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A Scanning Calorimetric Study of the Thermal Denaturation of the Lysozyme of Phage T4 and the Arg 96 → His Mutant Form Thereof†

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ABSTRACT: High-sensitivity scanning calorimetry has been employed to study the reversible thermal unfolding of the lysozyme of T4 bacteriophage and of its mutant form Arg 96 → His in the pH range 1.80–2.84. The values for $t_{1/2}$, the temperature of half-denaturation, in degrees Celsius and for the enthalpy of unfolding in kilocalories per mole are given by (standard deviations in parentheses)

wild type

$$t_{1/2} = 9.63 + 14.41\text{pH} \ (\pm 0.58)$$

$$\Delta H_{cal} = 5.97 + 2.33t \ (\pm 4.20)$$

mutant form

$$t_{1/2} = -19.84 + 21.31\text{pH} \ (\pm 0.51)$$

$$\Delta H_{cal} = -8.58 + 2.66t \ (\pm 4.48)$$

At any temperature within the range −20 to 60 °C, the free energy of unfolding of the mutant form is more negative than that of the wild type by 3–5 kcal mol^{-1}, indicating an apparent destabilization resulting from the arginine to histidine replacement. The ratio of the van't Hoff enthalpy to the calorimetric enthalpy deviates from unity, the value expected for a simple two-state process, by ±0.2 depending on the pH. It thus appears that the nature of the unfolding of T4 lysozyme varies with pH in unknown manner. This complication does not invalidate the values reported here for the temperature of half-completion of unfolding, the calorimetric enthalpy, the heat capacity change, or the free energy of unfolding.

The lysozyme of bacteriophage T4 is a small globular protein with a single polypeptide chain containing 164 amino acid residues and having a molecular weight of 18 700. The structure of this protein has been determined to a resolution of 1.7 Å, and the structures of several mutant forms have also been obtained (Grutter et al., 1979, 1983; Alber et al., 1987; Alber & Matthews, 1987; Matsumura et al., 1988). The thermal denaturation of T4 lysozymes has been carefully studied by Schellman and his colleagues (Hawkes et al., 1984; Elwell & Schellman, 1975, 1977, 1979; Schellman et al., 1981) using circular dichroism (CD) and fluorescence spectrophotometry to follow the course of the unfolding. It has been demonstrated that the thermal unfolding of T4 lysozyme and at least some of its mutants is completely reversible at low pH.

It is clear from this earlier work that the thermal unfolding of T4 lysozyme and its mutant forms is a very nearly ideal case for detailed study by means of high-sensitivity differential scanning calorimetry (DSC). Our colleagues at the University of Oregon have kindly agreed to supply us with the large amounts of proteins needed for such studies. This paper reports results obtained for the wild-type (WT) enzyme and the temperature-sensitive mutant Arg 96 → His (R96H).

The calorimetric method for studying thermal denaturation has a major disadvantage as compared with optical methods, namely, that concentrations higher by an order of magnitude or more are required, which in some cases may lead to increased difficulties due to aggregation, particularly of denatured proteins. On the other hand, a major advantage of the calorimetric method is that it gives values both for the apparent, or van't Hoff, enthalpy, the quantity derived from optical experiments, and for the true, or calorimetric, enthalpy and thus affords a direct check on whether the process under study shows simple two-state behavior, indicated by equality of these two enthalpies. It may be mentioned that it is quite possible for a process to adhere accurately to the van't Hoff equation, and thus appear to be two state, when in fact these two enthalpies differ significantly [cf. Sturtevant (1987)]. It is evident that the calorimetric method cannot distinguish

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Table I: Thermodynamic Data for the Thermal Denaturation of Wild-Type T4 Lysozyme[a]

pH	protein concn (mg mL^{-1})	$t_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$	ΔC_p (kcal K^{-1} mol^{-1})	dc_N/dt (cal K^{-2} g^{-1} × 10^3)[b]
1.60	1.77	32.92	78.5	(1.10)	(3.38)	2.65
1.80	0.82	35.51	83.0	1.01	2.38	2.59
	1.86	35.61	87.7	1.01	2.47	2.39
	3.69	35.50	87.5	1.01	2.80	2.53
	7.43	35.66	87.3	1.01	2.67	2.64
	7.46	35.72	87.9	1.01	3.03	2.12
2.00	0.89	39.10	95.7	0.94	1.93	2.92
	2.19	38.35	98.5	0.91	2.84	2.18
	3.11	39.17	98.5	0.91	1.99	2.80
	4.13	37.89	93.1	0.94	2.90	2.33
	4.38	38.71	99.7	0.91	1.95	2.08
	7.97	39.22	96.9	0.91	2.59	1.99
	8.77	38.81	96.9	0.91	2.59	1.92
2.20	7.29	40.55	101.9	0.89	2.27	2.45
2.34	4.35	42.94	111.3	0.84	2.53	2.24
2.40	1.22	43.50	114.3	0.90	2.44	3.01
	2.43	43.67	114.1	0.86	2.59	2.24
	4.86	43.93	114.3	0.86	2.43	2.07
2.50	1.61	44.18	114.3	0.88	2.56	(4.58)
	3.22	44.92	113.1	0.87	1.90	(4.08)
	4.84	45.22	118.4	0.82	1.78	(4.00)
	7.15	45.16	113.7	0.80	2.43	2.08
	8.07	45.66	113.3	0.81	2.11	3.06
2.70	0.88	48.37	117.2	0.92	2.16	2.54
	1.41	48.45	115.2	0.94	2.27	2.27
	2.82	48.65	116.7	0.87	2.52	2.20
	4.23	48.75	113.9	0.88	2.23	2.88
	5.64	48.90	110.7	0.91	2.47	2.50
	7.05	49.06	113.9	0.84	2.34	2.18
2.84	1.54	51.76	123.4	0.90	2.54	(5.10)
	3.90	51.59	123.8	0.83	2.46	3.02
				mean:	2.41	2.44
				SE:	±0.06	±0.07

[a] Values in parentheses are excluded from mean. [b] Apparent temperature dependence; see text.

between destabilization of the native form of a protein and stabilization of the denatured form, or a combination of these two.

Materials and Methods

As mentioned earlier, the proteins, wild-type T4 lysozyme and its R96H mutant, were prepared at the University of Oregon and were shipped to us at ice temperature. The proteins were exhaustively dialyzed at 5 °C against 20 mM KH_2PO_4 containing 25 mM KCl and 0.1 mM dithiothreitol adjusted with HCl to the desired pH in the range 1.8–2.84, the final dialyzate in each case being used as reference solution in the scanning calorimeter. Protein concentrations were determined spectrophotometrically at pH 7 and 280 nm with an absorption coefficient of 1.28 cm^2 mg^{-1}.

Calorimetric measurements were made in a DASM-4 scanning microcalorimeter (Privalov, 1980) purchased from Mashpriborintorg, Moscow, U.S.S.R. The scan rate in most experiments was 1 K min^{-1}, and it was ascertained that a decrease in the scan rate by a factor of 2 had no effect on the results.

The DSC curve obtained with a solution of the R96H mutant at a concentration of 8.29 mg mL^{-1} at pH 2.34 is shown in Figure 1. The large permanent increase in apparent specific heat results in the denaturational enthalpy increasing from 50 kcal mol^{-1} at 5% completion (23.4 °C) to 88 kcal mol^{-1} at 95% completion (37.5 °C). The enthalpy is thus not a linear measure of the extent of the reaction. In these circumstances we conclude that the only reliable way to obtain thermodynamic parameters from the experimental data is by fitting these data to a theoretical curve based on a suitable

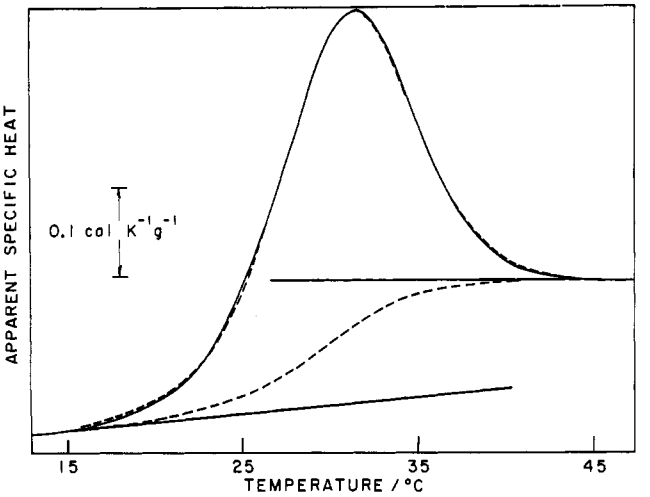

FIGURE 1: Tracing of a typical DSC curve for the thermal denaturation of the R96H mutant form of T4 lysozyme. Protein at a concentration of 8.29 mg mL^{-1} was dissolved in 20 mM potassium phosphate buffer, pH 2.34, containing 25 mM KCl and 0.1 mM dithiothreitol. Observed data and pre- and posttransition base lines (—); calculated curve and calculated base line (– –).

Table II: Thermodynamic Data for the Thermal Denaturation of the R96H Mutant Form of T4 Lysozyme[a]

pH	protein concn (mg mL^{-1})	$t_{1/2}$ (°C)	ΔH_{cal} (kcal mol^{-1})	$\Delta H_{vH}/\Delta H_{cal}$	ΔC_p (kcal K^{-1} mol^{-1})	dc_N/dt (cal K^{-2} g^{-1} × 10^3)[b]
2.00	1.71	23.44	48.2	1.23	2.66	2.46
	3.43	23.12	56.1	1.17	3.23	1.78
	4.16	22.53	47.3	1.23	2.90	2.40
	5.14	23.67	59.8	1.17	3.11	4.00
	7.74	22.64	50.9	1.09	2.67	2.59
	8.56	23.49	56.8	1.23	3.17	2.60
2.10	4.00	24.31	58.0	1.18	3.65	2.58
	8.01	24.31	54.0	1.18	3.01	2.09
2.20	1.14	26.81	69.0	1.05	3.27	4.55
	3.14	26.95	70.7	1.05	3.19	4.89
	7.84	27.56	72.6	1.00	2.81	(6.13)
2.34	4.14	29.35	64.7	1.13	2.88	2.31
	8.29	29.93	67.3	1.05	2.67	1.97
2.40	1.75	30.83	75.7	1.03	2.69	1.97
	3.51	30.63	72.0	1.02	3.30	2.05
	5.26	31.32	72.9	1.00	2.78	1.73
2.50	1.29	32.83	75.5	1.00	3.33	(–0.58)
	3.20	33.30	79.3	0.93	2.79	1.74
	8.66	34.19	73.7	0.96	2.54	1.75
2.70	1.29	36.99	95.4	0.92	2.36	2.88
	2.58	37.44	92.6	0.91	1.86	4.11
	3.87	37.41	92.6	0.90	2.49	3.33
	5.16	37.63	90.9	0.86	2.56	4.20
	6.45	37.85	93.1	0.86	2.56	3.01
2.84	1.34	40.80	104.9	0.81	2.38	2.50
	4.03	41.08	97.8	0.83	2.41	2.50
	6.71	41.51	95.4	0.83	2.46	2.39
	6.71	41.31	95.4	0.81	2.43	2.56
				mean:	2.79	2.65
				SE:	±0.08	±0.20

[a] Values in parentheses are excluded from mean. [b] Apparent temperature dependence; see text.

model. The method we have employed, which is based on the simple two-state model, is outlined in the Appendix. The agreement obtained between observed and calculated excess specific heats is illustrated in Figure 1.

Results and Discussion

The results obtained in 31 experiments with WT and 28 experiments with R96H are summarized in Tables I and II, respectively, and the calorimetric enthalpies ΔH_{cal} are plotted against the temperature $t_{1/2}$ of half-completion of denaturation

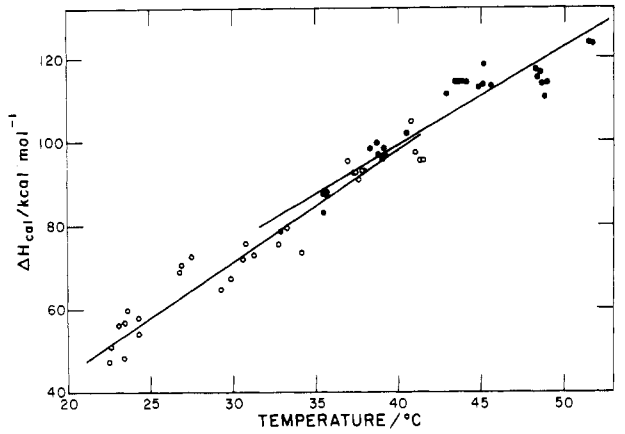

FIGURE 2: Molar enthalpy change on thermal denaturation of wild-type (●) and mutant R96H (○) T4 lysozyme plotted as functions of $t_{1/2}$, the temperature of half-completion of the denaturation. The lines shown in the figure were derived as described in the text.

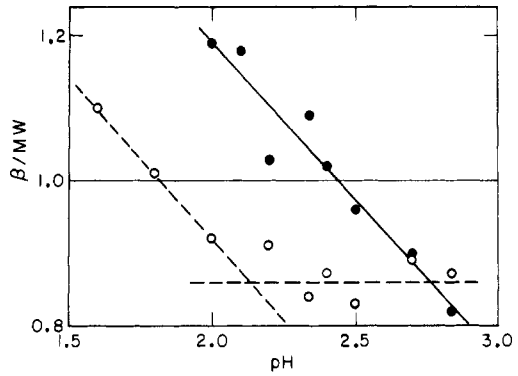

FIGURE 3: Mean values for the ratio $\beta/MW = \Delta H_{vH}/\Delta H_{cal}$ plotted as functions of pH. Wild type (○); R96H mutant (●).

in Figure 2. The values for $t_{1/2}$ (column 3 in the tables), $\Delta H_{cal} = \Delta h_{cal} \times MW$ (column 4), and $\Delta H_{vH}/\Delta H_{cal} = \beta/MW$ (column 5) were obtained by the curve-fitting process outlined in the Appendix. $\Delta C_p = (c_D - c_N) \times MW$ (column 6) was evaluated at $t_{1/2}$ from the observed pretransition base line, $c_N = A + Bt$, and the posttransition base line, $c_D = C + Dt$. $B = dc_N/dt$ is listed in column 7 in the tables; $D = 0$ within experimental uncertainty for all experiments reported here.

Reversibility of Denaturation. In preliminary experiments with WT at pH 2 in a buffer containing 100 mM KCl, it was found that the denaturation was less than 50% reversible as judged by the area of the DSC curve obtained on reheating the sample after the first scan. In 25 mM KCl, however, the denaturation was found to be >98% reversible over the pH range 1.6–2.84 provided the first heating was not carried above the temperature at which the denaturation was effectively complete. Whenever a reversibility check was performed, the posttransition base line was obtained in the second scan.

Adherence to Two-State Behavior. As pointed out in the Appendix, the parameters varied in fitting a theoretical curve to the experimental data were $t_{1/2}$, Δh_{cal}, and $\beta = \Delta H_{vH}/\Delta h_{cal}$. In the case of the simplest type of two-state process, β should be equal to the molecular weight of the substance under study. It has been observed with several globular proteins that $\beta/MW = 1.00 \pm 0.05$ (Privalov, 1979), and it is considered that values within this range do not indicate any significant deviation from two-state behavior. The mean of the values of β/MW observed at each pH is plotted as a function of pH for both proteins in Figure 3. Significant deviations from unity are evident, with a tendency for β/MW to decrease with increasing pH. It is thus clear that, at least under the conditions of our experiments, ΔH_{vH} as determined calorimetrically cannot be taken as a measure of the true molar enthalpy, ΔH_{cal}. A value of β/MW exceeding unity is usually assumed to indicate intermolecular cooperation, for example, involving oligomerization. The fact that the situation of $\beta/MW > 1$ is not accompanied by any significant change in $t_{1/2}$ with protein concentration suggests that the extent of oligomerization is essentially the same in the native and denatured forms of the proteins. When $\beta/MW < 1$, it may be concluded that one or more states intermediate between the native and denatured states are significantly populated. Inspection of the values for $t_{1/2}$ in the tables shows that this parameter increases slowly with increasing protein concentration in the pH regions in which $\beta/MW < 1$. This behavior is usually interpreted as being due to a decrease in the extent of oligomerization during the transition. Detailed explanations of these abnormalities are not currently available; these abnormalities do not, however, interfere with the reliable evaluation of the important thermodynamic parameters for the denaturations.

Effects of pH. The data in Tables I and II show that both $t_{1/2}$ and ΔH_{cal} increase sharply with increasing pH. Within experimental uncertainty $t_{1/2}$ was found to increase linearly with increasing pH for both proteins according to the following equations obtained by regression analysis:

WT

$$t_{1/2} = 9.63 + 14.41\text{pH} \quad (\text{SD} \pm 0.58) \tag{1}$$

R96H

$$t_{1/2} = -19.84 + 21.31\text{pH} \quad (\text{SD} \pm 0.51) \tag{2}$$

We attribute a significant fraction of the uncertainty in our thermodynamic results to uncertainty in pH measurement. For example, the standard deviation of the values for $t_{1/2}$ for WT corresponds to a variation of only ±0.04pH.

The fact that $t_{1/2}$ varies with pH shows that the extent of protonation of the protein changes on denaturation. If $\Delta\nu$ is the denaturational increase in the numbers of protons bound by the proteins, then

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

where $T_{1/2} = t_{1/2} + 273.15$. We have seen that for both proteins $dT_{1/2}/d\text{pH}$ is constant within experimental uncertainty. The calculated values of $\Delta\nu$ can be expressed as functions of pH:

WT

$$\Delta\nu = 1.59 + 0.752\text{pH} \quad (\text{SD} \pm 0.12) \tag{4}$$

R96H

$$\Delta\nu = -1.50 + 2.175\text{pH} \quad (\text{SD} \pm 0.22) \tag{5}$$

For the WT protein $\Delta\nu$ varies from 2.8 to 3.7 over the experimental range and from 2.9 to 4.7 for R96H. In view of the small enthalpy associated with the first ionization of phosphoric acid, we have not applied any corrections to the observed enthalpies to reflect buffer ionization heats caused by these proton transfers.

It has been shown (Privalov, 1979) for several globular proteins that the effect of pH on ΔH_{cal} can be considered as a heat capacity effect stemming from the variation of $t_{1/2}$ with pH. This is also true within experimental error for both WT and R96H. The mean value for ΔC_p of WT (Table I, column 6, excluding the value at pH 1.60) is 2.41 kcal K^{-1} mol^{-1} (SD ±0.32). Linear least squaring of ΔH_{cal} for WT as a function of $t_{1/2}$ gives $\Delta C_p = 2.25$ kcal K^{-1} mol^{-1} (SD ±0.47). Using the mean of these two values results in for WT

$$\Delta H_{cal} = 5.97 + 2.33t \text{ kcal mol}^{-1} \text{ (SD } \pm 4.20) \quad (6)$$

Similarly for R96H, the two values for ΔC_p are 2.79 kcal K^{-1} mol^{-1} (SD ±0.39) (Table II, column 6) and 2.52 kcal K^{-1} mol^{-1} (SD ±0.28) from least squaring ΔH_{cal} as a function of $t_{1/2}$, so that for R96H

$$\Delta H_{cal} = -8.58 + 2.66t \text{ kcal mol}^{-1} \text{ (SD } \pm 4.48) \quad (7)$$

The standard deviation is not significantly decreased in either case by inclusion of a term in t^2. The replacement of arginine by histidine decreases the denaturational enthalpy by 6.3 kcal mol^{-1} at 25 °C and by 1.2 kcal mol^{-1} at 50 °C.

Equations 1 and 6 lead to $t_{1/2} = 38.45 \pm 0.58$ °C and $\Delta H_{cal} = 95.6 \pm 4.2$ kcal mol^{-1} at pH 2.0. Values for these quantities, determined by optical methods, are $t_{1/2} = 41.9 \pm 0.4$ °C and $\Delta H_{vH} = 89 \pm 5$ kcal mol^{-1} (Matthews et al., 1987). Three factors may contribute to these differences: (1) The optical experiments were performed at protein concentrations an order of magnitude or more smaller than those used in the calorimetric experiments; (2) the optical experiments were performed in the presence of 100 mM KCl and the calorimetric experiments in the presence of 25 mM KCl; (3) the optical experiments measure ΔH_{vH}, which according to the calorimetric data is $0.91\Delta H_{cal}$ at pH 2.0.

It has been observed [Privalov, 1979; see also Baldwin (1986)] for a number of globular proteins that plots of specific enthalpy vs temperature extrapolate to a common value of approximately 13 cal g^{-1} at about 110 °C. Equations 6 and 7 extrapolate to 14 and 15 cal g^{-1}, respectively, at 110 °C.

As illustrated in Figure 1, the apparent specific heat of R96H before denaturation increases with temperature but is, within experimental uncertainty, independent of temperature after the transition. Similar observations were made in all the experiments reported here and have proven to be characteristic for several other proteins studied in this laboratory, including other mutant forms of T4 lysozyme (unpublished observations). The observed slopes (dc_N/dT in cal K^{-2} g^{-1}) are listed in column 7 in Tables I and II. The mean values, 2.44×10^{-3} cal K^{-2} g^{-1} for WT and 2.65×10^{-3} cal K^{-2} g^{-1} for R96H, are not unusual; for example, Privalov and Khechinashvili (1974) report $(2.2 \pm 0.5) \times 10^{-3}$ cal K^{-2} g^{-1} for five proteins.

The lack of temperature dependence of the apparent specific heats for many denatured proteins is surprising. Interpreted literally, this temperature independence, coupled with the temperature dependence of the apparent specific heats of native proteins, indicates a temperature dependence of the denaturational change in heat capacity ΔC_p which is much larger than actually observed. For example, for WT the observed slope of the initial base line, 2.44×10^{-3} cal K^{-2} g^{-1} or 0.0456 kcal K^{-2} mol^{-1}, corresponds to a decrease in ΔC_p of 0.78 kcal K^{-1} mol^{-1} over the temperature range 35–52 °C, whereas in fact no significant change in ΔC_p was observed over this temperature range. This discrepancy suggests that the apparent specific heat of some denatured proteins, although nearly independent of temperature, is larger the higher the temperature at which the denaturation takes place.

Free Energies of Unfolding. Since the free energy of unfolding ΔG_u vanishes at $t_{1/2}$, the Gibbs–Helmholtz equation

$$\frac{d(\Delta G/T)}{dT} = \frac{-\Delta H}{T^2} \quad (8)$$

may be employed to calculate ΔG_u at other temperatures. The results of such calculations, using $t_{1/2}$ and ΔH_{cal} as given by eq 1, 2, 6, and 7, are shown in Figure 4. The values of ΔG_u are subject to considerable uncertainty at temperatures far removed from $t_{1/2}$; the shaded area in Figure 4 was generated

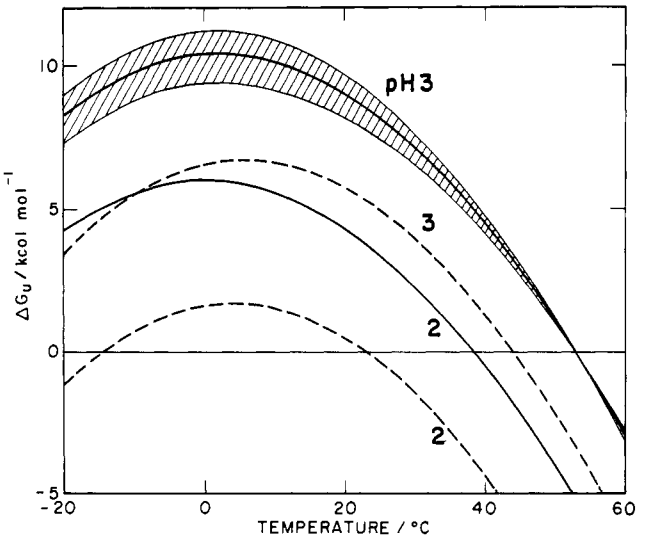

FIGURE 4: Molar free energy change on thermal unfolding of WT (—) and R96H mutant (– –) at pH 2 and 3 as a function of temperature, calculated on the basis of the Gibbs–Helmholtz equation (eq 8) and eq 1, 2, 6, and 7. The shaded portion of the pH 3 curve for WT was generated by adding (upper boundary) or subtracting (lower boundary) half a standard deviation of ΔH_{cal} and of ΔC_p.

by adding to or subtracting from eq 6 half a standard deviation of ΔH_{cal} (2.10 kcal mol^{-1}) and of ΔC_p (0.16 kcal K^{-1} mol^{-1}).

The differences in ΔG_u (WT – R96H) in the range of pH and temperature covered in Figure 4 vary from 5.4 kcal mol^{-1} at −20 °C and pH 2 to 3.21 kcal mol^{-1} at 40 °C and pH 3. Thus the replacement of a single fully charged arginine residue by a fully charged histidine residue produces a very significant apparent destabilization of the protein.

Weaver et al. (1988) have determined by X-ray crystallography the structure of R96H to a resolution of 1.9 Å and have discussed in detail, on the basis of comparisons with the structure of the WT protein, the possible sources of the decrease in the free energy of unfolding produced by the replacement of arginine by histidine. These authors concluded that the two most important sources are the loss of a helix–dipole interaction involving the C-terminus of helix 82–90 and significant strain caused by the introduction of the imidazole ring of histidine.

Appendix

We outline here the procedure we have employed for fitting a theoretical curve to the observed values of apparent specific heat in order to obtain the best values for the thermodynamic parameters. We have assumed simple two-state behavior:

$$N \rightleftarrows D \qquad K = [D]/[N] = \alpha/(1 - \alpha) \quad (A1)$$

K is the equilibrium constant for the denaturation, and α is the extent of conversion.

The apparent specific heat of the native protein c_N and that of the denatured protein c_D are assumed to be linear functions of the temperature:

$$c_N = A + Bt \qquad c_D = C + Dt \quad (A2)$$

where t is the temperature in degrees Celsius. The specific enthalpy is then

$$\Delta h_{cal} = \Delta h_o + (C - A)t + (1/2)(D - B)t^2 \quad (A3)$$

where

$$\Delta h_o = \Delta h_{1/2} - (C - A)t_{1/2} + (D - B){t_{1/2}}^2 \quad (A4)$$

with $\Delta h_{1/2}$ being the value of Δh_{cal} at $t_{1/2}$, the temperature of half-completion of the reaction. If we set

$$-A = \Delta h_o - 273.15(C - A) + (1/2)(273.15)^2(D - B) \quad \text{(A5)}$$

$$B = (C - A) - 273.15(D - B) \qquad C = (1/2)(D - B) \quad \text{(A6)}$$

then, since $K = 1$ at $t_{1/2}$, the integrated form of the van't Hoff equation can be written

$$\frac{R}{\beta} \ln K = A\left(\frac{1}{T} - \frac{1}{T_{1/2}}\right) + B \ln \frac{T}{T_{1/2}} + C(T - T_{1/2}) \quad \text{(A7)}$$

with $\beta = \Delta H_{vH}/\Delta h_{cal}$ and $T_{1/2} = t_{1/2} + 273.15$. The quantity β is assumed to be independent of temperature within a single experiment. K is evaluated at the temperature T, and α is obtained from $\alpha = K/(K + 1)$. Finally, the excess specific heat is given by

$$c_{ex} = \Delta h_{cal} \frac{d\alpha}{dT} \alpha(1 - \alpha) \frac{\beta \Delta h_{cal}^2}{RT^2} \quad \text{(A8)}$$

It may be assumed that the initial specific heat function changes to the final specific heat function in proportion to α, so that the calculated base line is

$$c_{base} = (1 - \alpha)(A + Bt) + \alpha(C + Dt) \quad \text{(A9)}$$

and the total specific heat to be compared with the observed values is

$$c_{tot} = c_{ex} + c_{base} \quad \text{(A10)}$$

We have employed a least-squares procedure in which the value of $\sum(c_{obsd} - c_{tot})^2$ is minimized by variation of the three parameters $t_{1/2}$, Δh_{cal}, and β, using either a "brute-force" procedure in which the parameters are varied in succession with a limitation of five variations in each cycle or a nonlinear least-squaring procedure based on either the SIMPLEX or the Marquhardt algorithm. By either type of procedure, standard deviations of 1% or less were obtained.

Registry No. Arg, 74-79-3; His, 71-00-1; lysozyme, 9001-63-2.

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