ (w/w), solutions of poloxamer 407 can form stiff gels as the temperature is increased. The process is highly cooperative, occurring within a narrow temperature range. This process can be monitored by observing the physical state of the solution or by spectroscopic methods, such as Fourier transform infrared spectroscopy.[19] Using a 22% gel solution, which gels at ~15 °C, the deamidation of VYPNGA was investigated under conditions analogous to those in solution (pH 9, 400 mM Tris). Whether the poloxamer solution was in the gel or sol state, there was a decrease in the rate of deamidation by $>40\%$ versus the peptide in aqueous solution. Above the gel transition temperature (35°C), the deamidation rate was $58.6 \pm 2.9\%$ ($n=3$) relative to solutions without poloxamer present. Below the gel transition temperature (5°C), the relative deamidation rate was $45 \pm 3\%$ ($n=2$).

In the presence of 22% poloxamer, the temperature dependence of the reaction was investigated for temperatures above the transition temperature of the gel, meaning all measurements were made on poloxamer solutions existing in the gel state. Under those conditions, the system displays Arrhenius behavior, and the activation energy was determined to be 12.1 kcal/mol, with an A-value of $10^{14.2}$ (Figure 6, $r^2=0.92$), similar to that determined for VYPNGA in buffer alone. Considering the variance in the data, the

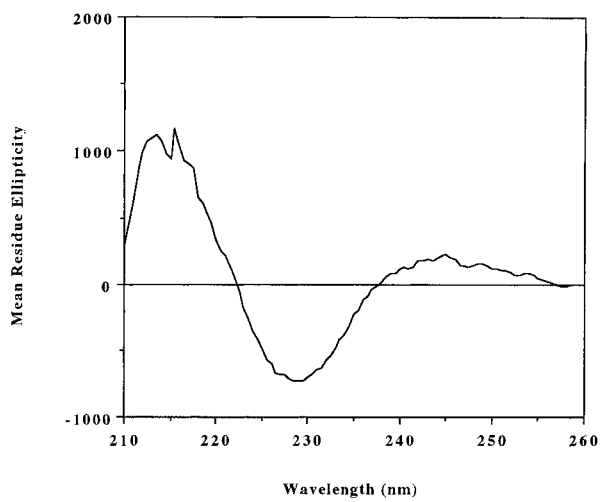

Figure 6. Arrhenius plot for deamidation of VYPNGA (1 mg/mL) at pH 9 (400 mM Tris buffer) in a 22 % (w/w) aqueous solution of poloxamer 407.

activation energies are probably not significantly different.

Given that sucrose appears to slow deamidation by altering the conformational preference of the peptide, similar CD studies were conducted looking for evidence of a structural change in the presence of poloxamer 407. Difference spectra showed a similar far UV CD pattern as with the addition of sucrose, but much weaker. Given that the solute concentration was much greater (22 versus 5%), it appears that although poloxamer can act as an excluded solute, it is much less effective than sucrose on a weight-to-weight basis.

SUMMARY

These results demonstrate that the addition of excluded solutes can alter the conformational preference of a flexible peptide while also altering its propensity to deamidate. The effect is analogous to the role of secondary structure on modulating deamidation by constraining backbone dihedral angles. In the case of VYPNGA, the addition of sucrose drives the conformational ensemble toward a greater preference for a type-II β-turn structure, most likely around the Pro-Asn residues.[28,29]

Furthermore, it is becoming clear that poloxamers, much like PEGs and other polymers, act as excluded solutes at high concentrations (as demonstrated herein and previously[19]). Possibly, the mechanism of stabilization is the same as with sucrose, although viscosity effects and small changes in pH cannot be ruled out. Certainly, one must consider the possibility for retardation of deamidation in such concentrated solutions is that it is simply a viscosity effect, even for poloxamer in the sol state.[23] However, the results with sucrose suggest that the effect is conformational, not a function of microviscosity, especially because the rate-limiting step is an intramolecular event. Furthermore, the fact that the extent of stabilization of poloxamer solutions was similar in both the sol and gel states, where the viscosity is dramatically different, also argues against viscosity playing a major role in slowing deamidation in flexible peptides. Together, these findings suggest that control of the conformational state of a peptide may not only retard physical processes, such as aggregation, but can also slow degradation due to hydrolytic reactions, such as deamidation.

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