Stability Mutants of Staphylococcal Nuclease: Large Compensating Enthalpy–Entropy Changes for the Reversible Denaturation Reaction†

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ABSTRACT: By use of intrinsic fluorescence to determine the apparent equilibrium constant K_{app} as a function of temperature, the midpoint temperature T_m and apparent enthalpy change ΔH_{app} on reversible thermal denaturation have been determined over a range of pH values for wild-type staphylococcal nuclease and six mutant forms. For wild-type nuclease at pH 7.0, a T_m of 53.3 ± 0.2 °C and a ΔH_{app} of 86.8 ± 1.4 kcal/mol were obtained, in reasonable agreement with values determined calorimetrically, 52.8 °C and 96 ± 2 kcal/mol. The heat capacity change on denaturation ΔC_p was estimated at 1.8 kcal/(mol K) versus the calorimetric value of 2.2 kcal/(mol K). When values of ΔH_{app} and ΔS_{app} for a series of mutant nucleases that exhibit markedly altered denaturation behavior with guanidine hydrochloride and urea were compared at the same temperature, compensating changes in enthalpy and entropy were observed that greatly reduce the overall effect of the mutations on the free energy of denaturation. In addition, a correlation was found between the estimated ΔC_p for the mutant proteins and the $d(\Delta G_{app})/dC$ for guanidine hydrochloride denaturation. It is proposed that both the enthalpy/entropy compensation and this correlation between two seemingly unrelated denaturation parameters are consequences of large changes in the solvation of the denatured state that result from the mutant amino acid substitutions.

The native state of most proteins is only marginally stable in aqueous solution relative to an alternative, less structured state of the protein chain referred to as the denatured state. For a number of proteins, the interconversion reaction between those two states appears to be reversible and can be analyzed by biophysical methods. For a smaller number of proteins, such analysis has yielded results that are consistent with the two-state approximation; i.e., the transition between the two alternative states behaves like a simple unimolecular reaction with no stable intermediate states detectable. For these proteins, basic physical-chemical parameters of the reaction can be obtained by determining how the ratio of the concentration of the two states, that is, the equilibrium constant K_d, changes when the reaction is perturbed by varying temperature, pH, or solvent composition (Tanford, 1971).

With the recent developments in recombinant DNA methodology, it has become possible to alter yet another fundamental variable of the denaturation reaction, namely, the amino acid sequence of the protein. Characterization of the effects of amino acid substitutions on the structure of the native and denatured states and on the energetics of their interconversion provides new opportunities for gaining fundamental insights into the physical-chemical processes that govern the folding of protein chains. If a mutant protein can be crystallized and its structure determined by X-ray diffraction methods, then definitive information regarding the effect of the amino acid substitution on the structure of native state can be obtained (Alber et al., 1986). To determine the energetic consequences of the substitution on the denaturation reaction, denaturation curve analysis and differential scanning calorimetry can be employed. Unfortunately, methods for characterizing the denatured state and alterations induced in its "structure" by amino acid substitutions have yet to be developed.

This laboratory has undertaken a study of the folding/stability behavior of a large collection of mutant forms of staphylococcal nuclease (Shortle & Lin, 1985), with initial emphasis being placed on characterization of the alterations in the energetics of denaturation. Since this small protein conforms rather closely to the two-state approximation in its behavior on reversible denaturation (Anfinsen & Taniuchi, 1971; Shortle, 1985), changes in K_d can be translated into changes in the apparent free energy for the reaction. A survey of over 40 different mutant nucleases by guanidine hydrochloride denaturation has revealed many amino acid substitutions that alter both the free energy of denaturation (ΔG_{app}) and the rate of change of the free energy of denaturation as a function of denaturant concentration [$d(\Delta G_{app}/dC$] (Shortle & Meeker, 1986). Single amino acid substitutions were found that reduced $d(\Delta G_{app})/dC$ for guanidine hydrochloride by as much as 22% relative to that of the wild type, whereas others increased this parameter by as much as 15%. When doubly mutant proteins were constructed from pairs of characterized single mutations, $d(\Delta G_{app})/dC$ deviated from the wild-type value by even larger amounts. From the patterns observed in these studies, the conclusion was reached that the altered values of $d(\Delta G_{app})/dC$ can be most readily explained as a manifestation of large changes in chain–chain or chain–solvent interactions which occur in the denatured state.

To more fully characterize the physical mechanism responsible for these observations, these same mutant nucleases have now been analyzed by thermal denaturation. Through use of the van't Hoff equation, values of K_{app} as a function of temperature can be translated into apparent enthalpy and entropy changes for the denaturation reaction. On the basis of the data presented in this paper, the conclusion that major changes have occurred in the denatured state as a consequence of only one or two amino acid substitutions receives additional

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support. In fact, most of the patterns observed for both solvent and thermal denaturation are consistent with modifications in the solvation of the denatured state.

Experimental Procedures

Purification of Proteins. Correctly processed wild-type and mutant nucleases were prepared from an alkaline phosphatase–staphylococcal nuclease gene hybrid carried on the pFOG405 plasmid and purified as previously described (Serpersu et al., 1986), except that Sephadex CM-25 was used in place of Bio-Rex 70 to avoid the leaching of an insoluble residue from the column. Protein preparations were confirmed to be at least 95% pure by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. Protein concentration was determined from the absorbance at 280 nm, with an extinction coefficient of 0.93 for a 1 mg/mL solution (Fuchs et al., 1967).

Thermal Denaturation. Stock solutions of protein were diluted to a final concentration of 50 μg/mL in 0.1 M NaCl plus either 25 mM Na_3PO_4, pH 7.00, or 20 mM sodium acetate with a specified final pH. Samples were heated to 37 °C for 15 min and then cooled to room temperature over 10 min, and 3.00 g was added to a stoppered fluorescence cuvette. The temperature of the sample was monitored with a YSI 511 probe (calibrated against ASTM 38C and 49C thermometers) inserted through the cap of the 1-cm-path fluorescence cuvette and immersed in the sample to a depth of 5 mm. Intrinsic tryptophan fluorescence was monitored on a SPEX Fluorog II with a water-jacketed cuvette holder by exciting at 295 nm and recording the emission at 325 nm. For experiments in which the T_m was greater than 40 °C, the temperature was increased at a uniform rate of 1 °C/min and the fluorescence signal averaged over 6-s intervals. For values of T_m less than 40 °C, the rate of temperature increase was 0.5 °C/min and the averaging time 12 s. Differential scanning calorimetry was performed on wild-type nuclease which had been dialyzed extensively against 100 mM NaCl–25 mM Na_3PO_4, pH 7.0, and diluted in dialysis buffer to 1.00 mg/mL. The instrument used was a Microcal MC2 interfaced to a microcomputer for automatic data collection and control via a 12-bit A/D converter (Data Translation DT2801). All experiments were performed at a scanning rate of 60 °C/h.

Data Analysis. In all cases, apparent thermodynamic parameters were obtained by nonlinear least-squares analysis of these denaturation curves. To summarize, the fluorescence intensity as a function of temperature is represented by

$$I(t) = [1 - f_D(t)]I_N(t) + f_D(t)I_D(t)$$

where $I(t)$ is the fluorescence intensity at any temperature t, $I_N(t)$ and $I_D(t)$ are the fluorescence intensities of the native and denatured states, respectively, and $f_D(t)$ is the fraction of molecules in the denatured state. $f_D(t)$ is given by the two-state equation

$$f_D(t) = e^{-\Delta G_{app}/RT}/(1 + e^{-\Delta G_{app}/RT})$$

where $\Delta G_{app} = \Delta H_{app,0} + \Delta C_p(T - T_0) - T[\Delta S_{app,0} + \Delta C_p \ln (T/T_0)]$. The fluorescence intensities of the native and denatured states were assumed to be linear functions of temperature within the transition region. The denaturation curves were fitted to the theoretical equations by a multivariate nonlinear least-squares fitting routine developed by us (Masserini & Freire, 1986). Data points equally spaced at 0.65 °C intervals were used in the analysis. In all cases the denaturation data did not show systematic deviations from the two-state behavior as indicated by the random scatter of the residuals. Convergence was achieved within 10–20 iterations.

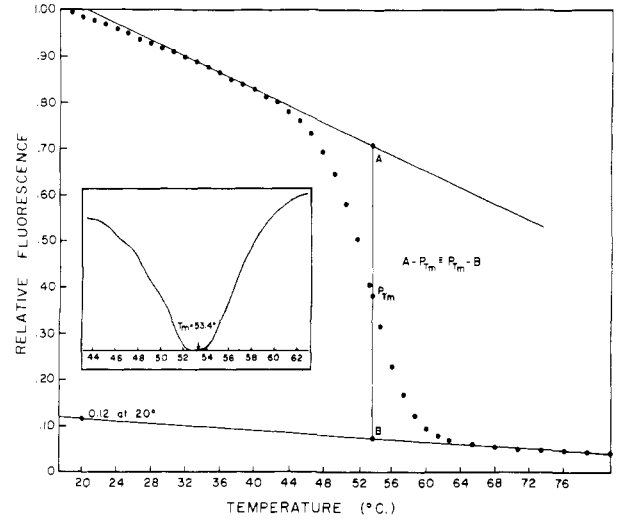

FIGURE 1: Graph of relative tryptophan fluorescence versus temperature for wild-type nuclease at pH 7.00. The midpoint temperature (T_m) is approximated as the temperature with the largest absolute value of the first derivative of fluorescence relative to temperature (insert). The base lines for the fluorescence of the native and denatured states are drawn so that K_{app} at T_m is equal to 1.00 (see Results). To obtain a better estimate of both the T_m and the ΔH_{app}, a nonlinear least-squares best fit to a two-state reaction is applied to the data.

The nonlinear least-squares analysis was initialized with estimates determined as follows: values of relative fluorescence were plotted manually as a function of temperature in 1.3 °C intervals. As described under Results, the base lines for the native and denatured states were drawn so that T_m equaled the temperature of maximum rate of change of the fluorescence with respect to temperature, determined by the first derivative of the fluorescence relative to the time axis. The apparent equilibrium constant K_{app} was calculated from (Pace, 1975)

$$K_{app} = (I_n - I)/(I - I_d)$$

where I is the fluorescence intensity of the sample, I_n is the estimated fluorescence of the native state, and I_d is the estimated fluorescence of the denatured state. The initial estimate of ΔH_{app} was obtained by van't Hoff analysis of the apparent equilibrium constant.

To estimate the ΔC_p, a linear least-squares straight line was fit to the values of ΔH_{app} as a function of T_m, by use of either all or part of the data set (see below).

Results

Analysis of Thermal Denaturation Curves. The reversible denaturation of staphylococcal nuclease was followed by monitoring the intrinsic fluorescence of the single tryptophan residue at position 140. As found in earlier studies, the relative fluorescence emission at 325 nm declines by 88% on unfolding of the protein at 20 °C (Anfinsen & Taniuchi, 1971; Shortle, 1985). As can be seen in Figure 1, the fluorescence of the native state declines more rapidly with increasing temperature than that of the denatured state. Consequently, the drop in relative fluorescence on denaturation is somewhat smaller at the higher temperatures required to completely denature the protein. Nevertheless, denaturation at temperatures above 55 °C still gives rise to a large reduction in intrinsic fluorescence that follows a sigmoidal curve.

To obtain the equilibrium constant as a function of temperature from such a curve, the fluorescence intensity of the native and denatured states in the transition region must be known or reasonably estimated. For the denatured state, the

Table I: Midpoint Temperatures and Apparent Enthalpies for Thermal Denaturation as a Function of pH

pH	T_m[a]	ΔH_{app}[b]	pH	T_m[a]	ΔH_{app}[b]	pH	T_m[a]	ΔH_{app}[b]
	Wild Type			V66L + G88V			V66L + G79S + G88V	
7.0	53.4	88.8	7.0	57.6	56.0	7.0	53.4	44.0
7.0	53.4	85.9	5.52	55.2	47.1	5.52	50.5	37.6
7.0[c]	53.2	86.7	4.95	52.6	44.4	4.95	47.8	35.4
7.0[d]	53.0	85.7	4.25	47.1	42.5	4.76	45.1	32.9
5.52	51.4	81.9	4.01	42.6	36.8	4.48	43.2	33.9
4.9	48.3	73.0	3.9	37.1	33.4	4.4	42.3	35.1
4.6	45.2	69.6	3.8	34.9	34.4	4.1	40.5	35.2
4.5	45.2	67.7	3.8	33.0	31.8	4.0	39.1	33.2
4.25	42.1	57.9	3.64	30.0	29.7	3.9	33.2	30.0
4.12	41.2	59.0				3.7	31.5	28.2
4.01	37.7	54.6		G88V		3.7	32.0	30.7
3.81	31.8	48.2	7.0	55.9	68.9			
3.63	24.6	39.3	7.0	56.2	73.2		A69T	
			5.52	55.0	65.4	7.0	41.2	63.4
	V66L		4.9	51.4	57.7	7.0	41.4	66.9
7.0	55.7	72.3	4.68	50.6	58.3	4.94	35.3	51.4
7.0	56.2	78.8	4.52	49.4	56.9	4.68	32.5	47.9
5.52	54.1	66.3	4.25	46.4	52.4	4.52	29.8	45.5
4.9	51.0	61.7	4.12	43.1	45.5	4.25	25.5	41.1
4.76	49.2	60.1	4.01	40.5	44.4			
4.48	45.6	55.8	3.9	37.5	39.6		I18M + A90S	
4.25	45.1	56.1	3.8	34.1	37.0	7.0	41.6	72.5
4.0	38.7	46.0	3.8	32.7	37.4	5.82	38.8	62.4
3.92	35.1	44.9	3.7	29.0	34.7	5.51	35.9	53.6
3.7	30.9	39.7	3.64	27.0	33.3	5.26	34.7	54.1
						4.9	31.1	47.9
						4.76	29.5	48.1
						4.55	26.8	42.4

[a] In kilocalories per mole. [b] In degrees Celsius. [c] Protein concentration was 10 μg/mL. [d] Heating rate was 0.5 °C per minute.

assumption was made that its fluorescence followed the same, nearly linear temperature dependence as *N*-acetyltryptophanamide (data not shown). Therefore, for this base line a straight line was drawn from a value of 0.12 at 20 °C to meet the linear portion of the curve at high temperatures, usually a value of 0.05 at 75 °C.

The fluorescence of the native state of nuclease, however, exhibits a slight downward curvature at temperatures below the onset of detectable denaturation, making the extrapolation of a native base line somewhat problematic. To achieve consistency in this important step, the first derivative of the fluorescence curve was determined, and the temperature at the minimum was used as an approximation of the midpoint temperature T_m. (This approximation is in error by a few tenths of a degree because temperature rather than inverse temperature was used as a variable and because of the linearly decreasing amplitude of the fluorescence change for the transition.) A vertical line was drawn from the point on the curve at this temperature to the denatured base line and then extended an equal distance above the curve. From the end of this line a tangent was then drawn to the low-temperature part of the curve (see Figure 1), thus constructing the native-state base line so that K_{app} equals exactly 1 at the temperature (T_m) of greatest rate of change of fluorescence. Only small changes in slope of the native base line drawn in this way were found among the seven different nucleases analyzed.

Values of K_{app} were determined graphically by calculating the ratio of the distance between the native-state base line and a point on the curve to the distance between the point and the denatured-state base line. The van't Hoff equation

$$d(\ln K_{app})/d(1/T) = -\Delta H_{app}/R$$

was solved by a least-squares fit of the data to a second-order polynomial, and the apparent enthalpy was determined from the first derivative of the polynomial equation at $T = T_m$. These values were then used to initialize the nonlinear least-squares optimization routine, as described under Experimental Procedures. For the 70 denaturation curves described below, the average change in T_m produced by the optimization routine was ±0.3 °C, and the maximal change was 0.8 °C; the average change in the ΔH_{app} was ±3% with a maximal change of 9%.

The ΔC_p was estimated by assuming that enthalpy of denaturation is independent of pH and determining the apparent enthalpy at different T_m through variation of the pH of the protein solution (Privalov, 1979). Straight-line and second-order polynomial linear least-squares fitting across part of or all of the temperature range were used to obtain the best estimate of ΔC_p and to interpolate values of ΔH_{app}.

Wild-Type Nuclease. The results of analyzing 13 denaturation curves of the wild-type protein are listed in Table I. From four separate curves at pH 7.0, a T_m of 53.3 ± 0.2 °C and an apparent or van't Hoff enthalpy of 86.8 ± 1.4 kcal/M were obtained. These values were unchanged when the protein concentration was lowered from the standard 50 μg/mL concentration to 10 μg/mL or when the heating rate was lowered from 1 °C/min to 0.5 °C/min. Although as much as 99% of the starting fluorescence could be recovered by rapid cooling at the end of a temperature ramp, a clear correlation between duration of incubation at temperatures above the T_m and loss of recoverable fluorescence was noted.

When the apparent enthalpies obtained in solutions of wild-type nuclease at lower pH were plotted as a function of the T_m to estimate the ΔC_p (see Figure 2), a plot was obtained that appears to be slightly upward curving. Similar results have been obtained for the calorimetric enthalpy as a function of T_m (Calderon et al., 1985; Table V). If ΔC_p is assumed to be constant, all of the data points should fit on a straight line. A linear least-squares fit of the data gives a line with a slope of 1.8 kcal/(mol K) (Table II). Much better correlation coefficients are obtained if the curve is arbitrarily divided into two equal halves and each half is fit separately by a straight line: a slope of 1.2 kcal/(mol K) for the four points in the range 25–42 °C and a slope of 2.4 kcal/(mol K) for the nine points in the range 42–53 °C.

The thermal denaturation of the wild-type nuclease was also studied by high-sensitivity differential scanning calorimetry.

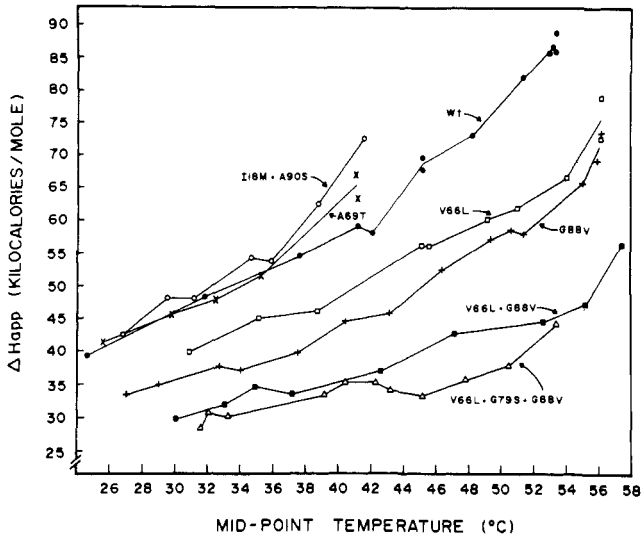

FIGURE 2: Graph of ΔH_{app} versus T_m for wild-type staphylococcal nuclease and six mutant forms (designated by their amino acid substitutions with the one-letter code). Numerical values are listed in Table I.

Table II: Estimated Values of ΔC_p[a]

protein	ΔC_p [kcal/(mol K)]	temp range (°C)	no. of points	correlation coefficient
wt	1.8	25–54	13	0.977
	1.2	25–42[b]	4	0.999
	2.4	42–54	9	0.991
V66L	1.4	30–56	10	0.968
	0.8	30–42	3	0.951
	1.8	42–56	7	0.937
G88V	1.3	27–56	14	0.973
	0.8	27–42	6	0.967
	1.9	42–56	8	0.976
V66L + G88V	0.8	30–57	9	0.961
V66L + G79S + G88V	0.5	31–53	11	0.895
A69T	1.6	25–42	6	0.972
I18M + A90S	1.9	26–42	7	0.963

[a] A linear least-squares fit to values of ΔH_{app} as a function of T_m was applied to data points over the listed temperature range. [b] The temperature range is divided into two approximately equal halves to obtain a better estimate of the ΔC_p at lower temperatures for wt, V66L, and G88V.

In this case, a transition temperature of 52.8 °C (defined as the temperature of the maximum in the heat capacity function), a calorimetric ΔH of 96 ± 2 kcal/mol, a van't Hoff ΔH_{vh} of 91 ± 3 kcal/mol, and a ΔC_p of 2.2 kcal/(mol K) were obtained. The ratio $\Delta H/\Delta H_{vh}$ of 1.05 agrees well with those found for other proteins (Privalov, 1979) and indicates that the transition deviates very little from two-state behavior. Previously, Calderon et al. (1985) obtain a value for $\Delta H/\Delta H_{vh}$ of 1.3. These experiments, however, used a variant of nuclease containing seven extra amino acids on the amino-terminal end and higher protein concentrations, concentrations at which nuclease exhibits a significant tendency to aggregate (Calderon et al., 1985).

Mutant Nucleases. (*A*) *Valine-66 to Leucine* (*V66L*). This mutation was originally isolated as a second-site suppressor of two different destabilizing mutations and was found to be a suppressor of at least five other mutations (Shortle & Lin, 1985). Initial characterization of the effect of this amino acid substitution on wild-type nuclease and several mutations by guanidine hydrochloride and urea denaturation (Shortle, 1985; Shortle & Meeker, 1986) has revealed a complex pattern of nonadditivity. In an otherwise wild-type protein, this substitution lowers the relative stability by an estimated 0.2 kcal, from a ΔG_{app} of +5.6 for wild-type nuclease to +5.4 kcal/mol. In combination with the G88V substitution described below, it reduces the stability by as much as 1.0 kcal/mol below that of the G88V substitution alone. However, in combination with A69T, it increases the stability by 0.8 kcal/mol above that of the mutant alone (Sondek and Shortle, unpublished data). One explanation for such nonadditivity is that the V66L substitution is inducing a "conformational change", either in the native state or in the denatured state (Shortle, 1985). However, the primary reason for further analysis of this mutant protein is the observation that V66L exhibits a reduced intrinsic sensitivity to denaturation by guanidine hydrochloride and urea as measured by $d(\Delta G_{app})/dC$, the rate of change of the free energy of denaturation as a function of denaturant concentration (Shortle & Meeker, 1986).

At pH 7.00, average values for a T_m of 56.0 °C and apparent ΔH of 75.1 kcal/mol were obtained. As can be seen in Figure 2, a graph of ΔH_{app} versus T_m also shows slight upward curvature. Fitting all 10 data points to a single straight line gives a value for the ΔC_p of 1.3 kcal/(mol K), whereas 0.8 and 1.8 are obtained from the low- and high-temperature ranges (Table II).

(*B*) *Glycine-88 to Valine* (*G88V*). This amino acid substitution was isolated as a second-site suppressor of the G79S mutation; its suppression spectrum has not yet been determined. At 20 °C, it is estimated by guanidine hydrochloride denaturation to have a free energy of stability of +4.6 kcal/mol. It also is of interest because it exhibits behavior similar to that of V66L on guanidine hydrochloride and urea denaturation, namely, a markedly reduced $d(\Delta G_{app})/dC$.

A T_m of 56.1 and a ΔH_{app} of 71.1 kcal/mol were obtained at pH 7.00. Thus, this mutant protein is more stable than wild-type at high temperatures but less stable than wild-type nuclease at 20 °C by 1.0 kcal/mol. The graph of ΔH_{app} versus T_m shows very marked curvature. A straight-line fit to all 14 data points gives a C_p of 1.3 kcal/(mol K). However, if the data are divided into the ranges 27–42 °C and 42–56 °C, values of ΔC_p of 0.8 and 1.9 kcal/(mol K) are obtained, respectively.

(*C*) *V66L plus G66V.* As mentioned above, when the V66L and G88V substitutions are combined, the resulting protein has a ΔG_{app} of 3.5 kcal/mol, which is 0.9 kcal/mol less than is predicted from additivity of the effects of the two mutations. At pH 7.00, the T_m was 57.6 °C and the ΔH_{app} was measured as 56.0 kcal/mol. The graph of ΔH_{app} versus T_m shows considerable scatter, making any intrinsic curvature less obvious. When the nine data points are fit to a single straight line, a value of 0.8 kcal/(mol K) is obtained for the ΔC_p.

(*D*) *V66L + G88V plus Glycine-79 to Serine.* This protein was constructed by recombining V66L + G88V with a third mutation, glycine-79 to serine. The triply mutant protein was chosen for study because it exhibits the smallest sensitivity to guanidine hydrochloride of any nuclease derivative studied to date, its $d(\Delta G_{app})/dC$ equaling only 0.51 of that of the wild-type protein. Nevertheless, it is not a very stable protein, an estimate of 2.6 kcal/mol being the value obtained by guanidine denaturation at 20 °C (Shortle & Meeker, 1986).

At pH 7.00, a T_m of 53.4 °C and a ΔH_{app} of 44.0 kcal/mol were obtained. The 11 points on the ΔH_{app} versus T_m plot can be fit by a straight line, although with considerable scatter, to give an estimated ΔC_p of 0.5 kcal/(mol K).

(*E*) *Alanine-69 to Threonine* (*A69T*). This mutant nuclease was chosen for study because, in contrast to the above proteins,

Table III: Thermodynamic Parameters of the Reversible Denaturation Reaction at pH 7.0[a]

nuclease	ΔG_{app} (321 K)	ΔH_{app} (321 K)[b]	ΔS_{app} (321 K)	ΔG (293 K)[c]	ΔG (293 K)[d]
wt	+1.4	73.3	0.224	+6.1	+5.6
V66L	+1.8	58.5	0.177	+5.6	+5.4
G88V	+1.7	53.7	0.162	+5.2	+4.6
V66L + G88V	+1.6	41.6	0.125	+3.9	+3.5
V66L + G79S + G88V	+0.6	37.2	0.114	+3.2	+2.6
A69T	−1.4	74.5	0.236	+3.3	+2.9
I18M + A90S	−1.6	80.2	0.256	+2.7	+2.8

[a] Values of ΔG and ΔH are in kilocalories per mole; values of ΔS are in kilocalories per mole per kelvin. [b] Estimated by fitting the ΔH_{app} data shown in Figure 2 to a second-order polynomial equation in T and solving at 47.9 °C. [c] Estimated from ΔH_{app} (321 K), ΔS_{app} (321 K), and ΔC_p (25–42 °C). [d] Estimated from guanidine hydrochloride denaturation at 293 K and linear extrapolation to zero denaturant (Shortle & Meeker, 1986).

it has a greater than wild-type value of $d(\Delta G_{app})/dC$ for both guanidine hydrochloride and urea. Its T_m at pH 7.00 was 41.2 °C, and an average of ΔH_{app} was 65.2 kcal/mol. Fitting a straight line to just six data points for this protein gave an estimate for the ΔC_p of 1.6 kcal/(mol K).

(*F*) *Isoleucine-18 to Methionine and Alanine-90 to Serine (I18M + A90S).* Like A69T, this doubly mutant protein has a greater than wild-type sensitivity to guanidine hydrochloride denaturation. In fact, the denaturation curves for I18M + A90S and A69T are virtually superimposable in both guanidine hydrochloride and urea, giving a ΔG_{app} of 2.8 kcal/mol. At pH 7.00, the T_m was measured at 41.6 °C, and ΔH_{app} was 72.5 kcal/mol. The plot of ΔH_{app} versus T_m seems adequately fit by a single straight line to give an estimated ΔC_p of 1.9 kcal/(mol K).

Discussion

In this analysis of the thermal denaturation of several different mutant forms of staphylococcal nuclease, the primary data consist of values of T_m and van't Hoff or apparent enthalpies of denaturation determined by monitoring tryptophan fluorescence as a function of temperature. Before proceeding to discuss the significance of these results, several issues concerning the merit of these data must be discussed. One major question is the reliability of tryptophan fluorescence as a parameter for quantitating the denaturation equilibrium constant as a function of temperature. For wild-type protein, the very good agreement between the calorimetric T_m versus the midpoint defined by fluorescence and between the ΔH_{app} and the calorimetric ΔH suggests that, for this nuclease, denaturation is the only significant reaction that leads to quenching, once the intrinsic temperature dependence of fluorescence of the native and denatured states has been subtracted. Although the possibility exists that, in addition to the denaturation reaction, a local unfolding or "breathing" reaction accounts for the broader transition found for many of the mutant proteins, the apparent enthalpy of the major quenching reaction is in all cases 50% or more of the wild-type value. Such a large value argues for a major structural change involving, presumably, at least 50% of the bonds broken on denaturation of the wild-type protein. In addition, none of the denaturation curves obtained showed systematic deviations from two-state behavior. Taken together, these two findings can be most simply explained by a single cooperative unfolding reaction involving exposure of the tryptophan residue.

A second important issue concerns the nature of reversible denaturation of proteins and the meaning of an apparent enthalpy for this reaction. In terms of the two-state approximation, denaturation represents the transformation of a polypeptide chain from a highly ordered native state (N) to a largely disordered denatured state (D), both of which are presumed to consist of a very large number of microstates (Lumry et al., 1966). As pointed out by Tanford (1969), there are considerable difficulties in rigorously defining the D state. One is forced to settle for an operational definition for denaturation, such as "...simply a major change from the original native structure, without alteration of the amino acid sequence..." (Tanford, 1969). Furthermore, as emphasized by Lumry, Biltonen, and Brandts (Lumry et al., 1966), the distribution of N and D microstates must change as the conditions of solution are altered, since microstates at opposite ends of the distribution will usually undergo different changes in free energy. When temperature is the variable, shifts to microstates of higher enthalpy occur as the temperature is increased, and given the greater heat capacity of the D state, the distribution of D microstates will exhibit significant dependence on temperature.

Given this inability to supply a precise standard definition of the D state that applies to all proteins or even one protein at all temperatures, the denaturation reaction must be viewed in operational terms, and thus the thermodynamic quantities that characterize the reaction also are operationally defined. The empirically determined van't Hoff enthalpy can only be expected to reflect the enthalpy change for the largest cooperative breakdown in N-state structure that is detectable by the spectroscopic parameter being monitored. From the definition of Tanford quoted above, this cooperative breakdown in structure is the denaturation reaction even though it may yield a more or less ordered D state. The important issue of intermediate states between the N and D states, how to define them and how to distinguish them from the more ordered microstates in the D-state distribution, will be considered below.

There are several significant results described in this paper. First of all, a series of mutant nucleases have been identified which, when denatured under identical conditions of temperature, salt, and pH, display either an increase or a decrease in the apparent enthalpy of denaturation. Since at T_m the entropy of denaturation is equal to the enthalpy divided by the T_m, this can only occur because the entropy of denaturation for the mutant proteins has also been reduced by an amount that almost exactly compensates for the reduction in the enthalpy. The values of ΔG_{app}, ΔH_{app}, and ΔS_{app} at 47.9 °C for the seven different proteins are compared in Table III. Relative to wild-type nuclease, the most striking disparity between changes in free energy and enthalpy is exhibited by the V66L + G79S + G88V mutant protein. Although at 47.9 °C this protein is only 0.8 kcal/mol less stable than wild-type protein, the apparent enthalpy change on denaturation is reduced by almost 35 kcal/mol. The quantitative relationship between the changes in ΔH_{app} and ΔS_{app} can be seen in Figure 3, where ΔH_{app} is found to be a linear function of ΔS_{app} with a proportionality constant of 303 K.

Enthalpy/entropy compensation is a common phenomenon for small molecule reactions in solution and has been observed in a number of reactions involving the binding of ligands to

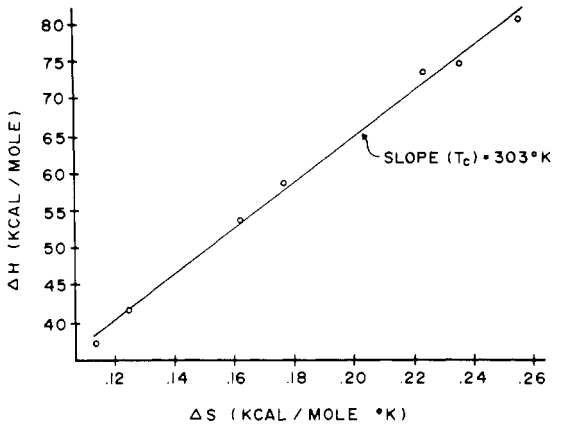

FIGURE 3: Plot of the ΔH_{app} versus ΔS_{app} at 47.9 °C (321 K) and pH 7.0 for the seven different nucleases. Values are taken from Table III. ΔH_{app} was obtained from a second-order polynomial equation fit to all of the points shown in Figure 2. The line is a linear least-squares fit to the data (correlation coefficient of 0.998), and its slope is defined as the compensation temperature T_c (Lumry & Rajender, 1970).

proteins [extensively reviewed by Lumry and Rajender (1970) and Lumry (1986)]. There are two general methods of generating values of ΔH and ΔS for a reaction in aqueous solution in order to detect this phenomenon. Either the solvent composition can be varied, usually by the addition of small amounts of alcohols, or the structure of the molecule undergoing reaction can be modified to produce a series of closely related reactants. The operational criteria for enthalpy/entropy compensation is the finding of a linear relationship between ΔH and ΔS for a series of modified reactions at a constant temperature.

Compensation behavior for the reversible denaturation reactions of proteins has been demonstrated by both of these methods. Brandts and Hunt (1967) measured the ΔH_{app} and ΔS_{app} for the denaturation of ribonuclease A at three different temperatures as a function of mole fraction of ethanol. When ΔH_{app} was plotted versus ΔS_{app}, straight lines of constant slope T_c = 285 °C were obtained at each temperature (Lumry & Rajender, 1970). One of the first reports of enthalpy/entropy compensation for a series of mutant forms of a simple protein was by Hawkes et al. (1984). In this study of T4 lysozyme, three out of the four single amino acid substitutions analyzed showed significant reductions in the ΔH_{app} for denaturation at 46.9 °C with compensating reductions in ΔS_{app}, leading to only very small changes in the free-energy change. For the N14 mutant (Ala-146 to Thr) which exhibited the most pronounced change, the denaturation enthalpy at 46.9 °C was reduced by 27% and the entropy by 25%.

In their discussion of the possible origins of the nearly equal reductions in the difference in enthalpy and entropy between the native and denatured states, Hawkes et al. (1984) put forward two possible mechanisms: (1) enhanced fluctuations in the mutant native state, perhaps through the formation of a partially unfolded loop; (2) distortion of local geometry in the native state that results in an increase in strain enthalpy and a higher vibrational entropy. Although these authors raised the possibility that the compensating enthalpy/entropy changes are a consequence of alterations in the denatured state of the mutant proteins, they considered this unlikely because of the magnitude of the changes produced by single, relatively conservative amino acid substitutions.

In their extensive review of the literature on enthalpy/entropy compensation phenomena, Lumry and Rajender (1970) proposed a unifying hypothesis for many, if not most, reactions

Table IV: Wild-Type and Mutant Nuclease Parameters of the Denaturation Reaction

protein	ΔC_p [kcal/(mol K)][a]	$d(\Delta G_{app})/d$[Gdn-HCl][b]
wild type	1	1
V66L	0.7	0.82
G88V	0.7	0.78
V66L + G88V	0.7	0.57
V66L + G79S + G88V	0.4	0.51
A69T	1.3	1.15
I18M + A90S	1.6	1.15

[a] Values taken from Table II, using the lower range temperature data for wt, V66L, and G88V. The wild-type value is 1.2 kcal/(mol K). [b] Data are taken from Shortle and Meeker (1986). The wild-type value is 6.8 kcal/mol per 1 M guanidine hydrochloride determined at 20.0 °C.

in aqueous solution that is based on the existence of two energetically different states of water. Suppose reaction

$$A \rightleftharpoons B$$

is coupled to a second reaction in which n water molecules in state W1 undergo a transition to state W2:

$$n(H_2O)_{w1} \rightleftharpoons n(H_2O)_{w2}$$

The overall enthalpy change for the reaction then becomes

$$\Delta H = \Delta H_{a\rightarrow b} + n\Delta H_{w1\rightarrow w2}$$

and the entropy change is

$$\Delta S = \Delta S_{a\rightarrow b} + n\Delta S_{w1\rightarrow w2}$$

If A corresponds to the native state and B the denatured state, then both the enthalpy and the entropy of denaturation are resolved into the sum of contributions from the breakdown of bonds and increased number of conformations of the polypeptide chain plus the thermodynamic consequence of n water molecules shifting from state W1 to state W2 in order to solvate newly exposed regions of the denatured protein.

One possible origin of the enthalpy/entropy compensation seen in protein denaturation could be parallel changes in $\Delta H_{a\rightarrow b}$ and $\Delta S_{a\rightarrow b}$, as suggested by Hawkes et al. (1984). However, as pointed out by these authors, it is rather difficult to explain almost exactly compensating changes in $\Delta H_{a\rightarrow b}$ and $\Delta S_{a\rightarrow b}$ without invoking special circumstances. On the other hand, compensation could arise from the coupled hydration reaction (Lumry & Rajender, 1970). If the number of water molecules n that take part in this reaction increases or decreases as a result of modifications in the structure of the reactant, the contribution water makes to ΔH and ΔS will increase or decrease in parallel; compensation would be almost complete. In their analysis of examples of compensation phenomena, Lumry and Rajender (1970) argue that a compensation temperature T_c in the range of 250–320 K may be interpreted as an indication of the role played by water in the underlying compensation mechanism.

There are two lines of evidence supporting the conclusion that large perturbations have occurred in the solvation of the denatured state, giving credence to the Lumry–Rajender mechanism of compensation. First of all, a general correlation can be seen between the estimated values of ΔC_p for the temperature range of 25–42 °C and the sensitivity to guanidine hydrochloride denaturation at 20 °C as measured by $d(\Delta G_{app})/dC$. As shown in Table IV, the direction of change and the rank order for the six mutant proteins are the same for both of these parameters.

It has been argued for many years that the principal contribution to the ΔC_p comes from the additional water of hydrophobic hydration bound to the denatured state (Kauzman, 1959; Privalov, 1979); some contribution is also expected from

the increase in the heat capacity of the denatured polypeptide chain itself (Sturtevant, 1977). Unfortunately, the precise chemical mechanism by which denaturants like guanidine hydrochloride promote denaturation has not been determined. However, a general thermodynamic formulation based on the statistics of many weak interactions between solvent and the surface of a protein leads to the conclusion that $d(\Delta G_{app})/dC$ should be proportional to the increase in solvent-accessible surface area ΔA on denaturation (Schellman, 1978). If this is the case, then the correlation between values of ΔC_p and $d(\Delta G_{app})/dC$ could be a consequence of changes in ΔA caused by the amino acid substitution. In other words, the component of ΔC_p arising from the number of water molecules n that solvate newly exposed regions of the protein may be proportional to the same change in solvent-accessible surface area that determines the solvent denaturation parameter.

The fact that the ΔC_p can be altered by one or two amino acid substitutions is a very surprising observation, since this number represents the difference between total heat capacities of the denatured state and the native state. Given the large number of bonds in both states, a change in ΔC_p argues for a major structural change in either the N state, the D state, or both. To the extent that hydrophobic hydration makes the dominant contribution to ΔC_p, one is forced to conclude that, for the V66L + G79S + G88V protein which has a ΔC_p that is only 30% of the wild-type value, there must be a major perturbation involving hydrophobic hydration. As has been argued elsewhere for the large changes in $d(\Delta G_{app})/dC$ for guanidine hydrochloride, it seems more plausible to attribute most of these changes to the D state rather than to the N state (Shortle & Meeker, 1986).

A second line of evidence that argues for the occurrence of major changes in the denatured state is the finding of two mutant proteins, A69T and I18M + A90S that exhibit greater than wild-type values of ΔH_{app} and ΔS_{app} even though they are *less stable* than the wild type at all temperatures. The amino acid substitutions in these two proteins all involve replacing a buried hydrophobic residue with a bulkier polar one. Given this fact plus their lower stability, it seems improbable that the larger increases in enthalpy and entropy on denaturation are a consequence of smaller starting values for the native state, for this would mean a more tightly bonded and more highly confined folded conformation. If the larger than wild-type increases in enthalpy and entropy cannot be attributed to changes in the native state, then the only alternative is to suppose a mutant denatured state with higher enthalpy and higher entropy. Some of this higher enthalpy and entropy could be a consequence of a greater disruption of intrachain interactions. But as argued earlier, it is not evident why the changes in both enthalpy and entropy should so nearly compensate each other, whereas the Lumry–Rajender mechanism provides a simple explanation. Clearly, a polypeptide chain with a higher entropy should require a larger number of water molecules for its solvation.

One last finding that warrants mention is that all of the proteins show an upward curvature of plots of ΔH_{app} as a function of T_m, which means that the ΔC_p is increasing with temperature. [A similar observation for the calorimetric ΔH of wild-type nuclease was made by Calderon et al. (1985).] Although the scatter in the data makes this conclusion somewhat uncertain for most of the proteins, the 14 data points for the G88V mutant nuclease do appear to generate a smooth curve. This feature of the data argues rather strongly that, at low temperature, this mutation lowers the ΔC_p by reducing the heat capacity of the denatured state. If instead the small ΔC_p was solely a consequence of a larger heat capacity for the native state, one would then be faced with the implausible conclusion that the larger ΔC_p at high temperatures is due to a *decrease* in the heat capacity of the native state as the temperature is raised. Since calorimetric data on ethane and propane dissolved in water show a decrease in the ΔC_p with increasing temperature for constant amounts of water of hydrophobic hydration (Naghibi et al., 1987), the most reasonable explanation for the results presented here is that the amount of new hydrophobic surface exposed to solvent increases as the T_m increases.

The van't Hoff enthalpy obtained in this analysis could be in error for at least two reasons. First of all, the assumption was made that pH has little or no direct effect on the enthalpy of denaturation. Privalov (1979) has shown this to be the case for several proteins. Since the pK_a's of the four histidine residues of staphylococcal nuclease are approximately 5.5, 6.0, 6.0, and 6.5 (Markley & Jardetzky, 1970), the residues undergoing protonation on denaturation at pH values lower than 5 are presumably only aspartates and glutamates. As argued by Privalov (1979), the ΔH for protonation of these residues is very small, so that use of a carboxylic acid buffer such as acetate should eliminate almost all heat changes due to proton transfer reactions on denaturation. In addition, the value of ΔC_p estimated by changing the T_m from 38.8 °C at pH 4.00 to 50.0 °C at pH 5.00 and measuring the changes in calorimetric enthalpy agrees well with the calorimetric ΔC_p (Calderon et al., 1985).

A second potential problem is that the amino acid substitutions in these mutant proteins are stabilizing one or more partially denatured intermediates which can subsequently undergo a cooperative transition to a "wild-type-like" denatured state. In keeping with the theoretical framework proposed by Lumry, Biltonen, and Brandts (Lumry et al., 1966), an intermediate state is most reasonably defined as a third distribution of microstates intermediate in structure between the N and D distributions. If there existed a single intermediate state I, then it can be shown that the apparent equilibrium constant K_{app}, as measured by fluorescence, for the thermodynamically coupled reactions

$$N \rightleftharpoons I \rightleftharpoons D$$

would be

$$K_{app} = K_1(1 + K_2 - F_i)/(1 + F_iK_1)$$

where K_1 and K_2 are the equilibrium constants for the N to I and I to D reactions and F_i is the relative fluorescence of the intermediate state with respect to a value of 1 for the native state and 0 for the denatured state. By differentiating ln K_{app} with respect to inverse temperature, the apparent enthalpy change is found to be

$$\Delta H_{app} = \Delta H_1[1/(1 + F_iK_1)] + \Delta H_2[K_2/(1 + K_2 - F_i)]$$

where ΔH_1 and ΔH_2 are then enthalpy changes for the N to I and I to N transitions, respectively. Since models for multiple intermediate states could be constructed along these same lines and since the data reported here would not permit testing of all of the different types of multiintermediate schemes, only the single-intermediate situation will be discussed.

The ability to discern the presence of an intermediate state in the thermal denaturation of one or more of these mutant nucleases would depend in part on its relative fluorescence F_i. If this value was approximately intermediate between those of the N and D states, then a maximum in the ΔH_{app} would be expected at the apparent T_m (Lumry et al., 1966). The possibility of this type of intermediate is effectively ruled out

by the finding that the van't Hoff plots of all 70 denaturation curves were indicative of an ΔH_{app} that increased monotonically with temperature.

If F_i was similar to that of the D state (i.e., approximately 0), the presence of an intermediate that is highly populated at lower temperatures and less populated at higher temperatures would also be reflected in the temperature dependence of ΔH_{app}, i.e., the apparent ΔC_p. From the equation above relating the ΔH_{app} to the enthalpies of the N to I and I to D transitions, it is apparent that, at lower temperatures where K_2 is small, the enthalpy of the observed reaction will be determined primarily by ΔH_1. At higher temperatures where K_2 is large do to conversion of I to D, the ΔH_{app} will approach $\Delta H_1 + \Delta H_2$, the enthalpy change for the N to D transition. The actual temperature dependence of ΔH_{app} is made more complicated by the intrinsic temperature dependence of ΔH_1 and ΔH_2, namely, $\Delta C_{p,1}$ and $\Delta C_{p,2}$. For most protein denaturation reactions, the calorimetric ΔC_p have been found to be more or less constant (Privalov, 1979). Assuming these two changes in heat capacity are constant, it can be shown that the net effect would be an apparent ΔC_p that increases from approximately $\Delta C_{p,1}$ to $\Delta C_{p,1} + \Delta C_{p,2}$. As discussed above, the apparent ΔC_p for several nucleases, particularly the G88V mutant, does increase significantly over the temperature range studied. Thus for these proteins, the occurrence of a significantly populated intermediate state is a formal possibility. With these data alone, it is not possible to distinguish between a uniform, noncooperative breakdown in the residual structure of the D state with increasing temperature and a cooperative structure breakdown in an I to D transition.

In the case of proteins A69T and I18M + A90S, however, intermediate states can be confidently ruled out, since these two proteins exhibit values of ΔH_{app} and ΔS_{app} that are larger than those of the wild type. This greater cooperative breakdown in structure cannot arise by transition to a more structured intermediate. Furthermore, the good agreement between the calorimetric and van't Hoff enthalpies argues that wild-type nuclease undergoes a two-state transition without significant population of intermediate states. Thus, the larger enthalpy and entropy change occurring in these two proteins cannot be explained by supposing that their amino acid substitutions destabilize an intermediate state normally present in the wild-type denaturation reaction.

The results presented in this paper suggest that one or two amino acid substitutions out of a total of 149 can have profound effects on how a simple protein like staphylococcal nucleases undergoes denaturation. Those proteins with lower values of the enthalpy and the estimated ΔC_p presumably do not lose as much order or structure on thermal denaturation. In other words, the initial breakdown of structure is incomplete. At still higher temperatures, presumably most of the remaining structure is eventually lost, either in local, noncooperative reactions or, if true intermediate states are present, in major cooperative reactions. By this same argument, the finding of two mutant proteins with a greater than wild-type ΔH_{app} suggests that the wild-type protein does not lose all structure in a single cooperative transition, even though there is good agreement between the van't Hoff and calorimetric enthalpies.

The changes in the estimated ΔC_p and the striking enthalpy/entropy compensation both suggest that major alterations in the hydration of the denatured state have resulted from the amino acid substitutions. Although this altered hydration produces large effects on the overall ΔH_{app} and ΔS_{app}, it produces only a small change in the ΔG_{app}. These data and conclusions point to the complex and central, but unfortunately poorly understood, role of water in the energetics of protein folding and stability.

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