Chemical Stability of Peptides in Polymers. 2. Discriminating between Solvent and Plasticizing Effects of Water on Peptide Deamidation in Poly(vinylpyrrolidone)

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Abstract □ The mechanistic role of water in the deamidation of a model asparagine-containing hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala) in lyophilized formulations containing poly(vinylpyrrolidone) (PVP) and glycerol was investigated. Glycerol was used as a plasticizer to vary formulation glass transition temperature (T_g) without significantly changing water content or activity. Increases in moisture and glycerol contents increased the rate of peptide deamidation. This increase was strongly correlated with T_g at constant water content and activity, suggesting that increased matrix mobility facilitates deamidation. In rubbery systems ($T > T_g$), deamidation rates appeared to be independent of water content and activity in formulations with similar T_gs. However, in glassy formulations with similar T_gs, deamidation increased with water content, suggesting a solvent/medium effect of water on reactivity in this regime. An increase in water content also affected the degradation product distribution; less of the cyclic imide intermediate and more of the hydrolytic products, isoAsp- and Asp-hexapeptides, were observed as water content increased. Thus, residual water appears to facilitate deamidation in these solid PVP formulations both by enhancing molecular mobility and by solvent/medium effects, and also participates as a chemical reactant in the subsequent breakdown of the cyclic imide.

Introduction

Many protein and peptide drugs are formulated as lyophilized or freeze-dried products to prolong shelf life.[1−3] Although the "dried" product is usually more stable than the aqueous formulation, chemical degradation reactions such as deamidation and hydrolysis can still occur.[1−3] Residual water in lyophilized protein formulations is known to promote chemical reactivity, leading to loss of biological activity and a shortened shelf life.[2−6] Although the destabilizing effect of water on proteins is widely acknowledged, the exact mechanistic role of water in facilitating chemical reactivity in the solid state is not well understood. This study will examine the mechanistic role of water in the deamidation of the model asparagine-containing hexapeptide Val-Tyr-Pro-Asn-Gly-Ala (Asn-hexapeptide) in lyophilized formulations containing poly(vinylpyrrolidone) (PVP).

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Water may affect the solid-state chemical reactivity of polymer-incorporated peptides and proteins through three possible mechanisms: 1) changing the dynamic mobility of the protein or peptide, 2) direct participation as a reactant, or 3) indirect participation as a medium/solvent.[2,7] The mobility mechanism is based on the premise that if chemical reactions require sufficient mobility to proceed, an increase in mobility would result in increased reactivity. In polymer matrixes, water can increase molecular mobility by acting as a plasticizer to increase free volume and decrease viscosity.[8,9] Second, water can increase chemical reactivity directly by acting as a reactant, as in hydrolysis.[2,7] Water may also affect chemical degradation by acting as a medium for the mobilization of reactants or by modifying the reaction environment, as when the effective solvent dielectric or polarity is altered.[2,7]

The plasticizing effect of water on molecular mobility of the matrix may be monitored by measuring the glass transition temperature (T_g), the temperature at which a glassy, brittle, dynamically constrained material becomes rubbery and soft, with increased molecular mobility. Many studies have correlated decreases in T_g with decreases in system viscosity and increases in mobility.[9,10] The Vogel−Tamman−Fulcher (VTF) equation and the Williams−Landel−Ferry (WLF) equation, which is a special case of the VTF, describe the changes in viscosity in terms of $T_{exp} - T_g$ or distance from the glass transition, where T_{exp} is the experimental temperature.[9−12] Thus, we can use T_g as a qualitative measure of the plasticizing effect of water on formulation mobility. Because the effective concentration of a reactant may be expressed in terms of its chemical potential, we can use formulation water activity to measure the role of water as a reactant. Last, water content can probably best describe the medium/solvent effect of water. Thus, we can use water content, water activity, and T_g as indictors of the various mechanistic roles of water.

A technical problem with using these formulation parameters is that they are interdependent; changing water content or water activity will also affect T_g. Unambiguous mechanistic interpretations of the data are therefore difficult to achieve.[13] In a previous study, we demonstrated a dependence of deamidation rate on matrix water content in poly(vinylpyrrolidone) and poly(vinyl alcohol) matrixes, but were unable to distinguish among the more fundamental effects of water as a solvent, reactant, or plasticizer because of this interdependence.[14] To overcome this problem in the present studies, we used glycerol as an additional plasticizer to vary formulation T_g without significantly affecting water content or water activity. We therefore could systematically determine the effect of water activity

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and content on deamidation kinetics when T_g is constant, as well as the effect of T_g on deamidation under constant water content and activity. Because water content and activity will not vary significantly between formulations, the role of water as a medium cannot be separated from its role as a reactant. In this study, these two roles of water will be combined under the role of solvent effects. Thus, using this strategy, we propose to deconvolute the mechanistic role of water as a plasticizer from its role as a solvent in solid-state deamidation.

Because deamidation is one of the most prevalent chemical degradations found in proteins and peptides,[15,16] we selected the Asn-containing hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala) (Asn-hexapeptide) as the model compound for this study. An advantage of using the Asn-hexapeptide is that its solution-state degradation kinetics and mechanisms are well understood.[16−18] This knowledge will provide a solid mechanistic basis with which we can better interpret the solid-state deamidation kinetic and product distribution data.

In this manuscript, we present our findings on the effect of water on Asn-hexapeptide deamidation in lyophilized PVP formulations. We show that glycerol was successfully used to modify formulation T_g without a significant effect on water content or activity. Then, we correlate the rates of Asn-hexapeptide deamidation with water content/activity and T_g to elucidate the role of water in solid-state deamidation. We also examine the Asn-hexapeptide deamidation product distribution to provide information regarding the mechanism of degradation and the effect of water on product distribution.

Experimental Section

Materials—L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala (Asn-hexapeptide) was synthesized by Dr. Madhup Dhaon (Abbott Laboratories, North Chicago, IL). The buffers and salts used in this study were purchased from Mallinckrodt Chemical, Inc. (Paris, KY). Organic solvents and trifluoroacetic acid were purchased from Fisher Scientific (Fair Lawn, NJ). The poly(vinylpyrrolidone), under the trade name of Kollidon K17 [MW = 10 000], was purchased from BASF Corporation (Parsippany, NJ). Glycerol was obtained from the manufacturing division of Pharmacia & Upjohn, Inc. (Kalamazoo, MI). Deionized and distilled water was used throughout.

Preparation of Formulations—Five formulations were prepared: four solid-state PVP formulations with 0, 10, 20, and 30% glycerol, and a liquid-state formulation in 100% glycerol. The detailed procedure has been described previously.[14] Briefly, the solid PVP formulations were prepared from solutions containing peptide, glycerol, and PVP in phosphate buffer (pH 6.8). The solutions were added in a dropwise manner to liquid nitrogen to form frozen spherical pellets, which were then lyophilized to remove residual moisture. The average peptide load, calculated on the basis of the maximum amount released,[14] was 0.017 g/g dry solid for 100% PVP, 0.022 g/g dry solid for 10% glycerol/PVP, 0.022 g/g dry solid for 20% glycerol/PVP, 0.014 g/g dry solid for 30% glycerol/PVP, and 0.24 g/g glycerol for 100% glycerol, with errors at 5% ($n = 3-5$). The approximate final composition of the formulations was 2% peptide, 2% buffer, and 96% (w/w) PVP and/or glycerol, with a residual water content of <0.1% (w/w).

Characterization of Water Sorption Behavior—Sorption isotherms relating formulation water content to relative humidity (water activity) at 50 °C were generated using a controlled atmosphere microbalance (CAM), according to a method reported previously.[14] As in the previous study, the water sorption data obtained with the CAM were fitted to the Guggenheim−Anderson−deBoer (GAB) equation which describes the sorption of water by heterogeneous sorbents or solids:[2]

$$W = \frac{W_m C_g K_{GAB}(\rho/\rho_o)}{[1 - K_{GAB}(\rho/\rho_o)][1 - K_{GAB}(\rho/\rho_o) + C_g K_{GAB}(\rho/\rho_o)]} \quad (1)$$

where W is the mass (mg) of water vapor adsorbed per mg of dry solid at (ρ/ρ_o), (ρ/ρ_o) is the relative vapor pressure, W_m is generally regarded as the amount of water vapor necessary to saturate the heterogeneous active sorption sites, and C_g and K_{GAB} are dimensionless constants that are related to the thermodynamic measures of sorption for strongly and weakly interacting water.[2,19,20]

Characterization of Formulation Glass Transition Temperature—Glass transition temperatures (T_g) were measured using modulated differential scanning calorimetry (DSC) according to the method described previously.[14] As in that study, the T_g data were fitted to the Gordon−Taylor equation (eq 2), which describes the T_g of two miscible components:

$$T_g = \frac{w_1 T_{g1} + K_{GT} w_2 T_{g2}}{w_1 + K_{GT} w_2} \quad (2)$$

where T_g is the glass transition temperature of the mixture, w_1 and w_2 are the weight fractions of the individual components, T_{g1} and T_{g2} are the intrinsic T_gs of each component, and K_{GT} is a constant of the system describing the true density of the materials and changes in the thermal expansivity.[21,22] In applying the Gordon−Taylor equation, we treated the ternary system PVP/glycerol/water as a binary system, with PVP/glycerol and water as the two components. This simplification is justified because the relative proportions of PVP and glycerol remained unchanged as water content varied in our studies. In fitting the data, we used the experimentally measured values for T_g, w_1 and w_2, and set the T_{g1} value for water to the reported value of 135 K, as in the previous study.[14] Values for T_{g2} (the glass transition temperature of "dry" PVP/glycerol systems) and K_{GT} were determined by regression.

Stability Study—Stability studies were conducted on 4 mg of pellets stored in glass lyophilization vials; details of the method have been reported previously.[14] Before beginning the stability study, the solid Asn-hexapeptide formulations were allowed to equilibrate in chambers at 20 °C for 12 h. After this initial equilibration period, formulations stored at 75% RH were removed and analyzed for Asn-hexapeptide content and integrity prior to beginning the stability study. No significant degradation products were observed. For the stability study, the samples were transferred to controlled relative humidity chambers in a 50 °C room. Samples were prepared in triplicate. Peptide composition was assayed by HPLC after various storage times to determine degradation kinetics and product distribution. At specified time intervals, triplicate samples were removed for peptide analysis, as described previously.[14] Observed rate constants (k_{obs}) for the disappearance of the parent Asn-hexapeptide were determined from the slopes of plots of ln(% peptide remaining) versus time, assuming first-order kinetics as previously observed for this peptide in solution[17] and solid[6,14] states.

Peptide Analysis—The analyses of Asn-hexapeptide and its degradation products were performed by reversed-phase HPLC, using a modification of a method described previously.[14] The HPLC analytical system consisted of a Shimadzu (Shimadzu Corp., California) LC-6A pump, a Shimadzu SPD-6A variable-wavelength UV detector, a Shimadzu CR601 Chromatopac integrator, and a Rheodyne 7161 manual injector outfitted with a 50-μL injection loop. The Asn-hexapeptide and its degradation products were separated on an Alltech Econosphere C-18 reversed-phase column (5 μm resin, 4.6 × 250 mm) by an isocratic method with a mobile phase of 10% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid (TFA) in 40 mM ammonium acetate at a pH of 4.5 and a flow rate of 0.8 mL/min. The detection wavelength was 214 nm. The identification of the degradation products was conducted by co-injection of standards.

Results

Physical Characterization—*Water Sorption Isotherms*—Water sorption isotherms at 50 °C are shown in Figure 1 for PVP formulations with different glycerol contents, and also for liquid glycerol. In all cases, the water content of the formulations increased as relative water vapor pressure increased. The curved lines in Figure 1 represent the nonlinear least-squares fits of the water sorption data to the GAB equation, which adequately described the water

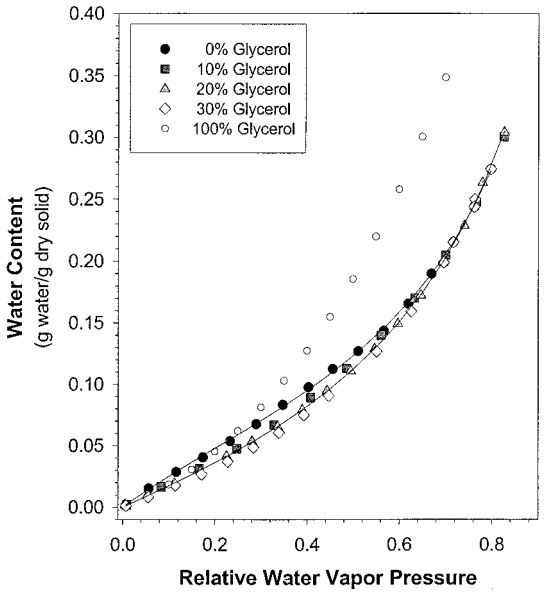


Figure 1—Water sorption isotherms for PVP formulations of various glycerol contents at 50 °C. The curved lines represent nonlinear least-squares fits to the Guggenheim–Anderson–deBoer equation (eq 1) for 0% glycerol–PVP and 20% glycerol–PVP formulations. The 100% glycerol data were taken from the literature.[24]

Table 1—Calculated Parameter Values and Standard Errors from Nonlinear Least Squares Fits of the Water Sorption Data (Figure 1) for PVP–Glycerol Formulations to the GAB Equation (eq 1) at 50 °C

PVP formulation % glycerol	W_m (g/g dry solid)	C_g	K_{GAB}
0	0.101 ± 0.003	3.13 ± 0.17	0.849 ± 0.009
10	0.129 ± 0.005	1.75 ± 0.09	0.809 ± 0.009
20	0.121 ± 0.004	1.65 ± 0.08	0.833 ± 0.008
30	0.129 ± 0.004	1.29 ± 0.05	0.838 ± 0.007

sorption behavior for all formulations. The calculated parameter values (Table 1) in conjunction with the GAB equation were used to describe formulation water content at specific relative humidities.

Figure 1 and the fitted GAB parameters (Table 1) suggest that the water sorption isotherms of the four solid PVP formulations were similar. At three of the four relative humidities studied (0, 11, and 75%), the presence of glycerol did not significantly affect formulation water sorption behavior, because the formulations (0−30% glycerol) have similar water contents. At 30% RH, the three PVP formulations with glycerol had similar water contents but contained 15% less water than the PVP formulation without glycerol. Thus, the maximum difference in water content is ~15%, and will be observed at the intermediate relative humidity (30% RH) for the PVP formulation without glycerol. The 100% glycerol, which was included as a control (freely mobile system), will also exhibit significantly greater water contents than the other formulations, particularly at 30% RH.

Glass Transition Temperature—The addition of plasticizers can decrease the T_g of amorphous polymers.[23] This plasticizing effect of water is evident in Figure 2. Figure 2a shows a representative DSC thermogram, and Figure 2b shows T_g as a function of water content for the different formulations studied. The curved lines in Figure 2b are the nonlinear least-squares fits of the data to the Gordon−Taylor equation, which appears to adequately describe the depression of formulation T_g by the absorbed water. The fitted parameter values are listed in Table 2. The horizontal dotted line in Figure 2 corresponds to the experimental

Table 2—Calculated Parameter Values and Standard Errors from Nonlinear Least Squares Fits of the T_g Data (Figure 2) to the Gordon–Taylor Equation (eq 2)

PVP formulation % glycerol	intrinsic T_g[a] (K)	fitted K_{GT} value
0	428.9 ± 2.9	0.281 ± 0.092
10	368.5 ± 1.4	0.224 ± 0.084
20	336.5 ± 2.0	0.164 ± 0.091
30	311.6 ± 1.3	0.153 ± 0.076

[a] The T_g of the "dry" formulation (water content < 0.004 g/g wet solid).

temperature (50 °C). During these stability studies, formulations with T_g > 50 °C will be in the glassy state, whereas those with T_g < 50 °C will be in the rubbery state. All "dry" PVP−glycerol formulations (water content <0.004 g/g dry solid), except for the one containing 30% glycerol, were in the glassy state at 50 °C.

Glycerol also acted as a plasticizer to lower the T_g of PVP. On a weight basis, water was a stronger plasticizer than glycerol. As shown in Figure 2b, an increase in water content from 0 to 20% (w/w) corresponded to a 120 K decrease in T_g, whereas a comparable change in glycerol content depressed the T_g by only 90 K. As the glycerol content increased, the amount of water required to decrease the formulation T_g to the experimental temperature decreased. With no glycerol added, 0.14 g water/g dry solid was needed to lower the PVP formulation T_g to 50 °C. With the addition of 10% (w/w) glycerol to the PVP formulation, less water (0.05 g water/g wet solid) was needed to achieve the same formulation T_g (50 °C).

By using glycerol to vary T_g, we have obtained PVP formulations with similar T_gs but different water contents. These formulations will allow us to study the effect of water content/water activity on deamidation in formulations with similar T_gs. As shown in Figure 2b, we also have generated PVP formulations with similar water contents or activities but significantly different T_gs. These formulations will help determine the effect of T_g on deamidation at constant water activity and water content. From these two sets of formulations, the dominant role of water in peptide deamidation in solid formulations may be inferred.

Physical Appearance—At 50 °C, the physical appearance of the "dry" PVP formulations (water content < 0.4% w/w) changed as the weight percentage of glycerol increased. Most notable was a decrease in pellet size and a change from a brittle to a soft, sticky texture. Formulations without glycerol were white, dry, powdery pellets. PVP pellets with 10% (w/w) glycerol were similar in appearance to the 0% glycerol formulation, except for a decrease in size. PVP pellets with 20% glycerol were approximately one-third the size of the 0% glycerol PVP pellets and had a shiny, opaque surface and a sticky texture. PVP pellets with 30% glycerol had melted into a sticky, viscous, opaque liquid with a yellowish color.

As the relative humidity and water content increased, the pellet size decreased with a concomitant increase in stickiness. At high relative humidity, the 20 and 30% glycerol formulations became viscous liquids. The changes in the physical appearance of the PVP formulations support the T_g data, which indicate that the formulations are undergoing the transition from a glassy state to a rubbery state with increases in water and glycerol content.

Degradation Profiles—Figures 3a and 3b show the disappearance of the Asn-hexapeptide over time for representative PVP−glycerol formulations at different relative humidities. Peptide degradation exhibited a pseudo-first-order dependence on peptide content in all formulations and at all relative humidities studied. This observation is consistent with previous reports of Asn-hexapeptide dea-

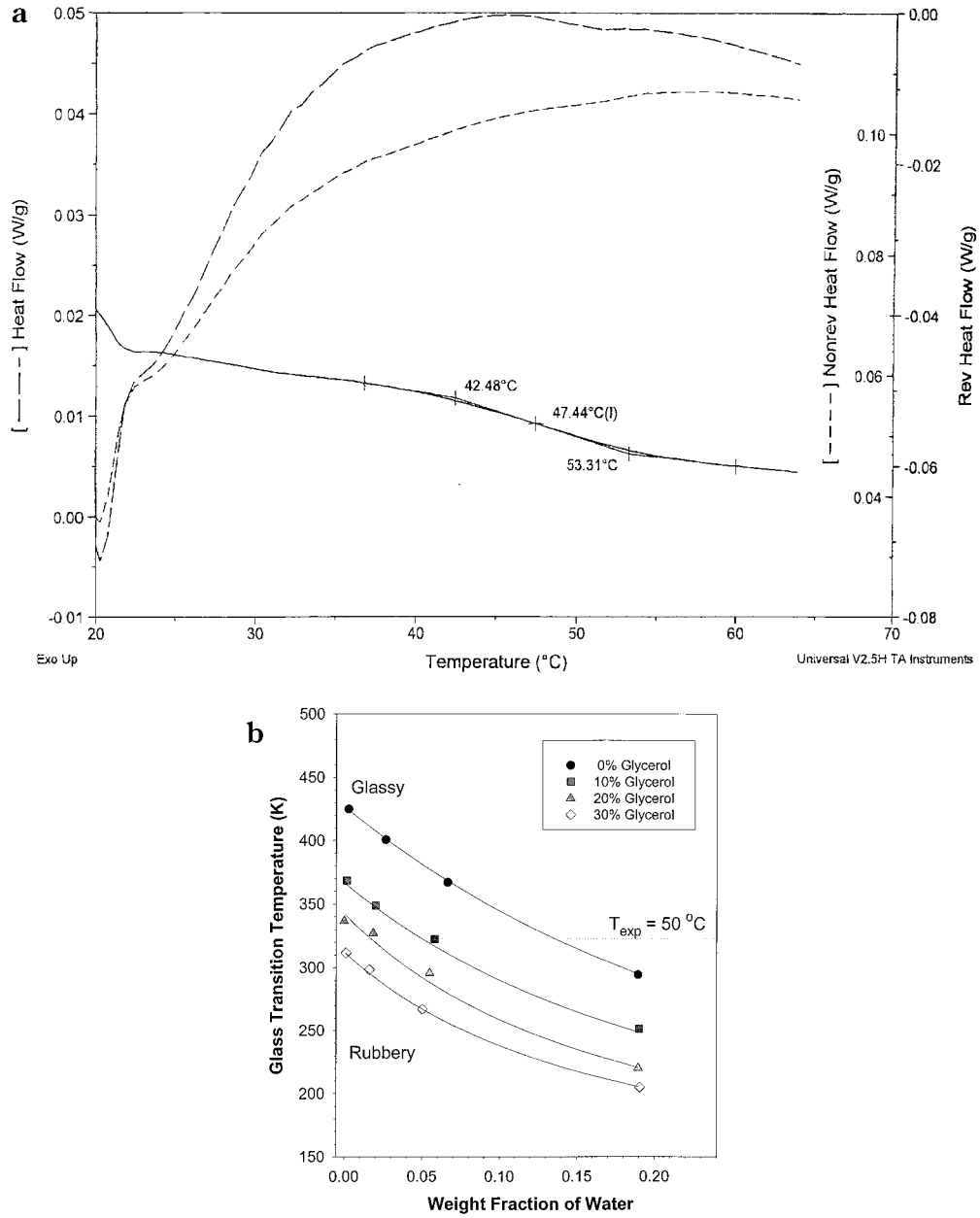


Figure 2—(a) Representative thermogram for a PVP K17 sample (containing 10% glycerol and stored at 30% RH) obtained with a scan rate of 2 °C/min and modulated at ± 1 °C/min. The endothermic T_g at 47.4 °C was calculated using the reversible heat flow curve. (b) Formulation glass transition temperature (T_g) as a function of water content at various glycerol contents ($n = 3$). The dotted line shows the experimental temperature used in the stability studies ($T_{exp} = 50$ °C, 323 K). The curved lines are the nonlinear least-squares fits to the Gordon-Taylor equation (eqn 2).

midation in lyophilized formulations.[6] The reaction order was not affected by glycerol or water. The apparent first-order deamidation rates (k_{obs}) were determined from the slopes of the lines shown in the figure, as described previously.[14]

Deamidation Kinetics—*Effect of Water Content and Activity*—Figure 4 shows the effect of water content on the decomposition of Asn-hexapeptide in lyophilized PVP with different glycerol contents. Because the PVP formulations had similar water sorption behavior (Figure 1), water activity affected deamidation rates in a manner similar to that observed for water content (data not shown). An increase in either water or glycerol content increased the rate of Asn-hexapeptide deamidation (Figure 4). The data suggest that at higher water or glycerol content, increasing the amount of either plasticizer did not affect rate as significantly as at lower levels. Increasing the weight fraction of water from 0 to 1 increased the rate of Asn-hexapeptide deamidation by 3 orders of magnitude in 0% glycerol formulations, by 2 orders of magnitude in PVP formulations with 30% glycerol, and by only 1 order of magnitude in 100% glycerol–0% PVP formulations.

The relatively rapid deamidation in glycerol when little water is present suggests that deamidation may not require water to proceed when the solvent environment allows for sufficient reactant mobility. Deamidation in glycerol was unaffected by increases in water content from <0.006 to 0.09 g/g wet glycerol. In contrast, much greater increases in Asn-hexapeptide reactivity were observed in polymer-containing formulations over the same region.

Effect of Glass Transition Temperature—Water can act as a plasticizer to decrease formulation T_g, with a corresponding decrease in viscosity and increase in molecular mobility.[9] This increase in molecular mobility may be the mechanism by which water promotes deamidation. By correlating deamidation kinetics to formulation T_g at constant water content/activity, we can determine the plasticizing effect of water on deamidation. Figure 5 shows

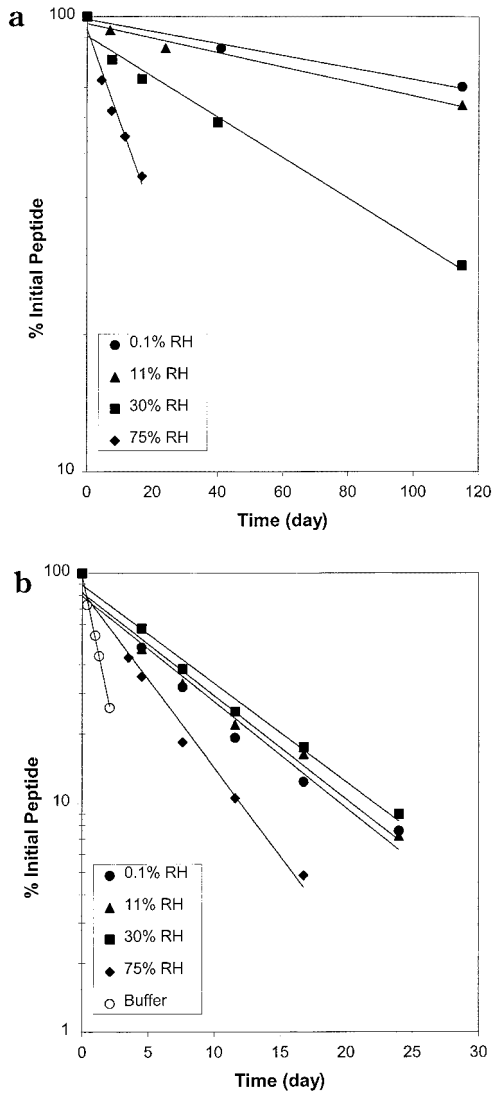


Figure 3—Disappearance of the Asn-hexapeptide as a function of time at 50 °C in (a) 10% glycerol–PVP formulation and (b) 100% glycerol at various relative humidities ($n = 3$).

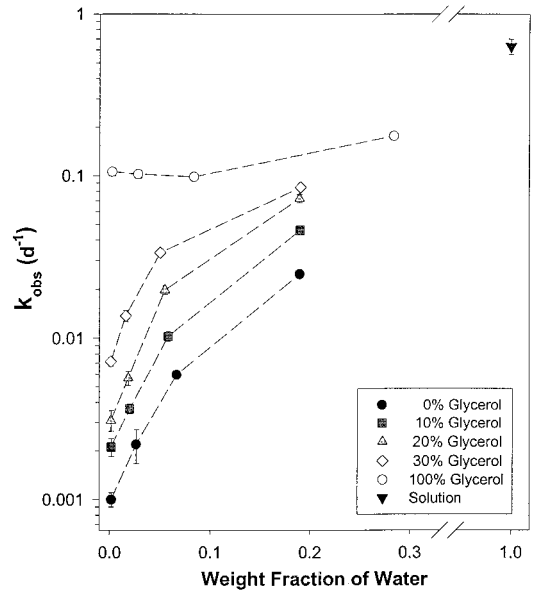


Figure 4—Observed rate constant (k_{obs}) of Asn-hexapeptide degradation at 50 °C as a function of water content in various PVP–glycerol formulations ($n = 3$). Lines have been added to clarify trends and do not represent regression.

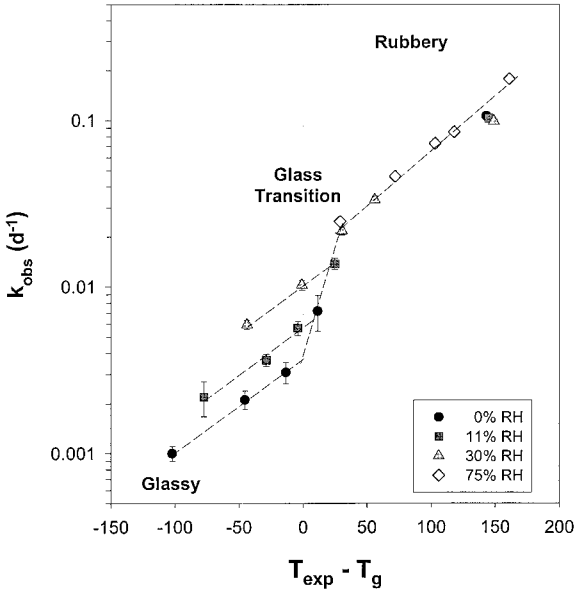


Figure 5—Observed rate constant (k_{obs}) of Asn-hexapeptide degradation as a function of T_g at $T_{exp} = 50$ °C at different relative humidities ($n = 3$). Water content (in g/g wet solid) at 0% RH is <0.004, at 11% RH is 0.02, at 30% RH is 0.06, and at 75% RH is 0.19. At each relative humidity, formulations with increasing ($T_{exp} - T_g$) (i.e., with lower T_g) were generated by adding increasing amounts of glycerol. The dashed lines denote trends in the data and do not represent curve fits to any equation.

the relationship between deamidation kinetics and T_g at different relative humidities for Asn-hexapeptide in 0−30% glycerol−PVP formulations and 100% glycerol.

When water activity was held constant, the rate of deamidation increased with decreasing T_g. For formulations in the rubbery state ($T_{exp} - T_g > 0$), the rate of deamidation appeared to be log linearly related to ($T_{exp} - T_g$), with no observed deviations for formulations with different water activities. This result suggests that the destabilizing effect of water in these formulations may be due to its role as a plasticizer. In the glassy state ($T_{exp} - T_g < 0$), decreases in T_g at constant water activity again resulted in increased deamidation rates. However, increasing water activity or content in formulations with similar T_gs also increased the rate of deamidation, suggesting that the role of water in the glassy state extends beyond that of a plasticizer. These results suggest that water may also facilitate deamidation through its role as a medium or reactant, especially in glassy PVP formulations.

Degradation Products and Their Distribution—In this study, the major degradation products observed for the deamidation of the Asn-hexapeptide in PVP solid formulations were the cyclic imide-hexapeptide (Asu), the isoAsp-hexapeptide (isoAsp), and the Asp-hexapeptide (Asp). In previous studies, these peptides have also been observed to be the products of Asn-hexapeptide deamidation in solution[16,17] and in the solid state.[6] The ratio of isoAsp to Asp was ~3, a value similar to that observed in solution at neutral pH.[16,17]

Figure 6 shows the effect of water on deamidation product distribution for the reaction in 100% glycerol. In this figure, the "% total degradation products" was calculated on the basis of the total area under the chromatographic peaks for the Asn-hexapeptide and its degradation products at each time point. Under low moisture conditions, the dominant degradation product is the cyclic imide-hexapeptide. As water content increases, the product distribution shifts toward increased formation of the isoAsp- and Asp-hexapeptides, with a corresponding de-

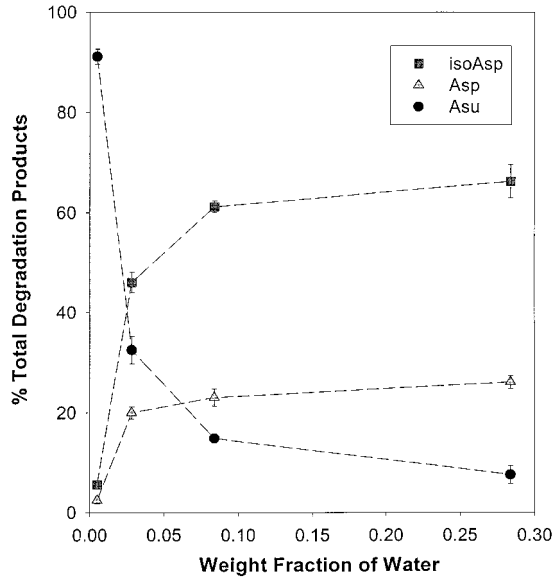


Figure 6—The Asn-hexapeptide degradation product distribution as a function of water content in 100% glycerol after 16 days at 50 °C ($n = 3$). The "% total degradation products" was determined on the basis of the total HPLC peak area at each time point.

crease in the cyclic imide. At higher water contents, isoAsp and Asp are the major degradation products with Asu present in minor quantities. These shifts in product distribution with increasing water content/activity were observed for all formulations (representative plots shown in Figures 7a and 7b, with "% total area" determined as in Figure 6).

When little or no water was present (<0.4% w/w), increasing glycerol content (and therefore decreasing T_g) did not noticeably affect product distribution. However, Figures 6, 7a, and 7b show that formulation mobility may affect the manner in which product distributions shift toward higher levels of isoAsp and Asp, with a reduction in the presence of the cyclic imide as water content increases. For example, at a water content of 0.03 g/g wet solid (water activity = 0.11), the fraction of Asu in the degradation products was lower in PVP formulations with higher glycerol contents. A higher water content was required to obtain the same product distribution in formulations with lower glycerol content and, thus, lower matrix mobility. Because of similar water activities for formulations of similar water content, water activity affected the product distribution (data not shown) in a manner similar to that observed for water content (Figure 6).

Discussion

Residual moisture can decrease the long-term stability of lyophilized protein formulations by promoting chemical degradation reactions such as asparagine deamidation. Numerous mechanistic interpretations for the destabilizing effect of water have been suggested based on relationships between chemical reactivity and formulation parameters such as water content, water activity, and T_g. However, the unambiguous interpretation of experimental results often is difficult because these parameters are interdependent and are affected by temperature.[13,14] To avoid this difficulty, we used glycerol as a plasticizer to change the T_g of the PVP formulation without significantly affecting water content or water activity. In this way, the effects of water content/activity and T_g on the deamidation of the Asn-hexapeptide can be determined independently in solid PVP formulations without a change in temperature.

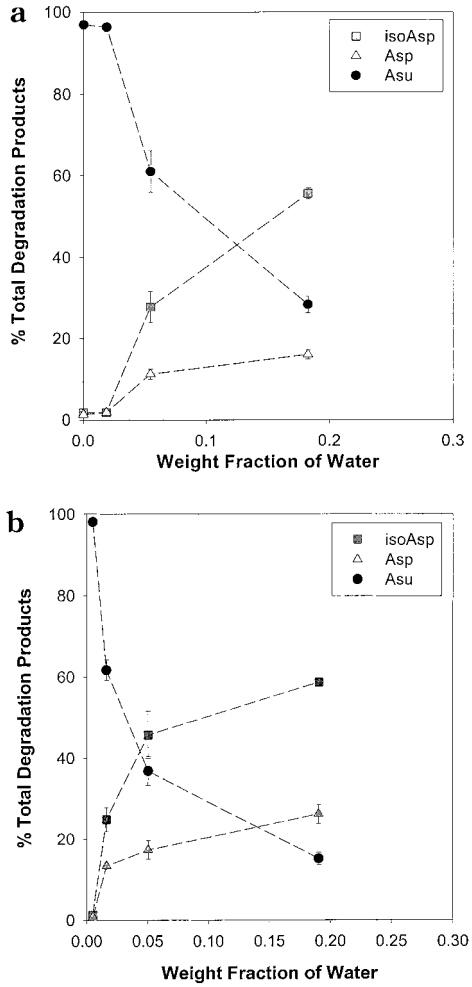
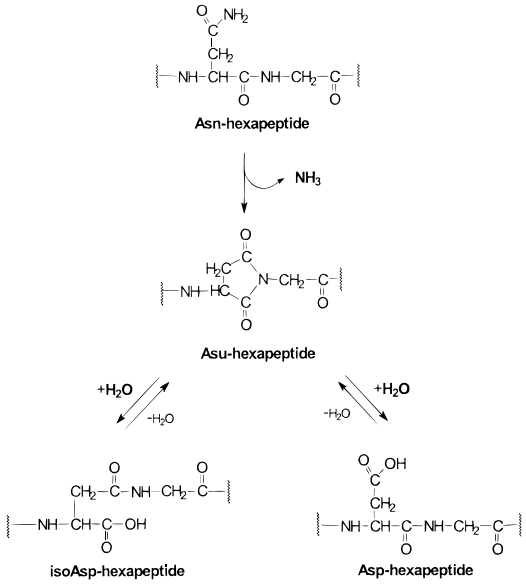


Figure 7—The Asn-hexapeptide degradation product distribution for PVP formulations with (a) 10% and (b) 30% glycerol contents as a function of water content ($n = 3$). The "% total degradation products" was determined on the basis of the total HPLC peak area at each time point.

Mechanism of Deamidation—The major degradation products of Asn-hexapeptide deamidation in PVP solid formulations are the cyclic imide hexapeptide (Asu), the isoAsp-hexapeptide (isoAsp), and the Asp-hexapeptide (Asp). These degradation products are identical to those observed for deamidation in solution, suggesting that the mechanism of deamidation in these polymer solids may be similar to that observed in solution. In solution at neutral pH, the deamidation of the Asn-hexapeptide occurs via intramolecular cyclization, which results from the nucleophilic attack of the succeeding peptide nitrogen onto the side-chain carbonyl of the Asn residue to form the cyclic imide (Scheme 1).[17] The cyclic imide hexapeptide is then rapidly hydrolyzed to form the isoAsp- and Asp-hexapeptides.[16,17] The observed ratio of isoAsp to Asp of ~3 in these solid formulations is also similar to the ratio observed for Asn-hexapeptide deamidation in solution and for the hydrolysis of the cyclic imide.[16,17] In solution, isoAsp is formed only from the hydrolysis of Asu.[16] The presence of this product in these solid formulations suggests its formation from Asu hydrolysis here as well. Taken together, these results support the hypothesis that the deamidation of the Asn-hexapeptide in these PVP–glycerol formulations proceeds via intramolecular cyclization to form the cyclic imide intermediate (Scheme 1), a mechanism similar to that observed in solution.

Other researchers have made similar observations. Oliyai et al. observed that Asn-hexapeptide degradation in lyophilized formulations containing either mannitol or



Scheme 1. —Solution-state mechanism for the deamidation of the Asn-hexapeptide at neutral pH (Adapted from Patel et al.[16]).

lactose yielded isoAsp, Asp, and Asu.[6] They concluded that the solid-state deamidation mechanism was similar to that in the solution state. Strickley and Anderson showed that the deamidation of insulin in the solid state was similar to that in the solution state in terms of degradation products, mechanism, and pH−rate profile.[5] The interpretation that the mechanism of Asn-hexapeptide deamidation in these solid systems is via intramolecular cyclization to form a five-membered cyclic imide hexapeptide is consistent with the results of this study and others.

Effect of Water on Deamidation Kinetics—Residual water appears to facilitate deamidation in lyophilized PVP formulations both by enhancing molecular mobility and by medium effects. Figures 4 and 5 show that increased mobility (decreasing T_g) and increased solvation (increased water content/activity) had a destabilizing effect on the Asn-hexapeptide in PVP. When water activity was held constant, the rate of deamidation increased with decreasing T_g, indicating that matrix mobility may be important in determining deamidation kinetics. Notably, water does not appear to act as reactant in the initial loss of the Asn-hexapeptide to form the cyclic imide. The rapid rate of deamidation in 100% glycerol at 0% RH suggests that deamidation can occur when very little water is present (<0.4% w/w). Furthermore, increases in water activity of up to 0.3 in glycerol did not affect the deamidation rate. If water was a reactant, deamidation would not be able to proceed in its absence, and the rate would be expected to vary with water content/activity. Therefore, it is likely that water is not directly affecting deamidation as a reactant. This explanation is also consistent with the deamidation mechanism determined in solution, in which water does not participate directly in the formation of the cyclic imide (Scheme 1).

Many researchers have proposed that increased dynamic mobility in solids leads to increased chemical reactivity.[1,2,7,8] The data in Figure 5 support this hypothesis. That the plasticizing role of water facilitates deamidation in lyophilized PVP formulations by increasing mobility is also consistent with the apparent mechanism of Asn-hexapeptide deamidation. In these polymer formulations, the deamidation of Asn-hexapeptide appears to proceed via intramolecular cyclization to form the cyclic imide hexapeptide. For cyclization to occur, the asparagine side chain and the peptide backbone require sufficient flexibility to assume the correct local conformation. Ota et al. have shown that decreased segmental flexibility decreases the deamidation rate in solution.[24] Because cyclization requires adequate segmental flexibility, deamidation would be expected to be sensitive to changes in formulation molecular mobility.

This explanation is supported by the trends in Figure 5. We observed a sharp increase in deamidation rate with decreasing T_g in the region of the glass transition ($T_g \sim T_{exp}$). This increase in deamidation rate appears to correspond with the sharp decrease in viscosity characteristic of the glass transition. Viscosity can decrease by as much as 4 orders of magnitude at the glass transition, leading to a large increase in matrix mobility.[8,10] The roughly 1 order of magnitude increase in deamidation rate shown in Figure 5 is significantly less than this potential viscosity decrease, which may suggest that deamidation and/or side-chain mobility is not completely coupled to overall matrix viscosity.

Figure 5 shows that the effect of water as a plasticizer and/or solvent on deamidation appears to depend on the physical state of the formulation. In the rubbery state, the strong correlation between reaction rate and T_g, regardless of water activity/content, suggests that water affects deamidation predominantly through its role as a plasticizer. In other words, water or its direct effect on deamidation is not the rate-limiting factor. However, in glassy formulations, water appears to affect deamidation via both mobility and medium/solvent effects. The mobility mechanism is supported by the increase in deamidation rates with decreasing T_g when water activity/content was held constant. In contrast, in formulations with similar T_g, increasing water content/activity increases Asn-hexapeptide reactivity, suggesting that water may also facilitate deamidation in the glassy state through its role as a solvent. If matrix mobility was the only factor dictating Asn-hexapeptide deamidation, then differences in water activity or content would not have affected degradation rates at constant T_g. Alternatively, even in a glassy matrix, the peptide has some degree of mobility. Peptide mobility thus may be influenced by increases in water content in a manner not coupled to matrix mobility.

Interestingly, the relationship between k_{obs} and T_g shown in Figure 5 is linear in both the rubbery and glassy states, with a slightly greater slope in the rubbery region. This relationship is reminiscent of so-called "cooling curves", which show the transition from a liquid to a glassy solid in terms of specific volume (or enthalpy) as a function of temperature, and display families of near parallel curves in the glassy state for materials cooled at different rates.[25] Although this similarity suggests that deamidation rate may be related to matrix specific volume in our studies, this claim must be regarded as speculative on the basis of the current data.

Because of the low amounts present, water technically is not a solvent in these solid systems. However, it is not unreasonable to postulate that water may affect the reaction environment in a manner that facilitates deamidation. The environment to which an asparagine residue is exposed can greatly affect its stability. Studies of deamidation in the solution state reveal that the crucial step for cyclic imide formation is deprotonation of the attacking peptide bond nitrogen to form a charged activated complex in the reaction transition state.[16−18] Brennan and Clarke have shown that deamidation at asparagine residues is markedly reduced in solvents of low dielectric strength because of decreased stability of the anionic peptide bond nitrogen.[26] Deamidation would be expected to be favored in polar environments, which can adequately stabilize the charged transition state during intermolecular cyclization. Although these findings are for the solution

state, water may affect deamidation in glassy formulations by increasing the polarity of the matrix. Water may also facilitate deamidation by serving as a medium for proton transfer during the cyclization process.[5,16]

In addition to having an effect on reactant mobility, matrix mobility may also affect the ability of a solvent to adequately solvate and stabilize charged reaction centers. In the glassy state, the solid polymer solvent may not have sufficient mobility to rearrange itself to "solvate" the charged transition state during Asu formation. Although the polymer may be dynamically constrained, Oksanen and Zografi have shown that water in solid PVP maintains a high degree of mobility relative to the polymer.[27] The more mobile water may be able to facilitate deamidation by "solvating" the charged transition state during Asu formation. In the rubbery state, PVP is more mobile than in the glassy state. With greater mobility, PVP may be more able to solvate and stabilize the development of a charged transition state during deamidation. Thus, matrix mobility may have an impact on chemical reactions beyond influencing reactant mobility.

Because water appears to have a solvent effect on deamidation in these solid systems, glycerol may have had a solvent effect on the reaction rates in addition to a plasticizing effect. We cannot rule out the possibility that the increase in deamidation rates with decreasing T_g (increasing glycerol content) is in part due to a solvent effect (Figure 5).

Effect of Water on Deamidation Product Distribution—Three major degradation products were observed in this study: the cyclic imide hexapeptide (Asu), the isoAsp-hexapeptide (isoAsp), and the Asp-hexapeptide (Asp). In these lyophilized PVP–glycerol systems, the Asn-hexapeptide appears to deamidate through intramolecular cyclization to form Asu, which may degrade to produce isoAsp and Asp. The deamidation product distribution would then depend on Asu formation (deamidation of Asn) and breakdown (hydrolysis to form isoAsp and Asp). In formulations with minimal moisture content (<0.004 g water/g wet solid), Asu is the dominant degradation product with little isoAsp or Asp observed at the sampling time evaluated. As water content increases, the product distribution shifts toward less Asu and greater fractions of isoAsp and Asp. These observations are consistent with the hydrolytic formation of isoAsp and Asp from Asu, as observed in solution (Scheme 1). In solution at neutral pH, Asu undergoes spontaneous hydrolysis to form isoAsp and Asp, where the attack of water or hydroxide ion on the cyclic imide is the rate-limiting step.[16,17,28]

Because the amount of Asu observed depends on the rates of Asn-hexapeptide deamidation and Asu hydrolysis, the decrease in the percentage of Asu among the degradation products with increasing water content suggests that Asu hydrolysis becomes more rapid than Asu formation under these conditions. The shift in product distribution with increasing glycerol content (Figures 6, 7a, and 7b) suggests that mobility may also affect Asu hydrolysis through its role as a plasticizer to increase formulation mobility, although direct evidence for a plasticizing role in this reaction was not obtained in these experiments. These differences in product distribution may be due to differences in the reaction time course. For example, reactions in formulations with higher glycerol contents occur at a faster rate. Therefore, these reactions would be more complete than slower reactions (lower glycerol content) at the time the reactions were sampled.

Conclusion

The mechanistic role of water in the deamidation of an Asn-containing model hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala) in lyophilized formulations containing PVP and glycerol was investigated. Increases in moisture and glycerol contents increased the rate of peptide deamidation. This increase was strongly correlated with T_g at constant water content and activity, suggesting that increased matrix mobility facilitates deamidation. In rubbery systems ($T > T_g$), deamidation rates appeared to be independent of water content and activity in formulations with similar T_gs. However, in glassy formulations with similar T_gs, deamidation increased with water content, suggesting a solvent/medium effect of water on reactivity in this regime. An increase in water content also affected the degradation product distribution; less of the cyclic imide intermediate (Asu) and more of the hydrolytic products, isoAsp and Asp, were observed as water content increased. Under low moisture conditions, the water-catalyzed hydrolysis of the cyclic imide intermediate to produce the isoAsp and Asp is suppressed. Thus, residual water appears to facilitate deamidation in these solid PVP formulations both by enhancing molecular mobility and by solvent/medium effects, and also participates as a chemical reactant in the subsequent breakdown of the cyclic imide.

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References and Notes

1. Carpenter, J. F.; Pikal, M. J.; Chang, B. S.; Randolph, T. W. Rational design of stable lyophilized protein formulations: Some practical advice. *Pharm. Res.* **1997**, *14*, 969–975.
2. Hageman, M. J. Water sorption and solid-state stability of proteins, In *Stability of Protein Pharmaceuticals, Part A*: *Chemical and Physical Pathways of Protein Degradation*; Ahern, T. J.; Manning, M. C. Eds.; Plenum: New York, 1992; pp 273–309.
3. Pikal, M. J.; Dellerman, K. M.; Roy, M. L.; Riggin, R. M. The effect of formulation variables on the stability of freeze-dried human growth hormone. *Pharm. Res.* **1991**, *8*, 427- 436.
4. Constantino, H. R.; Langer, R.; Klibanov, A. M. Moisture-induced aggregation of lyophilized insulin. *Pharm. Res.* **1994**, *11*, 21–29.
5. Strickley, R. G.; Anderson, B. D. Solid-state stability of human insulin. I. mechanism and the effect of water on the kinetics of degradation in lyophiles from pH 2–5 solutions. *Pharm. Res.* **1996**, *13*, 1142–1153.
6. Oliyai, C.; Patel, J.; Carr, L.; Borchardt, R. T. Solid-state stability of lyophilized formulations of an asparaginyl residue in a model hexapeptide. *J. Parenteral Sci. Technol.* **1994**, *48*, 167–173.
7. Shalaev, E. Y.; Zografi, G. How does residual water affect the solid state degradation of drugs in the amorphous state? *J. Pharm. Sci.* **1996**, *85*, 1137–1141.
8. Levine, H.; Slade, L. The glassy state phenomenon in food molecules. In *The Glassy State in Foods*; Blanshard, J. M. V.; Lillford, P. J., Eds.; Nottingham: Nottingham, U.K. 1993; pp 35–101.
9. Ferry, J. D. *Viscoelastic Properties of Polymers*, 3rd ed.; Wiley & Sons: New York, 1980; pp 486-544.
10. Angell, C. A. Formation of glasses from liquids and biopolymers. *Science* **1995**, *267*, 1924–1935.
11. Williams, M. L.; Landel, R. F.; Ferry, J. D. The temperature dependence of relaxation mechanisms in amorphous polymers and other glass-forming liquids. *J. Am. Chem. Soc.* **1955**, *77*, 3701–3707.
12. Hancock, B. C.; Zografi, G. Characteristics and significance of the amorphous state in pharmaceutical systems. *J. Pharm. Sci.* **1997**, 86, 1–12.
13. Bell, L. N.; Hageman, M. J. Differentiating between the effects of water activity and glass transition dependent mobility on a solid-state chemical reaction: Aspartame degradation. *J. Agric. Food Chem.* **1994**, *42*, 2398–2401.

14. Lai, M. C.; Hageman, M. J.; Schowen, R. L.; Borchardt, R. T.; Topp, E. M. Chemical stability of peptides in polymers. 1. Effect of water on peptide deamidation in poly(vinyl alcohol) and poly(vinylpyrrolidone) matrixes. *J. Pharm. Sci.* **1999**, *88*, 1073−1080.
15. Manning, M. C.; Patel, K.; Borchardt, R. T. Stability of protein pharmaceuticals. *Pharm. Res.* **1989**, *6*, 903−917.
16. Geiger, T.; Clarke, S. Deamidation, isomerization and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. *J. Biol. Chem.* **1987**, *262*, 785−794.
17. Patel, K.; Borchardt, R. T. Chemical pathways of peptide degradation. II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. *Pharm. Res.* **1990**, *7*, 703−711.
18. Capasso, S.; Mazzrella, L.; Sic, F.; Zagari, A.; Salvadori, S. Kinetics and mechanism of succinimide ring formation in the deamidation process of asparagine residues. *J. Chem. Soc., Perkin Trans. 2* **1993**, *2*, 679−682.
19. Guggenheim, E. A. *Applications of Statistical Mechanics*; Clarendon: Oxford, 1966.
20. van den Berg, C.; Bruin, S. Water activity and its estimation in food systems: Theoretical aspects. In *Water Activity: Influences of Food Quality*; Rockland, L. B.; Stewart, G. F., Eds.; Academic: New York, 1981; pp 1−61.
21. Gordon, M.; Taylor, J. S. Ideal copolymers and the second-order transitions of synthetic rubbers. I. Noncrystalline copolymers. *J. Appl. Chem.* **1952**, *2*, 493−500.
22. Hancock, B. C.; Zografi, G. The relationship between the glass transition temperature and the water content of amorphous pharmaceutical solids. *Pharm. Res.* **1994**, *11*, 471−477.
23. Sperling, L. H. *Introduction to Physical Polymer Science*; John Wiley & Sons: New York, 1986; p 278.
24. Ota, I. M.; Clarke, S. Calcium affects the spontaneous degradation of aspartyl/asparaginyl residues in calmodulin. *Biochemistry* **1989**, *28*, 4020−4027.
25. Craig, D. Q. M.; Royall, P. G.; Kett, V. L.; Hopton, M. L. The relevance of the amorphous state to pharmaceutical dosage forms: glassy drugs and freeze-dried systems. *Int. J. Pharm.* **1999**, *179*, 179−207.
26. Brennan, T. V.; Clarke, S. Spontaneous degradation of polypeptides at aspartyl and asparaginyl residues: Effect of the solvent dielectric. *Protein Sci.* **1993**, *2*, 331−338.
27. Oksanen, C. A.; Zografi, G. Molecular mobility in mixtures of absorbed water and solid poly(vinylpyrrolidone). *Pharm. Res.* **1993**, *10*, 791−799.
28. Xie, M.; Vander Velde, D.; Morton, M.; Borchardt, R. T.; Schowen, R. L pH-Induced change in the rate-determining step for the hydrolysis of the Asp/Asn-derived cyclic-imide intermediate in protein degradation. *J. Am. Chem. Soc.* **1996**, *118*, 8955−8956.

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