Effect of 'pH' on the Rate of Asparagine Deamidation in Polymeric Formulations: 'pH'–Rate Profile

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ABSTRACT: The rate of Asn deamidation of a model hexapeptide (L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala) was measured as a function of effective pH ('pH') in glassy and rubbery polymeric solids containing poly(vinyl pyrrolidone) (PVP) and in solution controls at 70°C. The reaction exhibited pseudo-first-order kinetics in all samples over a wide 'pH' range ($0.5 < \text{'pH'} < 12$); the formation of similar products suggests that the reaction mechanism is unaffected by matrix type. Rates of deamidation were comparable for the polymeric and solution samples in the acidic range ('pH' < 4). Solution-state rates were faster than those in polymeric solids at neutral 'pH' ($6 < \text{'pH'} < 8$), increasing to a > 10,000-fold difference in the basic range ('pH' > 8). Specific base catalysis was observed in solution and in the polymeric solids under neutral conditions ($6 < \text{'pH'} < 8$). In solution, the reaction exhibited general base catalysis for 'pH' > 8, whereas the reaction was 'pH'-independent in the polymeric solids in this range. The 'pH'–rate profile and supporting buffer catalysis data are consistent with a change in the rate-determining step in the basic range from 'pH'-dependent attack of the deprotonated backbone amide nitrogen on the Asn side chain in solution to 'pH'-independent ammonia expulsion in the polymeric solids. The results suggest that polymer matrix incorporation not only affects the magnitude of the deamidation rate constant but also the 'pH' dependency of the reaction and the rate-determining step in the basic 'pH' range. J Pharm Sci 90:141–156, 2001

Keywords: peptide stability; asparagine deamidation; solid state; polymer; poly(vinyl pyrrolidone); effective 'pH'

INTRODUCTION

Therapeutic peptides and proteins are often formulated in the solid state to prolong shelf life or to provide a platform for controlled release. Although many protein degradation reactions are appreciably slower in the solid state than in solution, reactivity is often sufficient to compromise drug potency. An understanding of the mechanisms of peptide and protein degradation reactions in the solid state and of the physical and chemical factors affecting their rate is therefore central to the rational formulation of these promising new drugs.

A number of physicochemical factors have been shown to influence protein degradation reactions in solids. These include temperature, pressure, the mobility of reactant or product species in the solid matrix, moisture content, the concentrations of various excipients, and the effective pH.[1,2] The studies presented here address the role of effective pH in determining the rate and mechanism of asparagine (Asn) deamidation for a model peptide in solid polymeric matrices. Because this reaction is known to be sensitive to pH in solution,[3–6]it is reasonable to expect a dependence on hydrogen ion activity in the solid state as well.

Assessing the role of effective pH in solid-state reactions is complicated by the fact that pH

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technically is defined only in aqueous solution. Although there have been numerous attempts to provide a comparable measure of hydrogen ion activity in solids, to date there is no single, generally accepted method. Methods that have been proposed for the measurement of hydrogen ion activity in solids include electron paramagnetic resonance (EPR),[7] confocal fluorescence microscopy,[8] Fourier transform infrared (FTIR)[9] or diffuse reflectance FTIR[10] spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy.[11] These methods generally employ a pH-sensitive probe molecule (e.g., a pH-sensitive spin probe[7] or fluorescent dye[8]) or isotopically labeled pH-sensitive functional group (e.g., ^{15}N-histidine[11]). An alternative approach adopted in many stability studies defines the "effective pH" of the solid as the pH measured in aqueous solution prior to lyophilization or following reconstitution of the solid.[12–14] The effective pH measured in this way is often termed the 'pH', where the quotation marks indicate an effective value.[12]

The recent literature provides several examples of 'pH'-dependent solid-state degradation reactions of peptides and proteins. Oliyai et al. have demonstrated that the rate of deamidation of lyophilized formulations of the hexapeptide L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala increases with 'pH' in the range 5–8.[15] This group also observed 'pH'-dependent isomerization of the lyophilized Asp-containing analog L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala.[14] Several groups have reported a 'pH' dependence for the degradation of lyophilized insulin, with degradation reactions involving covalent dimerization, cyclic anhydride formation, and aggregation.[12,13] Pikal et al.[16] have reported 'pH'-dependent aggregate formation in lyophilized human growth hormone, and Townsend and DeLuca[17] have observed a nonlinear 'pH'-dependent loss of RNase activity. These studies establish the importance of 'pH' in a variety of solid-state protein degradation reactions, but are limited in that the number of 'pH' values studied is often small and in a fairly narrow range. This limitation prevents effective analysis of the 'pH'–rate profile, which in solution-state reactions has provided valuable information on reaction mechanisms.[4,18] Similarly, although the use of large therapeutic proteins is of practical interest, these complex molecules are often subject to multiple simultaneous degradation reactions, which again complicates the mechanistic interpretation of 'pH' effects.

The studies reported here address the effect of 'pH' on the rate of Asn-deamidation in the hexapeptide L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala incorporated into lyophilized powders containing poly(vinyl pyrrolidone) (PVP). This peptide was selected because deamidation is its sole degradation reaction both in solution and in the solid state,[4,15] and because the kinetics and mechanisms of the reaction have been thoroughly characterized in solution, as shown in Figure 1.[4] In addition, the absence of higher-order structure common to larger proteins permits a focus on chemical rather than physical degradation. PVP, a linear vinyl polymer with a single pendant functional group, has been selected as a polymeric excipient and serves as a relatively inert amorphous matrix in which the peptide is embedded. Previous studies in our laboratories have established that lyophilized PVP can be plasticized with glycerol and/or sorbed water so that both glassy and rubbery solids can be produced in the temperature range of interest.[19,20] The 'pH' dependence of the deamidation reaction in glassy and rubbery solids and in a PVP-containing solution control are examined here. The results demonstrate that the 'pH'–rate profile is significantly altered in the polymeric media relative to the solution control and provide evidence for a matrix effect on the rate-determining step in basic media.

MATERIALS AND METHODS

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) and L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala (Asp-hexapeptide) was synthesized by Gemini Biotech (Woodlands, TX). The cyclic-imide-containing hexapeptide (Asu-hexapeptide) was isolated and purified from degraded Asn-hexapeptide by preparative high-performance liquid chromatography (HPLC). Poly(vinyl pyrrolidone) (PVP), under the trade name Kollidon K17 (MW = 10, 000), was purchased from BASF Corporation (Parsippany, NJ). Glycerol was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL). The salts and organic solvents used to make buffer solution and mobile phase, respectively, were obtained from Fisher Scientific

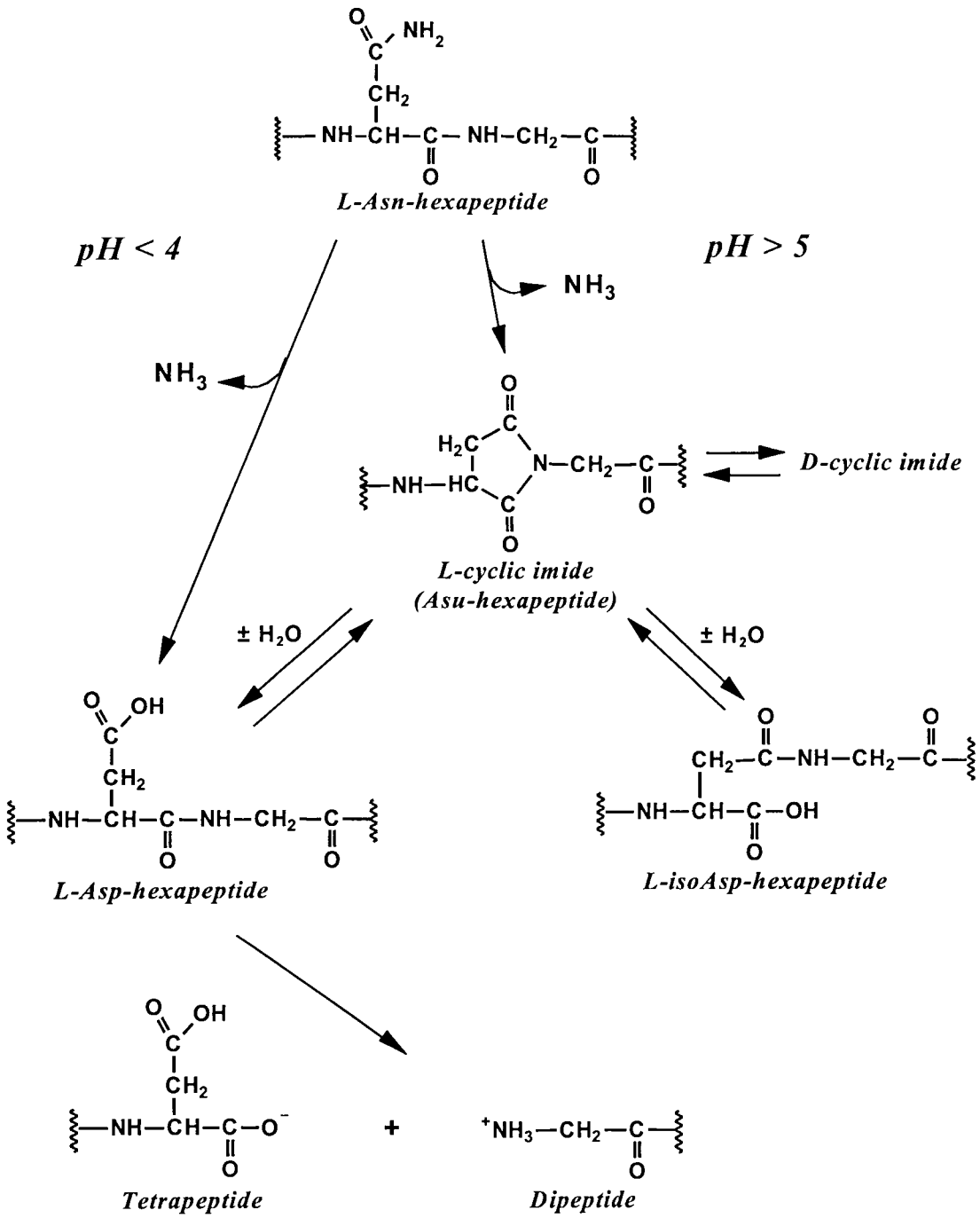


Figure 1. Pathways of L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala deamidation in aqueous solution. At 'pH' > 5, the reaction proceeds via the formation of a cyclic imide, which is subsequently hydrolyzed to form the L-Asp and L-iso-Asp hexapeptides. Racemization to the D-cyclic imide also occurs, which is hydrolyzed to the D-Asp and D-iso-Asp hexapeptides (not shown). At 'pH' < 4, the L-Asp hexapeptide is formed from the L-Asn hexapeptide by direct hydrolysis. The L-Asp hexapeptide ('pH' < 4) is then subject to cleavage of the Asp–Gly peptide bond, producing a tetrapeptide (L-Val-L-Tyr-L-Pro-L-Asp) and a dipeptide (Gly-L-Ala). Adapted from Ref. 4.

(Fair Lawn, NJ). All chemicals were analytical grade or better and used as received. All water used throughout the study was deionized with a Millipore MILLI-Q^{TM} water system.

PVP Purification

Prior to its use in formulations, PVP was dissolved in water and dialyzed for 48 h using Spectra/Pro molecular weight cut-off (MWCO) cellulose membranes (MWCO = 3500 Da; Spectrum Medical Industries, Houston, TX) to remove low MW impurities that may interact with the model hexapeptide. After dialysis, the polymer was freeze-dried for 3 days with a VirTis Freezemobile 5SL (Gardner, NY).

Preparation of Buffer Solutions

Since control of 'pH' in the solid state is crucial in a study of the effects of hydrogen ion activity on rate, preliminary studies were conducted to evaluate and select appropriate buffers. An effective buffer for these accelerated stability studies: (i) must be stable at the storage temperature (70°C); (ii) must not be subject to differential precipitation of salt species during lyophilization, as has been observed for sodium phosphate buffers;[21] (iii) must not contain volatile species (e.g., acetate, HCl), which may be lost during lyophilization and/or storage at elevated temperature; (iv) should show minimal interference with the HPLC assay for the peptide and its degradation products; and (v) should not interact with the peptide or polymer components of the formulation.

Formulations were prepared using a variety of salt species and evaluated for their ability to meet the criteria just outlined. Preliminary studies indicated that *p*-toluene sulfonic acid has a strong ultraviolet (UV) absorbance at 214 nm and co-elutes with the iso-Asp degradation product during HPLC analysis, and so is unsuitable for use in these studies. In a previous study, we noted that Tris buffer [tris-(hydroxy methyl) aminomethane] degrades at 70°C both in solution and in PVP-containing solids to produce formaldehyde, which then forms a covalent adduct with the Tyr residue in this peptide.[22] Tris buffer was omitted for this reason. Solution and solid formulations prepared with trichloroacetic acid showed a dramatic shift in 'pH' during processing and initial storage, from 'pH' 3.1 to 'pH' 10. Therefore, this buffer was also judged to be unsuitable.

As a result of these screening studies, the following salts were selected to maintain 'pH' in various regions of interest. In the very acidic region ('pH' 0.5–1.5), H_2SO_4 was used. Monobasic and dibasic potassium phosphates (KH_2PO_4/K_2HPO_4) in varying ratios were used to maintain 'pH' in the slightly acidic to neutral region ('pH' 3.0–7.0). Similarly, in the slightly basic region ('pH' 7.0–8.0), dibasic and tribasic potassium phosphates (K_2HPO_4/K_3PO_4) were employed. For 'pH' values between 9.0 and 10.5, mixtures of monobasic and dibasic sodium carbonate salts ($NaHCO_3/Na_2CO_3$) were used. Finally, for 'pH' values > 10.5, sodium hydroxide was added. With the exceptions of samples at extreme 'pH' (i.e., containing H_2SO_4 or NaOH) and those used in buffer catalysis studies, all buffer solutions were prepared at a concentration of 0.15 M. The ionic strength of all buffer solutions was maintained constant at 0.5 by the addition of sodium chloride. Buffer pH was measured with a pH-meter (Orion model 410A) equipped with a Ross combination electrode.

Preparation of Formulations in Solution and Solid State

Peptide stability was determined in glassy and rubbery polymeric solids at various 'pH' values, and in polymer-containing solutions. To prepare the formulations, a 5% (w/v) solution of dialyzed PVP was first prepared in one of the buffer solutions described above. The solution was then divided and glycerol added to half the solution (30% w/v); glycerol was used as a plasticizer to produce rubbery polymeric solids. The Asn-hexapeptide was then added to each solution to a concentration of ~0.5 mg/mL. After addition of the peptide, aliquots of each solution were placed in a 70°C oven to serve as solution-state controls in the stability studies. The remaining amounts of the glycerol-containing and glycerol-free solutions were placed into a series of 2-mL glass lyophilization vials, with 100 μL in each vial. The samples were then lyophilized using a VirTis BenchTop lyophilizer (Gardner, NY). The following lyophilization cycle was applied: (i) freezing at a shelf temperature of −35°C for 2 h; (ii) primary drying at a shelf temperature of −35°C for 10 h; and (iii) secondary drying at shelf temperatures of −15, −5, 5, 15, 20, and 25°C, for 10, 8, 6, 6, 12, and 10 h, respectively.

Accelerated Stability Studies

Accelerated stability studies were conducted in a 70°C oven. Solution-state samples were placed in closed containers. Solid samples were placed in open vials in a chamber containing a saturated solution of NaBr to maintain a constant relative humidity of ~50%.[23] Additional, supporting studies were performed at 70 and 95% RH, using saturated NaCl and K_2SO_4 solutions, respectively.[23] At appropriate time intervals, triplicate samples of each solid formulation or a 100-μL aliquot of the solution formulations were removed from the oven for analysis. Because water sorption is very rapid, with 95% of the equilibrium mass reached within 20 min,[19] sampling intervals of <20 min were considered impractical. All samples were either diluted (solutions) or reconstituted (solids) using 1 mL of 0.05 M ammonium acetate buffer solution (pH 4) because the Asn-hexapeptide is maximally stable between pH 3 and 5.[4] The samples were then analyzed by HPLC-UV, using the method described later. The pH values of the solutions and the reconstituted lyophilized solids were measured for each sample to verify that pH did not change during the studies. Stability studies were terminated when the concentration of Asn-hexapeptide was no longer detectable and the concentrations of the major degradation products showed no further change with time.

Sample Analysis

Analysis of the Asn-hexapeptide and its deamidation products was performed by isocratic reversed-phase HPLC with UV detection. Details of the method have been reported previously,[4] although there were minor modifications. Briefly, the method employed an Econosphere C_{18} reversed-phase column (4.6 × 250 mm, 5-μm particle size) from Alltech Associates, Inc. (Deerfield, IL) for separation. The chromatographic system consisted of a Shimadzu SCL-10Avp system controller, a SPD-10Avp UV-vis detector, a SIL-10ADvp autoinjector, and a LC-10ATvp pump. The mobile phase contained 8% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in 0.050 M ammonium acetate at pH 4.6. The flow rate was 0.8 mL/min and detection was at 214 nm. The method provided for separation and quantitation of the Asn-hexapeptide and its major deamidation products, including iso-Asp, D-Asp, L-Asp, the cyclic imide (Asu-hexapeptide), and the tetrapeptide, L-Val-L-Tyr-L-Pro-L-Asp. Approximate elution times of the hexapeptides were iso-Asp-hexapeptide, 8 min; D-Asp-hexapeptide, 10 min; L-Asp-hexapeptide, 10.5 min; L-Asn-hexapeptide, 13 min; and Asu-hexapeptide, 19 min. The tetrapeptide was eluted at ~6 min. The products of some side reactions were also detected and resolved with this method. The limit of detection of this assay is ~0.1 μg/mL for the Asn-hexapeptide.

On repeated injection of samples containing PVP, the retention times of the known compounds tended to decrease, compromising the separation of the iso-Asp- and Asp- hexapeptide degradation products. To regenerate the column, a high organic content mobile phase (50:50 methanol:-water) was flushed through the column after every 50 to 100 injections. Retention times and peak resolution were indistinguishable from those in a new C_{18} column following this procedure.

Previous studies employing this assay assumed that the concentrations of the Asn-, Asp-, and iso-Asp- hexapeptides were proportional to peak area, a reasonable assumption given that the principal contribution to UV absorbance at 214 nm is the peptide bond. Because peptide recovery and mass balance were of interest in the present studies, we determined the UV sensitivities of known compounds involved so that their concentrations could be determined with greater accuracy. Standard calibration curves were constructed by injecting different concentrations of authentic samples of the Asn-, Asp-, and Asu-hexapeptides and of the tetrapeptide; the respective UV absorbances were then determined. Because a synthetic standard for the iso-Asp-hexapeptide was not available, its theoretical sensitivity was determined by mass balance. The results are summarized in Table 1, and were used in calculating peptide concentrations from chromatographic peak areas.

Kinetic Analysis

In liquid and solid formulations, the observed rate constants (k_{obs}) for the disappearance of the Asn-hexapeptide were determined from first-order plots of the fraction of the initial Asn-hexapeptide concentration remaining versus time, according to eq. 1:

$$A = A_o \exp(-k_{obs} t) \quad (1)$$

where A is the amount of Asn-hexapeptide at time t, and A_o is the initial peptide concentration.

Table 1. Assay Sensitivities for Asn-Hexapeptide and Its Major Degradation Products

Parameter	Asn	Asp	Tetra	Asu	iso-Asp[a]
Sensitivity ± SD (peak area/(mg/mL) $\times 10^{-6}$)	7.16 ± 0.13	7.03 ± 0.11	5.94 ± 0.09	8.70 ± 0.20	5.62 ± 0.10

[a] Determined by calculation.

Previous studies have shown that the deamidation of the Asn-hexapeptide exhibits pseudo-first-order kinetics both in solution and in polymeric solids.[4,19,20] Values of the observed rate constants (k_{obs}) were determined by nonlinear regression using the software package Origin (Microcal Software, Inc., MA). In solution studies at pH $\geq$ 9.5, the reaction was too rapid to permit the frequent sampling and temperature re-equilibration necessary to determine k_{obs} values by this method. For these conditions, experiments were carried out at room temperature (22°C) and k_{obs} values at 70°C were estimated using the reported Arrhenius activation energy (*E*a) of 22 kcal/mol for the reaction in solution.[4]

In constructing the 'pH'–rate profile, k_{obs} values were measured at several buffer concentrations and linearly extrapolated to zero buffer concentration to obtain the reported k_o value. Exceptions to this procedure occurred in the very acidic ('pH' < 2) or very basic ('pH' > 10.9) regions in which buffer catalysis studies could not be conducted because strong acids or bases (H_2SO_4 or NaOH) were used to control 'pH'. In these cases, the reported k_o value is equal to the k_{obs} value measured at a single H_2SO_4 or NaOH concentration.

RESULTS AND DISCUSSION

Deamidation Kinetics

Figure 2 shows representative kinetic curves for the deamidation of the Asn-hexapeptide in aqueous solution, and in rubbery and glassy PVP solids at 'pH' 10.4 and 70°C. The lines represent nonlinear regression fits of the data to the pseudo-first-order model given in eq. 1. The data demonstrate apparent-first-order kinetics for the disappearance of the Asn-hexapeptide for all three formulations under these experimental conditions. Similar results were observed for all formulations and 'pH' values studied (data not shown). The data also demonstrate that the reaction rates (k_{obs}) decrease in the order solution > rubbery solid > glassy solid. This ordering of reactivity was observed throughout the neutral to basic 'pH' range, as discussed in detail later.

Products Formed

This section presents information on the products formed during deamidation, with the intent of establishing that the same reaction pathways are operative in solution and in polymeric solids. A complete comparative analysis of the kinetics of product formation and of the corresponding microscopic rate constants in solution and polymeric solids will be presented in a subsequent report.

Under acidic conditions ('pH' < 3), the deamidation products observed in the solution controls were those previously observed in acidic solution,[4] with the Asp-hexapeptide as the major initial degradation product, and the tetrapeptide (L-Val-L-Tyr-L-Pro-L-Asp) dominating at later times. Although the Asu- and iso-Asp-hexapeptides were also detected in these samples, they were present in smaller amounts, a finding consistent with direct hydrolysis as the dominant pathway of deamidation.[3,4,6] In polymeric solids at acidic 'pH', the Asu-hexapeptide was the major initial degradation product, whereas the tetrapeptide and iso-Asp-hexapeptides were the major products at later times. The Asp-hexapeptide was also detected in the acidic polymeric solids throughout the study, but in lesser amounts. These results suggest that the dominant pathway for deamidation at acidic 'pH' may shift from direct hydrolysis in solution to a combination of direct hydrolysis and cyclic imide formation in polymeric media. The ratio of the maximum concentration of Asu-hexapeptide to that of the Asp-hexapeptide at acidic 'pH' is shown in Figure 3A; higher ratios in the polymeric solids than in solution support the increased importance of the cyclic imide pathway. Possible alternative explanations for these observations include: (i) a difference between the "true" hydrogen ion activity in the polymeric solids and the effective 'pH' measured by reconstitution, with the "true" hydrogen ion activity being more

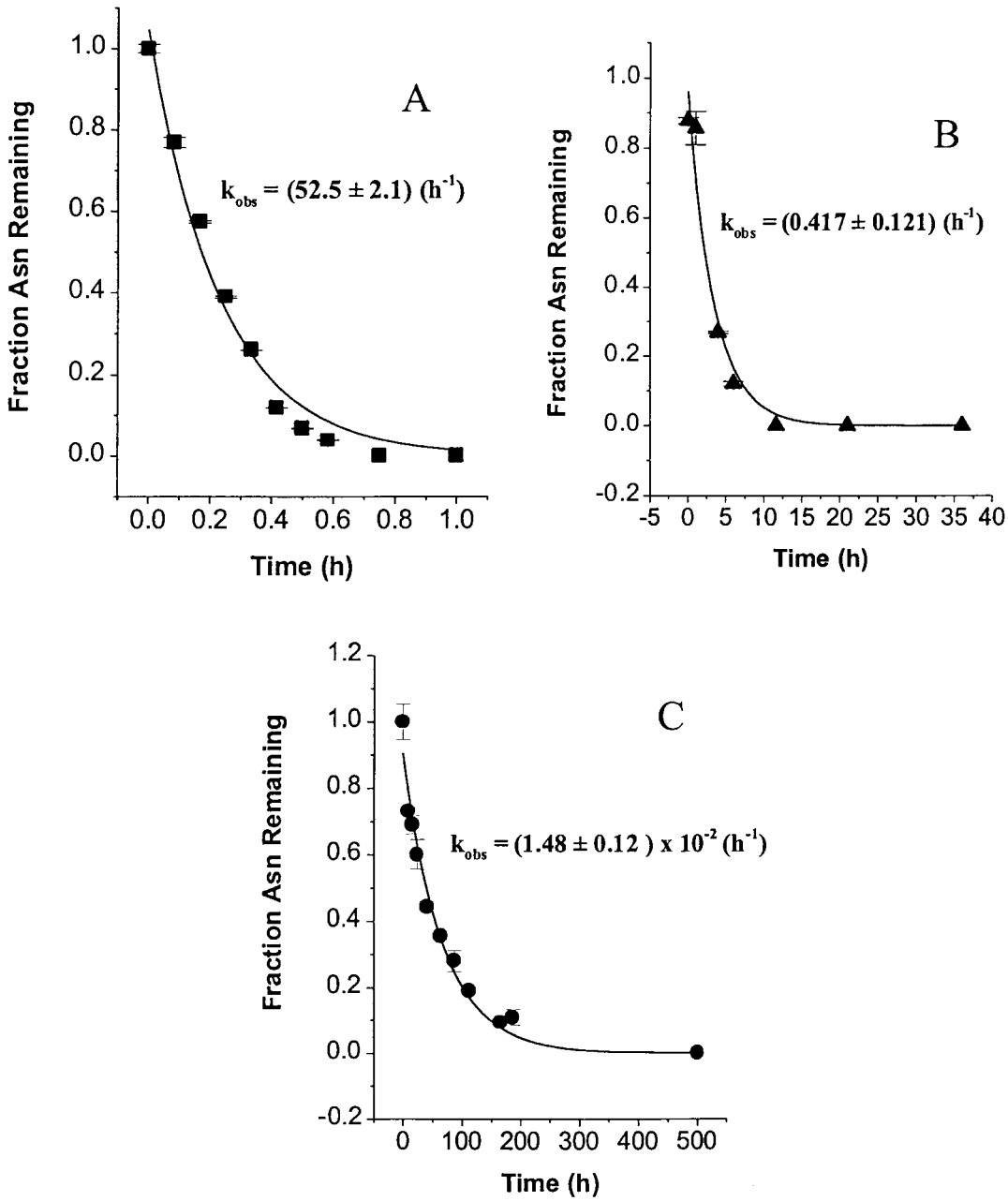


Figure 2. Representative kinetic profiles for Asn-hexapeptide loss in aqueous solution (■, Panel A), rubbery PVP solids (▲, Panel B) and glassy PVP solids (●, Panel C) at 'pH' 10.4 and 70°C. Apparent-first-order rate constants (k_{obs}) obtained by fitting the data to eq. 1 are shown in each panel. The line in each panel represents this regression. Error bars represent standard deviations; $n = 3$.

nearly neutral, and (ii) formation of the Asu-hexapeptide from the Asp-hexapeptide via back-reaction (Figure 1).

At acidic 'pH' in glassy polymeric solids, all the chromatographic peaks observed corresponded to known solution-state deamidation products of the Asn-hexapeptide, suggesting that no new reaction pathways contribute to deamidation in glassy solids. In rubbery polymeric solids at acidic 'pH', two additional peaks were observed, suggesting the formation of unknown products (data not shown). The cumulative areas of these new peaks corresponded to a maximum of 20–25% of the initial peak area for the Asn-hexapeptide, and

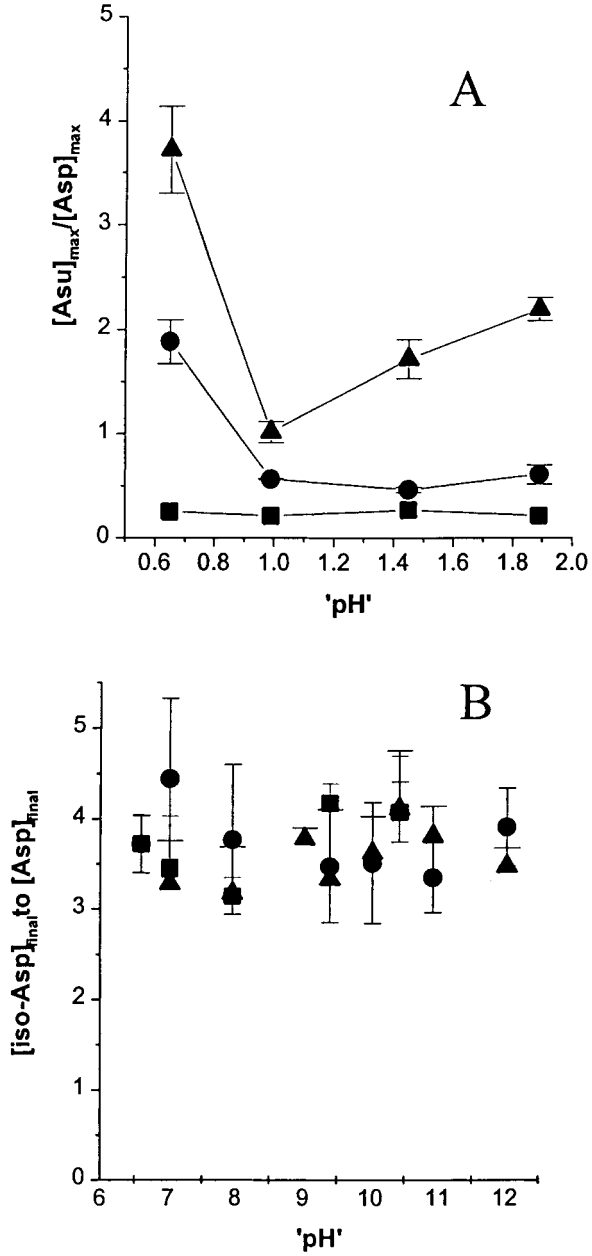


Figure 3. Product formation ratios for Asn-hexapeptide deamidation in solution (■), rubbery PVP solids (▲), and glassy PVP solids (●) at 70°C. Panel A: Ratio of the maximum concentrations of Asu- to Asp-hexapeptides at acidic 'pH'. Panel B: Ratio of the final concentrations of iso-Asp- to Asp-hexapeptides at neutral to basic 'pH'. The lines in Panel A are included to clarify trends, and do not indicate regression. Error bars represent standard deviations; $n = 3$.

were greatest toward the end of the stability studies. It is suspected that these products are the result of side reactions between the peptide and the glycerol plasticizer used in the rubbery samples. No correction for these unknown products was made.

At neutral to basic conditions ('pH' > 5), the dominant degradation products were the iso-Asp- and Asp-hexapeptides both in solution and in the polymeric solids. The final ratio of iso-Asp- to Asp-hexapeptide was in the range of 3.0 to 5.0 for all samples, as shown in Figure 3B, a value similar to that previously reported for the reaction in aqueous solution.[3,4,6] This result suggests that deamidation at neutral to basic 'pH' occurs via formation of the Asu-hexapeptide in the polymeric solids, as previously established for the reaction in solution.[3,4,6] No unknown chromatographic peaks were detected in the polymeric solids in this 'pH' range, indicating the absence of competing side reactions.

Because the degradation products detected in the PVP-containing solution controls and in the polymeric solids are comparable to those previously reported in polymer-free aqueous solutions, it is reasonable to assume that the dominant degradation mechanisms are similar

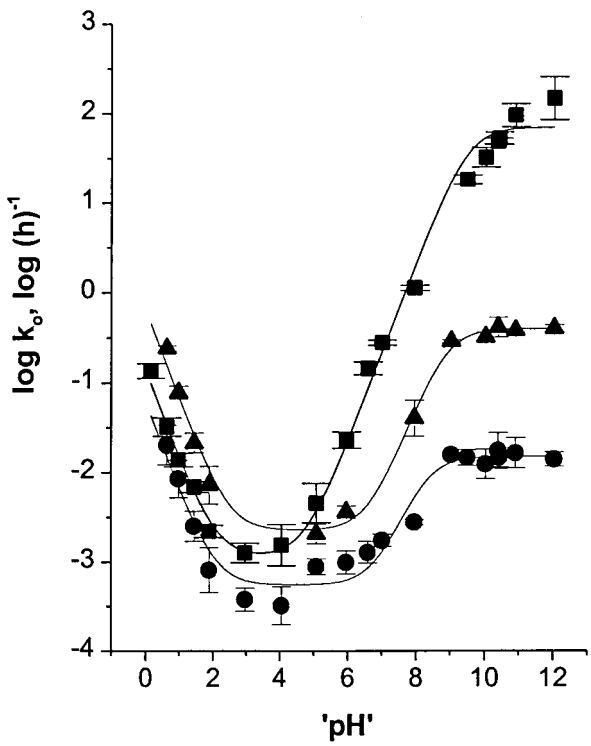


Figure 4. 'pH'–rate prole for Asn-hexapeptide loss in solution (■), rubbery PVP solids (▲), and glassy PVP solids (●) at 70°C. Lines represent regression of the data to eq. 2. Error bars represent standard deviations; $n = 3$.

Table 2. Rate Constants (k_o) for Deamidation of the Asn-Hexapeptide in Solution and in Polymeric PVP Solids[a]

		k_o (± SD), h^{-1}		
'pH'	Buffer Salt	Solution	Glassy PVP Solid	Rubbery PVP Solid
0.17	H_2SO_4	$1.34(\pm 0.11) \times 10^{-1}$		
0.65	H_2SO_4	$3.24(\pm 0.22) \times 10^{-2}$	$2.04(\pm 0.27) \times 10^{-2}$	$2.40(\pm 0.11) \times 10^{-1}$
0.99	H_2SO_4	$1.38(\pm 0.06) \times 10^{-2}$	$8.51(\pm 0.86) \times 10^{-3}$	$7.76(\pm 0.49) \times 10^{-2}$
1.45	H_2SO_4	$6.92(\pm 0.19) \times 10^{-3}$	$2.51(\pm 0.16) \times 10^{-3}$	$2.13(\pm 0.14) \times 10^{-2}$
1.89	H_2SO_4	$2.18(\pm 0.06) \times 10^{-3}$	$8.13(\pm 0.66) \times 10^{-4}$	$7.24(\pm 0.71) \times 10^{-3}$
2.97	KH_2PO_4/K_2HPO_4	$1.29(\pm 0.05) \times 10^{-3}$	$3.80(\pm 0.15) \times 10^{-4}$	
4.05	KH_2PO_4/K_2HPO_4	$1.55(\pm 0.10) \times 10^{-3}$	$3.24(\pm 0.19) \times 10^{-4}$	
5.08	KH_2PO_4/K_2HPO_4	$4.57(\pm 0.41) \times 10^{-3}$	$8.91(\pm 0.26) \times 10^{-4}$	$2.04(\pm 0.08) \times 10^{-3}$
5.97	KH_2PO_4/K_2HPO_4	$2.34(\pm 0.12) \times 10^{-2}$	$9.77(\pm 0.42) \times 10^{-4}$	$3.63(\pm 0.10) \times 10^{-3}$
6.6	KH_2PO_4/K_2HPO_4	$1.45(\pm 0.12) \times 10^{-1}$	$1.28(\pm 0.05) \times 10^{-3}$	
7.02	K_2HPO_4/K_3PO_4	$2.82(\pm 0.15) \times 10^{-1}$	$1.74(\pm 0.04) \times 10^{-3}$	
7.96	K_2HPO_4/K_3PO_4	$1.14(\pm 0.06) \times 10^{0}$	$2.82(\pm 0.03) \times 10^{-3}$	$4.07(\pm 0.59) \times 10^{-2}$
9.03	$NaHCO_3/Na_2CO_3$		$1.62(\pm 0.10) \times 10^{-2}$	$2.88(\pm 0.011) \times 10^{-1}$
9.5	$NaHCO_3/Na_2CO_3$	$1.83(\pm 0.072) \times 10^{1}$[b]	$1.48(\pm 0.09) \times 10^{-2}$	
10.03	$NaHCO_3/Na_2CO_3$	$4.90(\pm 0.087) \times 10^{1}$	$1.26(\pm 0.11) \times 10^{-2}$	$3.24(\pm 0.33) \times 10^{-1}$
10.43	$NaHCO_3/Na_2CO_3$	$5.25(\pm 0.21) \times 10^{1}$	$1.48(\pm 0.12) \times 10^{-2}$	
10.4	$NaHCO_3/Na_2CO_3$	$3.02(\pm 0.22) \times 10^{1}$[b]	$1.78(\pm 0.20) \times 10^{-2}$	$4.17(\pm 1.21) \times 10^{-1}$
10.91	NaOH	$9.55(\pm 0.62) \times 10^{1}$[b]	$1.66(\pm 0.15) \times 10^{-2}$	$3.80(\pm 0.18) \times 10^{-1}$
12.03	NaOH	$1.86(\pm 0.20) \times 10^{2}$[b]	$1.41(\pm 0.06) \times 10^{-2}$	$4.07(\pm 0.42) \times 10^{-1}$

[a] $n = 3$; 70°C
[b] Calculated from the k_{obs} at 22°C and $E_a = 22.2$ kcal/mol.

to those defined for the reaction in solution. In the remaining sections of this paper, we analyze the 'pH'-dependence of the pseudo-first-order disappearance of the Asn-hexapeptide adopting this assumption.

'pH'–Rate Profile

The relationship between the deamidation rate constants and 'pH' is shown in Figure 4; the corresponding rate constants are given in Table 2. The rate constants are designated "k_o" to indicate that the values have been extrapolated to zero buffer concentration for samples in which significant buffer catalysis was observed. Several features of the 'pH'–rate profile are noteworthy. Overall, the curve is U-shaped in solution and in the polymeric solids, suggesting a degree of catalysis by both acidic and basic species as reported previously for the reaction in solution.[4] The 'pH' of maximum stability occurs between 2 and 5 for all samples, and is relatively unaffected by the type of formulation.

In the acidic region ('pH' < 2.0), ko values are comparable in all three types of formulation. The curves are linear, with slopes approximately equal to -1 (Table 3). This result is consistent with specific acid catalysis, as reported previously

Table 3. Approximate Values of the Slopes of the 'pH'–rate Profile (Figure 4) in Selected Regions

	Slopes of the 'pH'–Rate Profile, log (h^{-1}) ± SE[a]		
Formulation Type	'pH' < 2.0	5 < 'pH' < 8	'pH' > 9
Solution	-1.00 ± 0.06	0.86 ± 0.06	0.39 ± 0.07
Rubbery solid	-1.22 ± 0.05	0.46 ± 0.06	0.05 ± 0.02
Glassy solid	-1.13 ± 0.006	0.18 ± 0.03	-0.005 ± 0.02

[a] Calculated by linear regression of the data in the 'pH' region indicated.

for the reaction in aqueous solution.[4] Note that both the k_o values and the absolute values of the slopes are slightly greater for the rubbery polymeric solids than for the other samples. These minor differences may reflect the formation of unknown products, as already noted.

In the neutral region ($5 < $'pH'$ < 8$), k_o values decrease in the order: solution > rubbery > glassy. The values in solution are ~3–10 times greater than in the rubbery solids; values in the rubbery and glassy solids differ by a similar proportion. The 'pH'–rate profile is approximately linear in this region, with slopes that decrease in the order observed for the k_o values (Table 3). The smaller slopes in the polymeric solids suggest a decreased sensitivity of the reaction rate to 'pH' changes in these samples relative to the solution control. Whereas the slope for the solution sample ($+0.86 \pm 0.06$ log (h^{-1})) approaches the value of $+1$ expected for specific base catalysis of the reaction, the values in the polymeric solids are significantly smaller, suggesting that such a mechanism cannot be invoked.

In the basic region ('pH' > 9), k_o values differ dramatically in the three types of samples. The k_o values in solution are as much as 1000-fold greater than that in the rubbery polymeric solid, which in turn is ~30-fold greater than that in the glassy solid. The 'pH'–rate profile reaches a plateau for the polymeric solids for 'pH' > 9 (Figure 4), suggesting that the reaction rate is 'pH' independent in this region. Calculated values of the slopes of the curves in the basic region confirm a decreasing sensitivity to 'pH' for all three types of samples. For the solution sample, the slope is ~0.40 log(h^{-1}), roughly half the value in the neutral region (Table 3). The slopes calculated for the rubbery and glassy solids are approximately equal to zero (Table 3), as expected given the plateaus shown in Figure 4.

The shape of the 'pH'–rate profile suggests that the reaction is subject to both acid and base catalysis. Accordingly, nonlinear regression was used to fit the data in Figure 4 to the following equation:

$$k_o = k_H[H^+] + k_W + k_B[OH^-]/\{K + [OH^-]\} \quad (2)$$

where k_H, k_W, and k_B are the rate constants for the acid, water, and base catalysis of the reaction, respectively, and K is a constant related to the charge of the transition state in the rate-determining step. The curves in Figure 4 represent the best fit for each type of sample; the excellent agreement between the data and the regression curves suggests that the model adequately describes the data for all three types of samples. Values of the regression coefficients are given in Table 4. Particularly noteworthy is the > 3000-fold variation in the value of k_B in the three samples. In solution at pH 10, the k_B term in eq. 2 is the dominant contribution to k_o, and is more than four orders of magnitude greater than the k_W term. However, in glassy solids, the k_B term is only 30-times greater than the k_W term, suggesting a decrease in the importance of base catalysis relative to water catalysis at basic 'pH'. The value of K is on the order of $0.1–1.0 \times 10^{-5}$ M for all three samples, and is consistent with the development of a plateau in the 'pH'–rate profile between 'pH' 9 and 10, with larger values of K corresponding to initiation of the plateau at higher 'pH' values. Values of k_W and k_H are comparable in the three types of samples.

Buffer Catalysis Studies

To further explore the effects of 'pH' on deamidation in the basic range, buffer catalysis was investigated in the range $6.5 < $'pH'$ < 10.5$ by measuring k_{obs} at varying buffer concentrations in both solution and solid formulations while maintaining constant 'pH' and ionic strength. The slope of the relationship between k_{obs} and 'pH' is

Table 4. Contributions of Acid (k_H), Water (k_W), and Base (k_B) Catalysis to the 'pH'–Rate Profile of Figure 4[a]

Formulation Type	$k_H \times 10^2$ ($M^{-1}h^{-1}$)	$k_W \times 10^4$ (h^{-1})	$k_B \times 10^2$ (h^{-1})	$K \times 10^5$ (M)
Solution	15 ± 2	11 ± 2	7000 ± 600	3.0 ± 0.9
Rubbery solid	70 ± 10	22 ± 5	40 ± 5	0.7 ± 0.3
Glassy solid	6 ± 2	6 ± 1	2 ± 0.2	0.2 ± 0.1

[a] Values determined by nonlinear regression of the data in Figure 4 to eq. 2.

defined as the catalytic rate constant, k_{cat}. The results are summarized in Figure 5 and Table 5.

In solution at 'pH' 6.6, there is no apparent buffer catalytic effect. The k_{obs} value is unaffected by buffer concentration (Figure 5A) and the k_{cat} value (Table 5) is near zero. Because the 'pH'–rate profile shows a dependence of k_{obs} on 'pH' in this range, this result suggests that the solution-state reaction is subject to specific base catalysis (i.e., by OH^-) in this region. In contrast, there is a distinct buffer catalytic effect at higher solution pH (9.5, 10.5; Figure 5A), with the value of k_{cat} increasing with increasing pH. When combined with the pH dependence demonstrated in the 'pH'–rate profile, this result suggests that the reaction is subject to general base catalysis (i.e., by the buffer anion) in this region, as reported previously.[4,6]

Like the solution-state samples, glassy polymeric solids at 'pH' 6.6 show no apparent buffer catalytic effect (Figure 5B). Again, because the 'pH' rate profile shows a 'pH'-dependence in this region, this result is consistent with specific base catalysis. At higher 'pH' values (9.5, 10.5), however, no buffer catalysis was observed and the k_{cat} value was not significantly different from zero. In addition, the 'pH'–rate profile is independent of 'pH' for the glassy solids in this region. The absence of both 'pH' and buffer catalytic effects suggests that the rate-determining step is 'pH' independent in glassy polymeric solids at basic 'pH'. This suggestion is in contrast to the general base catalysis of the solution samples, and is consistent with a change in the rate-determining step in the polymeric solids relative to the solution control.

Effect of Relative Humidity

Previous studies in our laboratories have demonstrated that the deamidation of the Asn-hexapeptide in PVP matrices is sensitive to the relative humidity (RH) of the surrounding gas phase,[19,20] an experimentally controllable parameter that determines the equilibrium moisture content of the polymer matrix. These studies also demonstrated that water serves as both a reaction medium and a plasticizer in these systems.[19,20] To determine whether the observed plateaus in the 'pH'–rate profile in basic polymeric solids were influenced by moisture content, additional stability studies were conducted at 70 and 95% RH in rubbery PVP matrices. The results are shown in Figure 6, and are compared with the

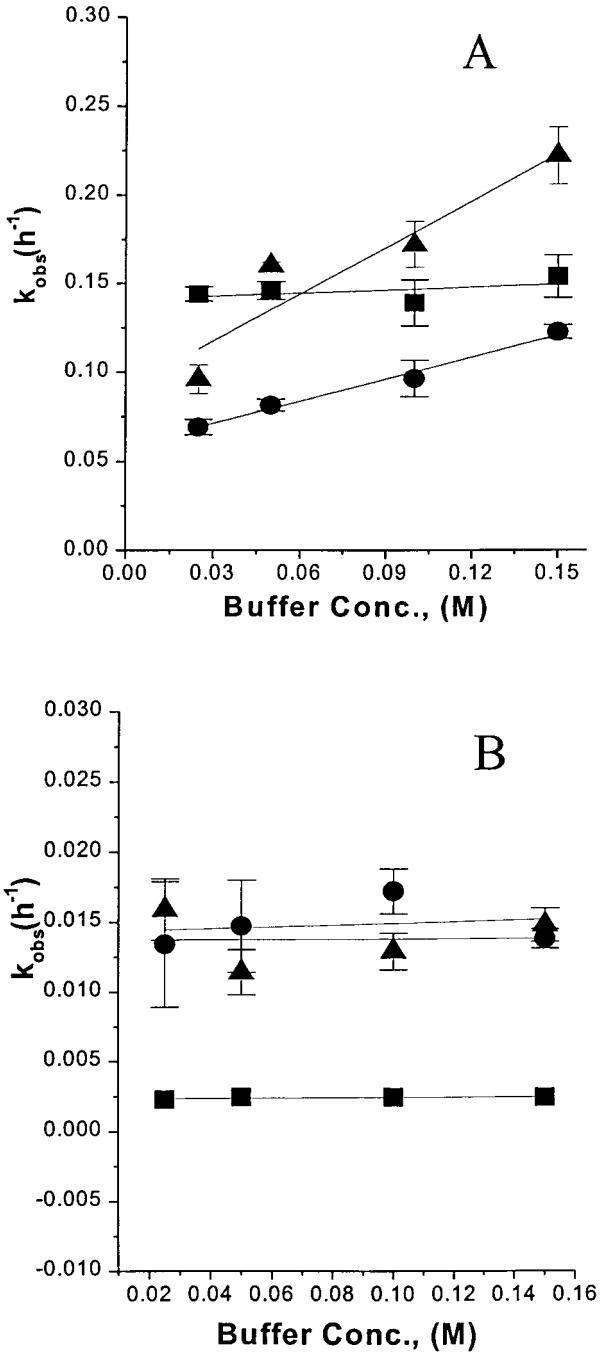


Figure 5. Effect of buffer concentration on k_{obs} values in solution (Panel A) and in glassy PVP solids (Panel B) at basic 'pH'. Symbols correspond to 'pH' values: ■, 'pH' 6.6; ●, 'pH' 9.5; ▲, 'pH' 10.4 in both panels. Studies were performed at 70°C, with the exception of solution samples at 'pH' 9.5 and 10.4 (●, ▲, Panel A), for which rapid reaction prevented effective sampling; these studies were performed at 22°C, and the k_{obs} values were extrapolated to 70°C. Catalytic rate constants (k_{cat}) determined from the slopes of the lines are listed in Table 5. Error bars represent standard deviations; $n = 3$.

Table 5. Catalytic Rate Constants (k_{cat})[a] for the Buffer Catalysis Studies of Figure 5

	$k_{cat} \times 10^2$ ($M^{-1}h^{-1}$)		
Formulation Type	pH 6.6	pH 9.5	pH 10.4
Solution	5.8 ± 1.4	41 ± 14[b]	88 ± 14[b]
Glassy solid	0.010 ± 0.002	0.23 ± 0.02	0.010 ± 0.002

[a]Determined by linear regression of the data in Figure 5.
[b]These studies at 22°C; others at 70°C.

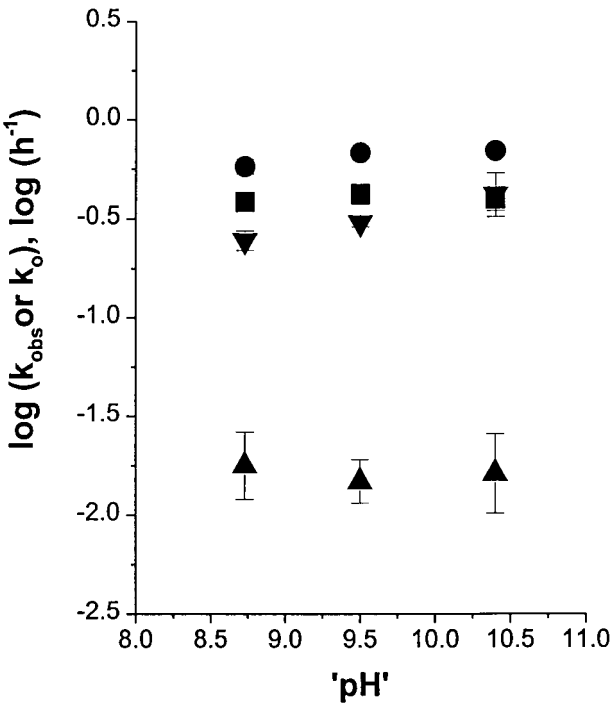


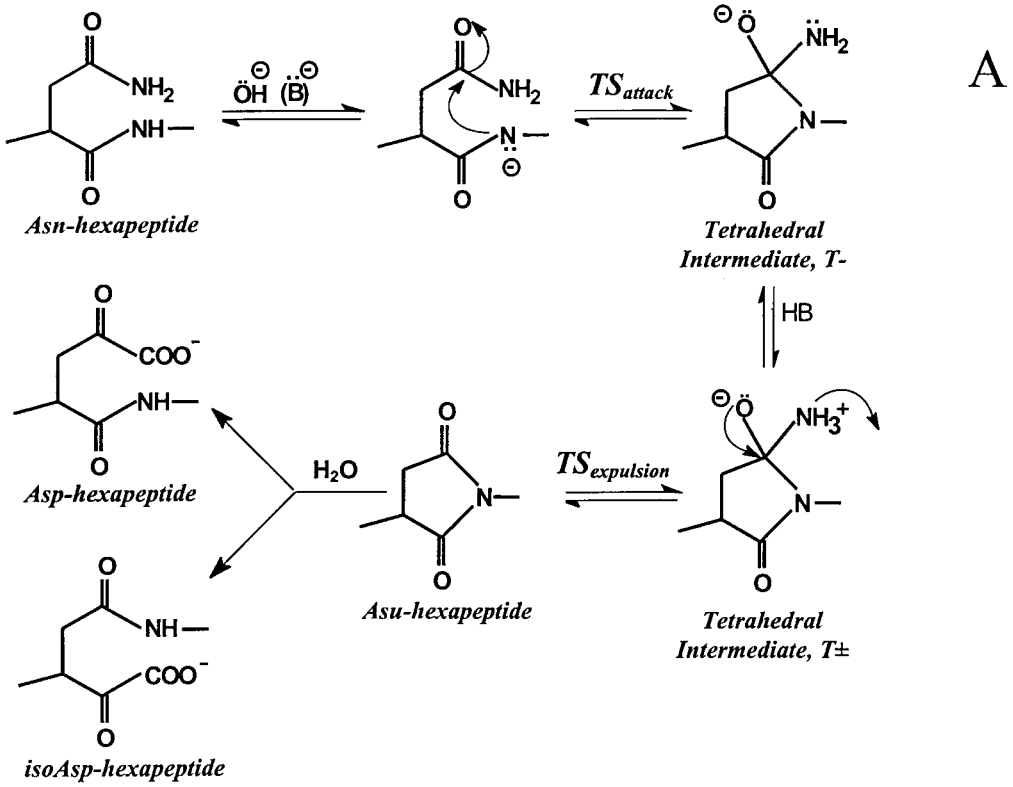
Figure 6. Effect of relative humidity (RH) on deamidation rate in solid PVP systems at basic 'pH'. Symbols indicate varying RH and/or glycerol content: ■, 70% RH, 0% glycerol; ●, 95% RH, 0% glycerol; ▲, 50% RH, 0% glycerol; ▼, 50% RH, 30% (w/w) glycerol. Rate constants are k_o values (i.e., extrapolated to zero buffer concentration) for 50% RH data, but are k_{obs} values (i.e., determined at a single buffer concentration of 0.15 M) for 70% and 95% RH data. Error bars represent standard deviations; $n = 3$.

corresponding 50% RH data from the 'pH'–rate profile for both glassy (0% glycerol) and rubbery (30% glycerol) samples. The k_o values increase with increasing RH, as observed previously,[19,20] but 'pH'-independent behavior is observed in all these basic polymer matrices. Thus, the plateau in the 'pH'–rate profile in the basic region is observed for both glassy and rubbery polymeric solids and at varying matrix moisture content.

Reaction Mechanism

The appearance of identical degradation products in solution and in polymeric solids and the similarities in the overall shape of the 'pH'–rate profile suggest that the mechanism of Asn-deamidation is fundamentally unaltered in the polymeric media. However, the notable differences in the 'pH'–rate profile in the neutral to basic 'pH' region are consistent with a shift in the rate-determining step of the reaction in the polymeric media. Specifically, in the polymeric solids: (i) the slope of the 'pH'–rate profile in the neutral to basic region (i.e., $5 <$ 'pH' < 8) decreases relative to that for the solution control (Figure 4); (ii) the corresponding values of k_B decrease (Table 3); and (iii) a distinct plateau appears in the 'pH'–rate profile for 'pH' > 9 (Figure 4). These observations suggest the lack of involvement of either the hydroxide ion or the basic form of the buffer species (Figure 5B) in the rate-determining step in basic polymeric solids, a feature present in the proposed mechanisms for the reaction in basic solution.[3,4,6] We propose that the polymeric media exert a strong and differential effect on the rates of the individual steps in the reaction, and that, in the basic region, the rate-determining step shifts from base-catalyzed ring formation to 'pH'-independent loss of ammonia.

Figure 7A shows the proposed mechanism of Asn-hexapeptide deamidation at neutral to basic 'pH'. Initially, a small amount of a highly reactive species is generated through the deprotonation of the backbone NH center. Further attack by this species on the side-chain amide functional group of Asn leads through the first transition state, labeled TS_{attack}, to a tetrahedral intermediate, T –. Because progress through TS_{attack} requires the anionic form of the backbone amide nitrogen,



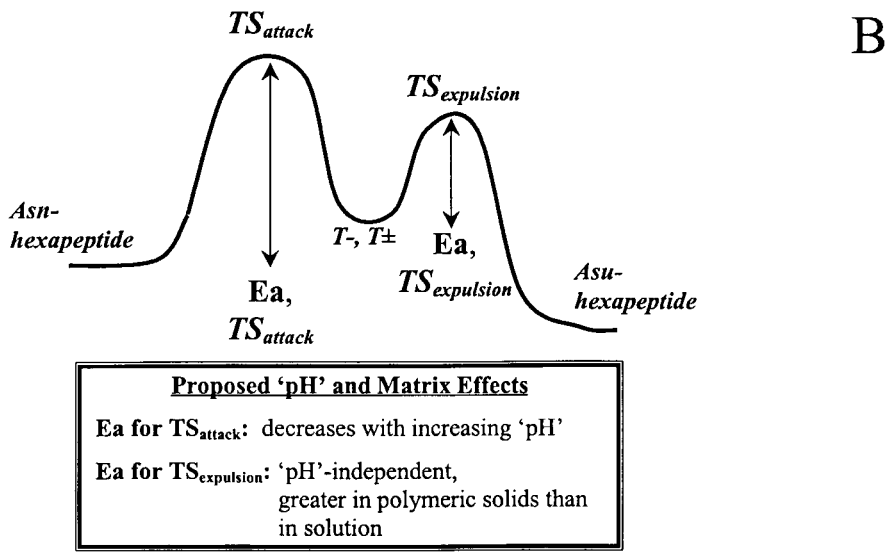


Figure 7. Proposed mechanism and energetics of Asn-hexapeptide deamidation in solution and in polymeric solids. Panel A: Proposed mechanism. TS_{attack} represents a transition state formed by attack of the amide nitrogen on the Asn-carbonyl. $TS_{expulsion}$ represents a second transition state for the expulsion of ammonia. Panel B: Proposed reaction energetics, summarizing 'pH' and matrix effects on activation energies (*E*a) for TS_{attack} and $TS_{expulsion}$. As shown, the diagram represents the relationship for the 'pH'-dependent reaction observed in basic aqueous solutions, in which (*E*a, TS_{attack}) > (*E*a, $TS_{expulsion}$). For the 'pH'-independent reaction in polymeric solids, (*E*a, $TS_{expulsion}$) > (*E*a, TS_{attack}) (not shown).

this step is hydroxide ion dependent. The tetrahedral intermediate $T-$ does not accumulate to appreciable levels, but either decomposes to regenerate the reactants or accepts a proton to form a second tetrahedral intermediate, $T\pm$. The intermediate $T\pm$ may lose a proton to regenerate $T-$, or progress through a second transition state, labeled $TS_{expulsion}$, forming the cyclic imide intermediate and releasing ammonia into the surrounding medium. This process is expected to be independent of medium 'pH'.

This mechanism provides a framework for interpreting the 'pH'–rate profiles in the neutral to basic region. If the activation energy for TS_{attack} is greater than for $TS_{expulsion}$ (Figure 7B), "attack" will be the rate-determining step and the overall reaction will be observed to be 'pH' dependent. If the converse is true, "expulsion" will be rate determining and the reaction as a whole will appear to be 'pH' independent. Because the formation of TS_{attack} is dependent on the concentration of base, the activation energy for this step is expected to decrease with increasing 'pH'. With regard to $TS_{expulsion}$, the loss of ammonia is expected to occur readily in solution because of the high mobility of both reactants and products, but may be restricted in the more rigid polymeric solids. The activation energy for $TS_{expulsion}$ is then expected to be less in solution than in the polymeric media. The plateaus observed in the 'pH'–rate profile in the polymeric media can then be understood to represent a shift in the rate-determining step, from amide anion attack (via TS_{attack}) to expulsion of ammonia (via $TS_{expulsion}$). This shift occurs at lower 'pH' in the polymeric samples, perhaps because mobility restrictions in the solid state increase the activation energy for $TS_{expulsion}$, so that a smaller hydroxide ion concentration is required to produce the condition $TS_{attack} < TS_{expulsion}$.

Limitations of the Method

The experimental data and mechanistic interpretations just presented should be viewed with some caution in light of the limitations in the methods. First, it is important to note that although the Asn deamidation rate in polymeric solids has been related to the 'pH' (effective pH), the relationship between this value and the "true" hydrogen ion activity in the matrix is unknown. Similarities in the general shape of the 'pH'–rate profiles of solution and polymeric samples and in the 'pH' of maximum stability suggest a correspondence between the two values. However, the high ratios of Asu- to Asp-hexapeptide (Figure 3) in polymeric samples at acidic 'pH' may suggest that the true hydrogen ion activity is less than the effective value (i.e., the "true 'pH'" is higher) because the Asu-hexapeptide is not formed in appreciable amounts in acidic aqueous solution (Figures 1 and 3).[3,4,6] Of course, the high ratios of Asu- to Asp-hexapeptide may also suggest that the reaction mechanism is altered in acidic polymeric media; discriminating between these explanations is not possible unless the "true" hydrogen ion activity is known. Similarly, the plateau in the 'pH'–rate profile in basic polymeric solids may be interpreted mechanistically as a shift in the rate-determining step, as already described, but may also indicate that the 'pH' value overestimates the "true" hydrogen ion activity (i.e., underestimates the "true pH"). Thus, the possible lack of correspondence between "effective" and "true" pH values should be borne in mind when considering our results. This difficulty also suggests that further development of methods for the direct measurement of hydrogen ion activity in solids is warranted.

A second important limitation involves the recovery of the Asn-hexapeptide and its degradation products from the polymeric solids. In the studies reported here, the observed degradation products were those recovered and detected following the sample preparation and HPLC assay methods already described. For the solution samples, the cumulative amount of the Asn-hexapeptide and its known degradation products detected was equal to the initial Asn-hexapeptide load at all times, so that the peptide recovery was 100%. In contrast, in the polymeric solids, peptide recovery decreased with storage time, with $<80\%$ of the initial peptide load recovered following 50 h of storage. The causes of this incomplete recovery are unknown, but may involve a change in polymer morphology during storage that affects peptide recovery, or an interaction between the polymer and the peptide and/or its degradation products that is not disrupted during sample preparation and analysis. In analyzing the data, we have assumed that when peptide recovery is incomplete it is not preferential, so that the compounds recovered are representative of the relative amounts present in the solid matrix.

Finally, by labeling the samples as "solution", "rubbery", and "glassy", we have implied that molecular mobility is of primary importance in controlling reactivity, but polymer matrix incor-

poration may influence the reaction in other ways. For example, inclusion in a polymer matrix may affect the conformation and/or higher order structure of peptides and proteins. Secondary structure has been shown to have significant effects on peptide deamidation rates in solution.[24–26] Although the small hexapeptide studied here is unlikely to be structured in solution, a degree of folding may be imposed by the polymer matrix. This possibility has not been investigated. In addition, the polarity of the polymer matrix may differ from that in solution. Deamidation occurs more rapidly in solutions of higher polarity (as measured by the solvent dielectric constant), a finding attributed to greater stabilization of the charged transition states (Figure 7A) by more polar solvents.[27,28] We have assumed that the solution and polymer samples are similar in this regard, but no attempt has been made to define and measure the "effective polarity" of the polymer matrices. Lastly, specific interactions between the peptide and the polymer may influence the deamidation rate in the polymer matrices. PVP has been shown to interact with compounds containing aromatic functional groups,[29,30] which suggests the possibility of an interaction between the polymer and the peptide tyrosine residue. The degree to which such an interaction occurs in our polymer samples and its effect on the rate of deamidation are unknown.

CONCLUSIONS

The rate of deamidation of the Asn-hexapeptide is sensitive to 'pH' both in polymer-containing aqueous solutions and in glassy and rubbery polymeric solids. The reaction follows pseudo-first-order kinetics over a broad 'pH' range (0.5–12) in all three systems; similarities in the products formed and their ratios suggest that the mechanism of reaction is unaffected by incorporation in polymeric media. In the acidic range, rates of deamidation are comparable in solution and in polymeric solids. The slope of the 'pH'–rate profile is approximately -1 for all three systems in acidic media, a result consistent with specific acid catalysis. In the neutral to basic 'pH' range, the deamidation rate is strongly dependent on the sample type, with as much as a 10,000-fold difference in rate between solution and glassy polymeric solids. The shape of the 'pH'–rate profile is also affected by matrix type in the neutral to basic range. The reaction in polymeric solids is less sensitive to 'pH' in the neutral range ($5 < $'pH'$ < 8$) than the solution control, as indicated by the slopes of the 'pH'–rate profile. In basic polymeric solids ('pH' > 10), a region in which the solution-state reaction shows general base catalysis, the reaction is 'pH' independent. These results, together with supporting studies of buffer catalytic effects, are consistent with a change in the rate-determining step of the reaction in the basic region, from 'pH'-dependent attack of the deprotonated backbone amide nitrogen on the Asn side chain in solution to 'pH'-independent expulsion of ammonia in the polymeric solids. As a whole, the results presented here challenge the notion that the 'pH' dependence of protein degradation reactions in the solid state simply parallels that in solution,[12] and provide strong evidence for a matrix effect on the relative rates of the mechanistic steps of the deamidation reaction.

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