Deamidation, Isomerization, and Racemization at Asparaginyl and Aspartyl Residues in Peptides

SUCCLNIMIDE-LINKED REACTIONS THAT CONTRIBUTE TO PROTEIN DEGRADATION*

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Aspartyl and asparaginyl deamidation, isomerization, and racemization reactions have been studied in synthetic peptides to model these spontaneous processes that alter protein structure and function. We show here that the peptide L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala undergoes a rapid deamidation reaction with a half-life of only 1.4 days at 37 °C, pH 7.4, to give an aspartyl succinimide product. Under these conditions, the succinimide product can further react by hydrolysis (half-time, 2.5 h) and by racemization (half-time, 19.5 h). The net product of the deamidation reaction is a mixture of L- and D-normal aspartyl and β-transpeptidation (isoaspartyl) hexapeptides. Replacement of the asparagine residue by an aspartic acid residue results in a 34-fold decrease in the rate of succinimide formation. Significant racemization was found to accompany the deamidation and isomerization reactions, and most of this could be accounted for by the rapid racemization of the succinimide intermediate. Replacement of the glycyl residue in the asparagine-containing peptide with a bulky leucyl or prolyl residue results in a 33–50-fold decrease in the rate of degradation. Peptide cleavage products are observed when these Asn-Leu and Asn-Pro-containing peptides are incubated. Our studies indicate that both aspartic acid and asparagine residues may be hot spots for the nonenzymatic degradation of proteins, especially in cells such as erythrocytes and eye lens, where these macromolecules must function for periods of about 120 days and 80 years, respectively.

A variety of spontaneous chemical reactions are known to affect proteins (1). Among the most prevalent of these at physiological temperature and pH are deamidation, isomerization, and racemization. In the deamidation reaction, the side chain amide linkage in a glutamine or asparagine residue is hydrolyzed to form a free carboxylic acid (2). In isomerization, the peptide backbone is transferred from the α-carboxyl of an aspartate, asparagine, glutamate, or glutamine residue to the side chain β- or γ-carboxyl (3). Finally, in the racemization reaction, the configuration about the α-carbon of the amino acid is inverted (4). For each of these reactions, the rate has generally been found to be more rapid with asparaginyl/aspartyl residues than glutaminyl/glutamyl or other residues. We were interested in these processes because they can account for the presence of L-isoaspartyl and D-aspartyl residues which have been implicated as substrates for mammalian protein carboxyl methyltransferases. The function of these enzymes is not well understood, but it has been hypothesized that they participate in the metabolism of proteins containing these altered residues (5).

Prior studies have suggested that the intermediate formation of a five-membered succinimide ring may contribute to both deamidation and isomerization events at aspartyl and asparaginyl residues in proteins and peptides. Succinimide formation entails an intramolecular cyclization in which the α-amino group of the carboxyl amino acid residue attacks the side chain carboxyl carbon of an aspartyl/asparaginyl residue (Fig. 1). Succinimide formation is the only known mechanism by which β-isomerization may occur and has been implicated in deamidation reactions because β-isomerized deamidation products can be detected from incubations of proteins and peptides (3, 6–10, 46). Additionally, it has been hypothesized that peptide succinimides may also be racemization prone and contribute to the formation of D-aspartyl derivatives in proteins (11).

Although succinimide formation can account for protein deamidation and isomerization, the extent to which this intermediate participates in these reactions has not been established. Very little is known about the rates of succinimide formation (and racemization) in specific sequences. As a first approach to this problem, we have investigated the pathways of spontaneous degradation of model hexapeptides at physiological pH and at a range of temperatures. We chose sequences based on residues 22–27 of adrenocorticotropic hormone (Val-Tyr-Pro-Asn-Gly-Ala) because previous work had implicated facile succinimide formation at the asparagine 25 residue in the intact 39-residue hormone (8, 9). Succinimide formation from asparagine, aspartic acid β-esters, and aspartic β-hydroxamic acids is most rapid when steric hindrance from the side chain of the adjacent carboxyl residue is minimized, as in the selected hexapeptide sequence above (12–17). Because of this and because of the conformational flexibility of short peptides, which lack the structural constraints of intact proteins, we expected that the rate of succinimide formation in the hexapeptide would approach the maximal rate possible in intact peptides and proteins.

In this study, we analyzed the processes of deamidation, isomerization, and racemization in several related peptides to understand the chemical and physiological significance of both succinimide formation and the rates of these reactions in cellular proteins. We have found that succinimide inter-
mediates play a central role in all three processes and that the rates of peptide deamidation, isomerization, and racemization are significant on a time scale relevant to cellular and macromolecular turnover.

**MATERIALS AND METHODS**

*Synthetic Peptides*—L-Val-L-Tyr-L-Pro-D-aspartylsuccinimide-Gly-L-Ala was synthesized and characterized by E. D. Murray, Jr. (University of California, Los Angeles) as described previously (18). L-Val-L-Tyr-L-Pro-L-aspartylsuccinimide-Gly-L-Ala was synthesized by Vega Biochemicals, while L-Val-L-Tyr-L-Pro-D-Asn-Gly-L-Ala was made by Peninsula Laboratories, Inc. L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala, L-Val-L-Tyr-L-Pro-L-Leu-L-Ala, and L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala were prepared by Dr. Janis Young at the UCLA Peptide Synthesis Facility using an Applied Biosystems model 430A instrument and hydrofluoric acid cleavage. L-Val-L-Tyr-L-Pro-D-Asp-Gly-L-Ala, L-Val-L-Tyr-L-Pro-D-isoAsp-Gly-L-Ala, L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala, and L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala and L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala were prepared by mild base hydrolysis of the appropriate succinimidyl peptide as described (18). All peptides were purified by HPLC using C18 reverse-phase columns. The actual pH of the phosphate buffer at 7.4 (measured at 22 °C) was added to each tube. The tubes were then sealed by a Bunsen burner flame. After addition of the peptide, the samples were frozen at -70 °C prior to sealing. Incubations were terminated by freezing the samples at -20 or -70 °C until HPLC analysis (see below). The actual pH of the phosphate buffer at elevated pH values can be estimated using a value of dpK_a/dT of -0.0028 °C^-1 (19).

**Peptide Purification and Analysis by HPLC**—Peptide purification and the determination of the composition of incubation mixtures were performed by HPLC using C18 reverse-phase columns. The HPLC system included two Waters model 510 pumps, a model 680 gradient controller, a U6K sample injector, a model 441 UV detector (214 nm), and a Shimadzu C-R3A integrating recorder. Separations were done on an Alttech Econosphere C18 column (5-µm resin, 4.6 mm diameter x 250 mm) at room temperature. Solvent A consisted of 0.1% trifluoroacetic acid (Fisher, HPLC grade), 0.1% trifluoroacetic acid, and 99.9% water. In all cases the column was pre-equilibrated in 100% solvent A. Analysis was performed under two elution conditions. The first, utilizing a linear 0–95% gradient of solvent B over 35 min at 1.0 ml/min flow rate resulted in the complete separation of the diastereomers of the l-Val-L-Tyr-L-Pro-D-Asp(Gly)-L-Ala and in the partial resolution of the d- and l-normal species (see Fig. 1 for structures). Typical elution times (min) for these compounds were as follows: l-iso, 24.7; d-iso, 26.0; l-normal and l-Asn, 27.4; d-normal, 28.4; d-imide, 30.8; l-imide, 33.3. A second system using a linear 0–95% gradient of solvent B over 40 min at 1.0 ml/min flow rate. This gradient also resulted in the separation of the diastereomeric d- and l-imide species although resolution of the diastereomers of the isoaspartyl and normal asparagyl peptides was not achieved. However, utilization of this gradient allows separation of the asparagine-containing peptide from the normal iso asparagine-containing peptide and imide and peptides was used in deamidation analyses (Fig. 2). For peptide purification, approximately 300 nmol or less of crude peptide was applied, while 2–5 nmol of purified peptides were analyzed in incubation mixtures.

**Characterization of Synthetic Peptides and Incubation Products**—The net charge of the peptides was determined by thin layer electrophoresis on 20 x 20-cm cellulose sheets. Samples (10 nmol) were electrophoresed at 20 V/cm for 30 min in 2% pyridine, 0.95% acetic acid (pH 5.3). Peptides and amino acids were detected by ninhydrin spray. The asparaginyl and succinimidyl peptides had mobilities corresponding to the neutral glycine and alanine amino acid standards while the asparyl and isoasparyl peptides had mobilities intermediate between the neutral amino acids and the aspartic acid and glutamic acid standards. Amino acid analysis was performed on lyophilized samples (less than 1 nmol) of purified peptides and incubation products after hydrolysis in 6 M HCl for 8 h at 108 °C using a Waters PicoTag Workstation (20). The resulting amino acids were derivatized with phenylisothiocyanate and analyzed as described by Bidlingmeyer et al. (20) or were derivatized with etho-phthalaldehyde as described by Jones and Gilligan (21). Analysis of D- and L-aspartate in these hydrolyses was done by the method of Aswad (22) with the modifications described in Ref. 45. Background racemization, measured in unincubated samples, was less than 2%, and the results were corrected for this amount. The results of these analyses, as well as the comigration of incubation products with known peptide standards and the detection of the expected hydrolysis products of the succinimide peaks, confirmed the attributed designations of the various incubation products.

**Kinetic Modeling by Computer Analysis**—The dynamics of the chemical reactions shown in Fig. 1 were described by differential equations representing the mass action terms and the rate constants indicated. These equations were solved by a computer-aided iteration procedure which resulted in mathematical stimulation of these reactions on a time scale. The iteration procedure was carried out using a Macro program on Excel electronic spreadsheet software for the Apple Macintosh Plus microcomputer. Kinetic analyses of data on the concentration of intermediates to determine the rate constants for the isomerization and racemization reactions were fit using models consisting of at least 1000 individual time points. The appropriate kinetic constant to be fit was modified until a least squares fit of the data and model was obtained (see text).

**RESULTS**

Pathways for the spontaneous degradation of the adrenocorticotropic hormone-derived asparaginyl and asparyl hexapeptides are shown in Fig. 1. To analyze which pathways predominate at physiological pH and temperature, we studied the rates of the component reactions. Once the rates for the individual reactions were known, we used computer modeling to illustrate the dynamics of these processes.
Deamidation of Val-Tyr-Pro-Asn-Gly-Ala—Incubation of the Asn-Gly-containing peptide resulted in the formation of several new products. Fig. 2 shows the distribution of these products. The D-asparagine-containing peptide was incubated at 70 °C in pH 7.4 phosphate buffer. At the zero time point, a single peak corresponding to the asparaginyl peptide was found. However, at 90 min, several new products appeared. A major peak, corresponding to the position of the isoaspartyl peptide, was found to elute 1.0 min before the asparaginyl peptide while lesser peaks corresponding to the normal aspartyl and imide peptides were found to elute 0.9 and 2.5 min afterward (see Fig. 1 for structures). Continued incubation results in an increase in the size of the iso and normal peptide peaks and a decrease in the size of the asparaginyl and imide peptide peaks.

The appearance of isomerized and imide peptide consequent upon deamidation demonstrates that succinimide formation is indeed a major pathway for deamidation of this peptide. Further, the ratio of the isomerized to normal peptide formed in the deamidation reaction (about 2:8:1) corresponds to that found when the purified succinimide peptide is hydrolyzed (about 3:1:1, see below). If there was significant participation of a deamidation pathway via direct solvent hydrolysis of the amide linkage (see Fig. 1), an increase in the proportion of normal peptide relative to isopeptide would be expected. This suggests that the deamidation reaction occurs almost exclusively through an intramolecular displacement resulting in a succinimide ring.

These studies were then extended to the L-asparagine form of this peptide and were performed at additional temperatures including 37 °C. The loss of the D- and L-asparaginyl peptide as a function of time at 37 °C, pH 7.4, is shown in Fig. 3A. The deamidation reaction displays first-order kinetics as is expected for such a reaction at constant pH. A significant difference in the rates of deamidation for the peptides containing D-asparagine (half-life = 50.9 h) and L-asparagine (half-life = 54.0 h) was found.

Deamidation data obtained at 70 and 85 °C are summarized in Fig. 3B. These experiments utilized the D-asparagine peptide because of the superior chromatographic resolution obtained with it in comparison to the L-asparagine peptide. From these data, it was possible to calculate an activation energy of 21.2 kcal/mol. This large value indicates that the rate of deamidation is temperature sensitive and that proteins exposed to high temperatures, as in thermophilic organisms, will be especially prone to this type of damage. For example, the half-life of the D-asparagine peptide is 1.9 h at 70 °C and only 30 min at 85 °C. At all temperatures, however, the same products were obtained, and there was no indication that significant reactions aside from those shown in Fig. 1 were occurring.

Hydrolysis of the Hexapeptide Succinimide—Because the deamidation of the asparagine peptide appeared to occur via a succinimide intermediate, we wanted to understand the characteristics of the imidyl peptide itself. Fig. 4A illustrates the result of incubating L-Val-L-Tyr-L-Pro-(D,L)-aspartylsuccinimide-Gly-L-Ala at pH 7.4 and 37 °C. The data show a first order loss of D-imide (half-life, 2.3 h) and D-imide (half-life, 4.6 h). The data for the L-imide can be compared with a half-life value of 3.0 h previously obtained for this peptide in 0.2 M sodium HEPES at pH 7.4, 37 °C (17). The increased stability of the D-imide relative to the L-imide has also been found for the succinimidyl forms of tetragastrin (Trp-Met-Asp-Phe-NH₂) (23).

Hydrolysis of the imidyl peptide appeared to exclusively produce the isoaspartyl and normal aspartyl peptides. Fig. 4B diagrams the corresponding rates of isoaspartyl and normal aspartyl formation from the succinimide at 37 °C. In addition to demonstrating the difference in hydrolysis rates for the D- and L-succinimide, these data suggest that the ratio of the products formed is slightly different in each case. The ratio of isopeptide to normal peptide formed at 37 °C from L-imide hydrolysis is 3.5:1 while that from D-imide hydrolysis is 3.1:1. Similar experiments were also performed at other temperatures. These data are summarized in Fig. 4C and indicate values of the activation energies for the reactions involved in isopeptide and normal peptide formation from the imide peptides. These values are 22.4 and 22.5 kcal/mol, respectively, from the L-imide and 25.9 and 25.4 kcal/mol from the D-imide. It is significant to note the large difference between the activation energies when L- and D-imide is hydrolyzed. Thus, although the rate of L-imide hydrolysis is 1.9 times that of the D-imide at 37 °C, at 74 °C it can be expected that they will be equal.

Racemization of the Hexapeptide Succinimide—Racemization at the α carbon of an amino acid residue is dependent upon the removal of this carbon atom's proton. The resulting carbanion is stabilized by resonance structures placing electron density on the α-carbonyl group of the peptide bond. This resonance is limited by a competing resonance in which the peptide bond nitrogen resonates with the α-carbonyl. In a succinimide-containing peptide, this peptide bond nitrogen is able to resonate not only with the α-carbonyl group, but with the β-carbonyl group as well. This additional resonance reduces competition between a potential carbanion and the nitrogen for placing electron density on the α-carbonyl. The increased ability of a carbanion to resonate with the α-carbonyl would be expected to enhance the rate of racemization at the succinimidylic residue.

To analyze the racemization rate of the imidyl peptides,
Spontaneous Degradation at Aspartyl and Asparaginyl Residues

A, deamidation of the L-Asn and D-Asn peptide at 37 °C, pH 7.4. The observed products consisted of the isoaspartyl and normal aspartyl derivatives (Fig. 1). The rate constants of deamidation (0.0204 ± 0.0023 h⁻¹ for the L-peptide and 0.0136 ± 0.0013 h⁻¹ for the D-peptide) were determined from the slope of the linear regression least squares line for the first-order process. Errors are given as 95% confidence limits determined by the Student's t test. Simultaneous incubations of the D- and L-peptides confirmed the differences in the rate of deamidation of these diastereomers.

B, rate constants determined at 70 and 85 °C are shown along with the 37 °C data as an Arrhenius plot. Error bars show the 95% confidence limits. The activation energy obtained from these data for the D-asparagine peptide is 21.2 kcal/mol.

Fig. 3. Kinetics of the deamidation of the Asn-Gly peptide at various temperatures. Incubations were performed and the products analyzed by HPLC as shown in Fig. 2 and described under "Materials and Methods." A, deamidation of the L-Asn and D-Asn peptide at 37 °C, pH 7.4. The observed products consisted of the isoaspartyl and normal aspartyl derivatives (Fig. 1). The rate constants of deamidation (0.0204 ± 0.0023 h⁻¹ for the L-peptide and 0.0136 ± 0.0013 h⁻¹ for the D-peptide) were determined from the slope of the linear regression least squares line for the first-order process. Errors are given as 95% confidence limits determined by the Student's t test. Simultaneous incubations of the D- and L-peptides confirmed the differences in the rate of deamidation of these diastereomers. B, rate constants determined at 70 and 85 °C are shown along with the 37 °C data as an Arrhenius plot. Error bars show the 95% confidence limits. The activation energy obtained from these data for the D-asparagine peptide is 21.2 kcal/mol.

Fig. 4. Analysis of imide hydrolysis kinetics. Both D- and L-succinimide-containing peptides were incubated at various temperatures. The resulting products were analyzed by an HPLC gradient of either 0–40% solvent B in 40 min or 0–15% solvent B in 35 min (see "Materials and Methods"). A, total peptide present as imide is plotted as a function of time at 37 °C, pH 7.4. Imide hydrolysis demonstrating first-order kinetics is observed with the formation of normal and isoaspartic acid products. Linear regression analysis of these data show a rate of hydrolysis with 95% confidence limits for the L-imide peptide of 0.239 ± 0.023 h⁻¹ and a corresponding rate for the D-imide peptide of 0.194 ± 0.010 h⁻¹. B, the rate of accumulation of normal and isoaspartic acid peptides from imide incubated at 37 °C is shown. The concentrations of iso and normal peptides as a function of time are given by the equation, \( \text{[normal, iso]}_{\text{t}} = \left( k_{\text{iso}} \right)_{\text{t}} \left( 1 - e^{-k_{\text{iso}} t} \right) + \left( \text{normal, iso} \right)_{0} \). In this equation, \( k_{\text{iso}} \) corresponds to the rate of imide hydrolysis (as determined in A) and \( k \) to the respective rate of either iso or normal aspartyl peptide formation. The slopes of the lines thus correspond to the respective rate constants, \( k \). Symbols are: ○, iso peptide derived from L-imide; □, normal peptide derived from L-imide; Δ, iso peptide derived from D-imide; Δ, normal peptide derived from D-imide. The respective rates of normal and iso peptide accumulation from L-imide at 37 °C are 0.0643 ± 0.0042 h⁻¹ and 0.236 ± 0.007 h⁻¹. Those from D-imide are 0.0375 ± 0.0029 h⁻¹ and 0.116 ± 0.008 h⁻¹. C, the temperature dependence of the rate constants for iso and normal hexapeptide accumulation upon imide incubation is shown in an Arrhenius plot.
samples of the L- and D-epimers were incubated at 37 °C, pH 7.4, and analyzed by HPLC under conditions where these diastereomers are resolved. Data are shown as the percentage of imide present in the epimerized form. The rate constant of imide racemization was determined from the least squares fit of a computer model (smooth curves) accounting for the imide hydrolysis reactions to the observed data. We used an iteration program with small time steps and the known rate constants of L- and D-imide hydrolysis to obtain solutions to the differential equations describing simultaneously imide racemization and hydrolysis. Both D- and L-imide peptides were found to epimerize with the same rate constant of 0.0178 h⁻¹. The racemization half-life is given by ln₂/2kₘᵣᵣ = 19.5 h. The deviations in the curve seen with time result from the differential stabilities of the two diastereomers to hydrolysis.

Isomerization of Val-Tyr-Pro-Asp-Gly-Ala—The results described above with the asparagine-containing hexapeptides indicated that deamidation occurred by facile succinimide formation. Succinimides can also form directly from aspartyl residues (Fig. 1), and we were interested in determining the relative rate of this reaction. For this reason, we incubated the hexapeptide corresponding in sequence to the asparagine peptide but which contained an aspartic acid residue in place of the asparagine residue. The results of these experiments showed that the aspartyl peptide was the major product. Since the formation of this peptide can only be explained by a mechanism involving a succinimide intermediate, the data were fit by modeling the following reaction.

\[
\text{Aspartyl peptide} \xrightleftharpoons[k_{\text{d,normal}}]{k_{\text{l,normal}}} \text{sucinimide} \xrightleftharpoons[k_{\text{l,iso}}]{k_{\text{d,iso}}} \text{isoaspartyl peptide}
\]

Because we found that the equilibrium ratio of normal peptide/isoaspartyl peptide formed in these experiments was approximately the same as that obtained when the purified succinimidyl peptide was hydrolyzed, we could assume that \( k_{\text{l}} \) was also approximately equal to \( k_{\text{d}} \).

\[
K_{\text{eq}} = \frac{(k_{\text{l,normal}})/(k_{\text{d,normal}})}{(k_{\text{l,iso}})/(k_{\text{d,iso}})}
\]

This approximation was confirmed experimentally by incubating the purified isoaspartyl peptide and comparing its rate of isomerization with that of the purified normal aspartyl peptide.

The loss of L-normal aspartyl peptide at 37 °C, pH 7.4, is shown in Fig. 6A. Based on these data, the rate of L-aspartyl peptide isomerization (half-time, 53 days) is about 38 times slower than the corresponding rate of the asparaginyl peptide’s deamidation (half-time, 1.4 days). HPLC analysis revealed that the loss of the normal aspartyl peptide was matched by an equal gain in the isoaspartyl peptide. The steady-state concentration of the succinimide intermediate (calculated to be about 0.2%) was too low to detect. The temperature dependence of the racemization reaction is shown in Fig. 6B for both the L- and D-aspartyl peptide.

Overall Simulation of Deamidation, Isomerization, and Racemization Reactions of the Aspartyl and Asparaginyl Hexapeptides—Using the rate constants summarized in Fig. 7, we used a computer iteration procedure to predict the course of these reactions over extended times. Fig. 8 shows the calculated appearance of degradation products of the L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala peptide at pH 7.4, 37 °C. Loss of the asparagine peptide is accompanied by the accumulation of the iso and normal peptide. This accumulation, however, occurs only after a lag period because the immediate product of the deamidation event is the succinimide, which accumulates to a maximal concentration of 5.9%. The racemization of this succinimide intermediate leads to the production of D-aspartyl and D-isoaspartyl peptides. After 6 days, the calculated composition included 1.2% D-aspartyl, 3.7% D-isoaspartyl, 19.1% L-aspartyl, and 70.2% L-isoaspartyl peptide.

Fig. 9 shows the results of an analogous model for the degradation of the aspartyl peptide, L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala at 37 °C, pH 7.4. Although the degradation of this peptide occurs more slowly than the asparaginyl peptide, significant amounts of racemized and isomerized products accumulate as well. After 6 days, 6.6% of the L-normal peptide has been converted to other species. The major product is the L-isoaspartyl peptide (6.0%). Other species include the D-isoaspartyl (0.8%), the L-imidyl (0.2%), the D-aspartyl (0.1%), and the D-imidyl peptide (0.02%). It is worthy to note that at equilibrium this model predicts that only 28% of the total concentration of peptides will be in the epimerized D-form.

This suggests that it may often be incorrect to presume a 1:1 equilibrium D/L ratio in analyses of racemization at aspartate residues in peptides and proteins.

The models shown in Figs. 8 and 9 assume that the succinimidyl peptide is the only species that undergoes racemization and that no racemization of the asparaginyl, aspartyl, isoaspartyl, or other possible species occurs. To test the validity of this assumption, we measured the content of D- and L-aspartic acid after hydrolysis of incubation mixtures of the aspartyl hexapeptide (Fig. 10). These data reflect the contribution of all racemization pathways. We then compared these experimental findings with the amount of D- and L-aspartic acid predicted to be present by a model in which racemization only occurs at the succinimide intermediate. The results show that greater than two-thirds of the total racemization can be attributed to the succinimide. The remainder of the observed
Fig. 6. Isomerization of the normal Asp-Gly hexapeptide. Incubations of the L- and D-normal aspartyl peptide at various temperatures were analyzed by HPLC as described under "Materials and Methods." Computer modeling was utilized to determine the value k = k(2) (see text). A, a least-squares fit of the data for isomerization of L-normal peptide incubated at 37°C, pH 7.4, is shown, in which k = 0.000551 hr⁻¹. B, an Arrhenius plot of the rate constants derived from incubations of L-normal peptide at 60 and 100°C and D-normal peptide at 100 and 70°C. The calculated activation energy for kL for the L-peptide was 21.7 kcal/mol and for the D-peptide was 21.1 kcal/mol. The calculated half-life for D-aspartyl peptide isomerization at 37°C, pH 7.4, was 40 days.

Fig. 7. Kinetics of spontaneous deamidation, isomerization, and racemization of the L-Val-L-Tyr-L-Pro-(Asn,Asp)-Gly-L-Ala hexapeptide at 37°C, pH 7.4. Kinetic constants were determined from extrapolations of the Arrhenius plots previously shown, except for those of imide racemization (which are derived from the data presented in Fig. 5) and those of asparagine deamidation (which are taken from the data of Fig. 3A). Values given in parentheses below each peptide indicate that peptide's calculated relative concentration if the system is allowed to reach equilibrium.

Deamidation, isomerization, and racemization may be the product of direct proton abstraction from the α-carbon of the aspartyl and isoaspartyl peptides or occur via the formation of small amounts of other racemization-prone intermediates such as oxazolones or isoimides (see "Discussion").

Asparaginyl Deamidation in Related Peptide Sequences—In the experiments presented so far we have utilized peptide sequences in which a glycyl residue follows the asparaginyl or aspartyl residue. We were then interested in examining the rates of the degradation reactions in sequences where bulky side chains may sterically hinder succinimide formation. To determine the magnitude of this steric effect, we measured the loss upon incubation of the peptide L-Val-L-Tyr-L-Pro-L-Asn-L-Leu-L-Ala. This peptide is identical to the asparagine hexapeptide previously used except for the replacement of the glycyl residue by a bulky leucyl residue. We found that the loss of this peptide at 100°C, pH 7.4, as measured by HPLC, follows first-order kinetics and has a half-life of 4.9 hr (Table I). This half-life can be compared to that extrapolated for the glycyl peptide of 0.15 hr under these conditions and suggests that a factor of about 33 can be expected for the steric effect. Interestingly, 14% of the reaction products was composed of a tetrapeptide sequence Val-Tyr-Pro-Nx. This peptide may represent the product of the attack of the α-amide nitrogen on the peptide bond carbonyl to release a Leu-Ala dipeptide and form a COOH-terminal succinimide (Fig. 11; cf. Refs. 16 and 24). This succinimide can then be further modified by hydrolysis and deamidation.

Finally, we measured the degradation rate of the peptide L-Val-L-Tyr-L-Pro-L-Asn-L-Pro-L-Ala at 100°C and pH 7.4. In this peptide, the replacement of the glycine residue by a proline residue has the consequence that succinimide formation from the attack of the peptide bond nitrogen, as was seen in the Asn-Gly peptide, may be limited both by the bulk of the side chain and by the absence of a hydrogen atom on this nitrogen. If this attack does occur, a charged quaternary amide group will result. Loss of this asparagine peptide at 100°C has a half-time of 7.5 hr (Table I). The major product of this reaction appears to be identical in composition to the tetrapeptide cleavage product found for the leucyl peptide. We do not detect such cleavage with the Asn-Gly peptide, probably because the deamidation reaction resulting in aspartyl and isoaspartyl residues is much more rapid and results in the loss of the side chain amide nitrogen which would participate in such a cleavage reaction.

DISCUSSION

Deamidation, isomerization, and racemization of aspartyl and asparaginyl residues appear to be major pathways of spontaneous structural damage in cellular proteins. Previous work has focused on deamidation because it can readily be detected by changes in the electrophoretic mobility of the protein. In a few cases, the deamidation of specific asparagine residues has been linked to changes in protein function. For example, deamidation at two Asn-Gly sequences in triosephosphate isomerase results in subunit dissociation (25), deamidation at an Asn-Gly site in hemoglobin Providence changes the oxygen affinity (26), and deamidation of an Asn-Asp site in human growth hormone alters its proteolytic cleavage properties (27). It has often been assumed that deamidation occurs via a direct hydrolytic mechanism that results in the formation of a normal aspartyl residue. However, the formation of β-isomerized aspartyl residues in peptides and proteins (3, 6–10) as well as the facile formation of
spontaneous degradation at aspartyl and asparaginyl residues

FIG. 8. Mathematical simulation of the degradation of L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala at 37 °C, pH 7.4. A computer program consisting of 30,000 iterative steps was used to model the deamidation, isomerization, and racemization reactions described in Fig. 7 (see "Materials and Methods"). At the initial time the model assumes all species are absent except for the L-Asn peptide which has a relative concentration of 100%.

FIG. 9. Mathematical simulation of the degradation of L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala at 37 °C, pH 7.4. A computer analysis, using the rate constants shown in Fig. 7, was performed similar to that shown in Fig. 8 but consisting of 90,000 steps. It was assumed that 100% of the material was initially present as the L-normal aspartyl hexapeptide.

Succinimides from peptide aspartyl β-esters (10, 12, 15, 17, 23, 28) suggests that succinimides, which can be hydrolyzed to a mixture of normal and isomerized proteins, may participate in deamidation reactions.

The results presented in this paper show that peptide deamidation under physiological conditions (pH 7.4, 37 °C) does in fact proceed essentially completely through a succinimide intermediate and suggest that this mechanism may be applicable to proteins as well. The rapid rate of deamidation apparent in the adrenocorticotropic hormone fragment measured here (1.4 days at 37 °C, pH 7.4) points to the inherent instability of asparagine residues. However, this rate probably represents the maximal rate of deamidation to be expected in proteins. The presence of a bulky residue following the asparagine residue can reduce the relative rate of degradation at 100 °C, as we have shown here, by a factor of 33–50. Additionally, the conformational flexibility of the short peptides studied allows for the peptide to assume the dihedral angles of $\psi = -120^\circ$ and $\chi_1 = +120^\circ$ necessary for succinimide formation. Succinimide formation in proteins may be limited if the main chain and side chain groups are not already in this conformation or if they do not have the flexibility to assume it. In fact, this particular conformation is relatively rare in proteins because both the main chain angle of $\psi = -120^\circ$ and the side chain angle of $\chi_1 = +120^\circ$ are energetically unfavorable, largely because of the steric hindrance between the hydrogen atom on the peptide bond nitrogen and the side chain γ carbon atom (29, 30). Finally, other features of protein structure, such as asparagine glycosylation or the structure of the amino acid preceding the asparagine residue, may influence the deamidation rate.

It is conceivable that glutamine residues can deamidate by a mechanism similar to that of asparagine residues and form a glutimide intermediate. The rate of glutamine deamidation in peptides, however, has been found to be slower than for asparagine peptides (2). Studies conducted with esters and hydroxamic acids of glutamic acid and aspartyl peptides also indicate that the corresponding reactions are slower for glutamyl residues (16, 28).

Isoaspartyl formation from aspartyl residues via a succinimide intermediate probably does not result in a change in the electrophoretic mobility of a protein. Thus, it may have previously escaped detection. The results of the present study indicate, however, that this process can also occur at significant rates, although much slower than with asparaginyl residues. The rate of this reaction may be enhanced by factors which increase the degree of protonation of the aspartyl $\delta$-carboxyl group such as an increase in its $pK_a$ value or its degree of hydrogen bonding.

Although there is no direct proof that isoaspartyl residues exist in cellular proteins, two lines of evidence suggest that these residues may be normally present in low amounts. First,
isoaspartate di- and tripeptides are constituents of normal urine and have been isolated from proteolytic digests of isolated proteins (3, 6, 31–33). Although other suggestions have been made for the origin of these peptides (3, 34, 35), it is reasonable to suggest that they at least partially represent the nondigestible portion of isoaspartyl-containing proteins. Second, the wide occurrence of cellular protein carboxyl methyltransferases which catalyze the methyl esterification of L-isoaspartyl residues but not of normal L-aspartyl residues in peptides suggests that these residues exist in cellular proteins (5, 9, 18).

The rate of racemization at aspartyl and asparaginyl residues has been extensively studied (4). Measurements have been made for a few classes of proteins in intact cells. Expressed in terms of the time required to generate 1% of these residues in the D-configuration, values of 3000–4000 days have been found for proteins from dentin, tooth enamel, and eye lens (36–38) and about 39 days for human erythrocyte membrane proteins (39). However, the mechanisms of these processes have not been determined. The results of the present study suggest that a major factor in this racemization is intermediate succinimide formation. Although these intermediates would be present at very low levels, their rapid rate of racemization (the time required for 1% racemization would only be 17 min at physiological pH and temperature for the L-hexapeptide succinimide (Fig. 5)) could account for the accumulation of D-residues. Based on succinimide racemization, incubation of the Asn-Gly hexapeptide at 37 °C, pH 7.4, would result in a 1% racemization in only 0.5 days, whereas incubation of the Asp-Gly peptide would form 1% racemized products in 14 days. If succinimides are the major racemizing species, one would expect that racemized aspartyl residues in proteins may be present largely as D-isoaspartyl residues which are the major products of D-succinimide hydrolysis. These D-isoaspartyl residues would be converted to D-aspartic acid when acid hydrolyzed for standard DL-aspartate analysis. Other mechanisms of racemization include direct abstraction of a protein from an aspartyl or asparaginyl residue, the formation of a five-membered isomide ring (created by the nucleophilic attack by the peptidyl carbonyl oxygen on the side chain carbonyl group), or the formation of an oxazolone ring (created by nucleophilic attack on the free carboxyl group of an isoaspartyl residue by the peptide oxygen of the preceding residue) (4, 40, 41). The extent to which each of these different mechanisms may contribute to racemization of aspartyl and asparaginyl residues in proteins has not been established.

**TABLE I**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>( t_{1/2} ) 100°C, pH 7.4</th>
<th>( t_{1/2} ) 37°C, pH 7.4</th>
<th>Relative degradation rate</th>
<th>Product distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala</td>
<td>0.15 ( \text{h} )</td>
<td>1.4</td>
<td>76</td>
<td>Isopeptide (73%), normal peptide (27%) ( a )</td>
</tr>
<tr>
<td>L-Val-L-Tyr-L-Pro-L-Asn-L-Leu-L-Ala</td>
<td>4.9 ± 0.7 ( \text{h} )</td>
<td>70</td>
<td>1.5</td>
<td>Isopeptide (64%), normal peptide (22%), tetrapeptide cleavage product(s) (14%) ( a )</td>
</tr>
<tr>
<td>L-Val-L-Tyr-L-Pro-L-Asn-L-Pro-L-Ala</td>
<td>7.5 ± 0.7 ( \text{h} )</td>
<td>106</td>
<td>1</td>
<td>Tetrapeptide cleavage product(s) ( a )</td>
</tr>
<tr>
<td>L-Val-L-Tyr-L-Pro-L-Asp-Gly-L-Ala</td>
<td>3.1</td>
<td>53</td>
<td>2</td>
<td>Isopeptide (79%), normal peptide (21%) ( a )</td>
</tr>
</tbody>
</table>

* Based on data for succinimide hydrolysis.

**FIG. 10. Comparison of observed and predicted racemization of the Asn-Gly hexapeptide.** L-Val-L-Tyr-L-Pro-L-Asn-Gly-L-Ala was incubated at 37 °C, pH 7.4. The circles represent data obtained by D/L-aspartate analysis of hydrolysates of the total reaction mixture. The line represents the predicted racemization from a model utilizing the kinetic constants shown in Fig. 7, where the only racemizing species is the succinimide form of the peptide. The shaded area represents the 95% confidence limits for this line, based on the error observed for the rate constants of imide hydrolysis (Fig. 4B).
The results of the present study suggest that deamidation, isomerization, and racemization reactions at aspartyl and asparaginyl residues is significant on a time scale relevant to cellular proteins. Although some proteins have half-lives short enough that significant accumulation of altered aspartyl residues would not occur, other proteins have half-lives on the order of the times shown in Figs. 8 and 9. At these times, significant amounts of deamidated, isomerized, and racemized products are present in the peptides studied and may likewise be present in cellular proteins. The effects of these reactions would be expected to be even more important in tissues such as human red blood cells and eye lens cells. The capacity for new protein synthesis in these cells is extremely limited, and the proteins must function for the entire 120-day lifetime of the erythrocyte or the age of the individual for the eye lens (42). It should be stressed that a typical protein of M, 100,000 may contain 100 aspartyl and asparaginyl residues, and an overall content of 1% damaged residues may reflect a population where every polypeptide chain is altered.

Our results indicate that the structural alterations observed in peptides (and presumably in proteins) due to the processes of deamidation, isomerization, and racemization are linked by a common succinimide intermediate. Thus, deamidation can be accompanied by simultaneous isomerization of the peptide linkage and racemization at the α-carbon. It may be important, therefore, to consider that all of these events have the same origin and lead to the accumulation of the same group of products.

The fate of deamidated, isomerized, or racemized aspartyl and asparaginyl residues in proteins is not clear. These species may accumulate and eventually result in the loss of the functional capacities of the cell. They may also be recognized by the cellular degradation apparatus, targeting the affected protein for specific proteolysis. Finally, it is possible that at least partial repair of these damaged residues may occur. One specific mechanism for repair involves the enzymatic methylation of α-carboxyl groups on L-isoaspartyl residues (9, 18) or of β-carboxyl groups of D-aspartyl residues (11). These protein methyl esters may rapidly demethylate to form succinimides (10, 17, 43) which can then be hydrolyzed to give normal aspartyl peptides. Evidence for this type of conversion of L-isoaspartyl peptides to normal aspartyl peptides has been recently presented (44, 45). The cellular accumulation of altered residues may also be limited by the evolution of protein structures to give conformations that cannot readily form succinimides. Indeed, deamidation, isomerization, and racemization due to succinimide formation may only occur once a protein is partially denatured. Nevertheless, evidence of racemization, isomerization, and deamidation in cellular proteins suggests that these spontaneous processes continuously affect living organisms and may require the repair or degradation of the affected protein molecules.

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Note Added in Proof—Recent work has demonstrated an in vitro deamidation half-life of 21.7–37.8 days for two Asn-Gly sequences in human triose phosphate isomerase at pH 7.0, 37 °C (47). Assuming that the deamidation rate is proportional to hydroxide ion concentration, these half-times would be 8.6–15.0 days at pH 7.4 and would be longer than the half-time of 1.4 days measured for the Asn-Gly hexapeptide in this study. Although it has not been demonstrated that the deamidation of this protein occurs via a succinimide mechanism, conformational restraints on imide formation in the protein might account for the different half-times.

REFERENCES
Spontaneous Degradation at Aspartyl and Asparaginyl Residues


