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

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ABSTRACT: In continuation of our earlier work on the effects of amino acid replacements on the thermodynamics of the thermal unfolding of T4 lysozyme [Kitamura, S., & Sturtevant, J. M. (1989) *Biochemistry 28*, 3788–3792; Connelly, P., Ghosaini, L., Hu, C.-Q., Kitamura, S., Tanaka, A., & Sturtevant, J. M. (1991) *Biochemistry 30*, 1887–1891; Hu, C.-Q., Kitamura, S., Tanaka, A., & Sturtevant, J. M. (1992) *Biochemistry 31*, 1643–1647], we report here a study by differential scanning calorimetry of the effects of five replacements at Ile3. Four of these replacements, those with Glu, Phe, Pro, and Thr, caused apparent destabilizations, while the replacement by Leu led to a small apparent stabilization. The largest observed destabilization (Ile3Pro) amounted to −3.0 kcal mol^{-1} in free energy at pH 2.00 and 38.8 °C (the denaturational temperature of the wild-type protein at this pH), and the largest stabilization amounted to +1.2 kcal mol^{-1} at pH 3.00 and 53.6 °C.

The importance of the role of hydrophobic interactions in protein structural stabilization continues to be a contentious issue (Dill, 1990; Kim & Baldwin, 1991; Shirley et al., 1992). Thermodynamic measurements of stability together with high-resolution X-ray crystal structures of mutant protein systems with single amino acid substitutions have provided much of the experimental evidence to fuel the debate.

T4 lysozyme has provided information on the effects of residues on their environments (Hawkes et al., 1984; Matthews, 1987; Klemm et al., 1991) and has been the subject of extensive study by differential scanning calorimetry (DSC) in this laboratory (Kitamura & Sturtevant, 1989; Connelly et al., 1991; Hu et al., 1992). The molecule is composed of a single polypeptide chain of 164 amino acid residues forming 2 domains connected by a long α-helix. The crystal structure for the wild-type enzyme has been refined to 1.7 Å (Weaver & Matthews, 1987), and the structures of several mutant forms of T4 lysozyme are also available [see, for example, Grutter et al. (1979), Matthews et al. (1987), Pjura et al. (1990), and Dao-pin et al. (1991)].

In wild-type T4 lysozyme, the isoleucine in position three (Ile3) contributes to the major hydrophobic core of the C-terminal lobe and also helps link the N- and C-terminal domains (Remington et al., 1978). Matthews and his colleagues (Matsumura et al., 1988) have employed site-directed mutagenesis to replace Ile3 by 13 different amino acid residues, and have reported the effects of these replacements on the temperature and free energy of unfolding as determined by the temperature dependence of the circular dichroism of the proteins.

The Ile3 side chain is 15–20% accessible to solvent. It is, however, sufficiently close to the surface to enable the accommodation of various substitutions without the need for major changes in the protein structures as judged by mutant crystal structures. The buried portion is in contact with the side chains of Met6, Leu7, and Ile100 as well as the main-chain portion of Cys97 (Matsumura et al., 1988). In the present work, the thermal denaturation of five replacements at the Ile3 position has been studied by DSC.

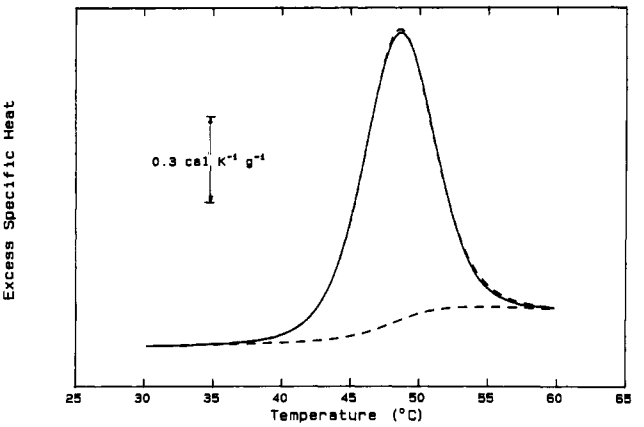

FIGURE 1: Typical DSC curve observed for a 3.36 mg mL^{-1} solution of the Ile3Leu mutant of T4 lysozyme at pH 2.46. Solid curve, observed data; dashes curves, calculated excess apparent specific heat and calculated chemical base line. The excess apparent specific heat was calculated with $t_{1/2}$ = 48.3 °C, ΔH = 6.95 cal g^{-1}, $\Delta H_{vH}/\Delta H_{cal}$ = 0.94, and ΔC_p = 0.131 cal K^{-1} g^{-1}. The calculated data deviate from the observed data with a standard deviation of 0.0058 cal K^{-1}g^{-1} (0.5% of the maximal excess specific heat).

MATERIALS AND METHODS

Materials. The mutants of T4 lysozyme protein were shipped at 0 °C from the University of Oregon. The proteins were dialyzed for several hours against two changes of a 20 mM potassium phosphate buffer containing 25 mM KCl and 0.5 mM dithiothreitol, and were used within 3 h of removal from the second dialysate. Protein concentrations were determined spectrophotometrically using an absorption coefficient at 280 nm of 1.28 cm^2 mg^{-1}.

Calorimetry. Calorimetric measurements were made using two different calorimeters: the MC-2 (Microcal, Inc., Northampton, MA) and the DASM-4 (Biopribor, Puschino,

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Table I: Variation with pH of the Temperature of Half-Denaturation of Ile3 Mutants of T4 Lysozyme

protein	pH range	no. of pH values	no. of expt	$A \pm$ SE (°C)[a]	$B \pm$ SE (°C pH^{-1})[a]	SD (°C)
WT	1.60–2.84	9	43	9.13	14.81	±0.6
Ile3Glu	1.98–3.05	10	12	−0.62 ± 0.13	−16.84 ± 0.05	±0.42
Ile3Phe	1.96–3.01	10	13	−2.29 ± 0.23	−18.18 ± 0.10	±0.75
Ile3Leu	2.00–3.19	9	21	11.43 ± 0.28	14.99 ± 0.12	±1.23
Ile3Pro	2.00–3.01	7	7	−14.02 ± 0.24	20.39 ± 0.10	±0.55
Ile3Thr	2.02–2.97	10	13	−7.67 ± 0.16	18.97 ± 0.06	±0.52

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

Table II: Variation with Temperature of the Enthalpy of Denaturation of the Ile3 Mutants of T4 Lysozyme

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 ± SE, (kcal K^{-1} mol^{-1})[b]	mean SD (curve fitting, %)[c]
WT	−10.51 ± 0.83	2.57 ± 0.02	±5.7	2.41 ± 0.08	
Ile3Glu	5.22 ± 1.14	2.51 ± 0.03	±3.6	2.61 ± 0.25	0.85 ± 0.11
Ile3Phe	21.54 ± 1.27	2.10 ± 0.03	±4.2	2.64 ± 0.26	0.72 ± 0.12
Ile3Leu	9.49 ± 1.25	2.28 ± 0.03	±5.4	2.36 ± 0.18	1.24 ± 0.18
Ile3Pro	−22.64 ± 2.58	2.96 ± 0.07	±5.8	3.70 ± 0.35	1.35 ± 0.24
Ile3Thr	−8.38 ± 1.14	2.79 ± 0.03	±3.8	2.98 ± 0.24	0.69 ± 0.07
		mean ± SE 2.54 ± 0.16	±4.8	2.78 ± 0.25	0.97 ± 0.18

[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. [c] Percent of the maximal excess heat capacity.

Russia), which gave closely agreeing results. A scan rate of 1 K min^{-1} was employed throughout. Instrumental base lines were determined prior to scanning each sample with both cells filled with dialysate which also served in the reference cell during scanning of the protein. The denaturations were found to be fully reversible provided that the first heating was not carried beyond 95% completion.

Data Analysis. The data, with instrumental base line subtracted, were fitted by a least-squares procedure to a modified two-state model with independent variation of ΔH_{vH}, the van't Hoff enthalpy, and ΔH_{cal}, the calorimetric enthalpy (Sturtevant, 1987; Kitamura & Sturtevant, 1989; Connelly et al., 1991). The standard deviation of calculated from observed points expressed as a percentage of the maximal excess heat capacity gives a measure of how close the fit is for each scan (as indicated in column 6 of Table II).

RESULTS

A typical DSC curve, obtained with the Ile3Leu mutant at pH 2.46 and protein concentration 3.36 mg mL^{-1}, is shown in Figure 1. The dashed curves are the calculated base line (Sturtevant, 1987) and the modified two-state curve which best fits the observed (solid) curve. The parameters for the theoretical curve in this case are $t_{1/2} = 48.3$ °C, $\Delta h_{cal} = 6.35$ cal g^{-1} at $t_{1/2}$, β/MW = 0.94, and $\Delta C_p = 0.131$ cal K^{-1} g^{-1} at $t_{1/2}$. The ratio β/MW is equal to the ratio $\Delta H_{vH}/\Delta H_{cal}$ and is equal to unity for a true two-state process. The standard deviation of the calculated from the observed points is 0.5% of the maximal value of the excess specific heat.

The results obtained in 66 experiments with the Ile3 mutants in this work and 43 with the wild-type (WT) protein (Connelly et al., 1991) are summarized in Tables I–III. The constants A and B listed in columns 5 and 6 of Table I were evaluated by linear regression analysis of the strong dependence of $t_{1/2}$ for each protein on the pH, with the standard deviations shown in the last column. As is generally the case in the thermal unfolding of proteins, large increases in the apparent heat capacity accompanied the unfolding of the proteins studied here, amounting on average to 2.5 kcal K^{-1} mol^{-1}. The values for the constants ΔH_0 and ΔC_p in the empirical equation

$$\Delta H_{cal} = \Delta H_0 + \Delta C_p t_{1/2} \quad (1)$$

obtained by linear least-squaring are given in Table II together with the standard deviations in the linear fits. The fifth column lists the mean values for ΔC_p evaluated at $t_{1/2}$ from each scan. These values are generally somewhat larger than those obtained from the variation of ΔH_{cal} with $t_{1/2}$, and are considered to be less reliable. The last column in Table II illustrates the fact that excellent fits were obtained in the curve-fitting calculations for all of the Ile3 mutants.

Table III: Variation with pH of the Ratio β/MW[a] for the Ile3 Mutants of T4 Lysozyme

protein	a	b (pH^{-1})	SD
WT	1.57	−0.24	±0.03
Ile3Glu	1.54	−0.25	±0.06
Ile3Phe	1.20	−0.11	±0.07
Ile3Leu	1.75	−0.31	±0.07
Ile3Pro	2.16	−0.46	±0.07
Ile3Thr	1.72	−0.33	±0.05

[a] β/MW = $a + b$pH.

WT T4 lysozyme and all the mutants thereof which we have studied to date, with the exception of Ala82Pro, Ala93Pro, Gly113Ala, and Cys54Thr:Cys97Ala (Hu et al., 1991), have exhibited a strong dependence of the ratio β/MW on pH, with values well above unity at pH 2 and well below unity at pH 3. This variation for the Ile3 mutants is shown in Table III, with values of slope ranging from −0.11 pH^{-1} for Ile3Phe to −0.46 pH^{-1} for Ile3Pro.

DISCUSSION

As in all our previous work with T4 lysozyme and its mutant forms, as well as with staphylococcal nuclease, ribonuclease T1, and other proteins, the pretransition slope of the apparent specific heat is significantly larger than the posttransition slope. The temperature dependence of ΔC_p which this phenomenon implies is in disagreement with the fact that with all these proteins plots of ΔH_{cal} vs temperature are linear within experimental uncertainity. No fully satisfactory explanation for this situation has been advanced [cf. Hu et al. (1991)].

The variation of $t_{1/2}$ with pH summarized in Table I shows that more protons are bound by the denatured than by the

Table IV: Changes Produced by the Ile3 Mutations of T4 Lysozyme in the Thermodynamics of Denaturation at $t_{1/2}$ for the Wild-Type Protein[a]

protein	Ile3Glu	Ile3Phe	Ile3Leu	Ile3Pro	Ile3Thr
		pH 2.00, 38.75 °C			
$\Delta t_{1/2}$ (°C)	−5.69 (−4.1)[b]	−4.68 (−4.0)[b]	2.66 (3.0)[b]	−11.99	−8.48 (−6.1)[b]
$\Delta\Delta G_d^\circ$ (kcal mol^{-1})	−1.77 (−1.1)[b]	−1.49 (−1.0)[b]	0.85 (0.9)[b]	−2.96	−2.45 (−1.7)[b]
$\Delta\Delta H_d$ (kcal mol^{-1})	13.41	13.84	8.76	2.98	10.66
$\Delta\Delta S_d^\circ$ (cal K^{-1} mol^{-1})	48.7	49.2	25.4	19.1	42.0
		pH 2.50, 46.16 °C			
$\Delta t_{1/2}$ (°C)	−4.68	−3.00	2.75	−9.20	−6.40
$\Delta\Delta G_d^\circ$ (kcal mol^{-1})	−1.71	−1.09	1.01	−2.97	−2.28
$\Delta\Delta H_d$ (kcal mol^{-1})	12.96	10.36	6.62	5.87	12.28
$\Delta\Delta S_d^\circ$ (cal K^{-1} mol^{-1})	46.0	35.9	17.6	27.7	45.6
		pH 3.00, 53.56 °C			
$\Delta t_{1/2}$ (°C)	−3.66	−1.31	2.84	−6.41	−4.32
$\Delta\Delta G_d^\circ$ (kcal mol^{-1})	−1.53	−0.53	1.16	−2.53	−1.81
$\Delta\Delta H_d$ (kcal mol^{-1})	12.52	6.88	4.47	8.76	13.91
$\Delta\Delta S_d^\circ$ (cal K^{-1} mol^{-1})	43.0	22.68	10.1	34.6	48.1

[a] Estimated average uncertainties: $\Delta t_{1/2}$, ±0.7 °C; $\Delta\Delta G_d^\circ$, ±0.4 kcal mol^{-1}; $\Delta\Delta H_d$, ±4 kcal mol^{-1}; $\Delta\Delta S_d^\circ$, ±10 cal K^{-1} mol^{-1}. [b] Matsumura et al. (1988).

native form of the protein. The mean values for the constants listed in Tables I and II substituted into the equation

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

where $\Delta\nu$ is the denaturational change in the protonation of the protein and $T_{1/2} = t_{1/2} + 273.15$ lead to the result

$$\Delta\nu = 0.27 + (1.68)pH \quad (3)$$

over the pH range covered here. Although this slope is considerably higher than previously observed, it leads to values for $\Delta\nu$ at pH 2 and 3 not markedly different from those observed before.

The decrease of β/MW with increasing pH is illustrated in Table III. A suggested qualitative explanation for this decrease of β/MW with increasing pH given in an earlier publication (Connelly et al., 1991) was based on the bilobar structure of T4 lysozyme and Brandts' (Brandts et al., 1989) model for domain interactions in proteins. It was found on the basis of simulations that a value for $t_{1/2}$ for one domain rising 30% more rapidly with pH than that for the other one led to values of $d(\beta/MW)/dpH$ in agreement with experiment. It is interesting that in the present series of experiments there is a rough parallelism between $d(\beta/MW)/dpH$ and $dt_{1/2}/dpH$, suggesting that mutations which have larger effects on the variation of the overall $t_{1/2}$ with pH also affect the pH rate of rise of $t_{1/2}$ for the N-terminal domain relative to that for the C-terminal domain. Since Ile3 is located in the N-terminal domain which contains two-thirds of the carboxyl groups in the protein, it is perhaps not surprising that mutations in this domain have especially large effects.

If lowering the pH from 3 to 2 significantly increases the population of the "molten globule" state of T4 lysozyme, this might account for $\Delta H_{vH} > \Delta H_{cal}$ at the lower pH. According to a recent study of apo-α-lactalbumin (Xie et al., 1991), addition of increasing concentrations of guanidinium chloride decreased ΔH_{cal} more than ΔH_{vH}; this change was attributed to the formation of a molten globule state.

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

$$\Delta\Delta G_d^\circ = \Delta G_d^\circ(\text{mutant}) - \Delta G_d^\circ(\text{WT}) \quad (4)$$

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.[1] It is obvious that according to the definition in eq 4 the term $\Delta G_d^\circ(WT) = 0$, so that destabilization of the native structure, or stabilization of the denatured structure, is indicated by a negative value for $\Delta\Delta G_d^\circ$. Table IV lists the values of $\Delta t_{1/2} = t_{1/2}(\text{mutant}) - t_{1/2}(WT)$, $\Delta\Delta G_d^\circ$, $\Delta\Delta H_d = \Delta H_d(\text{mutant}) - \Delta H_d(WT)$, and $\Delta\Delta S_d^\circ = (\Delta\Delta H_d - \Delta\Delta G_d^\circ)/T_{1/2}(WT)$ at pH 2.00 (38.75 °C), 2.50 (46.16 °C), and 3.00 (53.56 °C). the values in parenthesis for pH 2.00 are those reported by Matsumura et al. (1988), and agree reasonably well with the calorimetric values.

Matsumura et al. discussed in some detail the effects of replacements of Ile3 on the thermal stability of T4 lysozyme. They found an approximately linear relation between the residue hydrophobicity, expressed as the free energy of transfer of the side chain of an amino acid from water to ethanol (Tanford, 1962), and their value of $\Delta\Delta G_d^\circ$ at pH 2 for the corresponding replacement. This correlation is somewhat weakened for the replacements we have studied if our values for $\Delta\Delta G_d^\circ$ are used. Matsumura et al. noted that the aromatic replacements Phe, Tyr, and Trp deviated widely from the linear relation and attributed this to protrusion of the aromatic side chain into the solvent, as shown by their crystallographic structure for Ile3Tyr. This view is supported by our data for Ile3Phe. Our value of $\Delta\Delta G_d^\circ$ for the Pro replacement, which Matsumura et al. did not include in their study, deviates from the linear relation by an amount intermediate between the deviations for Tyr and Trp. The Pro replacement might be expected to reduce the configurational entropy of the unfolded form (Matthews et al., 1987), whereas it actually leads to large positive values for $\Delta\Delta S_d^\circ$. Similar entropic effects were previously observed with the replacements Ala82Pro and Ala93Pro in T4 lysozyme (Hu et al., 1992). On the other hand, the insertion of Pro near the end of the N-terminal α-helix would be expected to lead to some destabilization because of the low helical propensity of Pro, although it seems unlikely that this effect could produce such a large destabilization as actually observed.

[1] In previous publications, we have defined $\Delta\Delta G_d^\circ$ to be $\Delta G_d^\circ(WT) - \Delta G_d^\circ(\text{mutant})$. However, since nearly all other users of this quantity have employed the definition of eq 4, we have decided to comply. This change in sign for ΔX_d, where X is ΔG_d°, ΔH_d, ΔS_d°, or $T_{1/2}$, must be kept in mind when comparing data given here with those previously reported from this laboratory.

A general conclusion which may be drawn from the results presented here is similar to that mentioned in our earlier papers: the rationalization in terms of the structures of the folded and unfolded proteins of the thermodynamic results of single amino acid replacements presents great difficulties, especially in connection with the frequently large enthalpy and entropy changes, which in the present case are all positive.

ADDED IN PROOF

ΔH_{vH} for wild-type protein and for each mutant is temperature-independent with an average standard error of 2.1%. This constancy combined with the temperature dependence of Δh_{cal} can account for the variation of the ratio β/MW with pH (or temperature) noted in Table III.

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