Neighboring Side Chain Effects on Asparaginyl and Aspartyl Degradation: An Ab Initio Study of the Relationship between Peptide Conformation and Backbone NH Acidity

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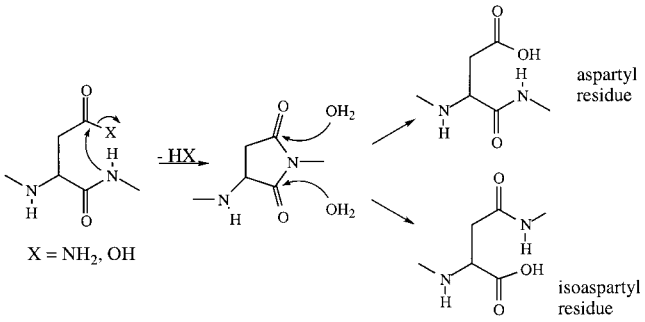
Abstract: The rate of spontaneous degradations of asparagine and aspartyl residues occurring through succinimide intermediates is dependent upon the nature of the residue on the carboxyl side in peptides. For nonglycine residues, we show here that this effect can largely be attributed to the electrostatic/inductive effect of the side chain group on the equilibrium concentration of the anionic form of the peptide bond nitrogen atom that initiates the succinimide forming reaction. However, the rate of degradation of Asn-Gly and Asp-Gly containing peptides is about an order of magnitude greater than predicted solely using this explanation. To understand the nature of the glycine effect, ab initio calculations were performed on model compounds. These calculations indicate that there is little to no change in the stability of the transition state or the tetrahedral intermediate of succinimide formation with Asn-/Asp-Gly and Asn-/Asp-Ala derivatives. However, we have found that the acidity of the backbone peptide nitrogen NH is highly dependent upon the conformation of the molecule. Since glycine residues lack the β-carbon common to all other protein amino acids, these residues can sample additional regions of conformational space where it is possible to further stabilize the backbone amide anion and thus increase the rate of degradation. These results provide the first rationale for the particular rate enhancement of degradation in peptidyl Asn-/Asp-Gly sequences. The results also can be applied to asparagine and aspartyl residues in proteins where the 3-dimensional structure provides additional constraints on conformation that can either increase or decrease the equilibrium concentration of the backbone amide anion and thus their rate of degradation via succinimide intermediates. Understanding this chemistry will assist attempts to minimize the deleterious effect of aging at the molecular level. The relationship between these results and proton exchange experiments is discussed in the Appendix.

Introduction

Asparaginyl and aspartyl residues in peptides and proteins can undergo spontaneous degradation reactions at neutral and alkaline pH to give isomerized and racemized derivatives.[1] Intramolecular formation of an aminosuccinimidyl peptide intermediate appears to be an obligatory first step in these reactions (Scheme 1).[2] The mechanism of succinimide formation involves deprotonation of the carboxyl-side backbone amide followed by attack of the now anionic nitrogen on the side chain carbonyl group (Scheme 2). General base catalysis of nucleophilic attack may also occur. Formation of the tetrahedral intermediate is followed by the rate determining loss of an amine to give the succinimide (Scheme 2).[3] For aspartyl residues, the side chain carboxyl group must be protonated for succinimide formation to occur, reducing the relative rate of aspartyl degradation compared to asparaginyl degradation.[2c,e] The succinimide intermediate is both racemization prone[4] and subject to spontaneous hydrolysis at either carbonyl group; these reactions generate the D/L-aspartyl and D/L-isoaspartyl residues as end products.[5] In a few cases, the succinimide residue is stable enough to be a major end product in itself, especially under mildly acidic conditions.[6] Recently, an X-ray crystal structure containing an *iso*-Asp residue generated by degradation was solved.[7]

Scheme 1



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(1) *Deamidation and Isoaspartate Formation in Peptides and Proteins*; Aswad, D. W., Ed.; CRC Press: Boca Raton, FL, 1995.

(2) (a) Meinwald, Y. C.; Stimson, E.; Scheraga, H. A. *Int. J. Pept. Protein Res.* **1986**, *28*, 79. (b) Geiger, T.; Clarke, S. *J. Biol. Chem.* **1987**, *262*, 785. (c) Stephenson, R. C.; Clarke, S. *J. Biol. Chem.* **1989**, *264*, 6164. (d) Patel, K.; Borchardt, R. T. *Pharm. Res.* **1990**, *7*, 703. (e) Capasso, S.; Mazzarella, L.; Sica, F.; Zagari, A.; Salvadori, S. *J. Chem Soc., Chem. Commun.* **1992**, 919. (f) Clarke, S.; Stephenson, R. C.; Lowenson, J. D. In *Stability of Protein Pharmaceuticals. Part A: Chemical and Physical Pathways of Protein Degradation*; Plenum Press: New York, 1992. (g) Brennan, T. V.; Clarke, S. *Protein Sci.* **1993**, *2*, 331.

(3) (a) Capasso, S.; Mazzarella, L.; Sica, F.; Zagari, A.; Salvadori, S. *J. Chem. Soc., Perkin Trans. 2* **1993**, 679. (b) Capasso, S.; Kirby, A. J.; Salvadori, S.; Sica, F. *J. Chem. Soc., Perkin Trans. 2* **1995**, 437.

(4) Radkiewicz, L. L.; Zipse, H.; Clarke, S.; Houk, K. N. *J. Am. Chem. Soc.* **1996**, *118*, 9148.

(5) Brennan, T. V.; Anderson, J. W.; Jia, Z.; Waygood, E. B.; Clarke, S. *J. Biol. Chem.* **1994**, *269*, 24586.

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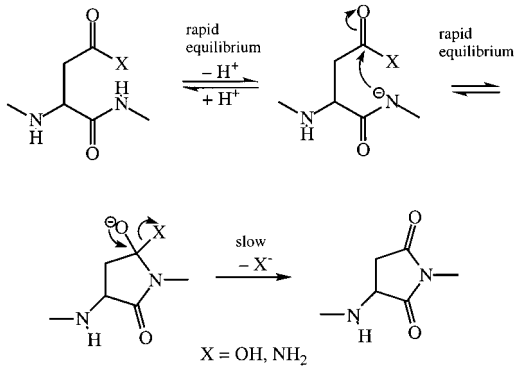
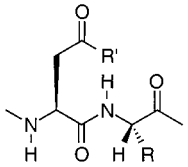
Scheme 2



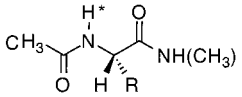
The mechanism of these degradation reactions is of interest for several reasons. In the first place, these reactions represent a potentially major side reaction in the production of polypeptide-based pharmaceuticals.[8] For example, the extensive degradation of a growth-hormone-releasing-factor analogue in solution has been shown to result from isoaspartate residue formation.[9] Second, the accumulation of deamidated, racemized, and isomerized derivatives of asparaginyl and aspartyl residues may be a major contributor to the loss of biological function in the aging process.[10] Finally, at least one enzyme present in most cells can specifically recognize L-isoaspartyl (and D-aspartyl) residues for a methyl esterification reaction leading to the regeneration of L-aspartyl residues in a protein repair pathway.[5] The absence of this enzyme in mice has recently been shown to lead to the accumulation of altered residues which is correlated with growth retardation and early death due to seizures.[11]

To identify peptides and proteins particularly susceptible to this type of spontaneous damage, it is important to be able to predict which asparagine and aspartyl residues would form succinimide residues most rapidly. A number of studies with small flexible peptides have indicated that the chemical nature of the neighboring amino acid side chain on the carboxyl side of the asparagine/aspartic acid residue has the largest effect on the rate of succinimide formation.[2b,c,f,12] For example, in a series of adrenocorticotropic hormone-derived hexapeptides of sequence Val-Tyr-Pro-Y-X-Ala where X is varied and Y is either asparagine or aspartate, the nature of the side chain of X can cause up to a 100-fold change in the rate of the reaction, with the most rapid rate of succinimide formation occurring when a glycine residue is placed at this position (Table 1). These and other results led to a general assumption that steric hindrance of the $n + 1$ side chain controls the rate of succinimide formation; however, this assumption was later disproven on the basis that the degradation rates are not well correlated with the size of the side chain.[2c,f]

Table 1. Comparisons of the Rate of Succinimide Formation of Asn/Asp Peptides and the Basicity of the h+l Amide Nitrogen[2b,c,g,11a,e] with the Half-Life and Relative Degradation Rates Given for Val-Tyr-Pro-Y-X-Ala at 37 °C, pH 7.4[a]



Asn/Asp Degradation



Base-catalyzed exchange of H*

Y	X	R	half-life (days)	rel log k_{degrad}	rel log k_{OH}
Asn	glycine	H	0.8−1.4[b]	1.23	0.27
Asn	serine	CH_2OH	5.6−8.0[c]	0.47	0.37
Asn	cysteine	CH_2SH	10.3	0.29	0.62
Asn	alanine	CH_3	20.2	0.00	0.00
Asn	histidine		24.7	−0.09	−0.10[f]
Asn	phenylglycine	Ph	68.5[d]	−0.53	N/A
Asn	leucine	$CH_2CH(CH_3)_2$	78	−0.59	−0.58
Asn	valine	$CH(CH_3)_2$	107	−0.72	−0.70
Asp	glycine	H	41−71[ed]	0.68	0.27
Asp	serine	CH_2OH	168	0.20	0.37
Asp	cysteine	CH_2SH	198	0.13	0.62
Asp	alanine	CH_3	266	0.00	0.00
Asp	histidine		266	0.00	−0.10[f]

[a] The relative basicity of the amino amide nitrogen in *N*-acetyl-X-*N*′-methylamide is taken from base-catalyzed hydrogen exchange measurements (H*). [b] Average value is 1.2. [c] Average value is 6.8. [d] Reference 7. [e] Average value is 55. [f] Value given for unprotonated species which is expected to predominate at pH 7.4.

Rather than using steric effects to explain these data, Brennan and Clarke proposed a relationship between the rate of degradation and the acidity of the backbone amide nitrogen.[12e] Any substituents that stabilize the nitrogen anion will increase its equilibrium concentration and directly affect the rate of the ring closure reaction (Scheme 2). Thus, the $n + 1$ side chain can affect the rate of succinimide formation by altering the acidity of the amide nitrogen. Brennan and Clarke converted the degradation half-lives of the Val-Tyr-Pro-Asn/Asp-Y-Ala peptide system to rate constants and compared them to the hydrogen exchange rates of the appropriate nitrogen in dipeptide models of the $n + 1$ residue.[12e] The latter rates are proportional to the basicity of the amide nitrogen. Here, we compare the degradation rates to a different set of experimental NMR exchange rates which correlate better with the degradation data (Table 1).[13]

The results in Table 1 show that the differences in the rate of succinimide formation of both the asparagine and aspartic acid peptides can be largely accounted for by the effect of the $n + 1$ residue on the basicity of the amide nitrogen. When the residue is Ser, Ala, His, Leu, or Val, the relative log rate constant for succinimide formation is similar to the relative log rate constant for base-catalyzed hydrogen exchange. Thus, for these side chains, the variations in the degradation rate are caused by changes in the nitrogen protonated/deprotonated equilibrium. The differences in the base-catalyzed exchange rates can be accounted for by two factors: increasing the electron-donating capability of the side chain decreases the acidity of the nitrogen, and increasing the bulk of the side chain decreases the accessibility of hydroxide to the nitrogen.

(6) (a) Violand, B. N.; Schlittler, M. R.; Kolodziej, E. W.; Toren, P. C.; Cabonce, M. A.; Siegel, N. R.; Duffin, K. L.; Zobel, J. F.; Smith, C. E.; Tou, J. S. *Protein Sci.* **1992**, *1*, 1634. (b) Senderoff, R. I.; Sootton, S. C.; Boctor, A. M.; Chen, T. M.; Giordani, A. B.; Julian, T. N.; Radebaugh, R. W. *Pharm. Res.* **1994** *11*, 1712.

(7) Capasso, A.; Di Donato, A.; Esposito, L.; Sica, F.; Sorrentino, G.; Vitagliano, L.; Zagari, A.; Mazzarella, L. *J. Mol. Biol.* **1996**, *257*, 492.

(8) (a) Liu, T. T.-Y. *Trends Biotechnol.* **1992**, *10*, 364. (b) Pearlman, R.; Nguyen, T. *J. Pharm. Pharmacol.* **1992**, *44* (Suppl. 1), 178. (c) Bischoff, R.; Kolbe, H. V. J. *J. Chromatogr. B* **1994** *662*, 261.

(9) Friedman, A. R.; Ichhpurani, A. K.; Brown, D. M.; Hillman, R. M.; Krabill, L. F.; Martin, R. A.; Zurcher-Neely, H. A.; Guido, D. M. *Int. J. Pept. Protein Res.* **1991**, *37*, 14.

(10) (a) Harding, J. J.; Beswick, H. T.; Ajiboye, Rl; Huby, R.; Blakytny, R.; Rixon, K. C. *Mech. Aging Dev.* **1989**, *50*, 7. (b) Stadtman, E. R. *Science* **1992**, *257*, 1220.

(11) (a) Kim, E.; Lowenson, J. D.; MacLaren, D. C.; Clarke, S.; Young, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 6132. (b) Lowenson, J. D.; Kim, E.; Young, S. G.; Clarke, S. *J. Biol. Chem.* **2001**, *276*, in press.

(12) (a) Patel, K.; Borchardt, R. T. *Pharm. Res.* **1990**, *7*, 787. (b) Tyler-Cross, R.; Schirch, V. *J. Biol. Chem.* **1991**, *266*, 22549. (c) Wright, H. T. *Crit. Rev. Biochem. Mol. Biol.* **1991**, *26*, 1. (d) Oliyai, C.; Borchardt, R. T. *Pharm. Res.* **1994**, *11*, 751. (e) Brennan, T. V.; Clarke, S. *Int. J. Pept. Protein Res.* **1995**, *45*, 547.

(13) Bai, Y.; Milne, J. S.; Mayne, L.; Englander, S. W. *Proteins* **1993**, *17*, 75.

However, the correlation between nitrogen basicity and succinimide formation rates is poor when the $n + 1$ residue is cysteine, and very poor when the $n + 1$ residue is glycine. In the former case, the rate of the degradation reaction is about 2- to 2.5-fold slower than one would expect from the nitrogen basicity, and in the latter case the rate of degradation is 3.5- to 7-fold faster than one would expect. In this paper, we focus on the rate enhancement of the glycine residue.

This difference between the rates of succinimide formation from Y-Gly and Y-Ala is much larger than expected from just inductive effects.[2c,12e] Several explanations for this discrepancy have been proposed. First, steric effects may account for part of the difference. The steric hindrance caused by the introduction of a β-carbon may slow the formation of the tetrahedral intermediate, or its decomposition to the succinimide product (Scheme 2). Further increases in the size of the side chain may be too distant from the reaction site to have a steric effect; thus, the correlation between nitrogen basicity is maintained for alanine and higher congeners. In addition, the more flexible glycine peptide could assume transition state geometries more readily, increasing degradation. Still, there has been no consensus on the cause of the rate enhancement of the Asn-/Asp-Gly peptides. Here we show the magnitudes of each of these effects.

In proteins, the situation is more complex than in the hexapeptides discussed so far. A compilation of the sites of deamidation in proteins, the bulk of which occur via succinimide mechanisms, indicates that Asn-Gly sequences are often not the major sites of damage as would be expected from the peptide results.[14] For structured proteins, it now appears that a major factor in the rate of succinimide formation is the local flexibility of the polypeptide chain at the asparagine/aspartic acid residue.[5,14] For example, in calmodulin ligated with calcium, isoaspartyl formation only occurs at Asp-Gln, Asp-Ser, and Asp-Thr sites in flexible regions at the N-terminus and in the central α-helical linker, while in the absence of calcium, the major sites are Asn-Gly residues in the now less-rigid calcium binding sites.[14b,c,d]

Perrin has explored the role of electrostatic effects on the rates of H(N) proton exchange in amides and has reviewed previous discussions of electrostatic interactions and environment on exchange rates.[15] Our work shows how electrostatic effects are altered in different conformations and how this is likely to alter amide acidities.

In this paper, we address the issue of why aspartyl glycine and asparaginyl glycine residues are particularly susceptible to spontaneous degradation. We use ab initio calculations to explore the mechanism of succinimide formation from asparagine and aspartic acid residues and how the conformation of the glycine residue can affect its NH exchange rate. For the two main steps of succinimide formation shown in Scheme 2 (deprotonation and formation of the tetrahedral intermediate), we analyze the effect of conformational flexibility and the effect of glycine versus alanine. This investigation led to results which are also applicable to proton exchange experiments. This connection is discussed in the Appendix.

Computational Methodology

The theoretical studies involved the optimization of molecular geometries using the Gaussian 92 program[16] and the RHF/3-21G and RHF/6-31+G* basis sets. Frequency calculations were performed on fully optimized structures to determine the nature of the stationary points. Tables in this paper give proton affinities. The proton affinity of a compound is calculated by subtracting the energy of the neutral compound from the energy of the anion. The compound with the lower proton affinity is the more acidic compound.

Results and Discussion

Ab Initio Calculations of Steric Effects of the $n + 1$ Residue on Succinimide Formation. We first investigated the importance of steric effects in the reactions leading to succinimide formation. We selected *N*-ethylsuccinamide as a model for a Asn-Gly sequence and *N*-isopropylsuccinamide as a model of the corresponding Asn-Ala sequence. In our model reaction of succinimide formation, the substituted amide anion attacks the unsubstituted amide leading to the tetrahedral intermediate (Figure 1). Using the RHF/3-21G level of theory, we calculated the optimized structures of the reactants, the deprotonated reactants, the transition state leading to the tetrahedral intermediate, and the tetrahedral intermediate itself (Figure 1, Table 2). This basis set is adequate for these calculations since the geometries and relative energies are similar to those for succinamide cyclization at the RHF/6-31+G* level of theory.[17]

For the Asn-Gly model, *N*-ethylsuccinamide, we found that the proton affinity for the substituted nitrogen is 372.0 kcal/mol and that the barrier to the ring closure of the anion is 19.4 kcal/mol. A stable tetrahedral intermediate was found at 4.7 kcal/mol below the transition structure. These results can be compared to those for the Asn-Ala model, *N*-isopropylsuccinamide, where the deprotonation requires 371.6 kcal/mol, and the transition state is 19.2 kcal/mol higher. The tetrahedral intermediate was 4.9 kcal/mol more stable than the transition structure.

The similarity in the energy values for the Asn-Gly and Asn-Ala model systems indicates that steric effects are not important in the ring-closure reaction. The energy barrier for ring closure decreases by a negligible 0.2 kcal/mol when the side chain hydrogen is replaced with a methyl group and the relative energies of the tetrahedral intermediates compared with the deprotonated reactants differ by only 0.4 kcal/mol. The alanine compound has the slightly lower gas-phase deprotonation energy due to the stabilizing effect of the electron-donating methyl side chain on the anion. Thus, the presence of the bulkier methyl group of the alanine residue compared to the hydrogen of the glycine residue actually slightly decreases the barrier for formation of the closed ring tetrahedral intermediate. This would correspond to a slight increase in the rate of degradation for Ala residues which is the reverse of what is observed experimentally. Hence, steric hindrance cannot explain the 20-fold decrease in the rate of succinimide formation of Val-Tyr-Pro-Asn-Ala-Ala compared to Val-Tyr-Pro-Asn-Gly-Ala (Table 1).

We attempted similar calculations on aspartic acid model systems without success. Transition states for cyclization could not be located, even at the RHF/6-31+G* level of theory.[17]

Ab Initio Calculations on the Effect of the $n + 1$ Side Chain on the Basicity of the Amide Nitrogen: Importance of Conformation. The relationship between the chemical nature of the $n + 1$ side chain and the equilibrium concentration of its unprotonated amide nitrogen was determined by

(14) (a) Clarke, S. *Int. J. Pep. Protein Res.* **1987**, *30*, 808. (b) Ota, I. M.; Clarke, S. *J. Biol. Chem.* **1989**, *264*, 54. (c) Ota, I. M.; Clarke, S. *Biochemistry* **1989**, *28*, 4020. (d) Potter, S. M.; Henzel, W. J.; Aswad, D. W. *Protein Sci.* **1993**, *2*, 1648.

(15) Perrin, C. L.; Chen, J.-H.; Ohta, B. K. *J. Am. Chem. Soc.* **1999**, *121*, 2448.

(16) Frisch, M. J.; Trucks, G. W.; Head-Gordon, M.; Gill, P. M. W.; Wong, M. W.; Foresman, J. B.; Johnson, B. G.; Schlegel, H. B.; Robb, M. A.; Replogle, E. S.; Gomperts, R.; Andres, J. L.; Raghavachari, K.; Binkley, J. S.; Gonzalez, C.; Martin, R. L.; Fox, D. J.; Defrees, D. J.; Baker, J.; Stewart, J. J. P.; Pople, J. A. *Gaussian 92*, Revision A.; Gaussian Inc.: Pittsburgh, PA, 1992.

(17) Radkiewicz, J. L.; Houk, K. N. Unpublished results.

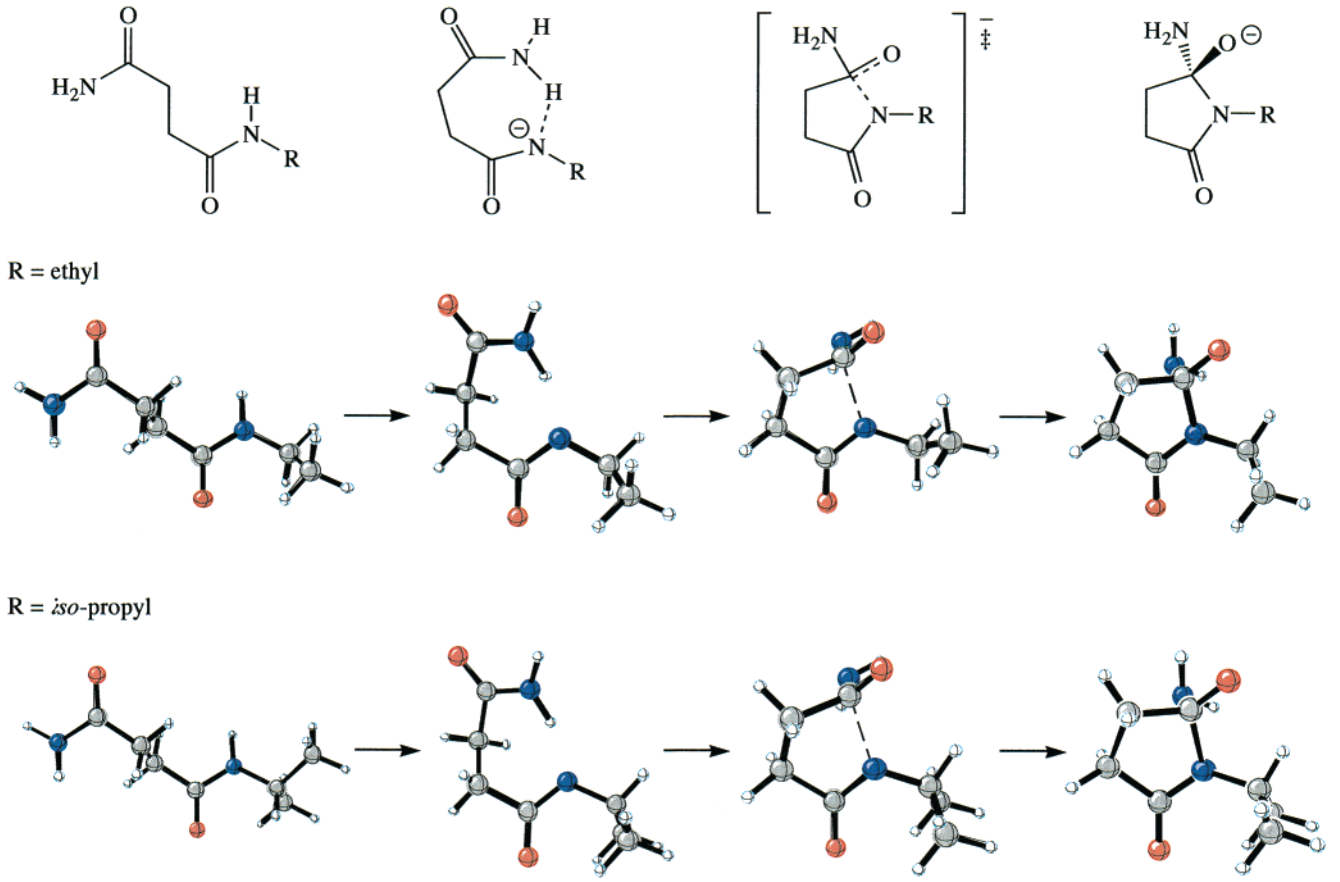
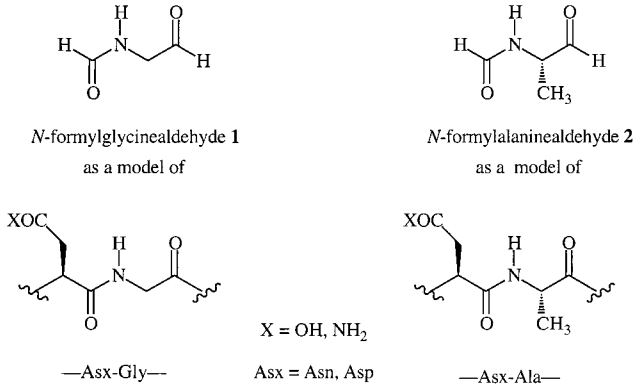


Figure 1. Optimized structures of the reactant, deprotonated reactant, transition state, and intermediate for *N*-ethylsuccinamide and *N*-isopropylsuccinamide.

calculations on *N*-formylglycinealdehyde **1** and *N*-formyl-L-alaninealdehyde **2** and their anions as models of glycine and alanine residues in peptides. Gas-phase proton affinities were



determined from the difference in the calculated energies of the unprotonated (anion) and protonated forms at the RHF/6-31+G* level of theory. Results from optimized conformational minima and transition states are shown in Table 3. These conformations were the only ones for which both the neutral and anionic species were either minima or transition states, allowing for complete optimization. The ϕ and ψ values in Table 3 are for the neutral structures, but the dihedral angles for the anions were within a few degrees. For the C_5 conformer, the proton affinity of **1** is 369.0 kcal/mol and that of **2** is 369.6 kcal/mol, a difference of only 0.6 kcal/mol. For the C_7^{eq} conformer, the proton affinity of both compounds is 360.5 kcal/mol. The replacement of glycine by alanine appears to have no effect on the acidity in the gas phase. However, it is important to notice

Table 2. Relative Energies (kcal/mol) of the Ground State, Deprotonated Ground State, Transition State, and Tetrahedral Intermediate for Cyclization of *N*-Ethylsuccinamide and *N*-Isopropylsuccinamide as Models of Asn-Gly and Asn-Ala Peptides[a]

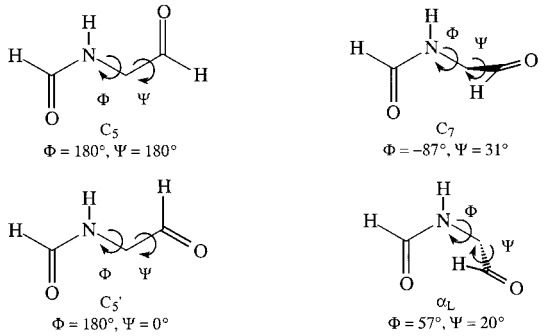
	energies	
	relative	cyclization
N-ethyl (Asn-Gly model)		
ground state	0.0	
anionic ground state	372.0	0.0
transition state	391.4	19.4
tetrahedral intermediate	386.7	14.7
N-isopropyl (Asn-Ala model)		
ground state	0.0	
anionic ground state	371.6	0.0
transition state	390.8	19.2
tetrahedral intermediate	385.9	14.3

[a] Calculations were done at the RHF/3-21G level of theory.

that the relative proton affinities for both model compounds are very dependent upon conformation. For example, the proton affinity of **1** in the C_5 conformer is 11.6 kcal/mol greater than in the $C_{5'}$ conformer, and differences of similar magnitude are seen for **2** when comparing the C_5 and C_7^{eq} conformers (Table 3). In each case the most acidic conformer is one where the C−O bond of the carbonyl group is oriented away from the N−H bond of the amide nitrogen. This maximizes the distance between the negative nitrogen and the partially negative oxygen in the anion.

We have previously reported that the conformation of *N*-formylacetamide can affect the carbon acidity by as much as 7 kcal/mol.[4] The electrostatic interactions between the two carbonyl group dipoles varies between conformers, causing

Table 3. Torsional Angles (deg) and Gas-Phase Proton Affinities (kcal/mol) of *N*-Formyl-glycinealdehyde and *N*-Formyl-L-alaninealdehyde Conformations as Models of X-Gly and X-Ala Peptides[a]



N-formylglycinealdehyde **1**	C_5	C_7	$C_{5'}$
Φ	180.0	87.4	180.0
Ψ	180.0	−30.7	0.0
gas-phase proton affinity	369.0	360.5	357.4
relative proton affinity	11.6	3.1	0.0
***N*-formyl-L-alaninealdehyde 2**	C_5	C_{7eq}	α_L
Φ	−159.2	−90.2	57.1
Ψ	177.0	40.0	20.1
gas-phase proton affinity	369.6	360.5	361.3
relative proton affinity	9.1	0.0	0.8

[a] Calculations were performed at the RHF/6-31G* level of theory.

Table 4. Relative Gas-Phase Acidities (kcal/mol) of Conformations of *N*-Formylglycinamide **3** at the RHF/6-31+G* and RHF/6-31+G*//RHF/3-21G Levels of Theory

Φ	Ψ	RHF/6-31+G*	RHF/6-31+G*//RHF/3-21G
180	180	12.7	13.3
90	180	10.8	10.5
0	180	9.7	10.2
−90	90	1.6	1.0
−90	0	0.0	0.0

changes in acidity. The changes reported here in Table 3 for compounds **1** and **2** confirm this relationship of electrostatic effects between the dipoles of the carbonyl groups and the amide nitrogen. These results suggest that the observed differences in the facility of deprotonation of alanine and glycine peptides may be a result of these peptides having different conformations in solution. It thus seemed advisable to further investigate the relationship between conformation and amide nitrogen acidity, especially since this could explain the enhanced rate of Asn-/Asp-Gly degradation: the presence of additional conformers with distinct electrostatic interactions could lead to a greater stability of the nitrogen anion and faster degradation.

Conformation and Amide Acidity: *N*-Formylglycinamide and *N*-Formyl-L-alaninamide as Models for ab Initio Calculations. Calculations were performed on *N*-formylglycinamide **3** and its anion. Compound **3** provides a better model of peptides than **1,** and the conformational energetics of the neutral compound have been studied previously at the RHF/3-21G and RHF/6-31+G* levels of theory.[18] Table 4 shows that the relative acidities from RHF/6-31+G* constrained geometry optimizations differ only slightly from RHF/6-31+G* single points on RHF/3-21G constrained geometries; thus, the remainder of the calculations were performed at the RHF/6-31+G*//RHF/3-21G level of theory. For each optimization, the Φ and Ψ dihedral angles were constrained. These angles were varied by either

(18) Head-Gordon, T.; Head-Gordon, M.; Frisch, M. J.; Brooks, C. L., III; Pople, J. A. *J. Am. Chem. Soc.* **1991**, *113*, 5989−5997.

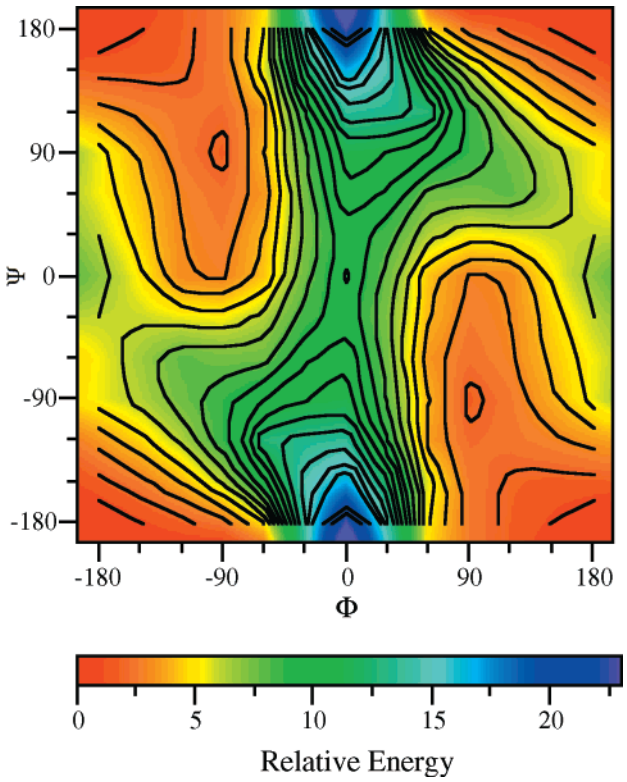
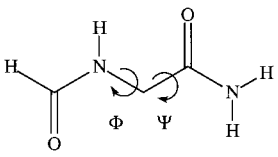


Figure 2. Relative energies (kcal/mol) of *N*-formyl-glycinamide as a function of the dihedral angles Ψ and Φ: RHF/6-31+G*//RHF/3-21G. Red indicates preferred conformers and purple indicates disfavored conformers. Contour lines indicate conformations with the same relative energy.

60° or 90°, so that each point of the 60° and 90° grids was calculated. These points were then used to make color-coded Ramachandran diagrams: Figure 2 depicts the relative energies (0−25 kcal/mol) of the neutral compound, Figure 3 depicts the relative energies (0−35 kcal/mol) of the anion, and Figure 4 depicts the relative proton affinities (0−20 kcal/mol) as a function of conformation. Figure 2 is very similar to that previously reported by Pople et al.[18]



3

Φ = 180°, Ψ = 180°

Comparison of the neutral potential energy surface (PES) (Figure 2) to the anionic potential energy surface (Figure 3) shows that the preferable anionic conformations are very different from those of the neutral compound. The three minima of the neutral compound are at Φ = 180°, Ψ = 180° (**3a**, C_5), approximately Φ = −90°, Ψ = 90° (**3b**, $C_{7^{eq}}$), and approximately Φ = 90°, Ψ = −90° ($C_{7^{ax}}$). The anion also has three minima, but they are at Φ = 180°, Ψ = 0° (**3c**, a saddle point on the neutral PES), approximately Φ = −90°, Ψ = 30°, and approximately Φ = 90°, Ψ = −30°. In both Figures 2 and 3, the area near Φ = 0°, Ψ = 180° is energetically unfavorable.

The large electrostatic effect of the neighboring carbonyl group dominates the anionic PES. For example, in the Φ = 180°, Ψ = 0° conformer **3c** (Figure 5), the anion formed by deprotonation of the amide nitrogen is highly stabilized by the

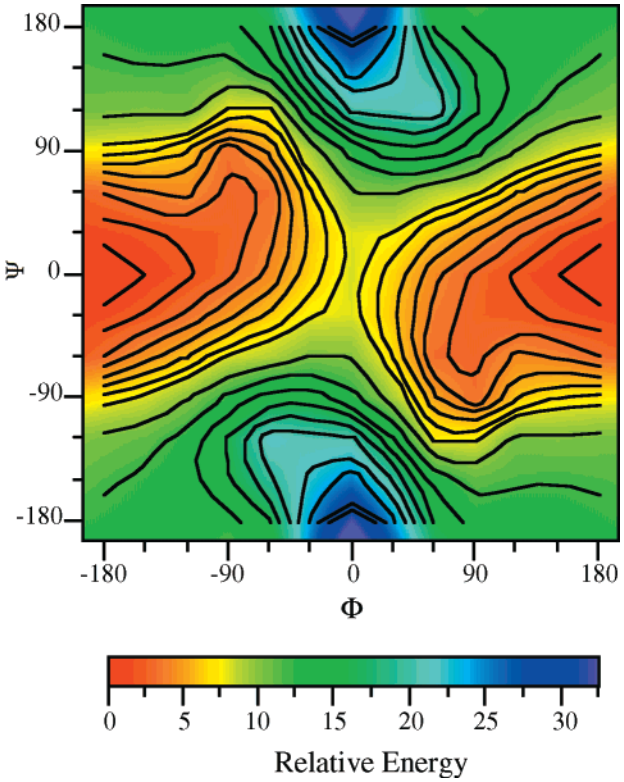


Figure 3. Relative energies (kcal/mol) of *N*-formyl-glycinamide anion as a function of the dihedral angles Ψ and Φ: RHF/6-31+G*//RHF/3-21G. Red indicates preferred conformers and purple indicates disfavored conformers. Contour lines indicate conformations with the same relative energy.

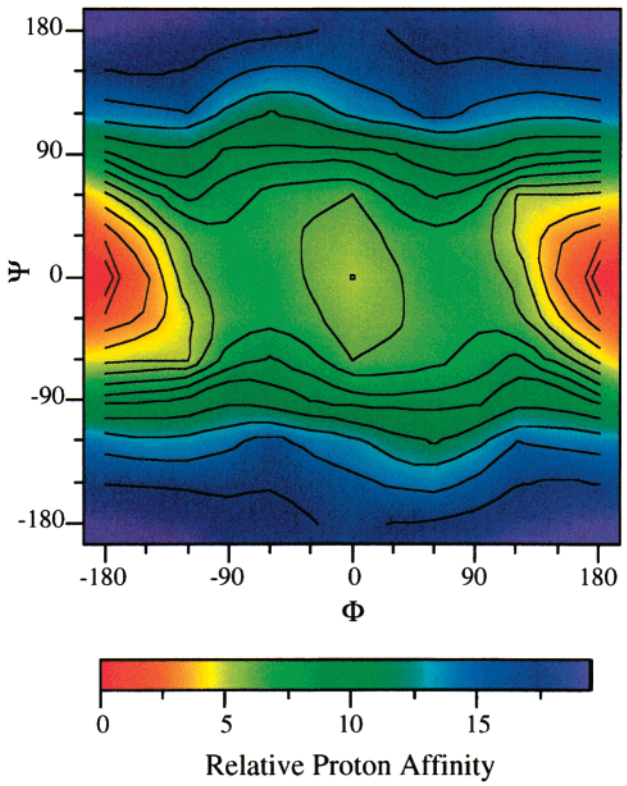


Figure 4. Relative proton affinities (kcal/mol) of *N*-formyl-glycinamide as a function of the dihedral angles Ψ and Φ: RHF/6-31+G*//RHF/3-21G. Red indicates a low proton affinity (high acidity) and purple indicates a high proton affinity (low acidity). Contour lines indicate conformations with the same proton affinity.

positive end of the C=O dipole and the hydrogen bond to the terminal NH_2, making **3c** the most stable conformer. The Φ = 180°, Ψ = 180° conformer **3a** (Figure 5) is 12 kcal/mol less stable; the anionic nitrogen is destabilized by the close proximity of the negative end of the CO dipole.

The conformation of the peptide has a profound effect on the amide acidity. The acidity graph, Figure 4, demonstrates that proton affinity is mainly dependent upon the value of Ψ; Φ has only a small effect. The largest variance in acidity over a range of Φ values and for a given value of Ψ is 10 kcal/mol at Ψ = 0°. For Ψ = 180°, the proton affinity only varies by ~2 kcal/mol upon varying Φ. On the other hand, the smallest variance over a range of Ψ values and for a given value of Φ is ~10 kcal/mol at Φ = 75°. For Φ = 180°, proton affinity varies ~20 kcal/mol, the largest difference observed.

Changing the side chain from glycine to alanine may indirectly affect the deprotonation equilibrium and, thus, the rate of degradation. Replacement of one residue with another will affect the conformational potential energy surface of the peptide; conformers that were unfavorable may become preferred. As can be seen in Figure 4, this will also cause changes in the acidity of the backbone amide. Variations in Φ and Ψ can cause up to an ~20 kcal/mol change in acidity. This will have a drastic effect on the equilibrium between protonated and deprotonated amide and thus it will also have a drastic effect on the rate of degradation.

The fact that proton exchange rates of dipeptides can explain the different degradation rates of alanine, serine, leucine, and valine hexapeptides indicates that these residues must have similar conformations in the dipeptides and hexapeptides. While the dipeptides can sample various conformations, they should mainly be in an extended conformation as indicated by

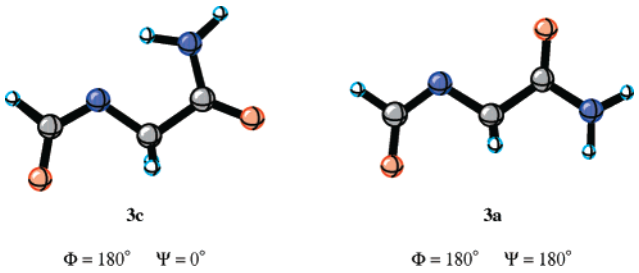


Figure 5. The most acidic and the most basic conformers of *N*-formyl-glycinamide RHF/3-21G. The anion is pictured.

calculations on glycine and alanine dipeptides.[18] Thus, the relative log *k* values for exchange rates reflect only the inductive and steric effects of the side chain. If alanine, serine, leucine, and valine existed in different conformations in the hexapeptide than in the dipeptide the relative log *k* values would also reflect the acidity of the particular conformation. Consequently, the rates would differ from the exchange log *k* values and this is not observed.

The extra flexibility of the glycine residue may not be important in assuming a transition state geometry, but rather, it may allow the formation of conformations which are more acidic. For example, glycine is much more liable to assume conformations in the region centered on Φ = 98, Ψ = 30 (α_L) than other residues.[19] This region is more acidic than other possible conformations such as the β-sheet conformation. It is interesting to note that all calculations also show that the α_R-helix conformation is much more acidic than the β-sheet

(19) (a) Gibrat, J. F.; Robson, B.; Garnier, J. *Biochemistry* **1991**, *30*, 1578. (b) Kamimura, M.; Takahashi, Y. *Comput. Appl. Biosci.* **1994**, *10*, 163.

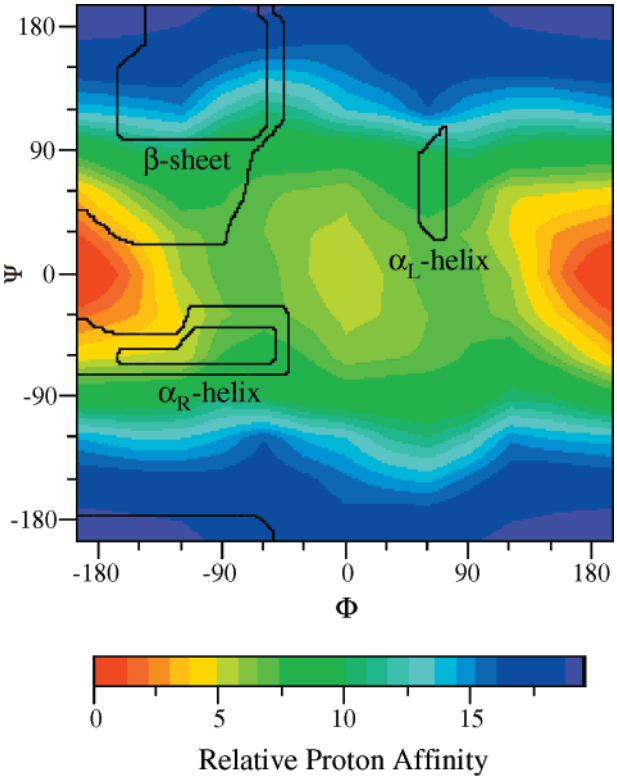


Figure 6. Relative proton affinities (kcal/mol) of *N*-formyl-glycinamide as a function of the dihedral angles Ψ and Φ, overlaid with a Ramachandran graph: RHF/6-31+G*//RHF/3-21G. Red indicates a low proton affinity (high acidity) and purple indicates a high proton affinity (low acidity).

conformation (Figure 6). In addition, formation of the anion can lead to conformational changes as depicted by the differences between Figure 2 and Figure 3. In the anion, the transition state geometry should be more stable, and extra flexibility will not be necessary to assume the transition structure. The steric calculations show that upon deprotonation the preference of the molecule switches from extended to cyclic. The formation of the hydrogen bond between the side-chain carbonyl oxygen and the deprotonated nitrogen may bring the side-chain carbonyl carbon close enough to the nitrogen for cyclization to occur.

Application to Proteins. Many proteins undergo degradation at their aspartyl or asparaginyl residues.[1,2f,5,14] However, the presence of an Asn-Gly or Asn-Ser sequence, which causes rapid hexapeptide degradation, does not guarantee the rapid degradation of native proteins.[14] Degradation has also been detected in proteins at sites where the neighboring side chain should disfavor succinimide formation.[14b] These results are presumably indicative of the role of protein three-dimensional structure in this process.

There are two possible ways in which the tertiary structure could be affecting degradation rates. First, the acidity of the amide will be determined in part by the tertiary structure. Changes in the neighboring side chain will not have as large an effect on conformation or acidity, due to the competing effects of other side-chain side-chain interactions. For the same reason, formation of the anion should also have only a small effect on the conformation. Second, the side chain, which should be able to hydrogen bond to the backbone in a small peptide, may be prevented from doing so in a protein. Without this hydrogen bond and lower flexibility, it then becomes much less feasible for the residue to assume a transition state-type geometry. Supporting both these ideas is the fact that loop structures are more susceptible to degradation, probably because of their increased flexibility.[5,14c,d] Since the rate of degradation depends on tertiary structure and flexibility, predicting this rate for residues in proteins would be nearly impossible without an X-ray structure and knowledge of the conformation flexibility. One could attempt to predict rates of degradation based on X-ray structures as long as the crystal structure corresponded to the preferred solution conformation.

Conclusions

We have explored the relationship between the neighboring residue and the rate of asparaginyl or aspartyl degradation. In most cases, the chemical nature of the side chain of the neighboring amino acid can directly affect degradation rates by affecting the equilibrium between protonated and deprotonated nitrogen. However, this is not true at least for glycine. Rather, new conformations induced by changes in the side chain may make the nitrogen atom more or less acidic than in the previous conformation and this will affect the rate of degradation. The flexibility of glycine provides an additional advantage to cyclization by making acidic conformations more accessible. This relationship between conformation and acidity indicates that α-helices are more acidic than *β*-sheets and that this relationship should be taken into consideration when interpreting proton exchange results.

Acknowledgment. We are grateful to the National Institutes of General Medical Sciences and Aging−NIH for financial support of this research and to the San Diego Supercomputer Center for computer time.

Appendix

Proton Exchange Experiments. The calculations reported in this paper can also be applied to the proton exchange experiments. This section discusses the implications these results have on the analysis of experimental proton exchange data.

The folding stability of proteins can be ascertained by measuring the rates of proton exchange of the peptide bond hydrogens.[20] Solvent inaccessible or hydrogen-bonded protons will only exchange after the protein has undergone a certain amount of unfolding.

$$E_{fld}(H) \underset{k_f}{\overset{k_u}{\rightleftharpoons}} E_{ufl}(H) \xrightarrow[D_2O]{k_{rc}} E_{ufl}(D)$$

Assuming the folded state is more stable than the unfolded state, i.e., $k_u/k_f \ll 1$, and that $k_f \gg k_{rc}$ gives the following equation for the observed proton exchange rate constant:

$$k_{ex} = K_{op} k_{rc} \qquad (1)$$

where k_{rc} is the rate for proton exchange in a random coil protein and $K_{op} = k_u/k_f$. This is called the EX_2 limit and most hydrogens in proteins belong to this category. The free energy of unfolding can then be calculated using

$$\Delta G_u = -RT \ln K_{op}$$

or

$$\Delta G_u = -RT \ln(k_{ex}/k_{rc}) \qquad (2)$$

The k_{rc} values have been determined for dipeptides and have

(20) For reviews see: (a) Wagner, G.; Wüthrich, K. *Methods Enzymol.* **1986**, *131*, 307. (b) Roder, H. *Methods Enzymol.* **1989**, *176*, 446.

been shown to have additive properties.[16,20] Thus, one can estimate k_{rc} values from the protein sequence and obtain the free energies of unfolding from proton exchange rate constants.

Electrostatic interactions have been postulated to affect the proton exchange rates of proteins.[21−23] Forsyth and Robertson investigated this theory by examining model compounds.[24] Their results demonstrate that electrostatics can have a dramatic effect on exchange rates. Diketopiperazine undergoes base-catalyzed exchange 740 times faster than 2-piperidone. Since previous experiments showed that the inductive effect of an additional amide group only causes a (4−17)-fold increase, the majority of the increase must be caused by electrostatic interactions. This implies that electrostatic interactions could also influence the exchange rates observed for proteins.

Our calculations on the relationship between conformation and proton affinity also support a relationship between hydrogen exchange and electrostatic interactions. Hydrogens restricted to a certain conformation will have different acidities than hydrogens in another conformation as demonstrated by our Ramachandran graph of proton affinities (Figure 5). As discussed previously, these changes in acidity are the result of electrostatic interactions. Thus, the conformation of the residues will determine the local environment of a hydrogen and this will affect its acidity and proton exchange rate.

Neglecting the effect of conformation on the acidity can lead to underestimations of the folding stability of certain residues. The k_{rc} values are determined from dipeptides which presumably randomly sample various conformations but are mainly in an extended conformation. This corresponds to the area of highest proton affinity on our Ramachandran graph (Figure 6). Thus, residues in more acidic conformations will have higher rates of exchange than predicted by the dipeptides. This underestimation of k_{rc} will result in an underestimation of ΔG_u for that proton. The k_{rc} values determined from peptides will only be valid for protons that are in completely unfolded areas of the protein.

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(21) (a) Kim, P. S.; Baldwin, R. L. *Biochemistry* **1982**, *21*, 1. (b) Matthew, J. B.; Richards, F. M. *J. Biol. Chem.* **1983**, *258*, 3039. (c) Eriksson, M. A.; Härd, T.; Nilsson, L. *Biophys. J.* **1995**, 329.

(22) Rohl, C. A.; Baldwin, R. L. *Biochemistry* **1994**, *33*, 7760.

(23) Dempsey, C. E. *J. Am. Chem. Soc.* **1995**, *117*, 7526.

(24) Forsyth, W. R.; Robertson, A. D. *J. Am. Chem. Soc.* **1996**, *118*, 2694.