Low-Temperature Unfolding of a Mutant of Phage T4 Lysozyme. 1. Equilibrium Studies†

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ABSTRACT: The mutant protein I3C-C97/C54T of phage T4 lysozyme is free of sulfhydryl groups and has a genetically engineered disulfide bridge between positions 3 and 97 (Perry & Wetzel, 1986). This protein has a maximum stability at 12 °C in 3 M guanidinium chloride and undergoes reversible high- and low-temperature melting at 28 and −3 °C, respectively, in this medium. The free energy of stabilization of the protein has been studied over a range of temperature that includes both melting transitions. The stability curve fits a constant ΔC_p model over the entire range, permitting an unusually complete determination of the thermodynamic parameters of the protein and demonstrating that the low-temperature unfolded form of the protein may be interpreted as an extrapolation with constant ΔC_p of the high-temperature unfolded form. The free energy of unfolding is a linear function of guanidinium concentration within experimental error which permits a rough estimate of the stability of the protein at low temperatures and of the differential interaction of the unfolded protein with guanidinium chloride. These equilibrium studies provide a basis for the interpretation of the kinetic studies reported in the following paper (Chen et al., 1989).

The main purpose of this paper is to present data on the equilibrium unfolding at high and low temperatures of a mutant of the protein phage T4 lysozyme. These results will be required for the interpretation of the kinetic data which follow in Chen et al. (1989). We have been interested in studying proteins which unfold at low temperatures for some time, but they have been rather elusive.

Christensen (1952) noted that β-lactoglobulin, which had been unfolded in 38% urea at 10 °C, refolded when the solution was heated to 30 °C. This observation was confirmed later by using optical rotatory dispersion (Schellman, 1958). The same behavior was observed in solutions of guanidinium chloride (Pace & Tanford, 1968). Evidently, Brandts (1964) was the first to note that a temperature of maximum stability was a general feature of globular proteins. Since then, there has been a continued search for low-temperature instability. Denaturation experiments have been carried out under high pressure for ribonuclease (Hawley, 1971) and for metmyoglobin (Zipp & Kauzmann, 1973), and their results suggested a possible cold unfolding below 0 °C. The cold denaturation of α-chymotrypsinogen in undercooled solution by the emulsion droplet technique (Franks & Hatley, 1985) and the cold denaturation of lactate dehydrogenase in cryosolvents (Hatley & Franks, 1986) have actually been observed. Cold denaturation has also been subjected to detailed thermodynamic analysis in the guanidinium chloride denaturation of phosphoglycerate kinases (Nojima et al., 1977) and in the acid denaturation of metmyoglobins (Cho et al., 1982; Privalov et al., 1986).

In our studies of mutants of phage T4 lysozyme, we occasionally encountered proteins whose stability curves (Becktel & Schellman, 1987) appeared to extrapolate to a low-temperature unfolding above 0 °C. Experiments to verify the instability were always negative, which only demonstrated the dangers of long extrapolations of data of modest accuracy. To initiate a study of the phenomenon, we decided to return to the β-lactoglobulin system in order to observe low-temperature unfolding and simultaneously to obtain data on the interaction of guanidine salts with this protein. Meanwhile, Perry and Wetzel (1984) had prepared a mutant of T4 lysozyme with cysteine replacing isoleucine at position 3. This permitted the formation of a disulfide bridge between cysteine-3 and cysteine-97 to produce a protein of higher stability and improved reversibility in the unfolding reaction. Further mutations of this class of proteins to replace cysteine-54 by threonine or valine generated proteins with one disulfide bridge and no free SH groups (Perry & Wetzel, 1986). These proteins show clean transitions and permit the study of equilibrium unfolding with high accuracy and reversibility (Wetzel et al., 1988).

The data on these proteins indicated that they should melt at temperatures slightly above 0 °C, but once again, this has not been observed. Possible reasons for errors in these long extrapolations have been discussed by Becktel and Schellman (1987). Our low-temperature melting studies of β-lactoglobulin were already under way in the laboratory, and W. J. Becktel and W. A. Baase decided to see if the low-temperature melting of the disulfide mutants of T4 lysozyme would also be facilitated in the presence of guanidinium chloride. They were successful in obtaining the low-temperature unfolding of I3C-C97/C54T[1] under these conditions and also in demonstrating that the kinetics of folding and unfolding at low temperature in guanidinium chloride were very slow (hours to days) under certain conditions. Similar observations had been made by Pace and Tanford (1968) for the unfolding and refolding of β-lactoglobulin in guanidine.

The studies which are presented here are a continuation of the preliminary study of Becktel and Baase which demonstrated the feasibility of equilibrium and kinetic studies on this

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[1] Abbreviations: I3C-C97/C54T protein, mutant phage T4 lysozyme with amino acid replacements of Ile-3 by Cys and Cys-54 by Thr and with a disulfide bond between Cys-3 and Cys-97; GdmCl, guanidinium chloride; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; CD, circular dichroism; temperature subscripts g, h, and s refer to processes for which ΔG, ΔH, and ΔS are zero, respectively.

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class of T4 lysozyme mutants. These proteins are much superior to β-lactoglobulin for this type of investigation, since the latter protein dimerizes in aqueous solution, contains both cystines and cysteine, and displays irreversible behavior.

Experimental Procedures

The purified mutant protein I3C-C97/C54T T4 lysozyme (EC 3.2.1.17) containing a C3–C97 disulfide bridge was a gift from L. Jeanne Perry and Ron Wetzel of Genentech, Inc. The mutant gene was constructed and the protein was purified at Genentech via methods described in Perry et al. (1985) and Perry and Wetzel (1986), respectively.

Ultrapure guanidine hydrochloride (Gdn·HCl; AMRESCO Co., lot number 350633) was used without further purification. Tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl) was reagent grade from Sigma Chemical Co. All other chemicals and reagents were Baker analyzed reagents.

Gdn·HCl concentrations of samples were adjusted by dilution from a 7.56 M stock solution; 2 mM sodium phosphate was used as a buffer to maintain pH. All the solutions were filtered through 0.22-μm Millipore filters (SLGV025LS).

Sample solutions for circular dichroism (CD) measurements contained 0.023 mg/mL I3C-C97/C54T T4 lysozyme, with a total volume of 2.5 mL. Samples were made by adding 44 μL of protein stock (1.30 mg/mL) to 2.45 mL of buffered Gdn·HCl. The concentration of the protein stock is established by the molar extinction coefficient of 24 170 L/(mol·cm) at 280 nm (Elwell & Schellman, 1975).

Circular dichroism at 223 nm was used to follow the kinetics of low-temperature melting and to determine the equilibrium unfolding data for this system. A Jasco J-500C spectropolarimeter with 1-cm optical length HELLMA Suprasil cuvettes was used for all CD measurements. Sample temperature control and stirring were provided by means of a Hewlett-Packard HP89100A thermal controller. Thermal accuracy was estimated to be ±0.2 °C and reproducibility to be ±0.1 °C. An HP87XM minicomputer directed temperature shifts and recorded the CD signal via a HP3478A digital multimeter. A Radiometer PHM84 pH meter with a GK2421C electrode calibrated by using Radiometer pH standards was used to measure the pH of the samples at room temperature. There is no correction made for salt and temperature effects on pH. At the end of each experiment, the density of the sample was determined by using an Anton Parr microdensimeter (Model DMA02C). The molarity of Gdn·HCl was calculated from the density by means of the formula of Kielley and Harrington (1960).

Equilibrium measurements at 3 M Gdn·HCl and pH 5 were done as follows: Above 12 °C, the reaction was fast enough to use the scanning mode of the temperature controller for the melting measurements; i.e., the CD signal change of the sample was monitored continuously as the temperature was increased at a rate of 2 °C/min. From −9.5 to 12 °C, the final equilibrium values from each kinetic run (Chen et al., 1989) at this solvent condition were used to calculate an equilibrium melting curve.

Theoretical Model

The Two-State Assumption. These experiments are interpreted with a two-state model, the folded or native state and the unfolded or denatured state, with negligible population of intermediates. Though only a few of the numerous mutants of T4 lysozyme have been subjected to a detailed investigation, evidence for the lack of intermediates has been cumulative. Unless otherwise stated, all of the denaturation curves quantitatively fit the two-state, constant $\Delta\bar{C}_p$ model described in our previous publications (Elwell & Schellman, 1977; Schellman & Hawkes, 1980; Schellman et al., 1981), but this is not a sufficient demonstration (Lumry et al., 1966). In addition, the mutants we have studied (Baase et al., 1986; Becktel et al., 1987; Becktel & Baase, 1987), except the class containing a cystine bridge, have the same change in heat capacity within experimental error, which is very unlikely if there is a stable intermediate in the folding pathway. For the protein discussed in this paper, there is very good experimental evidence that the kinetic pathway is describable as consisting of a single transition state and no stable intermediates (Chen et al., 1989). There is now NMR evidence confirming the two-state mechanism for several of the mutants (L. McIntosh, personal communication), and calorimetric investigations are in progress (Sturtevant, 1987). With this incomplete, but large, body of evidence, we feel that the two-state interpretation is valid for most of our experiments, but remain on guard for exceptions. These remarks and the two-state interpretation, of course, do not apply to all proteins. Exceptions are well-known in the literature, for example, those with domain folding (Privalov, 1982) or those like the α-lactalbumins (Kuwajima et al., 1976; Dolgikh et al., 1981), cytochrome *c* (Ohgushi & Wada, 1983), and carbonic anhydrase (Wong & Tanford, 1973) where stable intermediates have been found.

The unfolded or denatured state of a protein is frequently characterized by the procedure used to produce it. One sees references in the literature to the low- and high-temperature denatured states, the urea-denatured state, acid- or base-denatured states, etc. A qualitative distinction between these various forms of a denatured protein will not be made in this study, even though our experiments include the unfolding of proteins at high and low temperatures, in the presence of denaturing solvents. In this section, we state our point of view with respect to this problem.

In general, there are a number of pathways by which the native protein can be unfolded, depending on temperature and solution conditions. It is our premise that the unfolded states produced in these various processes are different manifestations of the same condition of the protein. It is unfolded. By this, we mean that (essentially) all of the residues have jumped from a structural probability distribution which is narrow in the native state, with long-range correlations, to broad distributions in the unfolded state, where most residues in the molecule will be distributed over an extensive area of the Φ–Ψ plane. Structural correlations may be still fairly strong over short distances for certain regions of the unfolded state, but long-range correlations are small or negligible. The distribution function for the native state is relatively insensitive to changes in solvent and temperature. This can be seen from standard spectroscopic properties such as UV, IR, and circular dichroism spectra, and from studies dependent on the detailed preservation of structure such as enzymatic activity, antigenicity, etc. Experiments with time resolution such as NMR, fluorescence, hydrogen exchange, etc. detect not only the equilibrium conformation but also kinetic fluctuations. These structural excursions are transient and make a negligible contribution to the equilibrium properties of the native structure.

The distribution functions describing the unfolded states vary depending on the conditions of unfolding. High-temperature denatured protein differs in its structural distribution from low-temperature denatured protein or protein in concentrated solutions of guanidinium salts. Tanford and coworkers have shown a number of years ago that the latter unfolded state is the most extended and has the least order

(Tanford, 1968). The evidence for treating all of these denatured forms on the same footing is that it is always possible to go from one type of unfolded state to another by a continuous process with no cooperative changes in thermodynamic or spectroscopic properties. It has been known for 50 years that the structural distribution of high polymers depends on temperature and solvent. For example, the structural distribution function of poly(methyl methacrylate) depends strongly on pH and on the presence of "good" solvents, which tend to minimize intramolecular contacts, or "poor" solvents, which tend to enhance them. Unfolded globular proteins obey the same rules but presumably have much more complicated structural distributions because evolution has tailored them to have the possibility of favorable, complicated tertiary interactions which can generate transient (or even long-lasting) local regions of structure. In addition, a solvent can be "good" for one pair of side-chain interactions and "poor" for another. Water is such a solvent, being "good" for hydrophilic interactions and "poor" for hydrophobic ones.

For the reversible melting reported here, low-temperature melting and high-temperature melting cannot be regarded as qualitatively different processes. In our work and that of Brandts (1964) and Privalov (1979), the low-temperature melting can be inferred from the extrapolation of data from the high-temperature melting. The parameters determined in this paper are capable of describing an unfolded state over a temperature region which embraces both high- and low-temperature meltings and as a function of guanidinium chloride concentration. This is true even though the thermodynamic properties of the unfolded state vary over an extraordinarily large range, even changing sign relative to the native form for the enthalpy and entropy.

The thermodynamic model which is used to interpret the data in the absence of destabilizing solvents has been described in several publications (Elwell & Schellman, 1977; Schellman, 1987; Becktel & Schellman, 1987). The unfolding reaction $N \rightleftharpoons U$ is assumed to be two-state. In addition, the change in partial molar heat capacity, $\Delta\bar{C}_p$, is assumed to be constant over the range of temperatures which are of experimental interest. There is a great deal of experimental evidence for the latter assumption (Privalov, 1979), a new version of which will be presented in this paper. Under these circumstances, the thermodynamics obey the relations:

$$\Delta\bar{G} = \Delta\bar{H} - T\Delta\bar{S} \quad (1)$$

$$\Delta\bar{H} = \Delta\bar{H}(T_0) + \Delta\bar{C}_p(T - T_0) \quad (2)$$

$$\Delta\bar{S} = \Delta\bar{S}(T_0) + \Delta\bar{C}_p \ln (T/T_0) \quad (3)$$

T_0 is any convenient reference temperature. Methods of evaluation of the parameters $\Delta\bar{H}(T_0)$, $\Delta\bar{S}(T_0)$, and $\Delta\bar{C}_p$ have been given elsewhere (Becktel & Schellman, 1987).

In the presence of a destabilizing substance like guanidinium chloride, these relations are altered. We assume that the change in free energy produced by the denaturing agent is linear in its concentration (Greene & Pace, 1974; Pace, 1975; Schellman & Hawkes, 1980). This is presumably only approximately true, but experimenal evidence appears to bear out this relation. The best test is to compare the stabilization free energies obtained by extrapolation of thermal denaturation with those obtained by isothermal extrapolation to zero concentration of one or more denaturing agents. The linear relation usually passes this test, though the experimental accuracy is not very high. Better data and the detection of nonlinearity would not invalidate the procedures which are used; it would only permit the use of a more sensitive model. Thermodynamic changes in the presence of a denaturant will be marked by a superscript prime. With the linear model, the change in free energy which accompanies unfolding is given by

$$\Delta\bar{G}' = \Delta\bar{G} + RT\Delta\beta_{23}C_3 \quad (4)$$

$\Delta\bar{G}$ is the change in free energy in the absence of denaturing agent, given by eq 1, and C_3 is the molarity of denaturant. The last term is the change in excess free energy of denaturation caused by the fact that the folded and unfolded forms interact differently with the denaturing agent. See Schellman (1978) for notation and other relations. The subscript 23 designates the excess free energy of component 2, the protein, caused by component 3, the added reagent.

The excess free energy can be written as a sum of enthalpic and entropic terms:

$$RT\Delta\beta_{23} = \Delta\bar{H}_{23} - T\Delta\bar{S}_{23} \quad (5)$$

The interaction enthalpy and entropy can also be represented by a model which assumes a constant change in heat capacity associated with the interaction

$$\Delta\bar{H}_{23} = \Delta\bar{H}_{23}(T_0) + \Delta\bar{C}_{p_{23}}(T - T_0) \quad (6)$$

$$\Delta\bar{S}_{23} = \Delta\bar{S}_{23}(T_0) + \Delta\bar{C}_{p_{23}} \ln (T/T_0) \quad (7)$$

Equations 6 and 7 can be combined with eq 1 and 2 to give relations for the enthalpy and entropy of unfolding in the presence of a destabilizing component:

$$\Delta\bar{H}' = \Delta\bar{H} + \Delta\bar{H}_{23}C_3 = [\Delta\bar{H}(T_0) + \Delta\bar{H}_{23}(T_0)C_3] + (\Delta\bar{C}_p + \Delta\bar{C}_{p_{23}}C_3)(T - T_0) \quad (8)$$

$$\Delta\bar{S}' = \Delta\bar{S} + \Delta\bar{S}_{23}C_3 = [\Delta\bar{S}(T_0) + \Delta\bar{S}_{23}(T_0)C_3] + (\Delta\bar{C}_p + \Delta\bar{C}_{p_{23}}C_3) \ln (T/T_0) \quad (9)$$

The free energy equation can be rewritten in terms of primed quantities:

$$\Delta\bar{G}' = \Delta\bar{H}' - T\Delta\bar{S}' = \Delta\bar{G} + \Delta\bar{G}_{23}C_3 = \Delta\bar{G} + RT\Delta\beta_{23}C_3 \quad (10)$$

This equation has the same form as eq 1 and permits us to treat the data in the standard way even in the presence of a denaturing agent. It will turn out that our experiments are not sufficiently accurate to evaluate separately the binding contributions to the enthalpy, entropy, and heat capacity. The reason that eq 5–10 have been written out explicitly is to demonstrate the way in which binding parameters enter into the unfolding equilibrium and to emphasize the fact that quantities like $\Delta\bar{H}'$, $\Delta\bar{S}'$, and $\Delta\bar{C}_p'$, which are determined from data in the presence of denaturing agents, can differ significantly from the equivalent quantities in aqueous solution. In terms of the equilibrium constant, we have

$$\ln K' = \frac{-\Delta\bar{G}'}{RT} = \frac{-\Delta\bar{H}'(T_0)}{RT} - \frac{\Delta\bar{C}_p'}{R}\left(1 - \frac{T_0}{T}\right) + \frac{\Delta\bar{S}'(T_0)}{R} - \frac{\Delta\bar{C}_p'}{R} \ln \frac{T_0}{T} \quad (11)$$

This is the relation that is used to interpret the effect of temperature on the unfolding reaction.

Results and Discussion

As discussed in the introduction, previous work in the laboratory had demonstrated that certain mutants of T4 lysozyme, namely, those with an internal disulfide bridge between positions 3 and 97, display a low-temperature unfolding in the presence of guanidinium chloride. This paper and the fol-

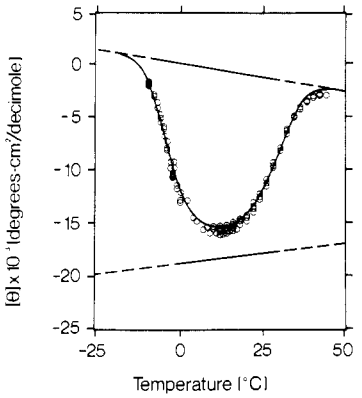

FIGURE 1: Thermal melting of protein T4 lysozyme 13C-C97/C54T. Molar ellipticity at 223 nm was plotted against temperature. Melting was done in 3 M Gdn·HCl at pH 5 with a protein concentration of 0.023 mg/mL. Below 12 °C, the reaction was very slow, so data points were obtained from the final equilibrium values of each kinetic measurement. Above 12 °C, the circular dichroism change was monitored continuously as the temperature was increased at a sufficiently low rate to reach equilibrium at each temperature. The top and bottom lines are for the unfolded and native form, respectively. The continuous line is the result of fitting the melting curve according to eq 12 in the text.

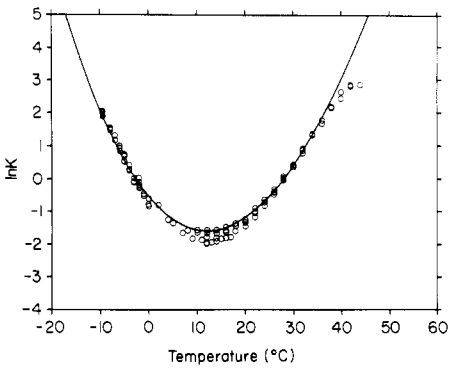

FIGURE 2: Stability curve of T4 lysozyme 13C-C97/C54T in terms of the ln K' equilibrium constant which was calculated from the melting data in Figure 1, with the continuous line as the curve-fitting result.

Table I: Coefficients of Equation 12 from the Fitting of the Melting Curve in Figure 1

T_0 (°C)	A	B	C
28.0	−1041	1041	−1099
−3.0	−1160	1160	−1099

lowing one (Chen et al., 1989) will show that guanidine plays three roles: (1) It lowers the stability curve so that the low-temperature instability is brought into the range of experimental accessibility; (2) it accelerates the unfolding reaction; and (3) it lowers the freezing temperature of water so that lower temperatures may be explored.

If one starts at high temperature with the unfolded form of the protein 13C-C97/C54T in 3.00 M guanidinium chloride and proceeds to lower temperatures, the protein first undergoes a partial refolding reaction at about 28 °C, goes through a stability maximum at about 12.5 °C, and then unfolds again at about −3 °C. This behavior is displayed in Figure 1, which shows the circular dichroism of the protein at 223 nm as a function of temperature. The folded protein is highly helical, and the signal at this wavelength is a sensitive indicator of the state of refolding. The upper base line for the fully unfolded protein is obtained by observing values for the circular dichroism in the plateau region above the transition concentration of guanidinium chloride (at 4.2 and 6.0 M). The lower base line for the fully folded protein was obtained in the plateau region below the transition concentration (at 0.2 and 1.0 M).

At the lower temperatures, both the unfolding and refolding reactions are very slow, and precautions had to be taken to see that the measurements represent equilibrium values. For example, at the lowest temperature of −9.5 °C, the half-time for approach to equilibrium was 98 min, and it was found advisable to continue the experiments for 10 half-times, or about 16 h. In general, it was not possible to study the equilibrium properties without first making kinetic observations. Many of the equilibrium values were taken from the final states of kinetic experiments, in which case the equilibrium condition was almost invariably checked by approaching it from both sides, i.e., by way of both the unfolding and refolding reactions.

It should be observed that at this concentration of guanidinium chloride, the protein is not completely refolded at 12.5 °C, the temperature of maximum stability.

Using the usual procedures for two-state transitions, we converted the CD curves of Figure 1 to fractions of folded and unfolded form, and the equilibrium constant was determined as a function of temperature. The results are shown in Figure 2. The data of this figure were fitted to the constant $\Delta\bar{C}_p$ model described above. Expressed in terms of constant coefficients, eq 11 may be written

$$\ln K' = A + B(T_0/T) + C \ln (T_0/T) \qquad (12)$$

with

$$A = [-\Delta\bar{C}_p' + \Delta\bar{S}'(T_0)]/R$$

$$B = [\Delta\bar{C}_p' - \Delta\bar{S}'(T_0)]/R - \Delta\bar{G}'(T_0)/RT_0$$

$$C = -\Delta\bar{C}_p'/R$$

where T_0 is an arbitrary reference temperature. Recall that the primes indicate that the thermodynamic functions refer to transitions in the presence of denaturing agents. Our normal practice for a single transition is to use the Gibbs–Helmholtz equation in the form $[\partial(\Delta\bar{G}/T)/\partial(1/T)] = \Delta\bar{H}$ where care must be taken because $\Delta\bar{H}$ is a strong function of temperature. The regression plot of ln K vs $1/T$ supplies best values for the transition temperature T_g as well as for $\Delta\bar{H}$ at the transition temperature. $\Delta\bar{C}_p$ is then obtained by comparing enthalpies at different temperatures.

In this paper, on the other hand, eq 12 will be used for the regression analysis of the entire curve of ln K' vs temperature to give values for all three parameters $\Delta\bar{C}_p'$, $\Delta\bar{S}'(T_0)$, and $\Delta\bar{G}'(T_0)$. It will be noted that if T_0 corresponds with either of the transition temperatures, then $\Delta\bar{G}' = 0$ by definition and $A = -B$. The data of Figure 2 were analyzed by using nonlinear least-squares, and T_0 was adjusted to values such that $A = -B$. This gave values for the transition temperatures and the associated values of A and B. These are shown in Table I. The transition temperatures were estimated by plotting points near −3 and 28 °C and locating the points where $\Delta G = 0$ by means of a visual interpolation. The results were −2.7 and 28 °C. Presumably, the latter values are the most reliable, but the comparison shows that estimates of the transition temperatures by looking at points near ln $K' = 0$ and estimates applying the constant $\Delta\bar{C}_p'$ model and using all the data in the entire curve gave results that are almost identical. This is a test of the validity of the model.

It should be noted that both the A and the C coefficients are dominated by $\Delta\bar{C}_p'$. This type of experiment, which extends over both a low- and high-temperature unfolding reaction, provides a stringent test of the assumption of constant

Table II: Thermodynamic Parameters of the Stability Curve Derived from Figures 1 and 2

	low-temp melting	high-temp melting
T_g (°C)	−3 (−2.7)	28.0
$\Delta H_g'$ (cal/mol)	−32700	34700
$\Delta S_g'$ [cal/(mol·K)]	−121	115
T_s (°C)	12.4	12.5
T_h (°C)	12.0	12.1
$\Delta G_s'$ (cal/mol)	926	949
$\Delta C_p'$ [cal/(mol·K)]	2180	2180

$\Delta C_p'$ and also an unusually precise experimental estimate of this quantity. Equations 11 and 12 are integrated forms of the van't Hoff equation. However, instead of evaluating slopes and minor curvatures over a small range of temperature, the data are evaluated over a temperature range in which ln K' changes sign twice and there is a drastic variation in slope from large positive to large negative values. We consider the good fit of the data shown in Figures 1 and 2 to be a confirmation of the constant $\Delta C_p'$ model. On the other hand, there are systematic deviations from the theoretical curve, especially in the central region of maximum stability. We are uncertain whether this reflects a small variation of $\Delta C_p'$ over the extended temperature, or a systematic error, e.g., an error in assigning a base line, always a vexing problem.

With the free energy known as a function of temperature, all of the interesting features of the stability curve can be calculated by the methods outlined by Becktel and Schellman (1987), and these are listed in Table II.

Of special note are the values of the entropy and enthalpy at the low-melting temperature. Both are large and negative. The unfolding of T4 lysozyme at this temperature is accompanied by a large *decrease* in entropy and in energy (enthalpy). It is the $T\Delta S'$ part of the free energy that stabilizes the folded form of the protein, so the conclusion to be drawn is that the protein is held in its ordered structure because of its high entropy! Restricted to the polypeptide itself, there is no valid statistical mechanical explanation for this kind of behavior, so it must be concluded that solvent entropies and energies are playing a strong role. The results are in line with the effect of temperature on the hydrophobic interaction (Kauzmann, 1959; Gill et al., 1976; Baldwin, 1986), but at low temperature, the hydrophobic effect appears to be totally dominant over other contributions to the stability, such as H bonds, London forces, and chain entropy. As a result, the normal signs are reversed except for $\Delta C_p'$. At the phenomenological level, there is nothing surprising about the negative entropies and enthalpies of unfolding. Both $\Delta G'$ and $\Delta G'/T$ increase with temperature on the low-temperature side of the maximum in $\Delta G'$, which establishes $\Delta S'$ and $\Delta H'$ as negative. On the other hand, this type of behavior has been rarely observed because of the difficulty in finding proteins which unfold reversibly at accessible low temperatures.

The $\Delta C_p'$ of 2180 cal/(deg·mol) found for this system agrees with the values obtained for most of the T4 lysozyme mutants, which almost invariably fall in the range of 2000–2200 cal/(deg·mol). The exceptions to this rule, however, are the proteins containing an internal disulfide bridge between positions 3 and 97. For these cases, ΔC_p is in the neighborhood of 2750 cal/(deg·mol) (W. J. Becktel, unpublished results). I3C-C97/C54T is a member of this class of proteins which displays an anomalously high ΔC_p when the high-temperature transition is studied as a function of pH (in the absence of guanidine), but it falls in the normal range when eq 11 is used for the unfolding reaction in guanidine which occurs at lower temperatures. We are uncertain of the origin of this difference,

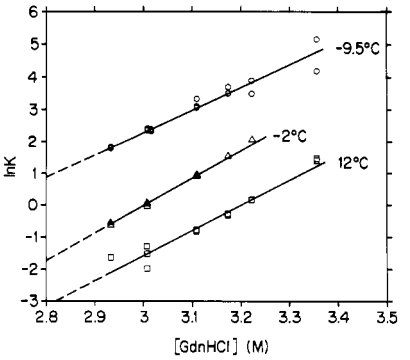

FIGURE 3: Natural log of the equilibrium constant of unfolding for T4 lysozyme I3C-C97/C54T as a function of concentration of Gdn·HCl at three temperatures: (○) −9.5, (△) −2, and (□) 12 °C. The lines are from the linear regression analysis.

Table III: Temperature Dependence of the Thermodynamic Parameters of Gdn·HCl Denaturation of I3C-C97/C54T at pH 5

T (°C)	C_m (M)	ΔG (cal/mol)	$RT\Delta\beta_{23}$ (cal/mol²)
−9.5[a]	2.68	9830	−3700
−2[a]	3.00	14000	−4700
5	3.07	13200	−4300
5	3.07	14200	−4600
5	3.13	12300	−4000
12[a]	3.20	14200	−4500
12	3.22	15300	−4800
12	3.09	14500	−4700
12	3.15	14700	−4600
15	3.14	14700	−4700
20	3.03	13200	−4400
25	2.93	12800	−4500
25	2.94	12900	−4400

[a] These three rows are from the kinetic measurements, and the rest are obtained from the isothermal Gdn·HCl titrations.

but there is no reason to discount either observation. Figure 1 and eq 11 provide a very accurate determination of $\Delta C_p'$ in the presence of 3 M guanidinium chloride. The measurements of the high-temperature transition in water are also reliable because they have been repeated numerous times. It may be that there is a variation of ΔC_p with temperature which makes extrapolation from one temperature region to another inadmissible; it may be simply a guanidine effect. There is not much data available on the effect of denaturing agents on $\Delta C_p'$ though it is known that the effect of guanidine on $\Delta H'$ can be very large (Pfeil & Privalov, 1976; Privalov, 1979). Pace and Tanford (1968) measured $\Delta C_p'$ as a function of urea concentration and found that there was no clear trend in the data.

Studies were also performed at constant temperature and with varying concentrations of guanidinium chloride. This permits an extrapolation to zero concentration to obtain approximate values for ΔG in aqueous solution and also provides information on the interaction of the denaturing agent with the protein. Within experimental error, ln K' is a linear function of the guanidinium molarity (Figure 3), and a linear extrapolation was used to obtain values for ΔG at zero concentration of guanidinium chloride. This is a long extrapolation, and in order to provide the reader with a general picture of the results and their accuracy, the results of all sets of experiments are recorded in Table III. The values for the intercept and slope were obtained by a weighted linear least-squares analysis. The data are also represented in Figure 4. C_m is the concentration of guanidinium chloride where the protein is half-unfolded, $K' = 1$.

The values obtained for the free energy in aqueous solution are accurate enough for a semiquantitative discussion of sta-

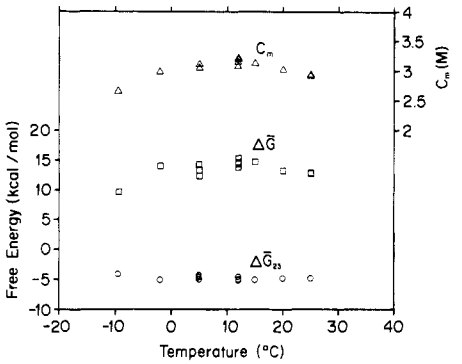

FIGURE 4: Temperature dependence of thermodynamic parameters of T4 lysozyme, I3C-C97/C54T from Gdn·HCl denaturation at pH 5. Curves are as follows: (△) C_m, which is the concentration of Gdn·HCl at the midpoint of the transition; (□) $\Delta\bar{G}$, which is the free energy change of unfolding extrapolated to zero molar concentration of Gdn·HCl; and (○) $\Delta\bar{G}_{23} = RT\Delta\beta_{23}$, which is the interaction term in eq 4.

bility but are not accurate enough for the analysis of the interaction parameter $\Delta\bar{G}_{23} = RT\Delta\beta_{23}$, which was one of the main purposes of this study. From eq 10, it can be seen that

$$\Delta\bar{G}_{23}C_m = RT\Delta\beta_{23}C_m = -\Delta\bar{G} \quad (13)$$

since at C_m, $\Delta\bar{G}' = 0$. This permits the calculation of $\Delta\bar{G}_{23}$ for each set of experiments. These values are given in Table III and in Figure 4. It was our purpose to analyze the temperature dependence of $\Delta\bar{G}_{23}$ to estimate the enthalpic and entropic contributions of the interaction of the protein with the guanidinium ion. Unfortunately, our data do not permit this type of interpretation. C_m, as can be seen from eq 13, depends both on the stability, $\Delta\bar{G}$, of the protein and on the change in the interaction parameter, $RT\Delta\beta_{23}$. These are both free energies and depend on temperature. It can be seen from Figure 4 that C_m has a maximum between 12 and 13 °C. $\Delta\bar{G}$ and $RT\Delta\beta_{23}$ are also plotted in Figure 4. Unfortunately, because of the long extrapolation, the data leave uncertain the variation of $\Delta\bar{G}_{23}$ and $RT\Delta\beta_{23}$ with temperature, which effectively rules out the sorting of $\Delta\bar{G}_{23}$ into its entropy and enthalpy contributions.

This problem can be resolved by independent measurements of the heat of transfer of the protein into guanidinium chloride solution. This would supply data on enthalpies of interaction, while the free energies can be estimated by studying the effect of guanidinium chloride on the stability of the protein. This method was not available to us during this investigation and would have made excessive demands on the supply of protein available. Others (Pfeil & Privalov, 1976; Lapanje, 1973; Lapanje & Wadso, 1971; Delben & Crescenzi, 1969; Atha & Ackers, 1971) have studied the interaction enthalpy and generally find that the enthalpy is large and negative. We could find few instances where both $\Delta\bar{H}_{23}$ and $\Delta\bar{G}_{23}$ have been evaluated under the same experimental conditions. We intend to obtain such data in future investigations.

Other aspects of these studies will be discussed in the following paper (Chen et al., 1989) after kinetic results have been presented.

Conclusions

We have shown that the addition of guanidinium chloride to solutions of the T4 lysozyme mutant I3C-C97/C54T induces a low-temperature melting. The conditions are such that by simply elevating the temperature from −9 °C, the protein can be caused to refold at about −3 °C, go through a region where the protein is predominantly folded, and then unfold again at about 28 °C. The reactions are reversible and conducive to quantitative study. The low-temperature unfolding has the expected thermodynamic anomalies. Both $\Delta\bar{H}'$ and $\Delta\bar{S}'$ are large and positive at the upper melting temperature of 28 °C; both are large and negative at the lower melting temperature. We assume, as do most workers in the field, that these results stem from the properties of the hydrophobic interaction which contributes more and more negative values to the enthalpy and entropy of unfolding as the temperature is lowered. Presumably at T_h (see Table II), the negative enthalpy of the hydrophobic interaction balances the positive energy of stabilization of the protein. Below this temperature, the hydrophobic enthalpy and entropy dominate, and all signs are changed.

We have interpreted these experiments by superposing the well-established constant $\Delta\bar{C}_p$ model (Privalov, 1979) with the linear model of solvent denaturation. Basically, this is decomposing a process involving changes in both temperature and guanidinium chloride concentration into processes at constant T and constant solvent composition. With this viewpoint, the effect of denaturing agents is to lower the whole stability curve until it crosses the $\Delta\bar{G} = 0$ axis in two places with a stable region in between. In addition to lowering the $\Delta\bar{G}$ curve, the denaturant may also move the position of maximum stability up or down in temperature and may change its shape, for example, if $\Delta\bar{C}_p'$ is greater or less than $\Delta\bar{C}_p$. Unfortunately, in our experiments, the interaction free energy was determined only by a rather long extrapolation, and information on the temperature behavior of the interaction of the protein with guanidinium chloride will have to await another investigation where $\Delta\bar{H}_{23}$ can be measured directly.

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Registry No. Gdn·HCl, 50-01-1; lysozyme, 9001-63-2.

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Low-Temperature Unfolding of a Mutant of Phage T4 Lysozyme. 2. Kinetic Investigations†

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ABSTRACT: A disulfide-bridged variant of bacteriophage T4 lysozyme has been found to undergo a low- as well as high-temperature unfolding transition in guanidinium chloride [see Chen and Schellman (1989)]. The kinetics for this process have been followed for several temperatures, a range of guanidinium chloride concentrations, and a number of values of pH. Microscopic rate constants for protein unfolding and refolding were extracted from these data to explore the nature of the cold unfolding transition. The data were interpreted using transition-state theory. It was found that the Arrhenius energy is temperature dependent. The transition state is characterized by (1) a high energy and low entropy compared to the native state, (2) a heat capacity which is closer to the native state than to the unfolded state, and (3) a low exposure to solvent compared to the unfolded state, as judged by its interaction with guanidinium chloride. With increasing concentration of guanidinium chloride, the low-temperature unfolding rate increases strongly, and the refolding rate decreases very strongly.

The background of this research was described in the previous paper (Chen & Schellman, 1989), hereafter referred to as paper 1, which discusses the equilibrium study of the reversible, low-temperature unfolding of the disulfide-bridged T4 lysozyme mutant I3C-C97/C54T. Moderately concentrated guanidinium chloride is required to bring the low-temperature transition into a temperature region where it can be observed. The rates of unfolding and refolding are very slow as temperatures of 0 °C and below are approached, and this suggested the possibility of an extensive kinetic investigation. The reaction rates are sufficiently slow so that the kinetic studies can be performed with a conventional circular dichrometer with no need for fast techniques.

Such a study has a number of attractive possibilities. With slow reaction rates, it is conceivable that one could see pro-

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