Succinimide Formation from Aspartyl and Asparaginyl Peptides as a Model for the Spontaneous Degradation of Proteins*

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Nonenzymatic intramolecular reactions can result in the deamidation, isomerization, and racemization of protein and peptide asparaginyl and aspartyl residues via succinimide intermediates. To understand the sequence dependence of these reactions, we measured the rate of succinimide formation in a series of synthetic peptides at pH 7.4. These peptides (Val-Tyr-Pro-X-Y-Ala) contained an internal aspartyl, asparaginyl, aspartyl β-methyl ester, or aspartyl α-methyl ester residue (X) followed by a glycyl, seryl, or alanyl residue (Y). The rates of succinimide formation of the asparaginyl peptides were found to be 13.1–35.6 times faster than those of the aspartyl peptides. The rates of succinimide formation for the glycyl peptides were 6.5–17.8 times faster than those of the alanyl peptides, while the rates for the seryl peptides were 1.6–4.5 times faster than those of the alanyl peptides. The overall 232-fold range in these reaction rates for aspartyl and asparaginyl residues suggests that sequence can be an important determinant in their stability in flexible peptides. In proteins, there may be a much larger range in the rates of succinimide formation because specific conformations may greatly enhance or inhibit this reaction.

Proteins are subject to a variety of spontaneous degradation reactions under physiological conditions that can limit their useful lifetime (1, 2). Aspartyl and asparaginyl residues are particularly susceptible to nonenzymatic degradation because intramolecular succinimide-forming reactions at these sites lead to their isomerization, racemization, and in the case of asparaginyl residues, deamidation and cleavage as well (3–7) (Fig. 1). Because these reactions occur slowly, their products are generally present in substoichiometric amounts making it difficult to chemically identify specific sites where such alterations have occurred in native proteins. For this reason, the relative importance of the factors that contribute to succinimide formation are not yet fully understood. These factors include both the nature of the residues adjacent to the aspartyl or asparaginyl residue and the three-dimensional conformation of these groups (8, 9).

Evidence from two types of model systems has been used in attempts to predict the potential sites of succinimide formation in proteins. The first model system involves the succinimide-dependent cleavage of proteins by hydroxylamine. When this reaction is performed under denaturing conditions at alkaline pH, succinimide formation in polypeptides is largely limited to the Asn-Gly sequence (10–12). This model would suggest that such sequences may represent the major sites of succinimide formation in native proteins as well. The importance of the flanking glycine residue has been attributed to the absence of steric hindrance in the attack of the peptide bond nitrogen on the side chain carbonyl required for succinimide formation (11).

The second model system involves the base-catalyzed hydrolysis of peptidyl aspartyl β-benzyl esters. Such peptides form succinimides much more readily than their asparaginyl or aspartyl counterparts and provide useful models for determining the chemical factors that control the rate of succinimide formation (13–15). Interestingly, in at least one series of experiments, aspartyl β-benzyl ester-containing peptides having a threonine or serine residue following the aspartyl ester formed succinimides three to six times faster than when the ester was followed by a glycine residue (15). These studies suggest that a hydroxyl group on the following residue may catalyze succinimide formation but do not explain why such interactions apparently do not occur in proteins subjected to hydroxylamine cleavage.

Neither of the above models can satisfactorily explain the distribution of deamidated and isomerized residues in cellular proteins. For example, deamidation has been found to occur not only in Asn-Gly (16, 18, 19) and Asn-Ser sequences (9, 17) but in a variety of other sequences including Asn-Asp (20, 21), Asn-Glu (22), Asn-His (23), Asn-Ala (24), Asn-Ile (25), and Asn-Met (24, 26). These results suggest that succinimide formation may not be limited to Asn-Gly or Asn-Ser sequences. Additionally, evidence for isoaspartyl residues derived from aspartyl residues in glucagon (Asp-Tyr (27)) and affinity-purified calmodulin (Asp-Gln, Asp-Thr (28)) suggests that succinimides may form from aspartyl as well as asparaginyl residues. Since the previous model studies were performed under different conditions, it is difficult to compare them directly. In this work, we have attempted to use a single peptide model system to explore the rates of succinimide formation at aspartyl, aspartyl ester, and asparaginyl residues. We wanted to understand why succinimides form at specific sites in peptides and proteins and not at others. While it is clear that specific conformations in proteins can markedly affect succinimide formation (8, 9), the rate of this reaction in peptides may provide an estimate for the propensity for succinimide formation at aspartyl and asparaginyl residues in the more flexible regions of proteins.

* This work was supported by Grant DMB-8602102 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
‡ Supported in part by United States Public Health Service Research Service Award GM 07185.

MATERIALS AND METHODS

Synthetic Peptides—Val-Tyr-Pro-Asn-Gly-Ala, Val-Tyr-Pro-Asn-Ala-Ala, and Val-Tyr-Pro-Asn-Ser-Ala were synthesized by Dr. Janis
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Young at the UCLA Peptide Synthesis Facility using an Applied Biosystems model 430A instrument and hydrofluoric acid cleavage. All amino acids except for Gly were in the L-configuration. Their compositions were confirmed by amino acid analysis. Val-Tyr-Pro-Asp-Gly-Ala, Val-Tyr-Pro-Asp-Ala-Ala, and Val-Tyr-Pro-Asp-Ser-Ala were prepared by base hydrolysis of the corresponding asparagine peptide at 37 °C in a 100 mM sodium borate buffer at pH 10.0. The Ser- and Ala-containing peptides were incubated for 90 h, and the Gly-containing peptide was incubated for 4 h. This procedure yielded two products from each asparaginyl peptide, the normal aspartyl and isoasparyl derivatives in a ratio of about 1:3. These were purified as described below.

Chemical Methylation—Aspartic acid β-methyl ester-containing peptides were synthesized by chemical methylation following the procedure of McPadden and Clarke (29). Purified asparyl peptides were dissolved in methanol, and 12 M HCl was added to a final concentration of 0.1 N HCl. This solution was incubated overnight at 23 °C in the dark. The mixture was then diluted with 5-6 volumes of 0.2% (w/v) trifluoroacetic acid, and the solvent was removed under vacuum in a Savant Speedvac apparatus at room temperature. This method results in the methylation of both the aspartyl side chain and COOH-terminal carboxyl group. These products were purified as described below.

Enzymatic Methylation—The isoasparyl-containing peptides were methylated at the α-carboxyl of the Asp residue by incubation with S-adenosyl-L-[methyl-3H]methionine and the ammonium sulfate fraction of protein L-isoasparyl/D-aspartyl methyltransferase from human erythrocyte cytosol (30). Enzyme (15 μl, 809 pmol of methyl groups transferred to ovalbumin per min per ml of enzyme) and S-adenosyl-L-[methyl-3H]methionine (10 μl, 50 μM, 500 cpm/pmol) in 200 mM sodium citrate buffer at pH 6.00 were added to the isopeptide fraction of protein (10 pl, 500 cpm/pmol) in 200 μl of HzO, and 4 ml of ASCII counting fluid (Amersham Corp.) was added. Radioactivity was determined by liquid scintillation counting to quantitate the amount of methyl groups remaining as esters in the peptides.

The elution patterns on reverse-phase HPLC for the products of Asn-Gly and Asp-Gly peptide incubations have been characterized previously (5). The -Asn-Gly- containing peptide cyclizes to form a more hydrophobic succinimide which is eluted several minutes later than the corresponding amide. The succinimide is hydrolyzed to give the less hydrophobic aspartyl and isoaspartyl derivatives in a ratio of 1:2.7. Similar results were obtained with the X-Ala (ratio = 1:3.0), and their breakdown products were identified by similar methods. Methylated peptides were found to elute about 5 min later for each methyl group incorporated. Typical elution times in minutes for the Val-Tyr-Pro-X-Y-Ala peptides on the Econosorb analytical column are given below (Me refers to the COOH-terminal α-monomethyl) ester, diMe refers to the dimethyl ester where both carboxyl groups were esterified: -Asn-Gly-, 21.5; -isoAsp-Gly-, 24.8; -IsoAsp-Gly-, 19.3; -Asp-Gly-, 20.6; -Asg-Gly-diMe, 34.0; -isoAsp-Gly-diMe, 33.0; -Asn-Ala-, 22.1; -isoAsp-Ala-, 20.1; -Asp-Ala-, 21.0; -Asg-Ala-diMe, 34.2; -isoAsp-Ala-diMe, 33.3; -Asg-Ser-, 19.1; -isoAsp-Ser-, 18.4; -Asp-Ser-, 17.2; -Asg-Ser-diMe, 31.5; isoAsp-Ser-diMe, 31.9. The succinimide peaks for the Ala- and Ser-containing peptides were small and were not directly identified. The elution times for the reaction products of the methylated aspartyl peptides on the Waters Resolve column are: -Asp-Gly-diMe, 22.5; -isoAsp-Gly-Me, 19.9; -Asp-Ala-diMe, 23.5; -isoAsp-Ala-Me, 29.9; -Asp-Ala-Me, 15.7; -Asp-Ser-diMe, 21.5; -isoAsp-Ser-Me, 23.7; isoAsp-Ser-Me, 14.2; -Asg-Ser-Me, 15.2. Integrated peak areas from the Shimadzu C-R3A integrating recorder (method

1 The abbreviation used is: HPLC, high performance liquid chromatography.
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RESULTS

Routes for the Spontaneous Degradation of Asp- and Asn-containing Peptides and Proteins—We have measured the rate of degradation via succinimide formation at pH 7.4 of a series of hexapeptides containing an asparagine, aspartyl, or aspartyl methyl ester residue. These peptides are of the sequence Val-Tyr-Pro-X-Y-Ala, where X represents an Asn, Asp, or Asp methyl ester residue and Y is a glycine, serine, or alanine residue. The degradation of the Asn-Gly and Asp-Gly-containing peptides has recently been shown to occur via the pathway diagrammed in Fig. 1 where the initial formation of succinimides is followed by spontaneous hydrolysis to give both normal and isoaspartyl derivatives (5). We have now prepared methyl ester derivatives of these peptides as well as peptides corresponding to substitutions of the glycine residue with serine and alanine and have characterized the rate of their degradation via succinimide intermediates.

Uracil Formation from Aspartyl Peptides—Incubations of these peptides lead to the disappearance of the starting peptide and the appearance of new products. The chromatograms shown in Fig. 2 represent the reaction course of Val-Tyr-Pro-Asn-Gly-Ala after incubating for 0, 18, and 78 h in 100 mM sodium phosphate at pH 7.4 at 37 °C. Products, including the aspartyl and isoaspartyl peptides in a 1:3 ratio, were identified using synthetic standards prepared as described previously (5, 31). The patterns of elution on HPLC of the corresponding derivatives for the Ser- and Ala-containing peptides were essentially identical except that the Ser derivatives eluted about 2.5 min earlier and the Ala derivatives eluted about 0.5 min later (see "Materials and Methods"). The deamidation rate for both the Ala- and Ser-containing peptides was slow enough that no significant amount of succinimide accumulated. Incubations were made over the course of 89 h, and the amount of each peptide was determined from the area under the peak. For each of the three peptides, the amount of the initial peptide was determined as a percentage of the total peptide and plotted on a log scale versus time (Fig. 3, left). The data was fit by a line whose slope represents the first order rate constant. The rate of succinimide formation for the -Asn-Gly- peptide was found to be 17.6 times faster than the -Asn-Ala- peptide, and the -Asn-Ser peptide was 2.50 times faster than the -Asn-Ala- peptide at 37 °C. The rate for the -Asn-Gly- peptide was similar to that measured previously by Geiger and Clarke (5).

Isoaspartyl Formation from Aspartyl Peptides—Incubations of the aspartyl peptides at 70 °C were made in 100 mM sodium phosphate at pH 7.4 for times up to 77 h. The percentage of Asp relative to both Asp and isoAsp was plotted in Fig. 3 (right). Unlike the essentially irreversible reaction of the asparagine-containing peptides, this reaction represents the progress toward an equilibration of the aspartyl- and isoaspartyl-containing forms via a succinimide intermediate (5). For this reason, the rate constant for the conversion of the aspartyl peptide to the succinimide was derived by fitting the data to a computer simulation of this reaction using iterative integration as described under "Materials and Methods." In Table I, we have converted the rates determined at 70 °C to 37 °C for comparison with the other peptides. This was done in each case using the experimentally determined Arrhenius activation energy of 21.7 kcal/mol for the Asp-Gly-containing peptide (5). The rate of succinimide formation for the -Asp-Gly- peptide was 6.52 times faster than the -Asp-Ala- peptide, and the -Asp-Ser peptide was 1.58 times faster than the -Asp-Ala- peptide at 70 °C.

Degradation of Aspartyl β-Methyl Esters—Aspartyl peptides were methylated in acidic methanol and purified by HPLC to form methyl esters at both the β-carboxyl group on the Asp residue and the alanine COOH-terminal carboxyl group. Incubations at 37 °C in 100 mM sodium phosphate at pH 7.4 were made. The course of this reaction was followed by HPLC, and the results can be seen in Fig. 4 (left). The major product observed is the succinimide containing the COOH-terminal methyl ester. The rate of this reaction for the -Asp-β-methyl ester-Gly peptide was 10.8 times faster than the -Asp-β-methyl ester-Ala peptide, and the rate for the -Asp-β-methyl ester-Ser peptide was 2.88 times faster than the -Asp-β-methyl ester-Ala peptide at 37 °C. The loss
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FIG. 3. Rate of loss of a series of asparaginyl- and aspartyl-containing peptides. Peptides of composition Val-Tyr-Pro-X-Y-Ala where X represents a Asn (left) or Asp (right) residue and Y represents a Gly (O), Ala (C), or Ser (Δ) residue were incubated in 0.100 M sodium phosphate at pH 7.4 as described under "Materials and Methods." The course of the reaction was followed by HPLC (cf. Fig. 2). Left, data for the asparaginyl peptides incubated at 37 °C was fit to a line using linear regression analysis, and the slope of each line gave the first order rate constants (k, Fig. 1) of 0.0225 h⁻¹ for the -Asn-Gly-containing peptide, 0.00358 h⁻¹ for the -Asn-Ser-containing peptide, and 0.00143 h⁻¹ for the -Asn-Ala-containing peptide. Right, data for the aspartyl-containing peptides incubated at 70 °C was fit by computer modeling the reversible reaction Asp ⇌ imide ⇌ isoAsp as described under "Materials and Methods" used an increment of 0.1 h⁻¹. A curve was fit to the data using the least squares method, and a rate constant for the formation of succinimide from aspartyl peptide was determined to be 0.0210 h⁻¹ for the -Asp-Gly-containing peptide, 0.00510 h⁻¹ for the -Asp-Ser-containing peptide, and 0.00322 h⁻¹ for the -Asp-Ala-containing peptide. Each plot contains data from two separate experiments.

FIG. 4. Succinimide formation from aspartyl β-methyl esters and isoaspartyl α-methyl esters. Peptides corresponding to those used in Fig. 3 in which the Asn or Asp residues were substituted with Asp β- or isoAsp α-methyl esters and where the carboxyl-terminal alanine residue was also methyl-esterified were incubated at 37 °C in 0.10 M sodium phosphate buffer at pH 7.4 as described under "Materials and Methods." Left, the decomposition of the normal aspartyl diesters was followed by HPLC, and the first order rates were determined as described in Fig. 3 (left) to be: 11.2 h⁻¹ for the -Asp-Gly-diester peptide, 3.00 h⁻¹ for the -Asp-Ser-diester peptide, and 1.04 h⁻¹ for the -Asp-Ala-diester peptide. During the course of the reaction no loss of carboxyl-terminal methyl ester was detected. Right, the decomposition of isoaspartyl diesters was followed by the loss of radioactivity as described under "Materials and Methods." The first order rates were determined to be 18.8 h⁻¹ for the -isoAsp-Gly-diester peptide, 5.64 h⁻¹ for the -isoAsp-Ser-diester peptide, and 1.24 h⁻¹ for the -isoAsp-Ala-diester peptide. Each plot contains data from two separate experiments.

Degradation of Aspartyl α-Methyl Ester Peptides—The isoaspartyl peptides synthesized as described under "Materials and Methods" were all substrates for D-aspartyl/L-isoaspartyl protein carboxyl methyltransferase. These peptides were methylated on the α-carboxyl of isoAsp with protein carboxyl methyltransferase and S-adenosyl-L-[methyl-³H] methionine. This was followed by chemical methylation at the carboxyl-terminal Ala in acidic methanol to make these peptides homologous with the Asp-β-methyl ester peptides. Peptides were incubated at 37 °C in 100 mM sodium phosphate at pH 7.4. Aliquots were removed, and radioactive methanol was removed by lyophilization. The percentage of radioactivity remaining on the peptide was plotted to determine the rates of succinimide formation (Fig. 4, right). The rate of succinimide formation for the -Asp-α-methyl ester-Gly peptide was 15.2 times faster than that of the -Asp-α-methyl ester-Ala peptide, and the rate for -Asp-α-methyl ester-Ser peptide was 4.54 times faster than that of the -Asp-α-methyl ester-Ala peptide at 37 °C.

DISCUSSION

Relative Propensity for Succinimide Formation by Asparaginyl, Aspartyl, and Aspartyl Methyl Esters—For each of the peptides tested here, we found that succinimide formation in
**TABLE I**

<table>
<thead>
<tr>
<th>Peptide Models for Nonenzymatic Protein Degradation</th>
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<tr>
<td><strong>Half-lives of asparaginyl, aspartyl, and aspartyl α- and β-methyl ester peptide degradation</strong></td>
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<td>via succinimide formation at pH 7.4, 37 °C</td>
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All data are from this study unless indicated otherwise.

<table>
<thead>
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<th>Peptide</th>
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<tr>
<td>Val-Tyr-Pro-Asn-Gly-Ala</td>
<td>1.14, 1.40&lt;sup&gt;a&lt;/sup&gt; days</td>
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<td>Val-Tyr-Pro-Asn-Ala-Ala</td>
<td>20.2 days</td>
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<tr>
<td>Val-Tyr-Pro-Asn-Ser-Ala</td>
<td>8.0 days</td>
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<tr>
<td>Val-Tyr-Pro-Asn-Leu-Ala</td>
<td>70&lt;sup&gt;a&lt;/sup&gt; days</td>
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<td>Val-Tyr-Pro-Asn-Pro-Ala</td>
<td>106&lt;sup&gt;a&lt;/sup&gt; days</td>
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<td>Acetyl-Val-Asn-Gly-Ala</td>
<td>3.3&lt;sup&gt;b&lt;/sup&gt; days</td>
</tr>
<tr>
<td>Val-Tyr-Pro-Asp-Gly-Ala</td>
<td>40.8, 53&lt;sup&gt;a&lt;/sup&gt; days</td>
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<tr>
<td>Val-Tyr-Pro-Asp-Ala-Ala</td>
<td>266 days</td>
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<tr>
<td>Val-Tyr-Pro-Asp-Ser-Ala</td>
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<td>Val-Tyr-Pro-Asp-Me-Ala-Ala-Me</td>
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<td>Val-Tyr-Pro-Asp-Me-Ala-Ala-Me</td>
<td>39.9 min</td>
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<tr>
<td>Val-Tyr-Pro-Asp-Me-Ala-Ala-Me</td>
<td>13.9 min</td>
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<tr>
<td>Trp-Met-Asp-Phe-NH₂</td>
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<td>Gly-Ala-Me</td>
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<td>4.1, 4.3&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>Lys-Met-Lys-Asp-Me-Gln-Leu-Thr-Glu-Gln-Ile-Ala-Glu-Phe-Lys</td>
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<td>Ac-Ala-Asp-Me-Leu-Ala-Lys-Tyr</td>
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<td>Trp-Met-Asp-Me-Tyr-Ser-Lys</td>
<td>23&lt;sup&gt;f&lt;/sup&gt;</td>
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</table>

<sup>a</sup>Data from Ref. 5.
<sup>b</sup>Data from Ref. 29.
<sup>c</sup>Data from Ref. 32.
<sup>d</sup>Data from Ref. 28.
<sup>e</sup>Data from Ref. 33.
<sup>f</sup>J. I. Ota and S. Clarke, unpublished data.
<sup>g</sup>J. Lowenson and S. Clarke, unpublished data.
an asparaginyl-containing peptide was 13–36 times more rapid than that of the corresponding aspartyl-containing peptide (Table I). These results would suggest that the bulk of the succinimides formed in proteins would be derived from asparaginyl residues. However, this does not appear to be the case in glucagon (27) and in affinity-purified calmodulin (28) where the primary sites are derived from aspartyl residues. Data obtained with the aspartic acid β-methyl ester-containing peptides can provide a rationale for these observations. These esters form succinimides 10,000–17,000 times faster than the corresponding aspartyl peptides, and similar rate enhancements were found for isoaspartyl β-methyl esters (Table I). Although the OH− group from the carboxylic acid is as good or better a leaving group as the OR− group from the ester (34), the carboxylate oxygens are generally unprotonated in aqueous solution and are thus very poor leaving groups (35). Given a pKa value of 3.9 for the aspartyl side chain, only 1 out of 3000 carboxyl groups will be protonated at pH 7.4 and available for succinimide formation. The rate of succinimide formation from a protonated aspartyl residue in our peptides would therefore approach that of an ester and would be much more rapid than the amide. Thus, the ability of an aspartyl residue to form a succinimide may depend upon the effect of the local chemical environment on its protonation (27, 35).

Effect of the Carboxyl Flanking Residue on Succinimide Formation in Peptides—We determined the effect on succinimide formation of the residue on the carboxyl side of the aspartyl derivative (Tables I and II). We found that the most rapid succinimide formation in each peptide occurred when a glycine residue was present at this site. For the asparagine-containing derivatives, the rate of imide formation with the glycine peptide was 7.0 times that of the serine peptide and 17.6 times that of the alanine peptide. Similar respective rate enhancements of 4.1 and 6.5 were found for the aspartyl derivatives and 3.7 and 10.8 for the aspartyl β-methyl ester derivatives (Table I).

It has been proposed that the unique reactivity of Asn-Gly sequences exists because the glycine residue lacks a side chain and cannot sterically interfere with the attack of the peptide bond nitrogen atom on the side chain carbonyl carbon atom (11). If this were the case, we would predict an order of reactivity of Asn-Leu < Asn-Ser < Asn-Ala < Asn-Gly. However, our results show that the Asn-Ser peptide reacts more rapidly than the Asn-Ala peptide and that there appears to be a much smaller difference in rate between the Asn-Leu peptide and the Asn-Ala peptide (3.5-fold) than between the Asn-Ala peptide and the Asp-Gly peptide (17.6-fold) (Table I). Clearly, the first (β) carbon atom in the side chain has the greatest effect whereas the influence of additional atoms is less. These considerations suggest that other properties of glycine residues may be important in the rate of succinimide formation.

One unique property of Gly is the flexibility that it imparts to the peptide main chain (36, 37). One might speculate that the glycine-containing peptides could adapt more conformations favorable for reaction compared to peptides where the presence of a β-side chain carbon restricts the range of motion. Another factor may involve the electron-inductive effect of the side chain on the deprotonation of the peptide bond nitrogen that has been postulated to be necessary for the nucleophilic attack that forms the succinimide (13). The absence of electron-donating substituents on the glycine side chain can thus also contribute to the reactivity of Asx-Gly peptides (14, 38).

Specific chemical interactions of the side chain of the carboxyl-flanking residue may facilitate succinimide formation. Serine-containing asparagine, aspartyl, aspartyl β-, and α-methyl ester peptides form succinimides more rapidly than their alanine-containing counterparts by factors of 2.5-, 1.6-, 2.9-, and 3.3- (monomethyl) to 4.5-fold (dimethyl), respectively (Table I). Two modes of catalysis by the side chain hydroxyl group have been suggested previously. The hydroxyl oxygen atom of the serine residue can help deprotonate the peptide bond nitrogen and enhance its nucleophilicity (13, 39), or the hydroxyl hydrogen can bond to the asparagine side chain oxygen or nitrogen atoms and enhance the electrophilicity of the carbonyl carbon (9). Thus whether flanking seryl (13, 15) or glycylic residues (10–12) are more favorable for succinimide formation may depend on the effectiveness of the side chain hydroxyl catalysis.

Sequence Dependence of Succinimide Formation in Proteins—An enzyme present in all cells examined so far has been shown to catalyze the S-adenosylmethionine-dependent formation of β-aspartic acid β-methyl ester residues in proteins and L-isoaspartyl α-methyl ester residues in both peptides and proteins (42). Since both of these abnormal amino acid residues can be derived from succinimides, the analysis of specific sites of methylation in proteins may indicate the preferred sites of succinimide formation. Based on the peptide results above, we would expect Asn-Gly and Asn-Ser sequences to be major sites of methylation in proteins and peptides. Although this is the case with adrenocorticotrophic hormone (43), this has not been confirmed with studies on glucagon (27) or calmodulin (28). In glucagon, the major site of methylation is derived from an Asp-Tyr sequence in preference to Asp-Ser and Asn-Thr sequences. In affinity-purified calmodulin, the major sites are also derived from aspartyl sequences flanked by either a glutaminyl or a threonyl residue even though several Asp-Gly, Asn-Gly, and Asn-Ser sequences are present.

The reactivity to form succinimides from the flexible asparaginyl and aspartyl peptides in Table II spans a range of about 232-fold. The effect of the flanking residue alone is no more than 93-fold within each group of the aspartyl derivatives shown in Table I. Since constraining reactive groups to a limited range of orientations can increase reaction rates by factors as much as 53,000-fold (40, 41), an optimal alignment of the peptide nitrogen and side chain carbonyl oxygen in a three-dimensional protein structure (8, 9) would allow rapid succinimide formation even in sequences where this reaction occurs slowly in peptides. On the other hand, a rigid suboptimal alignment of these groups in proteins would result in a corresponding large reduction in the rate of succinimide formation. In fact, orientations of aspartyl and asparaginyl residues in proteins conducive to succinimide formation appear to be relatively rare (8). Because the conformational factors may be much greater than the sequence-dependent factors, the rate of degradation of protein asparaginyl and aspartyl residues may reflect largely the possibility of orienting the peptide nitrogen atom near the carbonyl carbon atom. Thus,

### Table II

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Succinimide Formation</th>
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<tbody>
<tr>
<td>Val-Tyr-Pro-Asx-(Gly,Ser,Ala/-Ala)</td>
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<td>Val-Tyr-Pro-Asx-Gly-Ala</td>
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<tr>
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<tr>
<td>Val-Tyr-Pro-Asx-Gly-Gly-Ala</td>
<td>232</td>
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Data are taken from Table I.
the inherent chemical susceptibility of Asn-Gly and Asn-Ser sequences to succinimide formation in peptides may be over-riden in proteins by conformational restraints. Interestingly, the sites of isoaspartyl formation in calmodulin appear to be located in flexible regions of the polypeptide (28, 44).

Acknowledgments—We thank Jonathan Lowenson for kindly providing D-isopropyl/L-isopropyl methyltransferase and Dr. Janis Young of the UCLA Peptide Synthesis Facility for preparing the asparaginyl-containing peptides.

REFERENCES