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A Differential Scanning Calorimetric Study of the Thermal Unfolding of Seven Mutant Forms of Phage T4 Lysozyme†

Patrick Connelly, Lily Ghosaini, Cui-Qing Hu, Shinichi Kitamura, Akiyoshi Tanaka, and Julian M. Sturtevant*
Departments of Chemistry and of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511
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ABSTRACT: High-sensitivity differential scanning calorimetry has been applied to the study of the reversible thermal unfolding of the lysozyme of T4 bacteriophage in which the threonine residue at position 157 has been replaced by seven different residues. High-resolution structures derived from X-ray crystallography have been reported for these and six other mutants by Alber et al. [Alber, T., Dao-Pin, S., Wilson, K., Wozniak, J. A., Cook, S. P., & Matthews, B. W. (1987) *Nature 330*, 41–46]. At pH 2.5 the changes relative to the wild-type protein in the standard free energy of unfolding produced by these mutations indicate apparent destabilizations of 0.6 kcal mol^{-1} (T157R) to 1.9 kcal mol^{-1} (T157I), whereas the changes in enthalpy of unfolding range from −5.8 kcal mol^{-1} (T157N) to 11.9 kcal mol^{-1} (T157E). Since the denaturations are in all cases accompanied by large changes in heat capacity amounting to 2.5 kcal K^{-1} mol^{-1}, both the free energies and enthalpies are functions of temperature. An intriguing feature of the present results is the relatively large enthalpy changes and the corresponding compensating entropy changes. Our present understanding of the intramolecular energetics of proteins is insufficient to account for these changes.

The recent literature has reported studies of the effects of several single amino acid replacements in a protein on the energetics of thermal unfolding [λ repressor (Hecht et al., 1984, 1985); staphylococcal nuclease (Shortle et al., 1988; Tanaka et al., unpublished data); lysozyme of T4 phage (Alber et al., 1987; Kitamura & Sturtevant, 1989); tailspike protein of phage P22 (Sturtevant et al., 1989)]. In those studies the various replacements in a molecule were each at a different location. It was pointed out by Alber et al. (1987) that study of several different replacements at a single site in a protein might give detailed information concerning the contributions

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* Author to whom correspondence should be addressed.

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to the stability of the native conformation from the various parts of the side chain of the amino acid being replaced. These authors replaced the threonine residue at position 157 in T4 lysozyme by 13 other residues and investigated the effects on structure as revealed by high-resolution X-ray crystallography. Changes in apparent stabilities were estimated by measuring the molar ellipicity of the wild-type and each mutant protein at 223 nm as a function of temperature and determining the temperature of half-completion, $t_{1/2}$, of the cooperative transition as followed by the optical signal. At this temperature, assuming a two-state transition, the standard free energy of unfolding, $\Delta G°_d$, is zero, and the value for $\Delta\Delta G°_d = \Delta G°_d$(WT) $- \Delta G°_d$(mutant) at $t_{1/2}$ for the wild-type protein was approximated by the relation $\Delta\Delta G°_d = \Delta t_{1/2}\Delta S°$, where $\Delta t_{1/2} = t_{1/2}$(WT) $- t_{1/2}$(mutant) and $\Delta S°$ is the standard entropy change in the denaturation of the mutant. Values for $\Delta\Delta G°_d$ ranging from 0.45 kcal mol^{-1} with asparagine replacing threonine to 2.9 kcal mol^{-1} with isoleucine were found, and possible connections between these values and the corresponding structures were discussed in detail.

In the present paper we report a study by means of differential scanning calorimetry of the thermal unfolding of seven of these threonine 157 mutants, using samples generously supplied by Brain Matthews and Joan Wozniak of the University of Oregon.

Materials and Methods

Materials. Wild-type T4 lysozyme and seven mutants in which Thr157 is replaced by a variety of amino acids were shipped at ice temperature from the University of Oregon. The proteins were supplied at high concentration, were dialyzed before use against a 20 mM potassium phosphate buffer containing 25 mM KCl and 0.5 mM dithiothreitol, and were used in our experiments at concentrations of 1–12 mg mL^{-1}. Protein concentrations were determined spectrophotometrically by employing an absorptivity at 280 nm of 1.28 cm^2 mg^{-1}.

Calorimetry. Two different calorimeters were employed in the DSC experiments, the MC-2 (Microcal, Inc., Northampton, MA) and the DASM-4 (Biopribor, Moscow, USSR; Privalov, 1980). In all cases where a sample was run in both instruments, agreeing results were obtained. A scan rate of 1 K min^{-1} was employed throughout, it having been ascertained that essentially the same results were obtained at 0.5 K min^{-1}. In all experiments the reference cell was filled with the corresponding dialysis buffer, and instrumental base lines were determined with both cells filled with this buffer. Rescanning of the samples indicated that the denaturations were reversible provided that the first scan was not carried beyond about 95% completion.

Data Analysis. The data, with instrumental base line deducted, obtained in all the experiments were analyzed by a least-squares curve fitting procedure assuming a two-state transition (Sturtevant, 1987). The total apparent specific heat of a protein in solution may be broken up into two contributions, the average specific heat, c_{av}, and the excess specific heat, c_{ex} (see Figure 1):

$$c_{tot} = c_{av} + c_{ex} \tag{1}$$

For a two-state process the average specific heat is given by the sum of the specific heats of the native state, c_N, and denatured state, c_D, weighted by the fraction of each state present at a particular temperature:

$$c_{av} = (1 - \alpha)c_N + \alpha c_D \tag{2}$$

where α is the fraction in the denatured state. The excess specific heat is the heat capacity arising from the transformation of protein from the native to denatured state and, if the denaturation does not involve association or dissociation, is given by

$$c_{ex} = \alpha(1 - \alpha)\beta\frac{\Delta h_{cal}^2}{RT^2} \tag{3}$$

where Δh_{cal} is the enthalpy of the system relative to the native state and β is an empirical parameter expressing any deviation from true two-state behavior. The value of this parameter is equal to the molecular weight of the protein in the case of a true two-state transition in which the enthalpy and the equilibrium constant for the reaction native $\rightleftarrows$ denatured are related by the van't Hoff equation:

$$R\frac{\mathrm{d}\ln K}{\mathrm{d}(1/T)} = -\Delta H_{vH} \tag{4}$$

When the measured molar enthalpy, ΔH_{cal}, deviates significantly from ΔH_{vH}, a two-state transition is an inadequate model for the process. However, it has been observed that the specific heat profile for many proteins may be described in terms of a two-state model with a "non-ideal" constant correction factor to the van't Hoff relation. This factor, given by the ratio of the van't Hoff enthalpy to specific calorimetric enthalpy, $\Delta H_{vH}/\Delta h_{cal} \equiv \beta$, is a useful indicator of the underlying physical basis for the deviation from ideal two-state behavior, as has been discussed previously (Sturtevant, 1987).

The total specific heat, c_{tot}, would equal the average specific heat if the enthalpy of the transition were zero at all temperatures. Therefore, c_{av} is often referred to as a chemical base line. The quantity bounded by c_{tot} and c_{av} may be called the excess enthalpy, Δh_{ex}. Its value converges to a finite value, Δh_{cal}, at a temperature where denaturation is complete. The utility of Δh_{ex} is discussed below in terms of the analogy between β and the Hill slope. The latter quantity plays a dominant role in the analysis of ligand binding data (see Discussion).

The observed total apparent specific heat data were analyzed by selecting regions at low and high temperatures which were assumed to correspond to the apparent specific heats of native and denatured protein, respectively, and were fit as straight lines, $c_N = a + bt$ and $c_D = e + ft$. The transition portion of the curve was fit with eq 1 after substituting $K/(1 + K)$ for α in eqs 2 and 3 and the lines describing c_N and c_D into eq 2. The three parameters $t_{1/2}$, Δh_{cal} at $t_{1/2}$, and β were obtained in the fit. The standard deviation calculated from the observed points was generally well under 2% of the maximal value of $c_{tot} - c_{av}$.

Results

A typical DSC curve is shown in Figure 1. This was obtained with the mutant T157R[1] at pH 2.50 and a protein concentration of 6.01 mg mL^{-1}. The dashed curves are the calculated base line and the theoretical curve which best fits the observed (solid) curve. The parameters for the theoretical curve in this case are $t_{1/2}$ = 44.5 °C, Δh_{cal} = 5.60 cal g^{-1} at $t_{1/2}$, β = 17 000 daltons, and ΔC_p = 0.125 cal K^{-1} g^{-1} at $t_{1/2}$. The standard deviation of the calculated from the observed points is 1.2% of the maximal value of the excess specific heat.

The value of $t_{1/2}$ is strongly dependent on pH, changing an average of 17.5 °C per unit of pH change. This dependence, which is linear for each protein over the pH range covered in

[1] Abbreviations: A, alanine; E, glutamic acid; I, isoleucine; L, leucine; N, asparagine; R, arginine; V, valine.

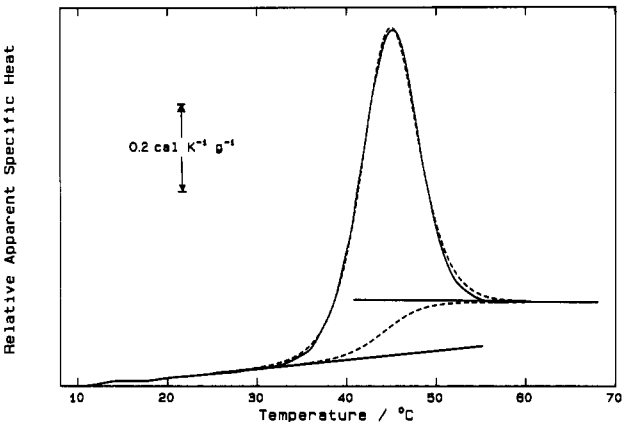

FIGURE 1: Typical DSC curve observed with T157R at a protein concentration of 6.01 mg mL^{-1} at pH 2.50. Solid lines, pre- and posttransition base lines; solid curve, C_{tot}, the observed data; dashed curves, calculated c_{ex} and c_{av}. c_{ex} was calculated with $t_{1/2}$ = 44.5 °C, Δh_{cal} = 5.60 cal g^{-1}, $\Delta H_{vH}/\Delta H_{cal}$ = 0.91, and ΔC_p = 0.125 cal K^{-1} g^{-1}. Calculation of c_{av} involved the pretransition base line $c_N = -0.208 + 0.00190t$ and the posttransition base line $c_D = 0.189 + 0t$ cal K^{-1} g^{-1}. c_{ex} deviated from c_{tot} with a standard deviation of 0.0084 cal K^{-1} g^{-1} (1.2% of $c_{ex,max}$).

Table I: Variation with pH of the Temperature of Half-Denaturation

protein	pH range	no. of pH values	no. of expts	A (°C)[a]	B (°C pH^{-1})[a]	SD (°C)
WT	1.60–2.84	9	43	9.13	14.81	±0.6
T157A	1.60–2.84	10	27	−0.18	17.49	±0.7
T157E	2.02–2.98	7	17	−5.87	19.06	±2.1
T157I	1.80–2.84	7	14	−6.26	18.64	±0.5
T157L	1.71–3.27	6	10	−1.29	16.61	±6.8
T157N	1.70–2.82	5	17	4.65	15.31	±1.8
T157R	1.80–2.84	6	14	−4.03	19.41	±0.7
T157V	1.60–2.84	8	15	−3.80	18.04	±0.8

[a] $t_{1/2} = A + B$pH.

this study, is summarized in Table I. The second column in the table gives the pH range covered; the third column shows the number of different pH values actually used in each case and the fourth column the total number of experiments run with each mutant. The coefficients listed in columns five and six were determined by linear least squaring, with the standard deviations shown in the last column.

The value of ΔH_{cal} is strongly dependent on temperature, as shown in Figure 2, for 27 experiments with T157A (solid circles) and 14 experiments with T157R (open circles). Included in the figure are the least-squared lines for T157A (---), for T157R (···), and for 43 experiments with WT protein (—). The least-squared results for all the proteins studied are summarized in Table II. Although the plots in Figure 2 are of values of ΔH_{cal} at $t_{1/2}$ vs $t_{1/2}$, it seems reasonable to assume that they describe the general dependence of denaturational enthalpy on temperature. The differences between the values for ΔC_p in the third and fifth columns of the table illustrate the relatively large uncertainties in the determination of ΔC_p in individual experiments.

Discussion

The DSC curve in Figure 1 illustrates a perplexing point which has been previously noted (Kitamura & Sturtevant, 1989) and has been observed by others (Tanaka et al., unpublished observations on staphylococcal nuclease; Dr. John Brandts, personal communication), namely, that the slope of the specific heat curve is positive before the transition and close to zero after the transition. This indicates that ΔC_p is temperature dependent, whereas the plot in Figure 2 shows that

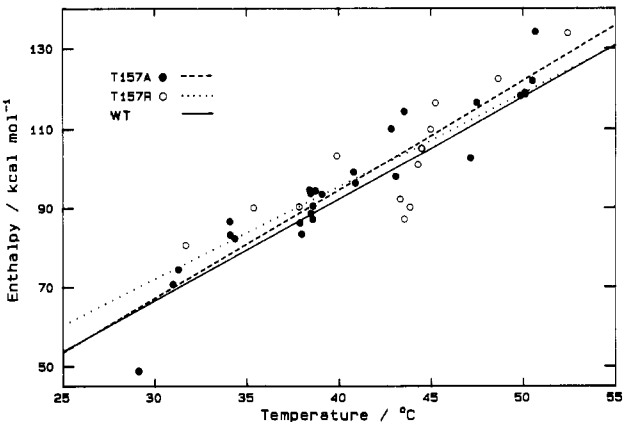

FIGURE 2: Variation with temperature of the enthalpies of denaturation of the mutants T157A (●; 27 experiments) and T157R (○; 14 experiments). Included in the figure are the least-squared lines for T157A (---), for T157R (···), and for wild-type protein (—; 43 experiments).

Table II: Variation with Temperature of the Enthalpy of Denaturation

protein	$\Delta H_0 \pm$ SE (kcal mol^{-1})[a]	$\Delta C_p \pm$ SE (kcal K^{-1} mol^{-1})[a]	SD (kcal mol^{-1})	ΔC_p(mean) (kcal K^{-1} mol^{-1})[b]
WT	−10.51 ± 0.83	2.57 ± 0.02	±5.7	2.41 ± 0.08
T157A	−14.97 ± 1.20	2.74 ± 0.03	±6.00	2.43 ± 0.12
T157E	−8.58 ± 2.15	2.27 ± 0.05	±9.78	2.30 + 0.27
T157I	−2.50 ± 1.52	2.42 ± 0.04	±5.25	3.11 ± 0.18
T157L	−7.68 ± 2.42	2.31 ± 0.06	±6.85	1.74 ± 0.45
T157N	−17.67 ± 2.82	2.85 ± 0.07	±10.99	3.37 ± 0.22
T157R	1.88 ± 2.48	2.34 ± 0.06	±8.58	2.65 ± 0.18
T157V	−14.15 ± 1.00	2.63 ± 0.02	±3.61	2.45 ± 0.16
mean ± SE		2.52 ± 0.09	±7.04	2.56 ± 0.20

[a] $\Delta H_{cal} = \Delta H_0 + \Delta C_p T_{1/2}$. [b] Mean of ΔC_p evaluated at $t_{1/2}$ from each scan ± standard error of the mean.

within experimental uncertainty ΔC_p is independent of temperature. We are unable to account for this apparent discrepancy.

The variation of $t_{1/2}$ with pH summarized in Table I shows that denaturation causes a change, $\Delta\nu$, in the number of protons bound per molecule of protein, according to

$$\Delta\nu = \frac{1000\Delta H_{cal}}{2.303RT_{1/2}^2}\frac{dt_{1/2}}{dpH} \quad (5)$$

where $T_{1/2} = t_{1/2} + 273.15$. Using the constants listed in Table I or those obtained by linear least squaring of all the observed values of $t_{1/2}$ as a function of pH ($t_{1/2} = -0.37 + 17.035$pH) leads to the result

$$\Delta\nu = 0.45 + 1.29\text{pH (SD} \pm 0.05) \quad (6)$$

over the pH range covered in this study.

Various sources of proton uptake during denaturation at low pH can be suggested. A carboxyl group involved in a salt linkage in the native protein would take up a proton during unfolding of the protein, as would also a free amino group on exposure to the solvent at low pH. Due to the large net positive charge on the protein at pH 2–3, some of the carboxyl groups may have an abnormally low value of pK (Anderson et al., 1990) in the native state and may become increasingly protonated in the denatured state with increasing pH. This could account for the positive value of the slope in eq 6.

As was found earlier with wild-type and R96H T4 lysozyme (Kitamura & Sturtevant, 1989), in only a few experiments was the ratio β/MW = 1.00 ± 0.05. In general this ratio was greater than unity at low pH and decreased with increasing

Table III: Changes in Free Energy and Enthalpy of Denaturation, at $t_{1/2}$ for the Wild-Type Protein, Produced by the Various Mutations[a]

protein	pH 2.0, 38.8 °C		pH 2.5, 46.2 °C		pH 3.0, 53.6 °C		pH 2.00, 42 °C
	$\Delta\Delta G°_d$	$\Delta\Delta H_d$	$\Delta\Delta G°_d$	$\Delta\Delta H_d$	$\Delta\Delta G°_d$	$\Delta\Delta H_d$	$\Delta\Delta G°_d$[b]
T157A	1.1	−2.1	0.9	−3.4	0.5	−4.6	1.4
T157E	1.5	9.7	1.3	11.9	0.8	14.1	1.1
T157I	2.1	−2.2	1.9	−1.1	1.5	0.0	2.9
T157L	1.7	7.2	1.7	9.2	1.7	11.1	1.3
T157N	1.0	−3.7	1.1	−5.8	1.2	−7.8	0.45
T157R	1.1	−3.5	0.6	−1.8	−0.3	−0.1	1.3
T157V	1.7	1.3	1.6	0.9	1.2	0.4	1.6

[a] $\Delta\Delta G°_d$ and $\Delta\Delta H_d$ (kcal mol^{-1}). Estimated uncertainties: $\Delta\Delta G°_d$, ±0.4 kcal mol^{-1}; $\Delta\Delta H_d$, ±4 kcal mol^{-1} (average value). [b] Alber et al., 1987.

pH, following roughly the relation $\beta/MW = 1.38 - 0.20pH$ (SD ±0.15). In other words, the denaturations deviated significantly from being strictly two state, i.e., with $\Delta H_{vH} = \Delta H_{cal}$. A convenient graphical representation of the data showing the deviation from two-state behavior comes from plotting

$$-\frac{R}{\Delta h_{cal}} \ln \frac{\Delta h_{ex}}{\Delta h_{cal} - \Delta h_{ex}} \text{ vs } \frac{1}{T} \tag{7}$$

The slope of such a plot is β, the effective cooperative molecular weight involved in the transition. If β equals the molecular weight, then the transition is two state. This plot is completely analogous to the Hill plot used in the analysis of ligand binding data in which one plots

$$\log \frac{\Delta x}{\Delta x^* - \Delta x} \text{ vs } \log a_x$$

where Δx is the amount of ligand bound per mole of macromolecule, Δx^* is the maximum amount of ligand bound (equal to the number of binding sites), and a_x is the activity of free ligand. The slope of this plot gives the effective cooperative binding unit whereas the slope of the plot we have employed here gives the molecular weight of the effective cooperative unit. The expressions for the slopes of the calorimetric plot and ordinary Hill plot, given by N_{Hcal} and N_H, respectively, emphasize the analogy between binding phenomena and thermal transition phenomena (Gill et al., 1985):

$$N_{Hcal} = \frac{RT^2 c_{ex}}{\Delta h_{ex}(\Delta h_{cal} - \Delta h_{ex})}$$

$$N_H = \frac{RTb\Delta x^*}{\Delta x(\Delta x^* - \Delta x)}$$

where b is the binding capacity (DiCera et al., 1988) and c_{ex} is the excess specific heat defined previously.

The usual interpretation of $\beta/MW > 1$ is the occurrence of aggregation in either the native or denatured form, or both, and since no significant variation of $t_{1/2}$ with concentration was observed, it would appear that the extent of aggregation is not much affected by denaturation. $\beta/MW < 1$ indicates that intermediate states are significantly populated. Computer simulations show that this situation can arise from domain interactions. The X-ray structure of T4 lysozyme (Weaver & Matthews, 1987) clearly shows it to be a bilobar molecule, with 89 residues in the C-terminal domain and 75 in the N-terminal domain. As shown by the simulations, which were based on the model for domain interaction proposed by Brandts et al. (1989), if the value of $t_{1/2}$ for the unfolding of one domain increases with increasing pH more rapidly than that of the other domain, β/MW as evaluated by curve fitting to a single transition will decrease with increasing pH as a result of the two-component transitions becoming increasingly separated in temperature. As seem in eq 6, the uptake of protons increases with increasing pH, suggesting the involvement of carboxyl groups. Since there are 12 Asp + Glu groups in the N-terminal domain and only 6 in the C-terminal domain, it seems likely that it is $t_{1/2}$ for the N-terminal unfolding which increases more rapidly with pH. In view of the large number of adjustable parameters involved in these simulations, such as the values for the individual unfolding enthalpies and the free energy and enthalpy of the domain interaction, it is not possible to arrive at definitive values for the present case. Suffice it to state that, with reasonable values and with one $t_{1/2}$ rising 30% more rapidly with pH than the other one, values for $d(\beta/MW)/dpH$ in agreement with experiment can be obtained.

A convenient measure of the change produced by a mutation in the apparent stability of a protein is the quantity

$$\Delta\Delta G°_d = \Delta G°_d(WT) - \Delta G°_d(mutant) \tag{8}$$

the difference in the standard free energies of unfolding for the wild-type and mutant proteins, evaluated at $t_{1/2}$ for the wild-type protein by means of the Gibbs–Helmholtz equation:

$$\Delta G°(\text{at } T_1) = \Delta H_2 \frac{T_2 - T_1}{T_2} - \Delta C_p \left(T_2 - T_1 + T_1 \ln \frac{T_1}{T_2} \right) \tag{9}$$

where T_1 and T_2 are $t_{1/2}$ + 273.15 for wild type and mutant, respectively, ΔH_2 is the denaturational enthalpy in kcal mol^{-1} of the mutant at T_2, and ΔC_p is the denaturational heat capacity change in kcal K^{-1} mol^{-1} of the mutant. It is obvious that in the present application of eq 8 the term $\Delta G_d(WT) = 0$, so that destabilization of the native structure, or stabilization of the denatured structure, is indicated by a positive value for $\Delta\Delta G°_d$. Table III lists the values for $\Delta\Delta G°_d$ at pH 2.0, 2.5, and 3.0 calculated from the constants given in Tables I and II and also lists the values for

$$\Delta\Delta H_d = \Delta H_d(WT) - \Delta H_d \text{ (mutant)} \tag{10}$$

at $t_{1/2}$ for the wild type. We estimate the uncertainty in the values for $\Delta\Delta G°_d$ to be ±0.4 kcal mol^{-1} and in the values for $\Delta\Delta H_d$ to average about ±4 kcal mol^{-1}.

The values for $\Delta\Delta G°_d$ at pH 2.0 reported by Alber et al. (1987) on the basis of their CD measurements are given in column 8 of Table III. Their values differ from ours on average by ±0.4 kcal mol^{-1}. Some of this discrepancy is due to the uncertainties in the measurements and, to a small extent, to the fact that ΔC_p was not included in the CD calculations, and some is probably due to the very much higher protein concentrations necessarily used in the DSC measurements. We also employed a lower ionic strength because we found more completely reversible denaturation, as judged by the behavior of rescanned solutions, under our experimental conditions at low than at high ionic strength.

Dang et al. (1989) have employed molecular dynamics/free energy perturbation calculations to obtain the value $\Delta\Delta G°_d = 1.91 \pm 1.1$ kcal mol^{-1} for the T157V mutation, in agreement with the experimental results at pH 2.

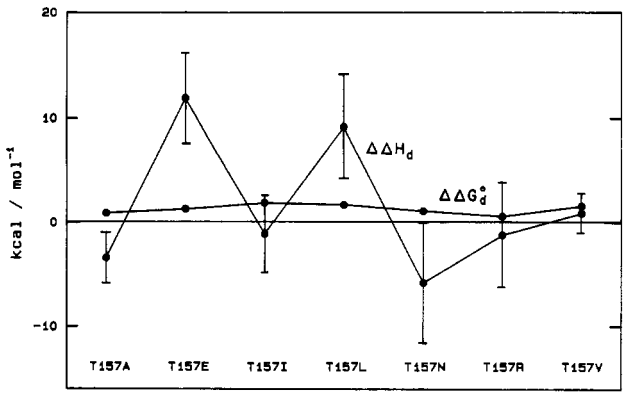

FIGURE 3: Plot of the data for the T157 mutants at pH 2.5 illustrating the lack of correlation between values for $\Delta\Delta G°_d$ and $\Delta\Delta H_d$ and the relatively large magnitude of the latter quantities.

It is important to note that the values of $\Delta\Delta H_d$ are in general much larger in magnitude than those of $\Delta\Delta G°_d$, and in some cases of opposite sign. This is illustrated in Figure 3 for pH 2.5 at the value of $t_{1/2}$ for the wild-type protein. It is obvious that there is no significant correlation between the values of these two quantities. This indicates that reaching a full understanding of the effects of mutations requires considerably more than statements concerning changes in free energies of denaturation. The observed enthalpy and entropy changes, in so far as they are concerned with the native structure, may be in part the results of numerous small contributions spread throughout the highly cooperative structure and will accordingly be difficult to identify and evaluate.

An example of these difficulties is given by the data for T157I and T157L at pH 2.5. This seemingly minor change in the geometry of the amino acid side chain leads, according to our data, to a decrease in $\Delta\Delta G°_d$ of only 0.2 kcal mol^{-1} but to an increase in $\Delta\Delta H_d$ of 10.3 kcal mol^{-1} and a corresponding decrease in entropy of 32 cal K^{-1} mol^{-1}. The X-ray data (Alber et al., 1987) show that introduction of Ile at position 157 forces the side chain of Asp 159 to move 1.1 Å away from its position in the wild-type protein, a much larger motion than caused by any of the other substitutions studied. It is accordingly difficult to understand why the substitution of Leu at position 157 causes a much larger change in enthalpy than the introduction of either Ile or Val.

The lack of correlation between changes due to mutations in free energies and enthalpies of denaturation has been observed with other systems, including λ repressor (Hecht et al., 1984), P_{22} tailspike protein (Sturtevant et al., 1989), and staphylococcal nuclease (Shortle et al., 1988; Tanaka et al., unpublished data).

An important feature of the data in Table III is the significant dependencies of both $\Delta\Delta G°_d$ and $\Delta\Delta H_d$ on pH. In the case of T157R, $\Delta\Delta G°_d$ changes from an apparent destabilization at pH 2.0 to a small apparent stabilization at pH 3.0. It is interesting that Alber et al. (1987) observed that the apparent destabilizing effect of the Thr to Arg mutation was significantly less at pH 6.5 than at pH 2.0, probably because of ionization of Asp 159 and the resulting formation of the Arg 157–Asp 159 hydrogen-bonded ion pair indicated by the crystallographic data. Our result suggests that significant ionization of Asp 159 has already taken place by pH 3.0. Figure 4 shows the variation with temperature of $\Delta\Delta G°_d$ at pH 2.50, as calculated from the data in Tables I and II. Since the uncertainties in $\Delta\Delta G°_d$ increase as extrapolation proceeds further from the temperature region where experiments were actually performed, caution must be observed in

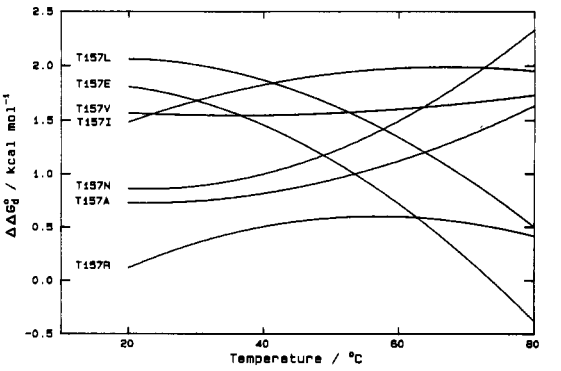

FIGURE 4: Variation with temperature of $\Delta\Delta G°_d$ at pH 2.5 for the T157 mutants calculated from the data in Tables I and II and the Gibbs–Helmholtz equation.

interpreting the large changes indicated in some cases at the extremes of temperature. For example, the uncertainty in $\Delta\Delta G°_d$ for T157E at 80 °C is about ±0.8 kcal mol^{-1}.

We have emphasized in this paper the difficulties in interpretation of DSC data imposed by our current limited understanding of protein intramolecular energetics. We view these difficulties as an indication of the importance of obtaining additional thermodynamic data on the thermal unfolding of a variety of proteins and their mutant forms.

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Registry No. A, 56-41-7; E, 56-86-0; I, 73-32-5; L, 61-90-5; N, 70-47-3; R, 74-79-3; V, 72-18-4; threonine, 72-19-5; lysozyme, 9001-63-2.

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