Pure & Appl. Chem., Vol. 70, No. 3, pp. 665–670, 1998.
Printed in Great Britain.

Thermal unfolding of hen egg-white lysozyme in the presence of 4-chlorobutan-1-ol

N. Kishore* and B. Sabulal

Department of Chemistry, Indian Institute of Technology, Powai, Bombay - 400 076, India.

Abstract: High sensitivity differential scanning calorimetry transitions for the highly irreversible thermal denaturation of hen egg white lysozyme in the presence of low concentration (< 250 mM) of 4-chlorobutan-1-ol are strongly scanning rate dependent, suggesting that the denaturation is at least in part, under kinetic control. The scan rate dependence can be examined by assuming that the thermal denaturation takes place according to the kinetic scheme $N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D \xrightarrow{k_3} F$; where $k_3 >> k_2$, thus the data fitting to the extreme case of this model, i.e. $N \rightarrow F$ where N is the native state, D is the unfolded one and F is the final irreversibly arrived state. However, the thermal denaturation in the presence of 3-chloro-propan-1-ol, 3-chloropropan-1,2-diol and their corresponding normal alcohols was earlier(ref.1) observed to be reversible two-state.

Alcohol-water mixtures as solvent are important due to their mixed hydrophobic-hydrophilic character. Alcohols have been used frequently to stabilize the unstable conformations of peptide fragments (ref. 2-4). In the recent past (ref. 5,6), the effect of 2,2,2-trifluoroethanol on the conformation of several intact proteins have been examined and their relationship with the molten globule intermediates accumulated in aqueous condition has been considered. In view of this, it is important to study the effect of chloro-substituted alcohols on the conformation of proteins.

We have recently reported (ref. 1) thermodynamics of the interactions of some chloro-substituted alcohols with hen egg white lysozyme. Among the various chloro-substituted and normal alcohols studied, the thermal denaturation in the presence of 4-chlorobutan-1-ol shows unusual behavior particularly at pH 4.5. The data showed partial irreversibility in the calorimetric transitions and the cooperativity ratio suggested intermolecular cooperation which increases as the concentration of the alcohol decreases in the mixture.

High sensitivity micro differential scanning calorimetry has been extensively employed in the last two decades to study the thermal denaturations of proteins (ref. 7). It has been shown that under different conditions of pH, ionic strength, added cosolutes, etc., the thermal denaturations of many globular proteins (ref. 8) and some complex proteins (ref. 9) is two-state and reversible. In order to check the reversibility of the denaturation in differential scanning calorimetric studies, the first scan of the sample is compared with a second one which is carried out after cooling down to temperature below the transition. If both the thermograms are identical or 'almost identical', the thermal denaturation is considered to be having calorimetric reversibility.

Experimental Methods

Micro differential scanning calorimetry was performed in a SETARAM, France instrument with a cell volume of 1 ml. The volume of the sample in the sample cell was kept at 0.85 ml and an equal weight of

the buffer containing alcohol was introduced in the reference cell. Scan rates were employed in the scan range of 18 to 60 K hr^{-1}. To verify the reversibility of thermal denaturations, the sample in the first scan was heated slightly above the complete denaturation temperature, cooled immediately and then re-heated. The calorimetric traces were also corrected for the effect for the finite time response of the instrument. In fact, the effect of this correction on the DSC traces is much smaller than the effect of the scan rate on them. An excess power versus temperature scan for lysozyme transitions was obtained by subtracting the power input of a thermal scan of solvent versus solvent from the power input scan of the solvent versus solution. The DSC data corrected for thermal lag of the instrument were analyzed by the EXAM program of W. H. Kirchhoff (ref. 10).

Results and Discussion

Figure 1 shows the differential scanning calorimetry traces for the thermal denaturation of hen egg white lysozyme at pH 4.5 in 20 mM acetate buffer at various scan rates. The results obtained by the deconvolution of the DSC traces studied at all scan rates employing EXAM program are summarized in Table1. Each value represents an average of three to four experiments. The ratio of van't Hoff to calorimetric enthalpy, (β value) or N the number of moles cooperative units per mole of the protein in the calorimetric cell, indicates increased degree of association in the protein in the presence of 63 mM 4-chloro-butan- 1- ol.

Table 1

scan rate (K/min)	t_m (°C)	N	ΔH_{cal} (kJ/mol)
1.0	74.88	0.364	387
0.7	71.71	0.404	503
0.5	68.50	0.447	577
0.3	64.47	0.461	640

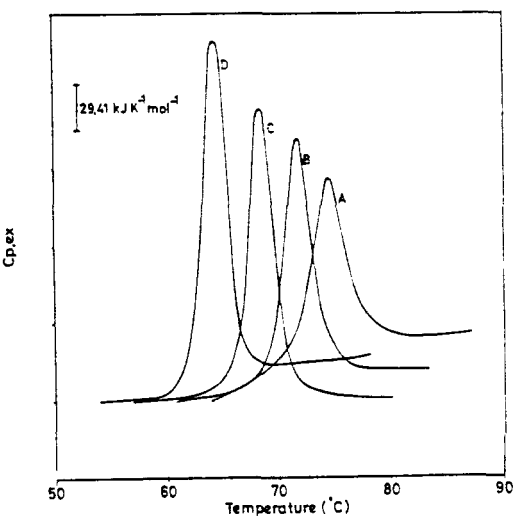

Fig. 1 DSC scans of hen egg white lysozyme in the presence of 63 mM 4-chlorobutan-1-ol, pH 4.5 at (A) 1 K min^{-1}; (B) 0.7 K min^{-1}; (C) 0.5 K min^{-1}; (D) 0.3 K min^{-1}.

All the differential scanning calorimetric traces were found to be highly irreversible. The rescan at each scan rate showed an endotherm at 53 °C, the area of which is only 5 % of the original scan (fig. 2). An interesting feature of the rescans is that all the rescan maxima were found to be at 53 °C (fig. 3). Also, the traces were highly scan rate dependent even after correction for the effect of instrumental response. It can therefore be concluded that the thermal denaturation of hen egg white lysozyme is strongly irreversible and kinetically controlled under the conditions employed.

Thermal denaturation of lysozyme in varying concentration of 3-chloropropan-1-ol, 3-chloro-propane -1,2-diol, n-propanol and n-butanol was earlier observed to be (ref. 1) two-state reversible process. The thermal denaturation of lysozyme at concentration of 4-chlorobutan-1-ol higher than 250 mM

was also observed to be approximate two-state reversible. The irreversibility in the thermal denaturation arises as the concentration of 4-chlorobutan-1-ol is lowered than 250 mM in the protein solution (ref. 1).

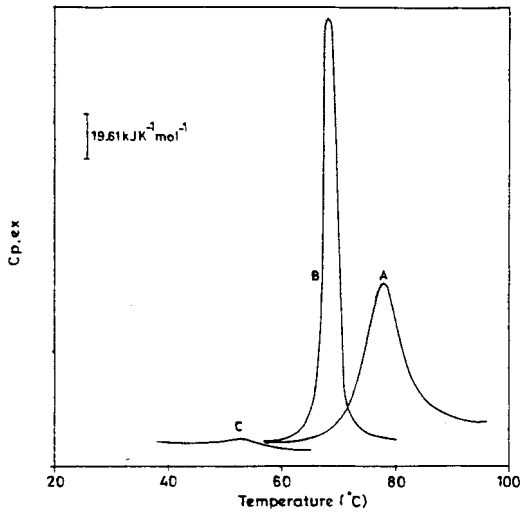

Fig. 2 Thermal denaturation of hen egg white lysozyme at pH 4.5 and 0.5 K min^{-1} (A) in 20 mM acetate buffer; (B) in 63 mM 4-chlorobutan-1-ol; (C) rescan of B.

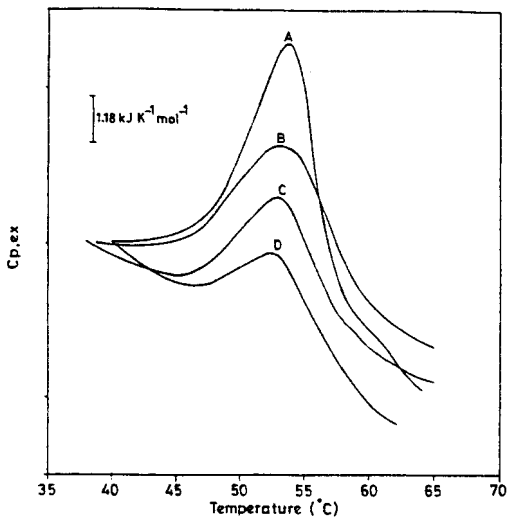

Fig. 3 Rescans of hen egg white lysozyme in 63 mM 4-chlorobutan-1-ol, pH 4.5 at (A) 1 K min^{-1}; (B) 0.7 K min^{-1}; (C) 0.5 K min^{-1}; (D) 0.3 K min^{-1}.

The irreversible thermal denaturation of soluble proteins is believed to involve at least two steps, (i) reversible unfolding of the native protein, and (ii) irreversible alteration to the unfolded protein to yield the final state that is unable to fold back to the native structure. Such a scheme that takes into account the two-step character of irreversible denaturation is well known Lumry-Eyring model (ref. 11).

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D \xrightarrow{k_3} F \qquad k_3 >> k_2 \quad \text{.....(1)}$$

In such a model, the reversible unfolding step might involve several significantly populated intermediate states (ref. 12,13). Since $k_3 >> k_2$, most of the D molecules are converted to the F state instead of returning to N through the process D N. The concentration of D always remains very low, which is the reason for about 5 % recovery of the endotherm but at different temperature. Thus the denaturation may be considered as an extreme case of the above model, i.e., $N \rightarrow F$. Hence the present differential scanning calorimetric data was fitted to a two-state irreversible model, $N \rightarrow F$. The mathematical elaboration of this model leads (ref. 14) to several methods of calculating the activation energy of this kinetic process.

Method 1

The rate constant of the reaction at a given temperature T can be obtained (ref. 14) by using,

$$k = \nu C_p/(Q_t - Q) \quad \text{..... (2)}$$

where ν in K min^{-1} is the scan rate, C_p is the excess heat capacity, Q_t is the total heat of the calorimetric transition and Q is the heat evolved at a given temperature. From the values of the rate constant k at several temperatures, the energy of activation E, can be obtained using an Arrhenius plot of ln k vs 1/T. The corresponding Arrhenius plot, lnk vs 1/T using the data from the four scan rates used is given in figure 4a. It

is observed that the different values obtained for the activation energy all agree supports the validity of this model. Although the variation of ln k with temperature is same, however, difference in k values could mean slight shift in the equilibrium constant of N D step at different scan rates. From the slope of Arrhenius plot in figure A, a value of 104 ± 6 kJ mol^{-1} for the energy of activation can be calculated.

Method 2

The two-state kinetic model predicts (ref. 14) that the temperature value corresponding to the maximum of the heat capacity, T_m changes with the scan rate according to

$$\ln (\nu/T_m^2) = \text{constant} - E/RT_m \quad \ldots\ldots (3)$$

The plot of ln (ν/T_m^2) versus $1/T_m$ for the DSC data is linear as shown in figure 4b in accordance with equation 3. The calculated activation energy from the T_m values obtained at four scan rates is 110 ± 4 kJ mol^{-1} which is in excellent agreement with the value obtained from the Arrhenius analysis of the k values.

Method 3

According to the two-state kinetic model, the dependence of the heat evolved with temperature is given (ref. 14) by

$$\ln [\ln Qt/(Q_t-Q)] = E/R\ [1/T_m - 1/T] \quad \ldots\ldots (4)$$

where T_m is the temperature corresponding to maximum heat capacity. Thus, a plot of ln [ln $Qt/(Q_t-Q)$] versus 1/T should give rise to straight lines. The slope of each plot should be -E/R. The plot obtained using the data at four scan rates is shown in figure 4c. The data fit to a straight line and the calculated average activation energy is 101 ± 7 kJ mol^{-1}. It is seen from equation 5 that x-axis intercept in these plots give the values of T_m. The T_m values obtained for the scan rates 0.3 K min^{-1}, 0.5 K min^{-1}, 0.7 K min^{-1} and 1.0 K min^{-1} are 64.5, 68.2, 71.5 and 74.4 °C. The corresponding ones obtained directly from the calorimetric traces are 65.9, 69.3, 72.2 and 74.8 °C. It is seen that both the calculated and experimental values compare very well.

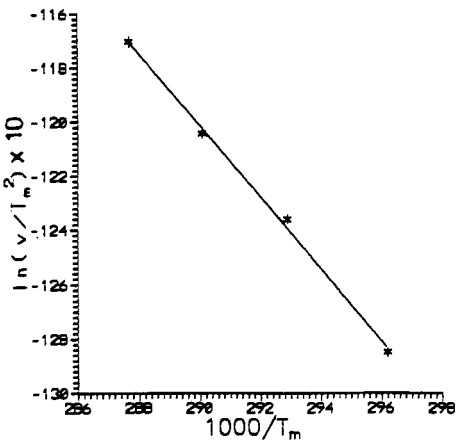

Fig. 4a The plot of ln (ν/T_m^2) versus $1/T_m$.

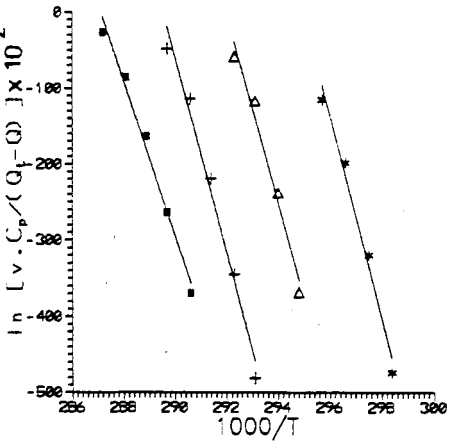

Fig. 4b Plot of ln $[\nu C_p/(Q_t-Q)]$ versus 1/T at (■) 1 K min^{-1}; (+) 0.7 K min^{-1}; (Δ) 0.5 K min^{-1}; (∗) 0.3 K min^{-1}.

Method 4

From the heat capacity at the maximum of the differential scanning calorimetric trace, the activation energy can also be calculated according to

$$E = e\,R\,C_p^{m}\,T_m^{2}/Q_t \quad \ldots\ldots (5)$$

From the four traces of figure 5, an average value of 108 ± 6 kJ mol^{-1} is obtained for the activation energy of the thermal denaturation of hen egg white lysozyme under these conditions.

Thus it is seen that different methods employed to obtain the activation energy involving different approximations (ref. 14) and different experimental information provide excellent agreement between the results obtained for the energy of activation.

4-chlorobutan-1-ol is a strong destabilizer of lysozyme (table 1). The cosolvent effects on a denaturation reaction depend upon a fine balance between patterns of interactions of the cosolvent with the native and the unfolded state of the protein, which involves a combination of exclusion and binding that changes during the course of the reaction (15,16). Possessing partly hydrophobic property,

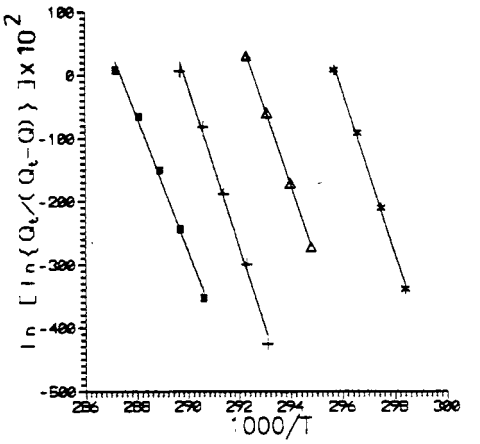

Fig. 4c The plot of ln [ln $\{Q_t/(Q_t-Q)\}$] versus 1/T at (■) 1 K min^{-1}; (+) 0.7 K min^{-1}; (Δ) 0.5 K min^{-1}; (∗) 0.3 K min^{-1}.

4-chlorobutan-1-ol interacts favorably with the hydrophobic side chains of the protein made available when lysozyme unfolds, thus leading to a preferential stabilization of the unfolded state and shifts the N D equilibrium towards D.

We propose that the kinetics of the thermal unfolding-refolding of hen egg white lysozyme in the presence of low concentrations of 4-chlorobutan-1-ol follow the scheme:

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D \xrightarrow{k_3} F \quad \ldots\ldots (6)$$

We assume that all the kinetic processes are of first order and $k_3 >> k_2$. Thus most of the D molecules are converted to the final state F instead of refolding to the native state trough the process D N. The concentration of D remains very low and a small fraction < 5 % returning to the native state N shows an endotherm (figure 2) on reheating. The change in the degree of association in this process may be the reason for the shift in the temperature of the endotherm on re-heating. Hence in this case, the rate limiting step is the unfolding N → D and the formation of F is determined by a first-order rate constant

equal to k, i. e., $N \xrightarrow{k} F$. Obviously, the data fits quite closely to the two-state irreversible model.

Conclusion

The main conclusion of the work done on the thermal unfolding of some globular proteins in the presence of 4-chlorobutan-1-ol is that under solvent conditions employed, the thermal denaturation of hen egg white lysozyme, is a rate limited process. Similar effect has been observed with ribonuclease A and α-lactalbumin (unpublished results). The interesting feature is that at higher concentration of the alcohol, two-state denaturation is observed (ref. 1). The extreme case of $N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D \xrightarrow{k_3} F$ [$k_3 >> k_2$] employed fits very good to the experimental data. It is also worth pointing out that not only the thermal denaturation of proteins at low concentration of 4-chlorobutan-1-ol needs to be understood, but also the thermodynamic properties of 4-chlorobutan-1-ol - water mixture at various concentration of this alcohol could help in better explanation of the results.

This work was funded by a grant-in-aid from the Department of Science and Technology, New Delhi, India.

References

1. B. Sabulal and N. Kishore. *J. Chem. Soc. Faraday. Trans.* **92**, 1905 (1996).

2. H. J. Dyson, J. R. Sayre, G. Murutka, H-C. Shin, R. A. Lerner and P. E. Wright. *J. Mol. Biol.* **226**, 819 (1992).

3. A. D. Kippen, J. Sancho and A. R. Fresht. *Biochemistry* **33**, 3778 (1994).

4. S. Segawa, T. Fukono, K. Fujiwara and Y. Noda. *Biopolymers* **31**, 497 (1991).

5. M. Buck, S. E. Radford and C. M. Dobson. *Biochemistry* **32**, 669, (1993).

6. A. T. Alexandrescu, Y-L. Nag and C. M. Dobson. *Biochemistry* **235**, 587 (1994).

7. P. L. Privalov. *Ann. Rev. Biophys. Biophys. Chem.* **18**, 47 (1989).

8. P. L. Privalov. *Adv. Protein. Chem.* **33**,167 (1979).

9. P. L. Privalov. *Adv. Protein. Chem.* **35**,1 (1982).

10. EXAM, W.H.Kirchhoff, US Department of Energy, Thermodyanmic Division, National Institute of Standards and Technology, USA.

11. R. Lumry and H. Eyring. *J. Phys. Chem.* **58**, 110 (1954).

12. E. Friere, O. W. W. van, O. L. Mayorga and J. M. Sanchez-Ruiz. *Annu. Rev. Biophys. Biophys. Chem.* **19**, 159 (1990).

13. J. M. Sanchez-Ruiz. *Biophys. J.* **61**, 921 (1992).

14. J. M. Sanchez-Ruiz, J. L. Lopez-L, M. Cortijo and P. L. Mateo. *Biochemistry* **27**, 1648 (1988).

15. T - Y Lin and S. N. Timasheff. *Biochemistry* **33**, 12695 (1994).

16. S. N. Timasheff. *Biochemistry* **31**, 9857 (1992).