The Endothermic Effects during Denaturation of Lysozyme by Temperature Modulated Calorimetry and an Intermediate Reaction Equilibrium

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To gain insight into the thermodynamics of protein denaturation, the complex heat capacity, C_p^* ($= C_p' - iC_p''$) of lysozyme−water system has been measured at pH 2.5 in the 293−368 K range by using temperature-modulated scanning calorimetry (TMSC), a technique in which the thermally reversible enthalpy changes are measured separately and simultaneously with the thermally irreversible enthalpy changes. The plot of C_p' against the temperature T shows a broad peak, which is similar to that observed in $C_{p,DSC}$, measured here and elsewhere by differential scanning calorimetry (DSC), a technique which gives the sum of both the reversible and irreversible contributions in the apparent heat capacity value. This peak in $C_{p,DSC}$ has been generally attributed to endothermic heat absorption on reversible and irreversible unfolding processes and irreversible thermal denaturation. It is shown that the observed C_p' peak results from heat absorption when the equilibrium constant between the native lysozyme state and a conformationally different intermediate state increases with T. The plot of C_p' versus T is subdivided into four regions, corresponding to the dominance of a certain process. Thermal denaturation of lysozyme was found to occur according to a scheme, native state ↔ unfolded (intermediate) state → denatured state. This conclusion is consistent with the general view that the first step of denaturation of small one-domain globular protein like lysozyme is a reversible conformational (unfolding) transition, and the second step is irreversible denaturation. It is shown that when kept isothermally at $T >$ 341 K, i.e., within the transition temperature range, C_p' of lysozyme decreases. This decrease is exponential in time and corresponds to a rate constant, which varies according to the Arrhenius-type equation, with a preexponential factor of 5×10^{20} s^{-1} and energy of 167 kJ/mol. The overall kinetics of the denaturation reaction is of the first order.

1. Introduction

In a variety of thermodynamic studies of protein denaturation reported since 1970, the net heat of denaturation has been measured by using the differential scanning calorimetry (DSC) techniques.[1−4] In general, it has been found that the heat capacity C_p of a protein first increases with temperature T, reaches a local maximum, and then decreases. The plot of C_p against T thus exhibits a relatively broad endothermic peak, whose area has been interpreted as being equal to the total enthalpy of transformation of the protein. Since the denaturation of proteins is an irreversible process, it follows that the equilibrium thermodynamic equations cannot be used for interpreting the DSC measurements. Despite that, equilibrium thermodynamic approach has been widely used in studying the temperature-induced changes that lead to denaturation of proteins.[1−6] It has also been suggested that part of the processes involved such as isomerization and clustering and unclustering in the denaturation process may be reversible.[1] This leads to concern as to whether the denaturation of proteins can be seen as entirely a thermodynamic transition that occurs gradually over a broad temperature range or whether it consists of a series of transformations some of which are reversible, but the consecutive nature of the transformation leads to irreversibility.

This study was initiated for the purpose of examining the nature of processes that lead to an endothermic peak in the denaturation of proteins. In this paper, we describe a thermodynamic study of denaturation of lysozyme, a one-domain globular protein. In the earlier DSC studies[7] of its dilute aqueous solutions only one endothermic peak was observed. This was attributed to its denaturation process which occurs in a single stage.[7] Since the DSC measurements provide information on both the reversible and irreversible heat effects of a time-, and temperature-dependent change, any reversible component of the denaturation process cannot be determined from this technique. To determine whether the denaturation process involves partial reactions that may be reversible with temperature, we use a new technique of calorimetry. In this technique, which is known as temperature modulated scanning calorimetry (TMSC), the temperature of the sample is sinusoidally modulated. The technique leads to determination of only the heat effects that are thermally reversible, such as a chemical equilibrium between the reactants and products. For lysozyme, the use of TMSC studies is expected to show whether certain reactions in its denaturation are reversible with temperature change, i.e., whether there is a chemical equilibrium in the intermediate states formed in its denaturation process.

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We use this TMSC technique here along with the conventional DSC technique, and examine the nature of the transitions that occur in the course of thermal denaturation of lysozyme in an aqueous solution. The results show that there is a chemical equilibrium between the native state of lysozyme and an intermediate state, and that the equilibrium constant increases with T. The intermediate state irreversibly transforms to the denatured state. This finding verifies Privalov's general suggestion,[1] but differs in detail in that there is a thermodynamic equilibrium between the native and the unfolded state of a protein.

2. Materials and Methods

Lysozyme from hen's egg white was purchased from Fluka Chemicals (catalogue no. 62970, lot 48622/1, filing code 34199). It was dissolved in 20 mM glycine−HCl buffer of pH 2.5 and dialyzed for 14 h against the buffer of the same composition. The protein solutions of desired concentration in amounts of 3−12% (w/w) were prepared by an appropriate dilution of a stock protein solution with the same buffer. Concentration of lysozyme in solutions was determined spectrophotometrically by using the known value of 26.4 for $E^{280}{}_{1\%}$, the light extinction coefficient at 280 nm, for 1% protein solution and pH 2.5.

The equipment used for these experiments is a multimode calorimeter named modulated adiabatic scanning calorimeter[8] (acronym MASC), which can be used for different operation modes and sets of thermal conditions. It had been designed for accurate measurements on specialized materials undergoing chemical and physical changes. Its operation for measurements in the mode of temperature-modulated scanning calorimetry (TMSC) has been described in an earlier paper, where it has been used for a study when the sample was heated at a fixed rate, cooled at a fixed rate or kept in a quasi-isothermal mode.[8] The data thus obtained from the calorimeter provide both the apparent heat capacity and the complex heat capacity C_p*.

Three sets of measurements were performed on the lysozyme solution. In one set the sample was heated at 12 K/h from 293 to 368 K, and the temperature was modulated during heating at a frequency of 3.3 mHz (modulation period = 300 s) with a modulation amplitude of 0.5 K. The sample was thereafter cooled at 12 K/h to 293 K with the same condition of temperature modulation. During both the heating and subsequent cooling of the sample, C_p* of the sample was measured at 1 K intervals: i.e., one data point was obtained for each modulation period, as described earlier.[9] From these measured data, the apparent value of heat capacity $C_{p,DSC}$, which is the quantity measured in the usual DSC measurements, was determined for the same heating and cooling rates. The method of analysis of the data obtained from the temperature modulation mode has been described earlier.[9]

In the second set of experiments, the sample was heated at 12 K/h from 293 K to a preselected mean temperature, while still being subjected to the above given temperature modulation, and kept at that temperature. Its C_p* was measured during the heating part of the experiment. It was also measured after the sample had reached at the pre-selected temperature, but now as a function of time while the sample's temperature was modulated in the above-given manner.

In a third set of experiments, 12% lysozyme solution was heated from 293 to 356 K in ∼3 min. It was then kept at 356 K for 1 h, and ultimately cooled to 293 K at 12 K/h. During the heating part, isothermal part and the cooling part of the cycle, C_p* measurements were performed with the calorimeter in the temperature modulation mode as described above.

3. Data Analysis and Significance of the TMSC Procedure

Most thermodynamic studies on proteins have been performed by using differential scanning calorimetry (DSC), a technique familiar to workers in this area. Since the relatively new technique for thermodynamic measurements by modulated scanning calorimetry (TMSC) may be unfamiliar to many workers in this area, it is appropriate to briefly recall: (i) how the information obtained from DSC and TMSC differs, (ii) how the various contributions to the heat capacity are determined from TMSC, and (iii) how the difference between the data obtained from TMSC and DSC techniques are used to deduce information on the irreversible thermodynamic changes and reversible ones. For further details, the reader may consult refs 9−12.

In the temperature modulated scanning calorimetry, i.e., in TMSC, the rate of that part of the heat stored or released which reverses with reversal of the temperature is measured. The components of the complex heat capacity oscillating in-phase C_p' and out-of-phase C_p'' with the temperature during the modulation cycle are calculated. The two components are related to the modulation amplitude, and the modulation period, $\tau = 2\pi/\omega$, where ω is the angular frequency of temperature modulation (for details see ref 9).

In DSC, the rate of heat flow is measured. From that rate, the heat capacity $C_{p,DSC}$ is calculated from the equation,

$$C_{p,DSC} = \left(\frac{1}{\beta}\right)\left(\frac{dH(T,t,x_i)}{dt}\right) \tag{1}$$

where $(dH(T,t,x_i)/dt)$ is the measured rate of enthalpy change and β is the temperature scanning (heating or cooling) rate. Equation 1 can be written in a more general form by taking into account the two time-dependent quantities: (i) the temperature T and (ii) the mole fraction of the material x_i undergoing a chemical change. Therefore, for C_p measured in a DSC experiment,

$$C_{p,DSC} = \left[C_{p,thermo} + \left(\frac{\partial H}{\partial x_i(T)}\right)\left(\frac{dx_i}{dT}\right)\right] + \left(\frac{1}{\beta}\right)\left[\frac{\partial H}{\partial t} + \left(\frac{\partial H}{\partial x_i(t)}\right)\left(\frac{dx_i}{dt}\right)\right] \tag{2}$$

where $C_{p,thermo} = \partial H/\partial T$ is the thermodynamic or true heat capacity. The term $[(\partial H/\partial x_i)(dx_i/dT)]$ in eq 2 for this case refers to a thermally reversible process that may be fast enough that equilibrium is maintained at every instant of the temperature modulation period and consequently the original state of the system would be restored after one modulation cycle.

Comparison of the data obtained from the DSC and TMSC techniques is more simple when $C_p'' = 0$, as is the case in these experiments on lysozyme solutions. When $C_p'' = 0$, the magnitude of C_p' is given by the first term in the square brackets in the right-hand side of eq 2. Hence,

$$C_{p,DSC} = C_p' + \left(\frac{1}{\beta}\right)\left[\frac{\partial H}{\partial t} + \left(\frac{\partial H}{\partial x_i(t)}\right)\left(\frac{dx_i}{dt}\right)\right] \tag{3}$$

According to eq 2, we identify three conditions for the occurrence of a chemical and/or physical process in terms of the enthalpy change as follows:

(i) When $(\partial H/\partial x_i) = 0$, or when $(dx_i/dT) = 0$, and the quantities $(dx_i/dt) = 0$ and $(\partial H/\partial t) = 0$, i.e., there is neither a chemical nor a physical process for producing a temperature- and time-dependent change in the enthalpy. In this case, the

measured value of C_p from a DSC experiment is given by

$$C_{p,DSC} = C_{p,thermo} = C_p' \quad (4)$$

(ii) When $(\partial H/\partial x_i) \neq 0$, $(dx_i/dT) \neq 0$, but $(\partial H/\partial t) = 0$ and $(dx_i/dt) = 0$, i.e., there is a fast reversible (chemical or physical) process that restores the original state of the sample at any time within the time period of the temperature modulation cycle, and there is no irreversible (physical or chemical) process. Therefore, the last term in the square brackets in eq 2 is zero. In this case, the measured value is given by

$$C_{p,DSC} = \left[C_{p,thermo} + \left(\frac{\partial H}{\partial x_i(T)}\right)\left(\frac{dx_i}{dT}\right)\right] = C_p' \quad (5)$$

(iii) When $(\partial H/\partial x_i) \neq 0$, $(dx_i/dT) \neq 0$, $(\partial H/\partial t) \neq 0$ and $(dx_i/dt) \neq 0$, i.e., there are slow and irreversible (chemical or physical) processes that occur at that T, i.e., the rate of these processes is slow such that the original state is not restored during the modulation cycle, and there is also a time-dependent enthalpy arising from an irreversible (chemical or physical) process. In this case,

$$C_{p,DSC} \neq C_p' \quad (6)$$

The results reported here will be discussed in the light of the these considerations, in particular by comparing $C_{p,DSC}$ and C_p', the two quantities simultaneously determined by the TMSC technique[8] for the dilute solutions of lysozyme.

From the data obtained from the temperature modulated calorimetry, the real (in-phase component) C_p' and the imaginary (out-of-phase) component C_p'' of the complex heat capacity C_p^* were calculated from the equation, $C_p^* = C_p' - iC_p''$. The data measured for the solution were used to determine the value of C_p^* for pure lysozyme from the equation,

$$C_p^*(\text{lysozyme}) = [C_p^*(\text{solution}) - w(\text{buffer})C_p^*(\text{buffer})]/w(\text{lysozyme}) \quad (7)$$

where w refers to the mass of the material written in parentheses. It is assumed that C_p^* of the buffer in the solution is equal to C_p^* of the buffer alone. The same assumption was made for determining the $C_{p,DSC}$ values. Therefore all calculated values here are provided in J K^{-1} g(lysozyme)$^{-1}$.

The errors in our measured C_p' values for a sample are within 0.15%. The errors resulting from the calculations of C_p' of lysozyme from its solution after taking into account the solvent contribution are estimated to be within 1.4% for the 12% solution, 4% for the 6% solution and 6.5% for the 3% solution.

4. Results

(i) General Features of the Denaturation Transformation. The quantity C_p' of 12% lysozyme solution was measured by TMSC technique during heating from 293 to 368 K at 12 K/h and its value is plotted against the temperature T as curve 1 in Figure 1A. Two sets of measurements, made independently on the same stock samples, are shown by open circles and crosses, and they agree within the experimental uncertainty. The sample appears have become completely denaturated on heating at 12 K/h to 368 K. Measurements of C_p' were then performed subsequently on cooling the denaturated sample to 293 K, and the data are plotted in curve 2 in Figure 1A. The C_p'' plot corresponding to the open circle curve in Figure 1A is presented in Figure 1B. It shows no significant features and its value is practically zero over the entire temperature range. The same behavior was observed for other curves not shown here.

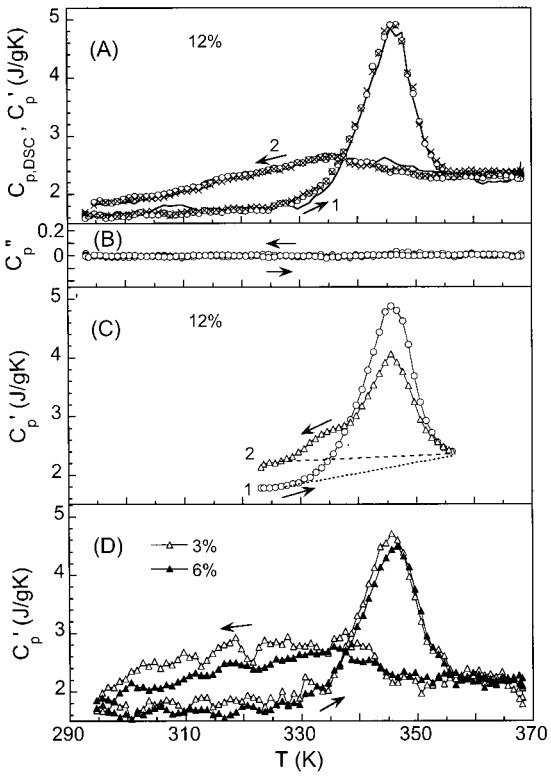

Figure 1. The measured C_p' *and* C_p'' of lysozyme solutions in buffer of pH 2.5, as measured by temperature modulated scanning calorimetry are plotted against the temperature. Arrows indicate the temperature scanning direction (1-heating scan or 2-cooling scan) of measurements. (A) Data for C_p' of lysozyme obtained from two different 12% solutions, one is shown by crosses and another by circles. Data for $C_{p,DSC}$ are shown by continuous line. (B) Data for C_p'' from the 12% solution. (C). Data for C_p' from 12% solution heated only to 356 K (circles) and then immediately cooled (triangles). (D) Data for C_p' of lysozyme from 3% and 6% solutions as denoted. The temperature of the peak and the area under it is not affected by the concentration of lysozyme solution.

In molecular relaxation terms, the almost zero value of C_p'' seen in Figure 1B indicates that relaxational effects are absent in those processes which cause the heat capacity of lysozyme to change with temperature at the modulation frequency of 3.3 mHz at the heating and cooling rates of 12 K/h. Therefore, the measured C_p' values correspond to the limiting low-frequency value of C_p^*. Since the data are obtained by TMSC technique, the change in the C_p' values are only due to processes in which the enthalpy changes occur reversibly on changing the temperature. With C_p'' being equal to zero, the C_p' value measured at different temperatures here is equal to the equilibrium or relaxed heat capacity at that temperature. (An analogous case is the equilibrium dielectric permittivity, which is equal to the in-phase or real component of the permittivity when the out-of-phase or imaginary component of the permittivity is zero at low frequencies.)

The continuous lines in Figure 1A show the plots of the $C_{p,DSC}$ data, as obtained here. They also agree with the C_p' values within the uncertainty of the data. The temperature of the peak at 346.2 K observed in these plots has an uncertainty of $\pm$ 1 K. These results clearly show that conditions (ii) and eq 5 are fulfilled by the process occurring in lysozyme.

To confirm the reversibility of the molecular process that leads to the C_p peak in the TMSC data, several lysozyme solutions were heated at 12 K/h to a temperature of lesser than 360 K, and their C_p' measured during heating. These were subsequently cooled, and C_p' was measured again during

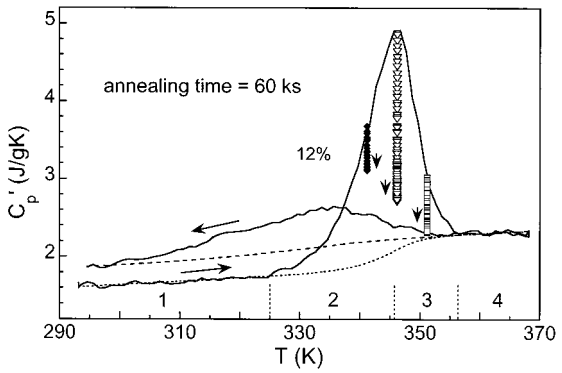

Figure 2. C_p' of lysozyme from 12% solution measured during the heating to different temperatures and then during isothermally keeping or annealing for a period of 60 ks. The vertically placed points present C_p' values measured at a fixed temperature.

cooling. In one such experiment, C_p' of 12% lysozyme solution was measured during its heating from 323 to 356 K, and then during its cooling to 323 K. The C_p' values are plotted against T in Figure 1C. In the 351 K−356 K temperature range, C_p' values measured during heating in curve 1 agree with those measured during cooling in curve 2. But, at temperatures below 351 K, the C_p' values measured on cooling in curve 2 are lower than those measured on heating (curve 1) the lysozyme solution. Also the height of the C_p' peak in curve 2 is lower than in curve 1, and an additional shoulder to the peak appears on its low-temperature side.

A further set of C_p* measurements was performed on several aqueous solutions, but with lesser concentration of lysozyme. The measured C_p' values of two such solutions, one containing 3% and the other 6%, were converted to J [g(lysozyme) K]$^{-1}$ from eq 7, and these values are plotted against T in Figure 1D. It is evident that both the temperatures of the peaks as well as the magnitude of C_p' in J [g(lysozyme) K]$^{-1}$ remains the same as those determined for the 12% lysozyme solution, within the experiment's uncertainty. We conclude that the temperature of the C_p' peak and their values do not depend on the lysozyme concentration in the solution.

(ii) Time Dependence of the Denaturation Transformation. To investigate the nature of the transformation that produces the C_p' peak at 346 K, a new set of experiments were performed by TMSC technique in which the effect of time on the transformation was investigated by keeping the samples at different temperatures. These were the peak temperature of 346.2 K, and two other temperatures: one above the peak temperature and the other below. In this set, three new samples of 12% lysozyme solution were heated from 293 K to (i) 341.2 K, (ii) 346.2 K, and (iii) 351.2 K at 12 K/h. Each of these samples was thereafter kept at its respective temperature of 341.2, 346.2, and 351.2 K, and C_p' of the sample was measured by TMSC as a function of time at intervals of 300 s, the temperature modulation period. The measured C_p' value was found to decrease with time. This decrease is shown by the data points, which were plotted along the vertical lines indicated by arrows in Figure 2. Here the plots of C_p' against T (full line) obtained for both the heating and cooling at 12 k/h rate are also provided.

To describe the decrease in C_p' more clearly, its value measured at each of the three temperatures, 341.2, 346.2, and 351.2 K, is plotted against time in Figure 3A. The shape of the curves in Figure 3A was found to fit the exponential equation,

$$C_p'(t) = C_p'(\infty) + [C_p'(0) - C_p'(\infty)]\exp(-kt) \qquad (8)$$

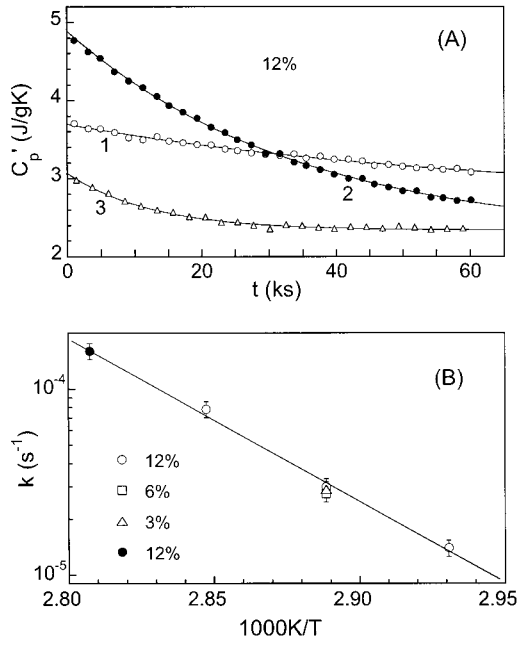

Figure 3. (A) The plots of C_p' against time during its spontaneous decrease at different temperatures: 1, 341.2 K; 2, 346.2 K; 3, 351.2 K. (B) The plot of k against $1/T$. Open circles refer to 12% lysozyme solutions shown in panel A. Open square and triangle refer to solutions at 6% and 3% concentrations annealed at 346.2 K. Full circle refers to solution at 12% concentration annealed 1 h at 356 K.

where $C_p'(t)$, $C_p'(\infty)$, and $C_p'(0)$, are, respectively, the values of C_p' at time t, at infinite time, and at zero time, and k is the reaction rate coefficient. The $C_p'(0)$ value was measured during the first modulation period, immediately after the temperature of the sample reached the pre-selected temperature value. The values of k, as obtained from fitting eq 8 to the data in Figure 3A, are 1.4×10^{-5} s^{-1} at 341.2 K, 3.0×10^{-5} s^{-1} at 346.2 K, and 7.8×10^{-5} s^{-1} at 351.2 K.

(iii) Concentration Effects on Denaturation Transformation. To determine the effects of concentration on the reaction rate coeffiecient k, isothermal measurements on the 3% and 6% lysozyme solutions were also performed at 346.2 K, and the k values were determined. These values were found to be the same as that for 12% solution within the experimental uncertainty. Therefore, the rate constant of the irreversible process is not affected by the concentration of lysozyme in the explored range. This also confirms that the overall kinetics of the processes occurring with time is of the first order in reaction rates.

To show the changes in the enthalpy H and entropy S, the integrals of $C_p'(T)\mathrm{d}T$, and of $C_p'(T)\mathrm{d}(\ln T)$, respectively, were obtained from $T_1 = 293.2$ K to a temperature T, and the values are plotted in Figure 4A,B. For both heating and cooling part of the experiment, the triangles are for the data referring to 12% lysozyme solution shown in Figure 1A. A second set of integrated values was obtained for the curves in Figure 1C, which are shown by the circles. For these, $T_1 = 323$ K and the highest value of T is 356 K. The plots clearly show that the rise in H and S occurs in a sigmoid shape manner as denaturation occurs. Also the plot is spread out over a broad temperature range. (It should be noted that the net enthalpy change on denaturation differs from the values indicated by the plots in Figure 4A because in calculating the net enthalpy change from Figure 2, one should subtract a weighted $C_p'(T)$ of the partially unfolded states, i.e., the dotted curve in Figure 2 from the measured data, and this was not done here.)

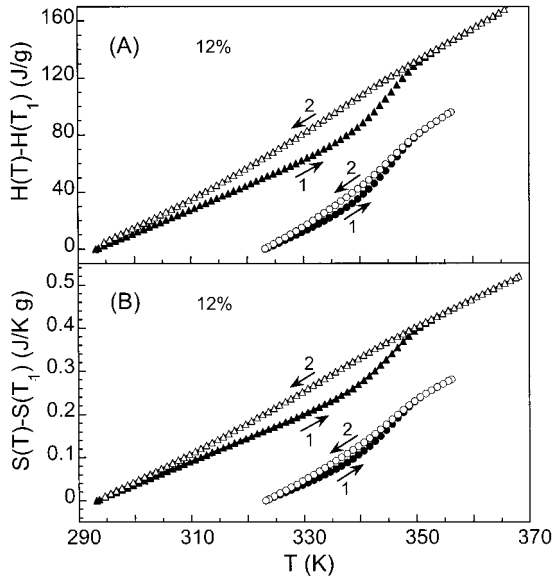

Figure 4. (A) The enthalpy difference is plotted against the temperature for 12% lysozyme solution. Solid triangles are the values obtained during heating and the hollow triangles obtained during cooling from the data in Figure 1A, and the difference is between 293.2 K and the relevant temperature. The solid circles are the values obtained from the heating curve 1 in Figure 1C, and hollow circles are those obtained from the cooling curve 2 in Figure 1C. For these curves, the difference is between 323 K and the relevant temperature. (B) Plots of the corresponding entropy difference.

5. Discussion

(i) The Thermodynamic Nature of the Process. According to the above-given discussion, when a (chemical or physical) process is reversible, its characteristic time is much less than the modulation period, and there are also no irreversible (chemical or physical) processes, $C_{p,DSC}$ is identical to C_p'. In cases when the (chemical or physical) process is reversible, but the characteristic time for the process is comparable or longer than the modulation period, $C_{p,DSC}$ is not equal to C_p'. Moreover when there is an irreversible process occurring, C_p' does not contain the effects of thermally irreversible enthalpy changes and again $C_{p,DSC}$ is not equal to C_p'. On that basis we discuss the results obtained here in terms of the processes involved in the unfolding of lysozyme that finally leads to its denaturation.

First we consider the mechanism behind the broad $C_{p,DSC}$ endothermic peak as seen in Figure 1A,C,D and in Figure 2. To recall the criterion, an irreversible transition is not observed in the TMSC measurements, but is observed in the DSC measurements. Therefore, if the C_p' endothermic peak at 346.2 K in Figure 1A,C,D and in Figure 2 was to be seen as an indication that an irreversible denaturation of lysozyme is occurring, as concluded in the earlier studies,[1−6] our TMSC experiments would not show the same broad C_p' endothermic peak. Instead, one would expect to observe values of C_p' which would correspond to equilibrium heat capacity of the mixture, that is, $C'_{p,mixture} = x_{native}C'_{p,native} + x_{denat}C'_{p,denat}$, where x_{native} is the weight fraction and $C'_{p,native}$ the equilibrium heat capacity of the native lysozyme, and x_{denat} and $C'_{p,denat}$ are the corresponding quantities of the denatured lysozyme. Accordingly, the change in C_p' expected from this transformation can be calculated. This calculated value is plotted against the temperature as the lowermost dashed line in Figure 2. This plot is quite different from the experimentally determined curve, thereby further substantiating that the observed peak is not due to an irreversible transformation.

However, there is also an unexpected behavior noted here. That is that the C_p' plot showing a broad endothermic peak in Figure 1A,C,D is qualitatively similar to the $C_{p,DSC}$ curve, which is shown in Figure 1A by a continuous line. It is also qualitatively similar to the already known shapes of the $C_{p,DSC}$ curves for lysozyme, α-chymotrypsin, ribonuclease-*A*, cytochrome-*C*, and myoglobin.[1] Moreover, integration of the observed C_p' peak yields 30.8 ± 0.5 J [g(lysozyme)]$^{-1}$ for the total absorbed heat, which agrees well with the value reported for lysozyme in the literature: 30.4 J [g(lysozyme)]$^{-1}$ estimated by Schwarz[13] or 33.6 J [g(lysozyme)]$^{-1}$measured by Privalov, et al.[14]

Since the only other condition that can produce identical C_p' and $C_{p,DSC}$ scans is condition (ii) in section 3, namely, that there be a fast reversible process that restores the original condition in a time period shorter than the temperature modulation period, the similarity of the C_p' and $C_{p,DSC}$ curves indicates that the chemical and physical changes observed on heating of the lysozyme−water solutions correspond to a reversible process with a characteristic time much shorter than the modulation period, and not entirely to an irreversible process of thermal denaturation.

(ii) The Unfolding-Intermediate Process during Thermal Denaturation. By using the C_p' curves labeled 1 in Figure 1A,C, we reinterpret thermal denaturation in terms of two processes: the first is thermally reversible and contributes to C_p' during both the heating and the cooling parts of the cycle, and the second is thermally irreversible and does not contribute to C_p'. The similarity between the shapes of the C_p' curves measured on heating to 356 K and then also measured on cooling, as seen in Figure 1C, supports the interpretation that there is a reversible equilibrium between the native state N and a conformationally distinguishable intermediate state I. It further shows that the N → I transformation is endothermic, with a characteristic time for reaching equilibrium much less than 300 s, which is the period of temperature-modulation. Therefore, as *T* is increased, the equilibrium constant K_A (= [I]/[N]) increases according to the vant-Hoff equation and the population of state I increases as the population of state N decreases with the absorption of heat. Concurrently, state I irreversibly converts to the more denatured state mD with a rate constant *k*. Thus the native state does not directly denature. Rather, it undergoes a rapid conformational change prior to postdenaturation. The complete process of denaturation may be written as

$$\text{native (N)} \leftrightarrow \text{intermediate state (I)} \rightarrow \text{irreversibly-denatured (mD)}$$

where the state I contains all states of an unfolded lysozyme. At low temperatures, both the weight fraction of state I and the value of *k* are vanishingly small, and therefore the denaturation occurs slowly or is absent. Hence the fractional amount of mD is negligible at relatively low temperatures. As lysozyme solution is heated, the amount of state I increases and concurrently *k* increases. The fraction of mD formed remains negligible during the early part of heating, when *k* is still small. In this case, C_p' is the sum of the $C_{p,thermo}$ of state N, and state I, and the measured value contains contributions from any thermal effects owing to the reversible and rapid N → I transformation. Since the heat effects of an irreversible process (of postdenatured transformation) are not observed in our TMSC study, the net C_p' is given by

$$C_p' = x_N C_{p,N} + x_I C_{p,I} + \Delta H_{N\rightarrow I}\left(\frac{dx_N}{dT}\right) \qquad (9)$$

where x_N and x_I are the weight fractions of state N, and state I, respectively, $C_{p,N}$ and $C_{p,I}$ their heat capacities, and $\Delta H_{N\rightarrow I}$ is the enthalpy of transformation of state N to state I.

In this interpretation, four regions of C_p' change with temperature can be identified, as indicated in Figure 2.

1. At T below 325 K, the initial slow increase in C_p' is due to the increase in the vibrational and configurational (and conformational) degrees of freedom of state N of lysozyme, as given by eq 4.

2. From 325 to 346 K, the relatively *rapid* increase of C_p' indicates a predominant conversion of state N to state I, and a relatively large contribution from the term in brackets in the right-hand side of eq 9, for the reason that K_A increases with T and thus the amount of state I at equilibrium increases.

3. From 346 to 356 K, the rapid decrease in C_p' is a result of a relatively *slow* increase in the amount of the intermediate state, a relatively rapid increase in postdenaturation which decreases C_p' and a partial change in $C_{p,thermo}$, according to the addition rule.

4. From 356 K to higher temperatures, a slight increase in C_p' caused by the increase in the vibrational and configurational (or conformational) contributions of the denatured lysozyme, and any further denaturation.

In none of these four temperature ranges does one observe the consequences of an irreversible change in the enthalpy as given by the second term in the square brackets on right-hand side of eq 2.

(iii) The Partial Denaturation and Change in C_p' on Cooling. We now discuss another aspect of the study shown in Figure 1C. In this study the 12% lysozyme solution was heated to 356 K, where the process N → I tended to reach completion and to attain the equilibrium amounts of state N and state I. The peak area under curve 2 in Figure 1C, which was obtained during the cooling from 356 K, is ~30% less than the peak area obtained from the curve measured during the heating (curve 1) to 356 K. This means that on heating to 356 K and cooling subsequently, ~30% of lysozyme had denatured irreversibly. Moreover, at 323 K in Figure 1C, C_p' in curve 2 remains higher than C_p' in curve 1, but still it remains below the C_p' of curve 2 in Figure 1A. This indicates that the sample of curve 2 in Figure 1C contains a certain amount of lysozyme irreversibly denatured (mD) at 323 K. Since C_p' of a sample now containing ~30% of denatured lysozyme and ~70% of state I is about the same as C_p' of the sample containing only the denatured state at 356 K, as seen by comparing its C_p' curve with C_p' of curve 2 in Figure 1A, this means that C_p' of state I is approximately the same as that of the denatured state, although their enthalpies may be very different.

(iv) The Isothermal Decrease of C_p'. For the quasi-isothermal condition of TMSC, the data obtained from which are provided in Figure 3A, K_A of the complete process, N ↔ I → mD remains constant. Therefore, the conversion of state N to state I would occur in such a manner that the ratio [I]/[N] remains constant. At high temperatures, the fraction of state I is large, approaching unity, and that of state N is small. As denaturation begins isothermally, the fraction of state I decreases and, to maintain K_A constant, the fraction of state N also decreases. As stated earlier here, thermal effects of denaturation as irreversible processes are not observable in TMSC, but other consequences of denaturation are observable. These are (i) a decrease in the contribution from the term $\Delta H_{N\rightarrow I}(dx_N/dT)$ of eq 9 with time, as the amounts of both state N and state I decrease with time, and (ii) a change in $C_{p,thermo}$ of the mixture according to the addition rule, as state I denatures and more state N transforms to state I. Since the equilibrium between state N and state I is established extremely rapidly, the rate at which C_p' decreases with time as indicated in Figure 2 would be controlled by the rate of denaturation, which is the slower process.

Thus, we conclude that the decrease in C_p' with time described in Figure 3A is mainly due to irreversible denaturation and its rate constant at a given temperature is equal to the rate constant of this process. This is in contrast with the earlier attribution of the denaturation rate obtained from the DSC measurements, where the entire calorimetric plot was used to analyze the process of denaturation.[1−4]

The effective rate constant, k, of denaturation determined in section 3 here is given by

$$k = \left(\frac{k_1 k_3}{k_1 + k_2}\right) = \left(\frac{k_3}{1 + K_A}\right) \tag{10}$$

where k_1, k_2, and k_3 are the rate constants of N → I, I → N, and N → mD reactions, respectively, and $K_A = k_1/k_2$. In Figure 3B are shown the rate constant k values already determined for 12% concentration at three temperatures together with those for three concentrations at one temperature.

We also determined k by a fourth experiment. For that purpose, a new sample of 12% lysozyme solution was heated from 298 to 356 K in ~3 min. It was kept at 356 K for 1 h, and ultimately cooled to 298 K at 12 K/h, while performing C_p' measurements by TMSC using the same temperature modulation parameters. The results showed that the area under the broad C_p' peak was ~ 56% of the area under curve 1 of Figure 1A. According to this analysis, 44% of lysozyme had irreversibly denatured on keeping for 1 h at 356 K. The rate constant at this temperature was calculated by assuming an exponential decrease with time according to eq 8, and its value was found to be 1.6×10^{-4} s^{-1}. Since k may be determined by either using the plots of the C_p' data against time, as was done here earlier, or else from the $C_p' - T$ integral data plotted against time, this value of 1.6×10^{-4} s^{-1} at 356 K may be combined with the values determined from direct measurements. All these values are plotted against $1/T$ in Figure 3B. The plot is a straight line and fits the equation $k = 5 \times 10^{20} \exp(-167000/RT)$. Evidently the overall kinetics of the irreversible denaturation process may be described by the Arrhenius equation with activation energy of 167 kJ/mol.

(v) The Enthalpy and Entropy Changes and the Reversibility of the Folding−Unfolding Process. We now consider the implications of the gradual increase in C_p' on heating from 300 K, as seen in Figures 1A and 2. Since C_p' of lysozyme does not depend on the lysozyme concentration in the solution, the increase may not be attributable to the water component and should therefore be related to the configurational entropy S_{conf}, i.e., the increase in the number of configurations accessible at a given temperature. S_{conf} increases because, as the stabilizing bonds of the folded-structure decrease on unfolding, steric hindrance for conformational changes decreases, and further because the number of configurations accessible to a given unfolded structure increases as T increases. But when all stabilizing bonds of the folded state vanish and the molecular length achieved on unfolding contributes to S_{conf}, any further contributions from the increase in unfolding vanishes and an increase in S_{conf} remains only due to an increase in T. A corresponding change is expected in the enthalpy H of the lysozyme solution. Therefore, the increase in C_p' on heating from 300 K is attributable mostly to the increase in S_{conf} resulting

from lysozyme's unfolding. Since a state of higher S_{conf} also has higher vibrational entropy, it is probable that the vibrational entropy also increases on lysozyme unfolding.

The changes observed in Figure 4A,B are a reflection of the complexity of the process by which denaturation of lysozyme occurs. Each transformation into a partially unfolded state opens the possibility of a variety of further transformations depending upon the nature of the protein and the conditions used. Also the reversibility of the folded and unfolded state may not lead to the exact original state in one step and in the same original time interval, thereby leading to steps of reaction in which the reaction to the native state is reversed. If that reversal requires a lower energy, then the step may occur at a lower temperature on cooling. This may be the reason for the broad shoulder observed below the peak's temperature in the cooling curve shown in Figure 1C. The unfolding may also be an equilibrium process with a set of other unfolded states dissimilar in their intermolecular bonding and that only through this equilibrium may the ultimate denaturing occurs. Nevertheless, if the equilibrium among a variety of such processes occurs in a time much shorter than the observation time of TMSC, only the ultimate changes resulting from the equilibrium would be observed. In this case, all intermediate processes between the native and the denatured states may be grouped under one intermediate state *I*, as done here. These intermediate processes may involve diffusion that requires overcoming of the potential energy barriers that are themselves time dependent, i.e., that the time required for the diffusion of a protein segment depends on the arrangements of the remainder of the molecule in the vicinity of that segment. As that arrangement changes with time, the height of the barrier changes and thus the diffusion time changes. This issue has been inferred from a calorimetric study of an apparently thermodynamically equilibrated state of beef proteins[15] and gliadin,[16] and was expressed in a potential energy diagram in a molecular group's configurational space, as represented by its coordinates. The details of such a process for lysozyme may be studied by varying the time scale of the experiments, i.e., by using either different heating rates and/or different frequencies of the temperature modulation.

Also the broad peak observed at ~335 K on cooling an apparently fully denatured state, as indicated by the data in Figure 1A, needs further study for understanding the nature of the process behind this peak. A calorimeter with a higher sensitivity has been built and new experiments on other proteins are in progress.

6. Conclusions

The comparison of DSC and the temperature modulated scanning calorimetry data, obtained with studies designed to investigate thermal denaturation of protein in aqueous solutions, shows that for lysozyme this process involves an intermediate reaction equilibrium. Thermal denaturation of lysozyme follows the scheme: native state ↔ intermediate (unfolded and possibly reversible) state → denatured (irreversibly) state. The $C_{p,DSC}$ peak alone does not give information on the transformations involved in the denaturation process. Our conclusions come close to supporting Privalov's suggestion[1] that an ancillary process may be involved prior to ultimate denaturation (unfolding plus aggregation), but they differ in the respect that a substantial heat is absorbed in the conversion of the native state to the intermediate state. The conversion rate of the intermediate to denatured state is found to be independent of the lysozyme concentration in the buffer, indicating that the overall kinetics of the process is of the first order. The rate constant of the overall process varies with temperature according to the Arrhenius equation with an energy of 167 kJ/mol.

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