Thermal unfolding of Staphylococcal nuclease and several mutant forms thereof studied by differential scanning calorimetry

ARTICLE *in* PROTEIN SCIENCE · APRIL 2008

Impact Factor: 2.85 · DOI: 10.1002/pro.5560020408 · Source: PubMed

Protein Science (1993), *2*, 567–576. Cambridge University Press. Printed in the USA.

Thermal unfolding of staphylococcal nuclease and several mutant forms thereof studied by differential scanning calorimetry

AKIYOSHI TANAKA,[1,3] JOHN FLANAGAN,[2] AND JULIAN M. STURTEVANT[1,2]

Departments of [1] Chemistry and [2] Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

(RECEIVED September 15, 1992; REVISED MANUSCRIPT RECEIVED December 8, 1992)

Abstract

The effects of eight mutations on the thermodynamics of the reversible thermal unfolding of staphylococcal nuclease have been determined over a range of pH and protein concentration by means of differential scanning calorimetry. Variation of the protein concentration was included in our study because we found a significant dependence of the thermodynamics of protein unfolding on concentration. Values for the change in the standard free energy of unfolding, $\Delta\Delta G_d^0$, produced by the mutations in the pH range 5.0–7.0 varied from 1.9 kcal mol^{-1} (apparent stabilization) for H124L to −2.8 kcal mol^{-1} (apparent destabilization) for L25A. As has been observed in numerous other cases, there is no correlation in magnitude or sign between $\Delta\Delta G_d^0$ and the corresponding values for $\Delta\Delta H_d$ and $T\Delta\Delta S_d^0$, the latter quantities being in most cases much larger in magnitude than $\Delta\Delta G_d^0$. This fact emphasizes the difficulty in attempting to correlate the thermodynamic changes with structural changes observed by X-ray crystallography.

Keywords: differential scanning calorimetry; mutations; staphylococcal nuclease; thermal unfolding

The thermal denaturation of staphylococcal nuclease (SNase) and several of its mutant forms has been studied by Shortle et al. (1988) using fluorescence emission to monitor the thermally induced conformational changes. The data were subjected to van't Hoff analysis to obtain the thermodynamic parameters for the denaturations. The denaturations were observed over a range of pH, which led to a variation in the mid-temperature of denaturation of approximately 30 °C and permitted evaluation of the permanent change in heat capacity accompanying the denaturations.

We have undertaken a reinvestigation of the thermal unfolding of five of these mutants—V66L, G79S, G88V, V66L + G88V, and V66L + G79S + G88V—using differential scanning calorimetry (DSC). We have also included in our study H124L, a naturally occurring form obtained from the V8 strain of *Staphylococcus aureus*, and the mutants L25A and A90S. It was also necessary to do a careful study of the wild-type (WT) enzyme for comparison purposes. We have employed experimental conditions similar to those employed by Shortle et al. (1988), with the important and unavoidable difference, enforced by the insensitivity of the DSC method, that we have used protein concentrations much above those studied by Shortle et al. We have actually included considerably higher protein concentrations than the calorimetric sensitivity would have required in order to study the significant dependence of the thermodynamics of denaturation on protein concentration. To counterbalance the inherent weakness of low sensitivity of the calorimetric method there are certain advantages. Chief among these is the fact that the denaturational enthalpy and heat capacity changes are obtained directly without resort to a van't Hoff treatment. Furthermore, calorimetry responds to changes taking place throughout the molecule, rather than at a few isolated locations as does fluorimetry.

Results

At the higher pHs studied, the DSC traces observed for WT and mutant proteins were similar to those observed with other small globular proteins such as T4 lysozyme (Kitamura & Sturtevant, 1989). This is illustrated in Fig-

Reprint requests to: Julian M. Sturtevant, Department of Chemistry, Yale University, New Haven, Connecticut 06511.

[3] Present address: Department of Chemistry, Mie University, Tsu, Mie 514, Japan.

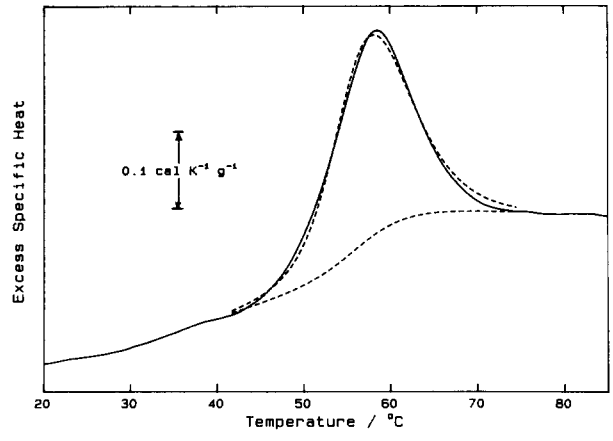

Fig. 1. Differential scanning calorimetry (DSC) curve observed with the double mutant V66L + G88V at pH 7.0. Protein concentration 10.9 mg mL^{-1}; 0.05 M cacodylate buffer containing 0.10 M NaCl. Solid curve, observed data; dashed curves, data calculated by means of program DIAS and calculated baseline (cf. text).

ure 1 for the double mutant V66L + G88V at pH 7.0. As the pH is lowered, the enthalpy decreases with the permanent heat capacity change remaining essentially unchanged. This leads to transition curves at low pH such as those shown in Figure 2. This figure illustrates the variation among mutants in sensitivity to changes in pH, with the V8 strain (H124L) being least affected. It is evident that in cases such as G79S at pH 3.90 the values obtained for Δh_{cal}, the specific enthalpy of denaturation, are highly dependent on the selection of pre- and posttransition baselines and are therefore subject to large uncertainties. This selection is especially arbitrary in those cases where the pretransition baseline is curved at all observable temperatures, presumably because of approach to cold denaturation at low temperatures (Griko et al., 1988).

As previously reported by Shortle et al. (1988), the denaturational enthalpy decreases rapidly as the temperature of denaturation is decreased by lowering the pH. This is illustrated in Table 1, which lists the data observed for the WT protein at nine different values of pH. The values for $t_{1/2}$, the temperature at which the denaturation is half completed, given in columns 3 and 8, and the calorimetric enthalpies listed in columns 4 and 9 were obtained by curve fitting with the program DIAS (see Data analysis below), while the van't Hoff enthalpies in columns 5 and 10 were obtained using program INDEP. This latter program gave values for $t_{1/2}$ and ΔH_{cal} agreeing closely with those given by DIAS. Planimeter integration also gave essentially the same enthalpy values. The values for ΔH_{vH} in the table can be well duplicated by means of the expression

$$\Delta H_{vH} = 4RT_{1/2}^2 C_{1/2}/\Delta H_{cal}, \quad (1)$$

where $C_{1/2}$ is the excess heat capacity at $T_{1/2} = t_{1/2} + 273.15$ and ΔH_{cal} is the molar calorimetric enthalpy at this temperature.

A plot of ΔH_{cal} as a function of $t_{1/2}$ is given in Figure 3 for the WT protein and the mutant H124L, and in Figure 4 for two of the single mutants. As can be seen in the summary in Table 2, the WT protein and the six single mutants have heat capacity changes that are essentially independent of temperature, at least over the temperature range covered in this work. Thus the plots of enthalpy vs.

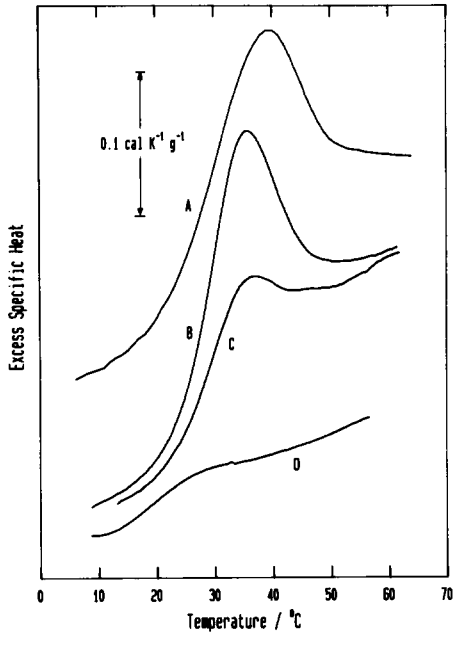

Fig. 2. DSC curves at low pH, illustrating that some mutants are more affected by pH changes than others. Curve A, H124L, pH 3.6; B, wild type, pH 4.0; C, G79S, pH 3.9; D, V66L + G88V, pH 4.0.

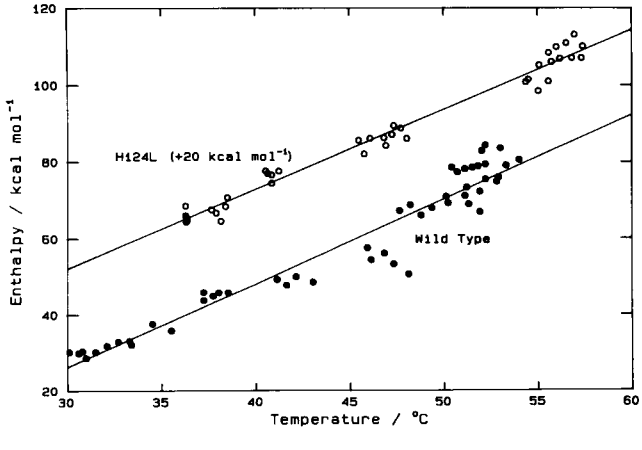

Fig. 3. The variation of ΔH_{cal} at $t_{1/2}$ with $t_{1/2}$ for wild-type protein (●; 50 experiments) and H124L (○; 53 experiments; displaced upward by 20 kcal mol^{-1}). The solid lines were obtained by unweighted linear least squaring. The heat capacity changes are nearly equal and independent of temperature within experimental uncertainty.

Table 1. *Detailed experimental results for wild-type staphylococcal nuclease*

pH	Conc. (μM)	$t_{1/2}$ (°C)	ΔH_{cal}[a] (kcal mol^{-1})	ΔH_{vH}[b] (kcal mol^{-1})
7.0	86.0	54.0	80.5	99.6
	131.0	52.9	75.9	97.7
	169.0	53.0	83.5	98.5
	176.7	53.3	79.0	102.5
	254	52.2	75.4	101.9
	287	52.0	82.8	103.3
	347	52.2	79.3	108.3
	483	51.5	78.5	112.3
	674	51.1	78.1	116.0
	904	50.4	78.5	121.9
6.5	85.4	52.8	74.8	91.5
	150.1	52.2	84.3	92.8
	291	51.8	78.8	103.0
	497	51.2	73.3	108.4
	673	50.7	77.3	110.6
6.0	163.7	51.9	72.2	95.0
	187.5	51.9	66.9	98.1
	314	51.3	68.9	104.0
	315	51.1	71.1	98.5
	635	50.2	69.2	108.2
	643	50.1	70.9	103.0
5.5	298	49.3	67.9	91.8
	492	48.7	66.0	91.7
	665	48.2	68.7	93.7
	944	47.6	67.2	96.6
5.0	272	48.1	50.7	90.3
	419	47.3	53.3	88.2
	580	46.8	56.1	88.6
	763	46.1	54.4	89.5
	970	45.9	57.5	90.0
4.5	361	43.0	48.7	71.1
	687	42.1	50.1	73.8
	895	41.6	47.9	73.8
	1,126	41.1	49.4	74.1
4.2	278	38.5	45.9	58.2
	461	38.0	45.9	60.0
	678	37.7	45.0	60.7
	919	37.2	43.8	61.2
	1,186	37.2	46.0	60.7
4.0	277	35.5	35.8	52.0
	464	34.5	37.6	47.9
	652	33.3	33.1	49.8
	892	33.4	32.1	49.4
	1,236	32.1	31.8	48.6
3.9	284	32.7	32.9	47.8
	426	31.5	30.2	44.8
	555	31.0	28.6	45.7
	707	30.8	30.4	44.8
	951	30.6	29.9	43.8
	1,279	30.1	30.2	43.8

[a] Evaluated using program DIAS.
[b] Evaluated using program INDEP.

temperature do not appear to show the pronounced upward curvature reported by Shortle et al. (1988). The enthalpy–temperature plots in Figure 5 for the double and triple mutants do show upward curvature, though with

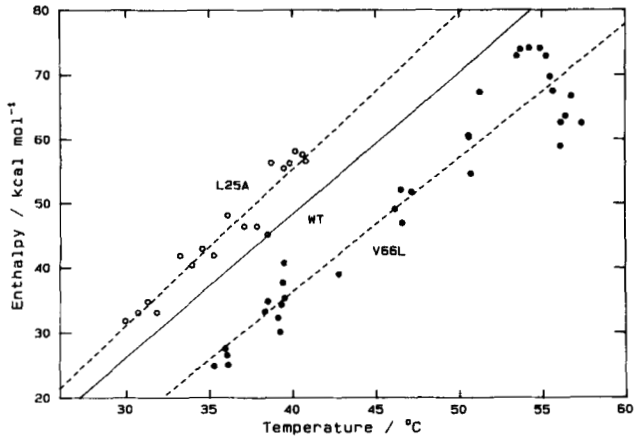

Fig. 4. The variation of ΔH_{cal} at $t_{1/2}$ with $t_{1/2}$ for the mutants V66L (●; 33 experiments) and L25A (○; 18 experiments). The solid line is the least-squared line for the wild-type protein (Fig. 3), and the dashed lines are the least-squared lines for the mutants. As in Figure 3, the heat capacity changes are constant over the observed temperature range.

enthalpy values remaining well below those in Figure 4. The enthalpy values observed for these two mutants are summarized in Table 3.

Statements concerning the variation of $t_{1/2}$ with pH are complicated by the variation of $t_{1/2}$ with concentration at fixed pH, as illustrated in Figure 6. We have arbitrarily selected 500 μM as a standard concentration and have reported values for $t_{1/2}$ at this concentration. In order to evaluate these quantities, each data set was least squared according to the van't Hoff type of equation

$$\ln(\text{conc}) = A + 1{,}000B/(t_{1/2} + 273.15), \qquad (2)$$

and the value of $t_{1/2}$ at 500 μM calculated using the resulting coefficients A and B. The resulting values for $t_{1/2}$

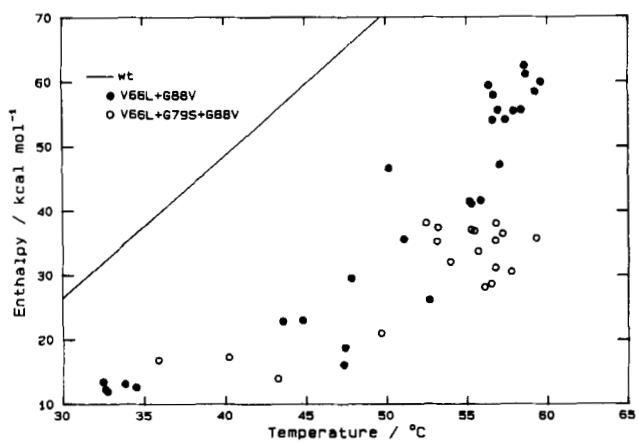

Fig. 5. The variation of ΔH_{cal} at $t_{1/2}$ with $t_{1/2}$ for the multiple mutants V66L + G88V (●; 28 experiments) and V66L + G79S + G88V (○; 19 experiments). The solid line is the least-squared line for the wild-type protein (Fig. 3). It appears that the heat capacity changes in these two cases decrease with decreasing temperature.

Table 2. *Summary of denaturational enthalpies ($\Delta H_{cal} = \Delta H_0 + \Delta Cp \cdot t\,°C$) of wild-type (WT) staphylococcal nuclease and six mutant forms thereof*[a]

Protein	Temp. range (°C)	No. of experiments	ΔH_0 (kcal mol^{-1})	ΔCp (kcal K^{-1} mol^{-1})	SD (kcal mol^{-1})	Coeff. of determination
WT	30.1–59.0	50	−40.0 (−43.9)	2.208 (2.847)	4.7 (6.7)	0.960 (0.925)
L25A	29.9–40.8	17	−44.2 (−58.4)	2.508 (3.485)	2.0 (6.1)	0.954 (0.815)
V66L	35.3–57.4	33	−46.7 (41.6)	2.076 (2.317)	5.1 (10.3)	0.911 (0.758)
G79S	26.7–45.6	22	−41.1 (−35.5)	1.962 (1.816)	3.2 (6.4)	0.938 (0.756)
G88V	34.8–56.3	30	−70.7 (−64.8)	2.478 (2.909)	3.9 (10.3)	0.955 (0.800)
A90S	26.2–41.8	23	−38.0 (−37.3)	2.430 (3.104)	3.6 (8.2)	0.910 (0.770)
H124L	36.3–57.4	53	−27.0 (−95.9)	2.000 (3.800)	2.7 (8.6)	0.971 (0.856)

[a] The quantities in parentheses were obtained by least squaring values for ΔH_{vH}, which were obtained by curve fitting with the program INDEP (see text) with the parameter $\beta = \Delta H_{vH}/\Delta h_{cal}$ free to vary.

are listed in Table 4, which includes some values for the triple mutant, which are based on single determinations. The variation of $t_{1/2}$ at 500 μM with pH is shown in Figure 7 for six of the mutants. In all cases $t_{1/2}$ is independent of pH at neutral pH and decreases with decreasing pH at lower pH. From the equation

$$\Delta\nu = \frac{\Delta H_{cal}}{2.303RT_{1/2}^2} \cdot \frac{dT_{1/2}}{d\ pH}, \tag{3}$$

one can estimate the values of $\Delta\nu$, the number of protons taken up by the protein during denaturation, given in Table 5. These small uptakes of protons could be due, for example, to the presence in the native protein of carboxyl groups with abnormally low pK values (Anderson et al., 1990). We have not applied corrections to the observed denaturational enthalpy changes based on these values for $\Delta\nu$ because of their small magnitudes and the likelihood that the protein groups involved and the buffers have similar ionization heats.

A convenient measure of the apparent stabilization or destabilization caused by a mutation is given by $\Delta\Delta G_d^0 = \Delta G_d^0$ (mutant) $- \Delta G_d^0$ (WT), the change in the standard free energy of denaturation produced by the mutation at the temperature of half-denaturation of the WT protein. Because the denaturational free energy of the wild type is zero at this temperature, the Gibbs-Helmholtz equation may be written

$$\Delta\Delta G_d^0 = \Delta H_2 \frac{T_1 - T_2}{T_2} + \Delta Cp_2\left(T_2 - T_1 + T_1 \ln\frac{T_1}{T_2}\right), \tag{4}$$

where T_1 and T_2 are the absolute temperatures of half-denaturation of the WT and mutant proteins, respec-

Table 3. *Summary of denaturational data for the mutants V66L + G88V and V66L + G79S + G88V*

Protein	pH	No. of experiments	Conc. range (μM)	$t_{1/2}$ range (°C)	ΔH_{cal} range (kcal/mol)	ΔCp[a] (kcal K^{-1} mol^{-1})
V66L + G88V	7.0	6	180–1200	56.4–59.2	54.1–62.5	1.22 ± 0.18
	6.0	4	335–1260	56.9–59.6	55.4–61.2	0.64 ± 0.17
	5.0	4	445–1240	55.1–58.4	41.5–55.6	0.97 ± 0.18
	4.5	3	545–1170	50.2–55.3	26.2–46.7	1.19 ± 0.52
	4.3	3	620–1050	47.3–47.8	16.1–29.6	1.19 ± 0.09
	4.1	2	915–1015	43.6–44.8	22.8–23.0	1.20 ± 0.49
	4.0	5	450–1450	32.5–34.5	11.9–13.4	0.59 ± 0.07
V66L + G79S + G88V	7.0	6	255–355	55.6–57.7	28.2–36.5	2.22 ± 0.20
	6.0	6	225–355	55.2–59.3	31.2–38.1	2.33 ± 0.29
	5.0	4	630–1390	52.4–53.9	32.1–38.1	1.78 ± 0.10
	4.5	1	1430	49.6	21.0	1.26
	4.1	1	1450	40.2	17.3	0.94
	4.0	1	500	43.2	13.9	0
	3.9	1	1130	35.8	16.8	0.42

[a] ΔCp values are the means ± SE of values determined in individual experiments.

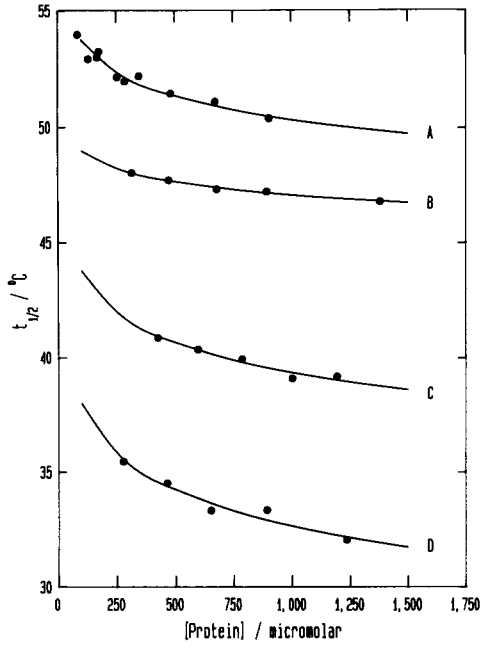

Fig. 6. Illustrating the variation of $t_{1/2}$ with concentration. Curve A, wild-type protein, pH 7.0; B, H124L, pH 4.1; C, G88V, pH 4.1; D, wild type, pH 4.0. The curves are drawn utilizing the parameters obtained by linear least squaring of $1/T_{1/2}$ as a function of ln (concentration) and illustrate the adherence of the data to the van't Hoff equation.

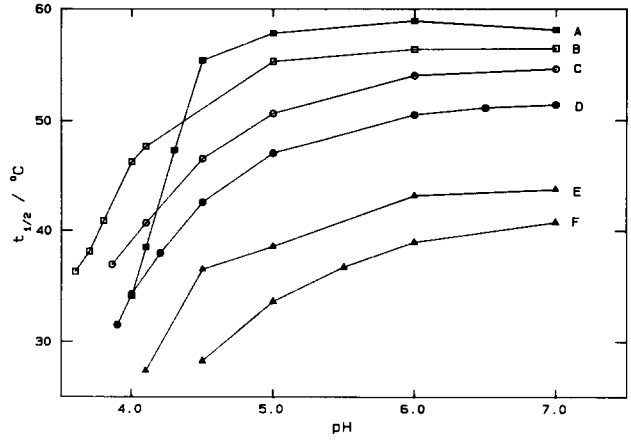

Fig. 7. The variation of $t_{1/2}$ with pH for six of the proteins studied. Curve A, V66L + G88V; B, H124L; C, G88V; D, WT; E, G79S; F, A90S. The values for $t_{1/2}$ were evaluated at a protein concentration of 500 μM using least-squared van't Hoff plots similar to those in Figure 6.

tively, ΔH_2 is the molar denaturational enthalpy of the mutant protein at T_2, and ΔCp_2 is the value at T_2 of the temperature derivative of ΔH_{cal}.

The values for $\Delta\Delta G_d^0$, $\Delta\Delta H_d$, and $\Delta\Delta S_d^0 = (\Delta\Delta H_d - \Delta\Delta G_d^0)/T_{1/2}$ at pH 7.0, 51.4 °C and pH 5.0, 47.0 °C listed in Table 6 for the single mutants were calculated using the constants ΔH_0 and ΔCp listed in Table 2 and the values for $t_{1/2}$ at 500 μM protein concentration given in Table 4. Because the values of ΔH for the multiple mutants did not show a linear variation with temperature, the values of ΔH and ΔCp for these mutants used in calculating the quantities in Table 6 were taken as the means of the individual values at each pH. Positive values of $\Delta\Delta G_d^0$ indicate apparent stabilization of the native state of a protein. As has been frequently pointed out, thermodynamics does not permit a choice between stabilization of the native state and destabilization of the denatured state. Shortle et al. (1988) have argued that in the case of the mutations of SNase studied by them, it is primarily the denatured state that is affected.

Table 4. *Temperature (°C) of half-denaturation at 500 μM protein concentration for wild-type and mutant staphylococcal nucleases*

	Protein								
pH	WT	L25A	V66L	G79S	G88V	A90S	H124L	V66L + G88V	V66L + G79S + G88V
7.0	51.4	39.9	55.3	43.8	54.3	40.7	56.5	57.8	56.1
6.5	51.1	–	–	–	–	–	–	–	–
6.0	50.5	37.8	54.7	43.1	53.7	38.9	56.4	58.9	56.7
5.5	48.6	34.4	–	–	–	36.7	–	–	–
5.0	47.0	31.2	50.8	38.5	50.6	33.5	55.3	57.9	54.3
4.5	42.5	23.5	46.8	36.5	46.3	28.1	–	55.8	49.6[a]
4.3	–	–	–	–	–	–	–	47.2	–
4.2	37.9	–	–	–	–	–	–	–	–
4.1	–	–	39.4	27.5	40.6	–	47.7	–	40.2[a]
4.0	34.2	–	39.2	–	–	–	46.3	34.2	43.2[a]
3.9	31.5	–	35.4	–	36.5	–	–	–	35.8[a]
3.8	–	–	–	–	–	–	40.9	–	–
3.7	–	–	–	–	–	–	38.1	–	–
3.6	–	–	–	–	–	–	36.3	–	–

[a] Single determinations not at 500 μM.

Table 5. *Denaturational change in the number of protons bound per molecule of staphylococcal nuclease with change in pH*

	$\Delta\nu$
Wild-type protein	
pH 4	2.0
pH 5	0.8
pH 6	0.3
pH 7	0
Wild-type and mutant proteins at pH 4.5	
WT	1.1
L25A	0.7
V66L	1.31
G79S	0.3
G88V	1.1
A90S	0.9
H124L	1.2
V66L + G88V	1.0
V66L + G79S + G88V	0.6

Discussion

The increase in $t_{1/2}$ with decreasing concentration at constant pH observed with all the proteins studied indicates that there is an increase in aggregation accompanying denaturation. Since the parameter $\beta = \Delta H_{vH}/\Delta h_{cal}$, as evaluated in curve fitting to a modified two-state model in which β is allowed to vary (program INDEP), is usually within the range of 1.3–2 times the molecular weight, the aggregation is presumably dimerization. As explained in the section on data analysis, we fitted the DSC data to an extended two-state model, which permits partial or complete dimerization of either the native or the denatured state or of both states (program DIAS, Appendix). It was found that in most cases the data were well fitted with dimerization constants, which at all concentrations gave negligible dimerization in the native state and extensive dimerization in the denatured state. In a few cases, especially at low values of pH, the variation of $t_{1/2}$ with concentration was less than usual or even not observable. The decreased tendency toward dimerization at low pH is presumably due to increased charge on the protein molecule.

In comparing our results with those of Shortle et al. (1988) there are difficulties because of the variation of $t_{1/2}$ with protein concentration. Their experiments were performed at a protein concentration of 3.0 μM while ours were at 80–1,500 μM. Because there are large uncertainties in extrapolating our data for $t_{1/2}$ to 3 μM, no useful comparisons of values for $t_{1/2}$ can be made. In order to compare enthalpy values, we have calculated ΔH_{cal} and ΔH_{vH} at the values for $t_{1/2}$ (T_m in the notation used by Shortle et al. [1988]) listed by Shortle et al., by means of the least-squared parameters ΔH_0 and ΔCp listed in Table 2, and the parameters for ΔH_{vH} given in parentheses in the table. Such a comparison for the WT protein and for V66L is given in Table 7. As mentioned earlier, the decrease in $t_{1/2}$ with decreasing pH leads to a decrease in ΔH_{cal}. A similar overall decrease observed in ΔH_{vH} is complicated at a single pH by the increase in dimerization with increasing concentration, which in turn leads to an increase in ΔH_{vH}. Thus the fit of ΔH_{vH} to a linear dependence on $t_{1/2}$ is considerably poorer than that of ΔH_{cal}.

Columns 1 and 2 in Table 7 list the values for $t_{1/2}$ and enthalpy reported by Shortle et al. (1988) at various values of the pH, the latter quantities being van't Hoff enthalpies. The ratio of these quantities to the calculated values for ΔH_{cal} down to 40 °C in column 3 averages 1.12 for WT and 1.10 for V66L, reasonable values in view

Table 6. *Changes in thermodynamic parameters at pH 5.0 and 7.0 produced by various mutations of staphylococcal nuclease, calculated at $t_{1/2}$ for the wild-type protein ($t_{1/2}$ = 47.0 °C at pH 5.0 and 51.4 °C at pH 7.0 for the WT protein)*[a]

	pH 7.0				pH 5.0				
Protein	$\Delta t_{1/2}$	$\Delta\Delta G_d^0$	$\Delta\Delta H_d$	$\Delta\Delta S_d^0$	$\Delta t_{1/2}$	$\Delta\Delta G_d^0$	$\Delta\Delta H_d$	$\Delta\Delta S_d^0$	$\Delta\Delta Cp$
L25A	−11.5	−2.6	11	43	−15.8	−2.8	10	40	300
V66L	3.9	0.8	−14	−44	3.8	0.6	−13	−42	−130
G79S	−7.6	−1.3	−14	−38	−8.5	−1.2	−13	−36	−250
G88V	2.9	0.5	−17	−53	3.6	0.6	−18	−58	270
A90S	−10.7	−2.5	13	49	−13.5	−2.6	12	47	220
H124L	5.1	1.3	2	3	8.3	1.9	3	4	−210
V66L + G88V	6.4	1.0	−24	−77	10.9	1.2	−28	−91	(−1,300)[b]
V66L + G79S + G88V	4.7	0.3	−52	−161	7.3	0.5	−41	−130	(−100)[b]

[a] Estimated uncertainties: $\Delta t_{1/2}$, ±0.5 °C; $\Delta\Delta G_d^0$, ±0.4 kcal mol^{-1}; $\Delta\Delta H_d$, ±4 kcal mol^{-1} (average value); $\Delta\Delta S_d^0$, ±10 cal K^{-1} mol^{-1}; $\Delta\Delta Cp$, ±200 cal K^{-1} mol^{-1}.

[b] Based on the mean of the values for pH 5.0, 6.0, and 7.0 obtained in individual experiments.

Table 7. *Comparison of calorimetric and fluorimetric denaturational enthalpies for wild-type and V66L staphylococcal nuclease*

$t_{1/2}$ (°C)	ΔH_{vH} (Shortle et al., 1988) (kcal mol^{-1})	ΔH_{cal} (this work) (kcal mol^{-1})	$\frac{\Delta H_{vH} \text{ (Shortle et al., 1988)}}{\Delta H_{cal} \text{ (this work)}}$	ΔH_{vH} (this work) (kcal mol^{-1})	$\frac{\Delta H_{vH} \text{ (this work)}}{\Delta H_{vH} \text{ (Shortle et al., 1988)}}$
			Wild type		
53.4	87.4	78.3	1.12	108.1	1.24
53.2	86.7	77.8	1.11	107.6	1.24
53.0	85.7	77.4	1.11	107.0	1.25
51.4	81.9	73.9	1.11	102.4	1.25
48.3	73.0	67.0	1.09	93.6	1.28
45.2	68.7	60.1	1.14	84.8	1.23
42.1	57.9	53.3	1.09	76.0	1.31
41.2	59.0	51.3	1.15	73.4	1.24
37.7	54.6	43.5	1.26	63.4	1.16
31.8	48.2	30.4	1.58	46.6	0.97
24.6	39.3	14.5	2.71	26.1	0.66
			V66L		
56.2	78.8	70.0	1.13	88.6	1.12
55.7	72.3	68.9	1.05	87.5	1.21
54.1	66.3	65.1	1.01	83.7	1.26
51.0	61.7	59.2	1.04	76.6	1.24
49.2	60.1	55.4	1.08	72.4	1.20
45.6	55.8	48.0	1.16	64.1	1.15
45.1	56.1	46.9	1.20	62.9	1.12
38.7	46.0	33.6	1.37	48.1	1.05
35.1	44.9	26.2	1.72	39.7	0.88
30.9	39.7	17.4	2.28	30.0	0.76

of the small extents of dimerization expected in the experiments of Shortle et al. In contrast, the ratio of our calculated values for ΔH_{vH} to those of Shortle et al. (column 6 in Table 7) averages 1.26 for WT and 1.19 for V66L, presumably because of the larger extent of dimerization at the high concentrations used in our work. The marked changes in these ratios below 40 °C are due to the fact that Shortle et al. found values for ΔCp much smaller than ours, especially at the lower temperatures. Sizeable deviations in ΔCp are perhaps not surprising when one considers that ΔCp is the first temperature derivative of the enthalpy determined calorimetrically, whereas it is the second derivative of the free energy, which is essentially the quantity determined by analysis of the melting curves obtained by the fluorimetric technique employed by Shortle et al.

The paper by Shortle et al. (1988) includes a calorimetric determination of the denaturational enthalpy of WT SNase at pH 7.0, yielding the value 96 ± 2 kcal mol^{-1} for ΔH_{cal} and 91 ± 3 kcal mol^{-1} for ΔH_{vH}. The source of the discrepancies with our calorimetric data is unknown.

Three other papers have reported denaturational enthalpies for SNase. Two of these (Calderon et al., 1985; Griko et al., 1988), using a form of the WT protein having seven extra residues at the amino-terminus, employed DSC and obtained values agreeing only moderately well with ours, those of Calderon et al. being somewhat smaller than ours and those of Griko et al. somewhat larger. A comparison of these values with our values is summarized in Table 8. Eftink et al. (1991) observed the unfolding of H124L at pH 7.2 using fluorimetry and obtained a value for ΔH_{vH} smaller than ours by roughly the same factor as listed for V66L at the higher temperatures in Table 7.

The values for $\Delta\Delta G_d^0$ in Table 6 show apparent stabilizations produced by the amino acid replacements V66L, G88V, H124L, and the double and triple replacements. The mutations L25A, G79S, and A90S cause apparent destabilizations. It is interesting that replacement of His 124 by Leu, to form the enzyme produced by the V8 strain of *S. aureus*, leads to a distinct stabilization, which is significantly larger at pH 5.0 than at pH 7.0 presumably because of increased positive charge on the histidine side chain in the WT protein. As might be expected for this naturally occurring form, the enthalpy and entropy changes during unfolding are practically indistinguishable from those for the WT protein. The values in Table 6 for the double and triple mutants indicate approximate additivity in the mutational changes in free energy, enthalpy, and entropy.

As has been observed in several other cases of mutant proteins (α-subunit of tryptophan synthase [Matthews

Table 8. *Comparison of calorimetrically determined enthalpies of denaturation at pH 7 of wild-type staphylococcal nuclease*

	Calderon et al., 1985			Griko et al., 1988			Shortle et al., 1988			This work		
pH	$t_{1/2}$ or t_m (°C)	ΔH_{cal} (kcal/mol)	ΔH_{vH} (kcal/mol)	$t_{1/2}$ or t_m (°C)	ΔH_{cal} (kcal/mol)	ΔH_{vH} (kcal/mol)	$t_{1/2}$ or t_m (°C)	ΔH_{cal} (kcal/mol)	ΔH_{vH} (kcal/mol)	$t_{1/2}$ or t_m (°C)	ΔH_{cal} (kcal/mol)	ΔH_{vH} (kcal/mol)
7	53.3	72.5	103.6	57.7	84.4	77.2	52.8	96	91	50.9–53.7	79.1	108.3
6.8	–	–	–	50.0	79.4	74.6	–	–	–	–	–	–
6.5	–	–	–	–	–	–	–	–	–	50.5–52.7	77.1	101.3
6	53.0	67.9	93.7	–	–	–	–	–	–	49.8–51.7	69.9	101.1
5	50.0	52.8	78.8	48.3	69.3	65.0	–	–	–	45.7–47.9	54.1	89.3
4.5	–	–	–	43.3	64.1	56.9	–	–	–	40.9–41.8	48.8	73.2
4	38.8	29.5	62.8	35.0	44.5	38.0	–	–	–	31.7–35.1	34.1	49.5

et al., 1980]; phage λ repressor [Hecht et al., 1984]; P22 tailspike protein [Yu et al., 1989]; T4 lysozyme [Kitamura & Sturtevant, 1989; Connelly et al., 1991; Hu et al., 1992; Ladbury et al., 1992]), the values for $\Delta\Delta G_d^0$ and $\Delta\Delta H_d$ in Table 6 show no correlation in either size or sign. Because the changes in free energy caused by mutations are generally much smaller in magnitude than the corresponding changes in enthalpy, it necessarily follows from the Second Law that in many cases large compensating changes in the entropy of denaturation will be observed. An extreme example of this is shown by V66L + G79S + G88V, with the total entropy of unfolding at pH 7.0 reduced from 228 to 68 cal K^{-1} mol^{-1}! What might appear to be a minor disturbance of the energetics of the molecule, as judged by the value of $\Delta\Delta G_d^0$, obviously involves profound changes in enthalpy and entropy, which are probably distributed throughout the molecule and presumably involve both the folded and unfolded forms. If this entropy change is attributed entirely to a change in the number of accessible, energetically similar, bond rotational and vibrational configurations, the value 160 cal K^{-1} mol^{-1} corresponds to a change by a factor of 10^{35}, either an increase in the number of conformations available in the folded state or a decrease in the unfolded state, or a combination of the two. On a per residue basis, with 149 residues per molecule, this factor becomes 1.72. Although Karplus et al. (1987) have estimated the residual per residue entropy of a folded polypeptide chain to be 35 cal K^{-1} mol^{-1}, it is obviously difficult to imagine a set of changes in rotations or vibrations that would produce an average entropy change of 1.1 cal K^{-1} mol^{-1} in every residue in the molecule. Significant entropy effects can also arise from changes in hydration, but such changes are very difficult to specify.

It appears that our current level of understanding of the intra- and intermolecular forces that stabilize the structures of globular proteins is inadequate to serve as the basis for a useful analysis of the large mutational effects on the enthalpy and entropy of unfolding reported in this paper.

Materials and methods

Preparation of proteins

Wild-type SNase and all of the mutant nucleases studied in this paper were purified from *Escherichia coli* overexpressed from a plasmid in which the nuclease gene was under control of either the λpL promoter (the plasmid was a derivative pAS1 in AR120 cells, and was a gift from David Shortle), or the T7 ϕ10 promoter (the plasmid was a derivative of pET3a [Studier et al., 1990] in MGT7 cells derived from MG1655 and contains the gene coding for T7-RNA polymerase under the control of the *lac* UV6 promoter, and was a gift from David LeMaster). Genetically equivalent nucleases, expressed from either promoter, yielded purified proteins that were indistinguishable by DSC.

The nuclease gene, contained on a modified version of pAS1, was expressed constitutively, presumably due to the numerous modifications made to the parent vector (David Shortle, pers. comm.). Consequently, the nuclease gene contained on this plasmid is unstable when grown at 37 °C in AR120 cells, and reproducible high levels of SNase production could be obtained only if great care was taken in maintaining and growing these strains, which was done as described by Flanagan et al. (1992). Large-scale growth and extraction of SNase from pET3a-derived plasmids in MGT-7-pLYS-S cells were also carried out at 37 °C.

Purification of wild-type and mutated versions of SNase

The soluble fractions from lysed cells (supernatant from high-speed centrifugation [Flanagan et al., 1992]) were brought to 15 mM EDTA to chelate excess Ca^{2+} and loaded onto 40 mL of a phosphocellulose (Whatman) column previously equilibrated in 0.3 M NH_4OAc. The column was washed with 200 mL of the equilibration buffer

and the nuclease eluted with a 2 M NH_4OAc bump. Fractions containing nuclease were pooled (~60 mL) and dialyzed against 2 L of distilled water. A white precipitate, which forms during dialysis, was removed by centrifugation at 10,000 × *g* for 30 min. Further purification was achieved by chromatography on a 2.5 × 100-cm AcA54 column equilibrated in 50 mM $(NH_4)_2CO_3$. The column was operated at a flow rate of 1 mL/min, and the eluent was collected in 8-mL fractions. The bulk of SNase eluted as a sharp peak in a volume consistent with it being a monomer, although a small fraction of the nuclease eluted earlier; this species is probably dimeric. Concentration and rechromatography of the monomeric species resulted in a single peak at the monomer position (data not shown). For WT nuclease, the amount of the early peak was about 1–2% of the monomeric species, although, for some thermally less stable mutants, the higher molecular weight material accounted for as much as 10% of the total nuclease. In general, the amount of the higher molecular weight species increased with decreasing thermal stability of the protein. Surprisingly, the apparently dimeric peak on rechromatography showed a single peak again indicating a dimeric species, and the monomeric and higher molecular weight species gave essentially identical DSC traces (data not shown). In the present study all the reported DSC scans were obtained with the monomeric species. For DSC samples, the fractions that eluted at the monomer position were pooled, concentrated to ~25–30 mg/mL using an Amicon flow cell (YM10 membrane), and dialyzed extensively against the appropriate buffer. Typically the yield of purified protein was between 100 and 350 mg/L of original culture. The purity of these samples was sufficient to allow crystals to be grown for WT and several of the mutated versions (data not shown).

Scanning calorimetry

DSC experiments were run in two different microcalorimeters, the DASM-4 (Biopribor, Puschino, Russia [Privalov, 1980]) and the MC-2 (Microcal, Inc., Northampton, Massachusetts). These two instruments gave entirely consistent results. A scan rate of 1 K min^{-1} was employed in all experiments, it having been established that essentially the same results, except for a small decrease in t_m, the temperature of maximal excess specific heat, were observed at a scan rate of 0.5 K min^{-1}. Rescans of previously scanned samples showed the denaturations to be reversible, provided the initial scan was not carried beyond 90% completion.

The protein samples were exhaustively dialyzed before use against the appropriate buffer, and the final dialysate was used as a reference solution. The buffers employed were acetate, cacodylate, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), or phosphate, usually at a concentration of 0.05 M containing 0.1 M NaCl.

Data analysis

The original data after subtraction of the instrumental baseline, which were essentially records of the excess power fed to the sample cell as a function of time, were converted to listings of excess specific heat as a function of time. When attempts were made to fit these data to a modified two-state model (computer program INDEP) as outlined earlier (Sturtevant, 1987; Connelly et al., 1991), it was found that the value of the adjustable parameter $\beta \equiv \Delta H_{vH}/\Delta h_{cal}$ was in all cases considerably larger than the molecular weight. This suggests extensive dimerization in either the native or the denatured form, or in both forms. We have therefore modified the fitting model to the form

$$\frac{1}{2}\,N_2 \overset{K_N}{\rightleftharpoons} N \overset{K}{\rightleftharpoons} D \overset{K_D}{\rightleftharpoons} \frac{1}{2}\,D_2. \tag{5}$$

Utilization of this model with computer program DIAS is outlined in the Appendix. Because the values of $t_{1/2}$, the temperature of half conversion, decreased with increasing protein concentration, it may be concluded that the denatured proteins are more fully dimerized than the native proteins. In the curve-fitting process the automatically adjusted parameters were $t_{1/2}$, Δh_{cal}, and β. Values for K_N and K_D were selected to give good fits with β/MW as close as possible to unity, it being assumed that there are no significant enthalpy changes accompanying the dimerization reactions, so that K_N and K_D are independent of temperature. It was found that K_N could in all cases be assigned a small value corresponding to negligible dimerization in the native state, and that K_D for each protein could be assigned a range of values with equally satisfactory fits. The values for K_D finally selected ranged from 0.1 $\mu M^{1/2}$ for the wild-type protein, corresponding to 80% dimerization at 1,000 μM total concentration, to 5 $\mu M^{1/2}$ for H124L, leading to essentially complete dimerization at the higher protein concentrations. It was surprising that the values for K_N and K_D for a particular protein did not have to be varied over the pH range covered.

Acknowledgments

Many of our earlier DSC runs were performed using proteins generously supplied by Dr. David Shortle of the Johns Hopkins University. As expected, no differences in thermodynamic properties were detected between proteins prepared here and those sent to us by Dr. Shortle. This research was supported in part by grants GM-22788 (D.M.E.) and GM-04725 (J.M.S.) from the National Institutes of Health, and DMB-8810329 (J.M.S.) from the National Science Foundation. The travel expenses of A.T. were paid by the Ministry of Education, Science and Culture of Japan.

References

Anderson, D.E., Becktel, W.J., & Dahlquist, F.W. (1990). pH-induced denaturation of proteins: A single salt bridge contributes 3-5 kcal/mol to the free energy of folding of T4 lysozyme. *Biochemistry 29*, 2403-2408.

Calderon, R.O., Stolwich, N.J., Gerlt, J.A., & Sturtevant, J.M. (1985). Thermal denaturation of staphylococcal nuclease. *Biochemistry 24*, 6044-6049.

Connelly, P., Ghosaini, L., Hu, C.-Q., Kitamura, S., Tanaka, A., & Sturtevant, J.M. (1991). A differential scanning calorimetric study of the thermal unfolding of seven mutant forms of phage T4 lysozyme. *Biochemistry 30*, 1887-1892.

Eftink, M.R., Ghiron, C.A., Kantz, R.A., & Fox, R.O. (1991). Fluorescence and conformational stability studies of *Staphylococcus* nuclease and its mutants, including the less stable nuclease-concanavalin A hybrids. *Biochemistry 30*, 1193-1199.

Flanagan, J.M., Kataoka, M., Shortle, D., & Engelman, D.M. (1992). Truncated staphylococcal nuclease is compact but disordered. *Proc. Natl. Acad. Sci. USA 89*, 748-752.

Griko, Y.V., Privalov, P.L., Sturtevant, J.M., & Venyaminov, S.Y. (1988). Cold denaturation of staphylococcal nuclease. *Proc. Natl. Acad. Sci. USA 85*, 3343-3347.

Hecht, M.H., Sturtevant, J.M., & Sauer, R.T. (1984). Effect of single amino acid replacements on the thermal stability of the NH_2-terminal domain of phage λ repressor. *Proc. Natl. Acad. Sci. USA 81*, 5685-5689.

Hu, C.-Q., Kitamura, S., Tanaka, A., & Sturtevant, J.M. (1992). Differential scanning calorimetric study of the thermal unfolding of mutant forms of phage T4 lysozyme. *Biochemistry 31*, 1643-1647.

Karplus, M., Ichiye, T., & Pettitt, B.M. (1987). Configurational entropy of native proteins. *Biophys. J. 52*, 1083-1085.

Kitamura, S. & Sturtevant, J.M. (1989). A scanning calorimetric study of the thermal denaturation of the lysozyme of phage T4 and the Arg96 → His mutant form thereof. *Biochemistry 28*, 3788-3792.

Ladbury, J.E., Hu, C.-Q., & Sturtevant, J.M. (1992). A differential scanning calorimetric study of the thermal unfolding of mutant forms of phage T4 lysozyme. *Biochemistry 31*, 10699-10702.

Matthews, C.R., Cristanti, M.M., Gepner, G.L., Velicelebi, G., & Sturtevant, J.M. (1980). Effect of single amino acid substitutions on the thermal stability of the α subunit of tryptophan synthase. *Biochemistry 19*, 1290-1293.

Privalov, P.L. (1980). Scanning microcalorimeters for studying macromolecules. *Pure Appl. Chem. 52*, 479-497.

Shortle, D., Meeker, A.K., & Freire, E. (1988). Stability mutants of staphylococcal nuclease: Large compensating enthalpy-entropy changes for the reversible denaturation reaction. *Biochemistry 27*, 4761-4768.

Studier, F.W., Rosenberg, A.H., Dunn, J.J., & Dubendorff, J.W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol. 185*, 60-89.

Sturtevant, J.M. (1987). Biochemical applications of differential scanning calorimetry. *Annu. Rev. Phys. Chem. 38*, 463-488.

Yu, M., Haase-Pettingell, C., King, J., & Sturtevant, J.M. (1989). Thermostability of temperature-sensitive folding mutants of the P22 tailspike protein. *J. Biol. Chem. 264*, 10693-10698.

Appendix

In fitting DSC data to the model given in Equation 4, we have assumed that the dimerization enthalpies to form N_2 and D_2 can be neglected in comparison with the denaturational enthalpy, and that variation in K_N and K_D can accordingly also be neglected. As detailed earlier (Connelly et al., 1991), linear portions of the pre- and posttransition baselines are expressed, in cal K^{-1} g^{-1}, as $c_N = a + bt$ and $c_D = c + dt$, so that the denaturational change in specific heat is $\Delta c_p = c_D - c_N$, and the specific heat if there were no denaturational enthalpy would be $c_{av} = (1 - \alpha)c_N + \alpha c_D$, where α is the total extent of denaturation. c_{av} is the so-called chemical baseline. The excess apparent specific heat due to the specific denaturational enthalpy, Δh_{cal}, is $c_{ex} = \Delta h_{cal}\, d\alpha/dT$, and the total observed apparent specific heat is $c_{av} + c_{ex}$.

If we define $KN \equiv K_N^2[A]_0$ and $KD \equiv K_D^2[A]_0$, where $[A]_0$ is the total protein concentration expressed in monomer units, then the equilibrium constant for the N to D step is

$$K = \frac{1 - (1 + 8\alpha KD)^{1/2}}{1 - (1 + 8(1 - \alpha)KN)^{1/2}} \cdot \frac{K_N^2}{K_D^2}. \tag{1A}$$

Differentiation of $\ln K$ gives an expression for $d\alpha/dT$:

$$\frac{d \ln K}{dT} = \left[\frac{4KD}{1 + 8\alpha KD - (1 + 8\alpha KD)^{1/2}} + \frac{4KN}{1 + 8(1 - \alpha)KN - (1 + 8(1 - \alpha)KN)^{1/2}}\right] \times \frac{d\alpha}{dT} \tag{2A}$$

$$= \beta \Delta h_{cal}/RT^2. \tag{3A}$$

Here $\beta \equiv \Delta H_{vH}/\Delta h_{cal}$ is a parameter expressing the deviation of the observed unfolding transition from strictly two-state behavior, for which β = molecular weight. Integration of Equation 3A gives a form of the van't Hoff equation, which can be expressed as

$$\ln \frac{K}{K_{1/2}} = \boldsymbol{A}\left(\frac{1}{T} - \frac{1}{T_{1/2}}\right) + \boldsymbol{B} \ln \frac{T}{T_{1/2}} + \boldsymbol{C}(T - T_{1/2}), \tag{4A}$$

where the factors $\boldsymbol{A}$, $\boldsymbol{B}$, and $\boldsymbol{C}$ involve the constants a, b, c, and d (Kitamura & Sturtevant, 1989). We thus obtain the value of K at a specified temperature, and solution of Equation 1A at that temperature by successive approximations gives α. Minimization of the differences between the observed specific heats and values for c_{tot} calculated as outlined here, by means of nonlinear least squaring, leads to values for the parameters $t_{1/2}$, Δh_{cal}, and β, with values for K_N and K_D selected to give β/MW as near to unity as possible.