Analysis of Backbone Hydrogen Bonding in a *β*-Turn of Staphylococcal Nuclease

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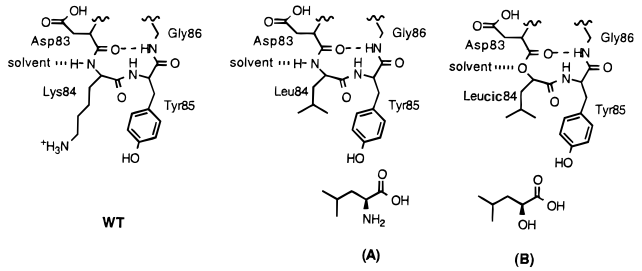
The reverse or *β*-turn is a common structural element in proteins that facilitates folding by reversing the direction of the polypeptide chain. Because *β*-turns are frequently found at the protein surface and are comprised of mostly polar residues,[1] they are widely believed to act as molecular recognition sites for many biological processes.[2] *β*-Turns involve four residues with a distance of ≤7 Å between the α carbons of residues *i* and *i* + 3, and are classified into different types depending on the backbone torsional angles of residues *i* + 1 and *i* + 2.[3] Several solid state and solution studies have suggested that the hydrogen bond between the carbonyl group of residue *i* and the NH group of residue *i* + 3 may not contribute as much to the overall stability of proteins as the analogous hydrogen bonds in *β*-sheets and α-helixes.[3b,4] To investigate the energetics of hydrogen bonds in *β*-turns, we have determined the difference in stabilities of staphylococcal nuclease (SNase) and a mutant in which the amide group at residue *i* of a *β*-turn is replaced with an ester group.

Residues 83−86 of SNase form a solvent-exposed type I *β*-turn between *β*-strands 4 and 5. The carbonyl oxygen of Asp^{83} is intramolecularly hydrogen-bonded to the amide NH of Gly^{86} as illustrated in Scheme 1.[5] The side chains of the *i* + 1 and *i* + 2 residues of the turn, Lys^{84} and Tyr^{85}, form hydrogen bonds with the 3′-phosphate of the inhibitor in the protein−inhibitor complex. To estimate the energetic contribution of the hydrogen bond between Asp^{83} and Gly^{86} to overall protein stability, the backbone amide linkage between Asp^{83} and Lys^{84} was replaced with an isosteric ester linkage using unnatural amino acid mutagenesis, which allows the site-specific substitution of amino acid side chains[6] as well as polypeptide backbones.[7] The ester bond, like the amide bond, favors the *trans* conformation and has a significant *cis*−*trans* rotational barrier.[8] However, this substitution results in a decrease in the basicity of the carbonyl oxygen[9] and a concomitant decrease in the strength of the $O_i\cdots HN_{i+3}$ hydrogen bond. Because the backbone NH group of Lys^{84} is solvent-exposed, the substitution of this group by oxygen is not expected to significantly affect the overall protein stability.

Scheme 1. Schematic Representation of Leucic Acid Incorporation at Position 84 in a *β*-Turn of SNase



The ester-containing protein was synthesized in vitro using a SNase gene containing an amber codon at position 84 in the presence of suppressor tRNA derived from *E. coli* $tRNA^{Asn}$ that was chemically acylated with 6-amino-2-hydroxyhexanoic acid,[10] a lysine isostere. SNase was expressed with a C-terminal hexahistidine tag[11,12] and purified to homogeneity by nickel affinity column chromatography.[13] The suppression efficiency of 6-amino-2-hydroxyhexanoic acid was less than 5% in a large-scale in vitro protein synthesis reaction, affording inadequate quantities of purified protein for biophysical studies. We therefore attempted in vitro suppression with leucic acid, a leucine isostere, since hydrophobic amino acids are generally incorporated in higher yield than hydrophilic ones.[14] Indeed, the efficiency of incorporation of leucic acid was 20−25% at 8.8 mM $Mg(OAc)_2$.[15] As a control, the natural mutant, Lys ^{84} → Leu, was also prepared by in vitro synthesis.

To measure the difference in stabilities ($\Delta\Delta G°_{H_2O}$) of the mutants, an apparent equilibrium constant for denaturation (K_{app}, Table 1) was determined with purified proteins by monitoring the intrinsic fluorescence of the single tryptophan residue at position 140 as a function of denaturant concentration (Figure 1).[16] The value of K_{app} was extrapolated to a denaturant concentration of zero; $\Delta G°_{H_2O}$ is given by the equation $\Delta G° = -RT \ln K_{app}$. Substitution of Lys^{84} by leucine decreases the

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(1) (a) Rose, G. D. *Nature* **1978**, *272*, 586. (b) Kuntz, I. D. *J. Am. Chem. Soc*. **1972**, *94*, 4009. (c) Lewis, P. N.; Momany, F. A.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 2293.

(2) (a) Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512. (b) Dyson, J. J.; Cross, K. J.; Houghten, R. A.; Wilson, I. A.; Wright, P. E.; Lerner, R. A. *Nature* **1985**, *318*, 480.

(3) (a) Sibanda, B. L.; Thornton, J. M. *Methods Enzymol*. **1991**, *202*, 59. (b) Rose, G. D.; Gierasch, L. M.; Smith, J. A. *Adv. Protein Chem.* **1985**, *37*, 1. (c) Richardson, J. S. *Adv. Protein Chem*. **1981**, *34*, 167. (d) Smith, J. A.; Pease, L. G. *CRC Crit. Rev. Biochem*. **1980**, *8*, 315.

(4) (a) Karle, I. L. In *The Peptides* Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1981; Vol. 4, pp 1−54. (b) Gierasch, L. M.; Deber, C. M.; Madison, V.; Niu, C.; Blout, E. R. *Biochemistry* **1981**, *20*, 0. 4730. (c) Pease, L. G.; Niu, C. H.; Zimmerman, G. *J. Am. Chem. Soc*. **1979**, *101*, 184.

(5) (a) Hynes, T. R.; Fox, R. O. *Proteins: Struct., Funct. Genet.* **1991**, *10*. 92. (b) Loll, P. J.; Lattman, E. E. *Proteins: Struct., Funct. Genet.* **1989**, *5*. 183.

(6) (a) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182. (b) Ellman, J. A.; Mendel. D.; Anthony-Cahill, S. J.; Noren, C. J.; Schultz, P. G. *Methods Enzymol*. **1991**, *202*, 301. (c) Robertson, S. A.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc*. **1991**, *113*, 2722. (d) Cornish, V. W.; Benson, D. R.; Altenbach, C. A.; Hideg, K.; Hubble, W. L. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2910. (e) Kanamori, T.; Nishikawa, S−I.; Shin, I.; Schultz, P. G.; Endo, T. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *91*, 2910.

(7) Ellman, J. A.; Mendel. D.; Schultz, P. G. *Science* **1992**, *255*. 197.

(8) Wiberg, K. B.; Laidig, K. E. *J. Am. Chem. Soc.* **1987**, *109*, 5935.

(9) Jaffe, H. H.; Freedman, L. D.; Doak, G. O. *J. Am. Chem. Soc*. **1953**, *75*, 2209.

(10) Although the unnatural amino acid was racemic, only the L-amino acid is accepted by the protein biosynthetic machinery (D. Mendel, unpublished material).

(11) (a) Cload, S. T.; Liu, D. R.; Pastor, R. M.; Schultz, P. G. *J. Am. Chem. Soc*. **1996**, *118*, 1787. (b) Liu, D. R.; Cload, S. T.; Pastor, R. M.; Schultz, P. G. *J. Am. Chem. Soc*. **1996**, *118*, 1789.

(12) Chapman, E.; Thorson, J. S.; Schultz, P. G. *J. Am. Chem. Soc*. **1997**, *119*, 7151−7152.

(13) Hochuli, E.; Dobeli, H.; Schrocher, A. *J. Chromatogr*. **1987**, *411*, 177.

(14) (a) Cornish, V. W.; Mendel, D.; Schultz, P. G. *Angew. Chem., Int. Ed. Eng*. **1995**, *34*. 621. (b) Mendel, D.; Cornish, V. W.; Schultz, P. G. *Annu. Rev. Biophys. Biomol. Struct.* **1995**, *24*. 435.

(15) Incorporation of the α-hydroxy acid was verified by alkaline hydrolysis (1:1 = concentrated NH_4OH:10% SDS, 40 min, 95 °C) followed by denaturing polyacryamide gel analysis of [^{35}S]Met-labeled protein fragments. Selective cleavage was observed at the site of the ester bond; no cleavage was observed for proteins containing the amide backbone.

(16) Denaturation studies were carried out in 25 mM sodium phosphate, 100 mM sodium chloride, pH 7.0 buffer at 25 °C, according to the known procedure of Shortle and Meeker with a Hitachi F-4500 fluorescence spectrophotometer.[20] The apparent equilibrium constant (K_{app}) was determined for a series of guanidinium chloride concentrations by using the equation $K_{app} = (I_n - I)/(I - I_d)$ where I is the measured intrinsic fluorescence, I_n is the extrapolated fluorescence for the native state, and I_d is the extrapolated fluorescence for the denatured state. To obtain ΔG_{H_2O} and m_{GuHCl}, a straight line was fit to log K_{app} versus [GuHCl] by using a linear least-squares method.

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Table 1. Relative Thermodynamic Stabilities of Proteins Containing Amide and Ester Backbone Linkages and Their Kinetic Constants

protein	K_m[a]	V_{max}[b]	C_m[c]	m_{GuHCl}[d]	$\Delta\Delta G_{H_2O}$[e]
WT	107	0.81	0.69	1.00	0.62
Lys84 → Leu (**A**)	159	0.42	0.59	1.02	
Lys84 → leucic acid (**B**)	190	0.38	0.30	1.25	−1.55

[a] K_m is expressed in units of μg/mL. The kinetic constants were obtained by measuring the change of absorbance at 260 nm with varying amounts of single-stranded calf thymus DNA (1−50 μg/mL) in 10 mM Ca^{2+} and 40 mM sodium glycinate at pH 9.9. [b] V_{max} is expressed in units of $A_{260}/(\mu gmin)$. [c] Midpoint concentration of GuHCl in molarity. [d] Units are relative to the wild-type in vitro expressed (WT) which is normalized to 1.00. [e] $\Delta\Delta G_{H_2O}$ is expressed in units of kcal/mol. A negative value denotes decreased stability relative to the Lys^{84} → Leu mutant.

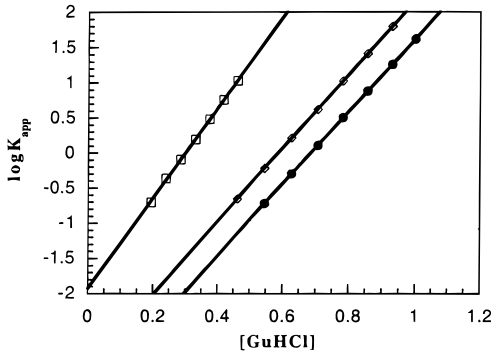

Figure 1. Denaturation plots of log K_{app} versus guanidinium chloride concentration for the following SNase proteins: (●) wild-type; (◇) Lys 84 → Leu (**A**); (□) Leu 84 → leucic acid (**B**).

overall protein stability by 0.62 kcal/mol and results in a 2-fold decrease in V_{max}. These results suggest that the Lys^{84} → Leu mutation does not significantly affect the overall structure of the protein. The catalytic activity of the Leu^{84} → leucic acid mutant is almost identical with that of the corresponding amide-containing mutant Lys^{84} → Leu, suggesting that the leucic acid substitution, like the leucine substitution, does not result in a significant structural perturbation (Table 1). Substitution of Leu^{84} by leucic acid results in a decrease in protein stability by 1.55 kcal/mol, indicating that this reverse turn hydrogen-bonding interaction contributes significantly to the overall stability of the protein.

This value of $\Delta\Delta G°_{H_2O}$ reflects the energetic differences between the amide interactions in the folded and unfolded states and the ester interactions in the folded and unfolded states. If structural perturbations are insignificant and both the amide NH and ester O groups are solvent-exposed, $\Delta\Delta G°_{obs}$ is largely determined by two factors: (1) the different strengths of the hydrogen bonds formed by the amide and ester in the folded proteins and (2) the energetic differences between the interactions of the amide and ester carbonyl groups with water molecules in the unfolded proteins. The latter value can be estimated from the difference in the water−octanol partition coefficients of *N*-methylacetamide and methyl acetate ($\Delta\Delta G° = -1.7$ kcal/mol),[17] which provides an upper limit to the difference in solvation energies of the ester and amide bonds. On this basis, the difference in the strength of the backbone amide and ester hydrogen bonds is less than or equal to 3.25 kcal/mol in favor of the amide.

This study can be compared to similar experiments involving related substitutions of amides by esters in other protein secondary structures, such as β-sheets[12] and α-helixes.[18] The corresponding main chain amide-to-ester substitution in an internal β-sheet of SNase (Leu^{14} → leucic acid) destabilized the protein by 2.5 kcal/mol, while the same substitution near the N-terminus of a β-sheet (Leu^{72} → leucic acid) decreased protein stability by 1.5 kcal/mol.[12] Leu^{72} and Leu^{84} appear to be in similar hydrophilic environments, and mutation of each to the corresponding α-hydroxy acid destabilizes the protein to the same degree. This result suggests that the energetic contribution of the backbone hydrogen bond in this type I β-turn can be very similar to that of main chain to main chain hydrogen bonds in antiparallel β-sheets. The $\Delta\Delta G°$ value for the β-turn in SNase can also be compared to the value of 1.6 kcal/mol which was obtained from a similar amide-to-ester substitution in a peptide β-turn mimetic,[19] again showing good agreement.

The energetic contributions of backbone hydrogen bonds in α-helixes to overall protein stability have been studied in T4 lysozyme.[18] Mutation of an amide group to the corresponding ester group at the N-terminus (Leu^{39} → leucic acid) and C-terminus (Ile^{50} → isoleucic acid) of an α-helix, where only one hydrogen-bonding interaction is perturbed (the carbonyl and NH groups, respectively), resulted in destabilization of the protein by 0.9 and 0.7 kcal/mol, respectively. The decreased energetic contribution of the α-helical hydrogen bonds relative to those in a β-sheet or β-turn may be a result of increased solvent accessibility to the α-helix backbone or electrostatic effects associated with the α-helix dipole. Altogether, these studies suggest that backbone hydrogen-bonding interactions in the three major secondary structures commonly found in protein structures can contribute significantly to the overall stability of proteins.

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(17) Leo, A.; Hansch, C.; Elkins, D. *Chem. Rev. (Washington, D.C.)* **1971**, *71*, 525.
(18) Koh, J. T.; Cornish, V. W.; Schultz, P. G. *Biochemistry*, in press.
(19) Gallo, E. A.; Gellman, S. H. *J. Am. Chem. Soc.* **1993**, *115*, 9774.
(20) Shortle, D.; Meeker, A. K. *Proteins: Struct., Funct. Genet.* **1986**, *1*, 81.