Mutational Analysis of Backbone Hydrogen Bonds in Staphylococcal Nuclease

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The overall contribution of hydrogen bonds to the stability of proteins remains an unresolved issue.[1,2] Site-directed mutagenesis has been used to experimentally determine the energetic consequences of deleting one member of a hydrogen-bonded pair.[2] More recently, a linear free energy relationship between protein stability and hydrogen bond strength was demonstrated by analyzing a series of mutants containing isosteric tyrosine analogues.[3] While these studies provide important information on side chain−side chain interactions, many of the hydrogen bonds in proteins, such as those in α-helices and β-sheets, involve main chain amide groups. Detailed analysis of these hydrogen-bonding interactions, which are also thought to play a key role in protein folding, is complicated by the fact that conventional site-directed mutagenesis provides no replacements for the backbone amide bond. In order to address this problem, we have used unnatural amino acid mutagenesis to independently replace the amide groups of two different main chain to main chain hydrogen-bonded pairs in an antiparallel β-sheet with the corresponding esters. These substitutions, which modulate the pK_A[4] and, consequently, the hydrogen bond strength of the corresponding carbonyl groups, result in a 1.5−2.5 kcal/mol decrease in protein stability.

Both backbone amide groups (Thr 13/Leu 14 and Lys 71/Ile 72) are located in the five-stranded β-barrel of Staphylococcal nuclease (SNase), and each participates in the formation of a main chain to adjacent main chain intramolecular hydrogen bond in an antiparallel β-sheet.[5] The carbonyl group of Thr 13 (β-sheet 2) is hydrogen bonded to the amide NH of Met 26 (β-sheet 3), while the carbonyl group of Lys 71 (β-sheet 5) is hydrogen bonded to the amide NH of Asp 95 (β-sheet 6). These sites were selected from a pool of optimal β-sheet main chain−main chain hydrogen bonds with respect to length and linearity.[5] Mutation of the residues adjacent to Thr 13 and Lys 71 (Leu 14 and Ile 72) to the α-hydroxy acid (**2**) replaces a good hydrogen bond acceptor, the amide carbonyl group (*N*,*N*-dimethylacetamide·H^+; $pK_A \approx 0.4$), with a considerably weaker hydrogen bond acceptor, the ester carbonyl (ethylacetate·H^+; $pK_A = -4.5$).[6] The amide and ester groups are structurally similar, and both exist predominately in the *trans* configuration.[7] Sites have been selected where the adjacent amide N−H and corresponding ester -O- groups are hydrogen bonded to solvent to minimize the concommitant effects of the backbone N−H to O substitution (Scheme 1).

Wild-type SNase was used as the control for the Leu 14 substitution while an Ile 72 → Leu mutant (with stability and activity similar to WT enzyme) was generated for comparison to the substitution of **2** at site 72. Incorporation of α-hydroxy acid (**2**) and L-leucine (**1**) at both sites was accomplished by *in vitro* suppression of a Leu 14 → TAG or Ile 72 → TAG *amber* mutation with a chemically aminoacylated suppressor tRNA derived from yeast $tRNA^{Phe}$.[8] The suppression efficiencies of **2** were 18% and 10% (6.6 mM $MgOAc_2$) at sites 14 and 72, respectively, while **1** was incorporated with a suppression efficiency of 65% (10 mM $MgOAc_2$) at site 72. Wild-type and mutant proteins were purified to homogeneity from 5.0 mL *in vitro* protein synthesis reactions.[9,10] The homogeneity of protein containing α-hydroxy acid **1** at either site was confirmed by site-specific alkaline hydrolysis of the ester linkage.[11] Separation of the hydrolysis products by SDS-PAGE revealed quantitative cleavage at Leu 14 and Ile 72, respectively.

The ester-containing mutants have K_m and V_{max} values that are within of factor of 3 of those displayed by the corresponding amide containing SNase, suggesting that the ester substitution does not lead to significant configurational abnormalities (Table 1). The K_{app} values, an apparent equilibrium constant for denaturation, were determined with purified protein by monitoring the intrinsic fluorescence of the single tryptophan residue at position 140 as a function of denaturant concentration in 25 mM sodium phosphate, 100 mM NaCl, pH 7.0 buffer at 20 °C (Figure 1).[12] The values of *m* (Table 1), the rate of change of the free energy of denaturation with respect to the denaturant concentration, are in relatively close agreement for the wild-type and the corresponding mutant protein as well.[13] Substitution of Leu 14 with α-hydroxy acid **2** decreases the stability of

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(10) To simplify the purification of SNase, a pAED4 derived vector was constructed that allows the efficient expression of a C-terminal His-Tag SNase under T7 control. Purification of the corresponding *in vitro* protein was accomplished using a nickel affinity column or via batch method.[18] This system provides reproducible *in vitro* expression of >80 μg mL^{-1} SNase after purification.

(11) Mutants were incubated in concentrated ammonium hydroxide for 10 min at 90 °C. The resulting products were separated by SDS-PAGE and visualized by Coomassie blue staining; quantitative cleavage was observed at the site of substitution.

(12) Denaturation experiments were performed in triplicate in 25 mM sodium phosphate, 100 mM sodium chloride, pH 7.0, according to the procedure of Shortle and Meeker with a Hitachi F-4500 fluorescence spectrophotometer.[19] Protein concentrations used in the denaturation measurements were 6 mg mL^{-1}. The apparent equilibrium constant, K_{app}, for reversible denaturation, assuming a two-state model, was determined for a series of GuHCl concentrations incremented in steps of 0.047 M by using the equation $K_{app} = (I_n - I)/(I - I_d)$ where *I* is measured intrinsic fluorescence, I_n is the extrapolated value of fluorescence for the native state and I_d is the extrapolated value 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. From this plot, the value of K_{app} at [GuHCl] = 0 was extrapolated and ΔG_{H_2O} was determined from the equation $\Delta G = -RT \ln K_{app}$.

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Scheme 1. Schematic Representation of Amino Acid Substitutions for Leu14 and Ile72 in SNase

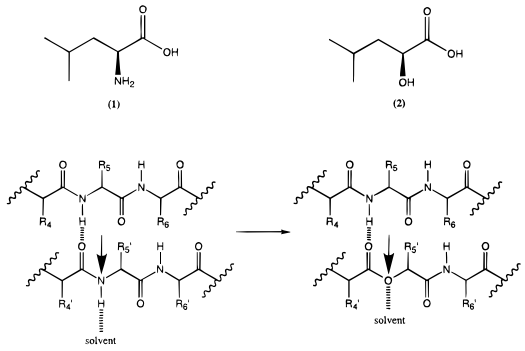

Table 1. Amino Acids Incorporated for Leu-14 and Ile-72 of SNase and the Experimental Thermodynamic Stabilities and Kinetic Constants of the Resulting Enzymes

mutant	C_m[a]	m_{GuHCl}[b]	$\Delta\Delta G°_{H_2O}$[c]	K_m[d]	V_{max}[e]
WT	0.60	1.0		163.4	0.89
Leu-14 → **2**	0.17	1.56	2.5	50.3	0.32
Ile-72 → **1**	0.63	1.0		66.9	0.30
Ile-72 → **2**	0.37	1.14	1.5	24.7	0.10

[a] Midpoint concentration of GuHCl in molarity. [b] Units for Leu-14 → **2** are relative to the wild-type *in vitro* suppressed (WT) and units for Ile-72 → **2** are relative to Ile-72 → **1**, normalized to 1.0, respectively. [c] $\Delta\Delta G°_{H_2O}$ is expressed in units of kcal mol^{-1} (a positive value indicates that the WT protein is more stable than the ester-containing mutant) relative to the wild-type *in vitro* suppressed value of 4.3 kcal mol^{-1} (Leu-14 → **2**) or the Ile-72 → **1** value of 4.6 kcal mol^{-1} (Ile-72 → **2**). [d] K_m is expressed in units of μg mL^{-1}. The assay mixture for kinetic determinations contained 40 mM sodium glycinate at pH 9.5, 10 mM Ca^{2+}, with varying amounts of single-stranded calf thymus DNA (1 to 50 μg mL^{-1}). [e] V_{max} is expressed in units of A_{260} μg^{-1} min^{-1}.

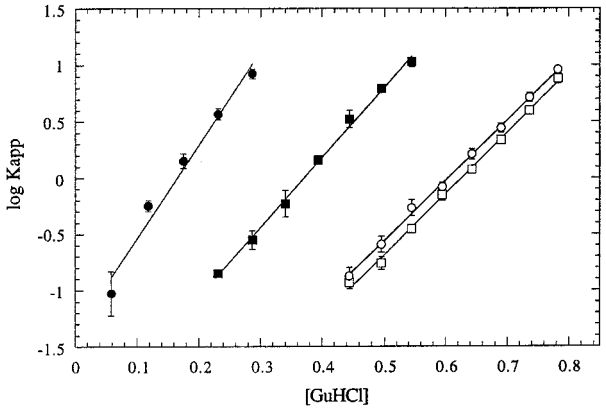

Figure 1. Denaturation plots of log K_{app} versus guanidine hydrochloride concentration for the following SNase variants: (○) Leu14 → L-leucine (**1**, wild-type); (□) Ile72 → L-leucine (**1**); (●) Leu14 → α-hydroxy acid (**2**); (■) Ile^{72} → α-hydroxy acid (**2**).

the mutant protein by 2.5 kcal mol^{-1} with respect to wild type SNase. Likewise, substitution of Ile 72 with **2** leads to a decrease in protein stability of 1.5 kcal/mol relative to the corresponding Ile 72 → Leu mutant.

The experimental $\Delta\Delta G°$ values are in the range of those reported for the deletion of a hydrogen-bonding interaction between neutral side chains in a protein.[2,3] In our case, the value of $\Delta\Delta G°$ reflects the energetic differences between (i) the amide interactions in the folded and unfolded states and (ii) the ester interactions in the folded and unfolded states, i.e., $\Delta\Delta G°_{obsd}$ = [$\Delta G°$ amide (U) − $\Delta G°$ amide (F)] − [$\Delta G°$ ester (U) − $\Delta G°$ ester (F)]. Given the isostructural nature of these groups and the fact that the NH- and O- groups are solvent exposed, $\Delta\Delta G°$ is largely determined by the difference in the backbone carbonyl hydrogen bond strengths in the folded WT and ester-containing proteins, together with the difference in the free energies of the amide and ester carbonyl hydrogen bonds to water in the unfolded states. If the difference in the water−octanol partitioning coefficients of *N*-methylacetamide and methylacetate ($\Delta\Delta G° = -1.7$ kcal/mol)[14] is taken as an upper limit for the energetic difference between the amide and ester hydrogen bonds to water, an upper limit for the difference in the strength of the amide and ester hydrogen bonds in the protein backbone would be on the order of 3.2−4.2 kcal/mol. Other effects such as solvent accessibility, the cooperativity of β-sheet formation, local electrostatic effects, as well as the possibility of structural perturbations, may also contribute to this difference.

The $\Delta\Delta G°$ values for the backbone amide to ester substitutions determined here can be compared to those reported for protein-inhibitor complexes. For example, it has been shown that chemical substitution of a backbone amide group with an ester at the $P'_1 - P'_2$ site in a semisynthetic BPTI leads to a 1.9 kcal/mol decrease in binding affinity for trypsin.[15] In this case, the mutation substituted a hydrogen-bonding NH group with an ester O group. Similarly, substitution of a backbone amide group with an ester in a semisynthetic protein inhibitor, turkey ovomucoid third domain, led to a 1.5 kcal/mol decrease in binding to its cognate enzyme.[16] Again, the mutation substituted a hydrogen-bonding NH group with an oxygen, although alternative hydrogen-bonding schemes are possible. A difference of 2.7 kcal/mol has been determined for an amide to ester substitution in an amide-containing enzyme inhibitor that binds a charged protein side chain in thermolysin.[17] In this case, both the C═O and NH groups of the amide were involved in hydrogen-bonding interactions in the complex. Although these chemical substitutions affect the hydrogen bonding interactions in different ways, generally the amide to ester substitution results in a 1.5−2.7 kcal/mol loss in binding affinity, comparable to the decrease in protein stability determined here.

In conclusion, the substitution of a weaker hydrogen bond acceptor for a backbone amide group in a β-sheet secondary structure leads to a 1.5−2.5 kcal/mol decrease in protein stability, similar to those values reported for deletion of a protein side chain−side chain interaction. It will be of considerable interest to compare these values with those determined for ester mutations at other sites in β-sheets and in other secondary structures, as well as to obtain structural information on these mutant proteins.

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