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4-Chlorobutanol induces unusual reversible and irreversible thermal unfolding of ribonuclease A: thermodynamic, kinetic, and conformational characterization☆

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Abstract

The thermal denaturation of ribonuclease A has been studied by differential scanning calorimetry in the presence of 4-chlorobutan-1-ol. The thermal transitions were observed to be reversible at pH 5.5 in the presence of low concentration (up to 50 mM) of the alcohol, irreversible in the intermediate (50 mM $< c <$ 250 mM) and again reversible in the presence of 250 mM and higher concentrations of 4-chlorobutan-1-ol. In the presence of 50 mM 4-chlorobutan-1-ol, ribonuclease A is present in two conformational states unfolding at different temperatures. The reversible thermal transitions have been fitted to a two-state native-to-denatured mechanism. Irreversible thermal transitions have been analyzed according to two-state irreversible native-to-denatured kinetic model. Using the irreversible model, rate constant as a function of temperature and energy of activation of the irreversible process have been calculated. Circular dichroism and fluorescence spectroscopic results corroborate the DSC observations and indicate a protein conformation with poorly defined tertiary structure and high content of secondary structure in the presence of 50 mM 4-chlorobutan-1-ol at a temperature corresponding to the second transition. Similar results have been observed at pH 3.9.

Keywords: Ribonuclease A; 4-Chlorobutan-1-ol; Thermal denaturation; Differential scanning calorimetry; Enthalpy; Activation energy

1. Introduction

An understanding of protein–solvent interactions is essential to elucidate the nature of forces that stabilize the native conformation of proteins under a given environment. Due to mixed hydrophilic–hydrophobic character, alcohols especially fluoro-substituted have been widely used to generate partially folded states in the proteins and peptide fragments with an aim to contribute to the area of protein folding and understanding of the conformation of proteins in aqueous organic solutions [1–8]. However, a study on the interaction of other halogen-substituted alcohols with proteins may also provide useful information on the conformation of proteins which could be linked to the intermediate states in the protein folding. Alcohols are known to affect the proteins in three distinct ways: destruction of the rigid native structure, induction of α-helices and dissolution of peptide aggregates. Apart from an understanding of protein-folding, alcohol–protein interactions are also important in a wide range of applications such as dissolution of aggregates that sometimes occur during peptide synthesis, investigation of prior diseases [9,10] and Alzheimer's amyloid peptides [11–13].

In this paper, we have quantitatively demonstrated that 4-chlorobutan-1-ol induces reversible thermal transitions in ribonuclease A at low concentration, irreversible at intermediate concentrations and again reversible transitions at further higher concentration of the alcohol. The reversible and irreversible thermal transitions have been analyzed using appropriate models and conformation of the protein under these conditions discussed.

2. Experimental

2.1. Materials

Bovine pancreatic ribonuclease A was obtained from Sigma Chemical Company and used without further

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purification. 4-Chlorobutan-1-ol was procured from Fluka. Sodium acetate, potassium phosphate (both monobasic and dibasic), acrylamide, and 8-anilino-1-naphthalenosulfonic acid (ANS) were procured from Sigma Chemical Company. Urea, hydrochloric acid, and sodium hydroxide used for buffer preparation were all reagent grade. All the solutions were prepared in double distilled, de-ionized water. The protein solution was prepared in 20 mM acetate buffer at pH 5.5 and 3.9 and then dialyzed at 4.0 °C overnight in a large volume of the same buffer with at least two changes. Protein concentration was determined on a Shimadzu-260 double beam spectrophotometer using molar absorption coefficient of 9800 M^{-1} cm^{-1} at 278 nm [14].

2.2. Differential scanning calorimetry

Differential scanning calorimetric measurements were done with a micro Differential Scanning Calorimeter (Setaram, France), the procedure of which and extraction of thermodynamic parameters accompanying the denaturation process have been described earlier [15]. The concentration of the protein was kept the same (0.32 mM or 0.29 mM) in all the experiments except for the protein concentration variation studies.

2.3. Circular dichroic measurements

CD measurements were done on a Jasco J-600 spectropolarimeter at room temperature. A 1-mm cell was used for recording the far-UV CD (200–250 nm) and a 1-cm cell was used for the near-UV CD (240–320 nm) experiments. All the spectra were baseline corrected. The CD spectra were measured at a protein concentration of 15 μM for ribonuclease A at both pH 5.5 and 3.9. The protein solutions were heated wherever required in a Remi temperature controlled water bath with an accuracy of ±0.1 °C.

2.4. Fluorescence measurements

The Spex 1681 0.22 m spectrofluorimeter was used to perform the intrinsic fluorescence measurements of ribonuclease A in presence of varying concentrations of 4-chlorobutan-1-ol. The concentration of ribonuclease A was 7 μM and the samples were excited at 280 nm. All the spectra were buffer subtracted. ANS binding to the exposed hydrophobic residues of proteins was studied and ANS concentration was determined by using molar absorption coefficient of 5.0×10^3 M^{-1} cm^{-1} at 350 nm [16]. The concentrations of ribonuclease A and ANS were maintained at 7 μM and 5.8×10^{-5} M, respectively. The excitation wavelength was 365 nm.

3. Results and discussion

3.1. Differential scanning calorimetry of ribonuclease A in presence of 4-chlorobutan-1-ol at pH 5.5

The thermodynamic parameters: temperature at half denaturation ($t_{1/2}$), calorimetric enthalpy (ΔH_{cal}), heat capacity of denaturation (ΔC_p), and β (ratio of van't Hoff to calorimetric enthalpy) accompanying the denaturation process in the absence and presence of 4-chlorobutan-1-ol are reported in Table 1. A well behaved two-state transition centered at 62.0 °C characterized by a calorimetric enthalpy of 402 kJ mol^{-1} and $\beta = 1.03$ is observed in 20 mM acetate buffer at pH 5.5, heated at a scan rate of 0.5 K min^{-1}, in good agreement with those reported in literature [17]. With an increase in concentration of the alcohol, the thermal stability of the protein was found to decrease as reflected by its denaturation at lower temperatures. The calorimetric enthalpy also decreased in a non-linear fashion. In the presence of 10 and 20 mM 4-chlorobutan-1-ol, reversible thermal transitions of the protein were observed with $t_{1/2}$ and ΔH_{cal} values less than that without the alcohol. The

Table 1
Thermodynamic parameters of thermal unfolding of 0.32 mM ribonuclease A in the presence of 4-chlorobutan-1-ol at pH 5.5 (scan rate 0.5 K min^{-1})

Alcohol (M)	$t_{1/2}$ (°C)	ΔH_{cal} (kJ mol^{-1})	ΔS (kJ K^{-1} mol^{-1})	ΔC_p (kJ K^{-1} mol^{-1})	β ($\Delta H_{VH}/\Delta H_{cal}$)
0.0	71.6	360.3	7.35	0.92	0.96
0.000	62.0	402	1.20	7.0	1.03
0.010	61.0	377	1.13	5.1	1.05
0.020	60.5	370	1.11	5.7	1.03
0.050[a]	58.5	332	1.00	5.9	0.72
0.100	55.0	337	–	–	–
0.125	54.6	322	–	–	–
0.150	51.9	337	–	–	–
0.175	45.5	323	–	–	–
0.200	43.9	292	–	–	–
0.250	35.4	294	0.95	5.9	1.03
0.400	26.8	235	0.78	6.1	1.05

Each value in the table represents an average of three to five experiments. All thermodynamic parameters reported only for reversible transitions. For irreversible transitions, only $t_{1/2}$ and ΔH_{cal} have been reported.

[a] Only first peak included in deconvolution.

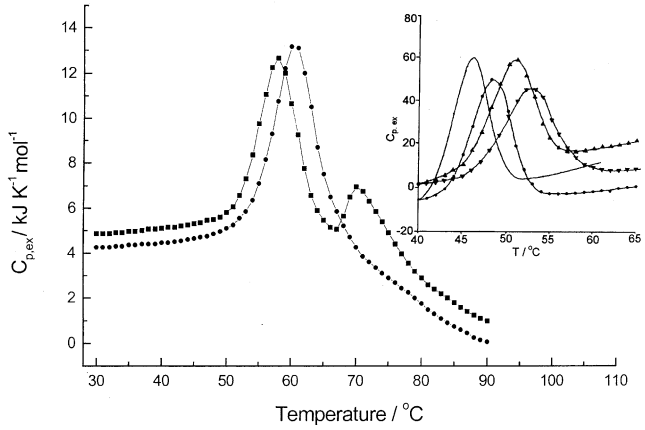

Fig. 1. DSC profiles of 0.29 mM ribonuclease A at pH 5.5 in 20 mM acetate buffer (●), and in the presence of 50 mM 4-chlorobutan-1-ol (■). Inset: DSC profiles of thermal denaturation of 0.32 mM ribonuclease A at pH 5.5 in the presence of 175 mM 4-chlorobutan-1-ol heated at the scan rates of 0.3 (■), 0.5 (●), 0.7 (▲), and 1.0 (▼) K min^{-1}.

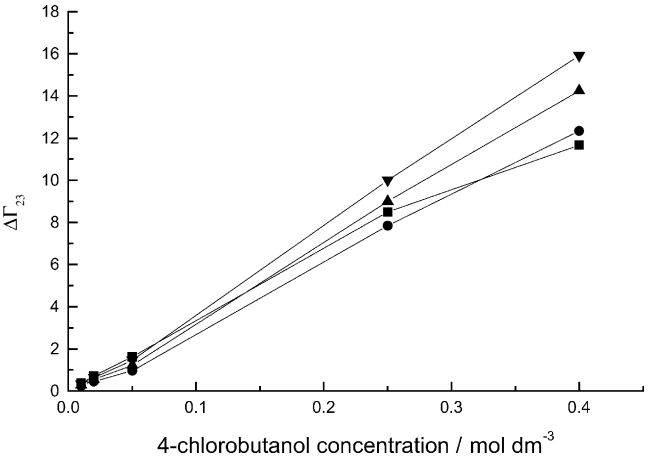

Fig. 2. Denaturational change in the preferential solvation of the protein at transition temperature (■), 303.15 (●), 313.15 (▲), and 323.15 (▼) K.

values of β were 1.05 and 1.03, respectively, suggesting that the protein undergoes a two-state reversible denaturation.

When the concentration of 4-chlorobutan-1-ol was 50 mM in the protein solution, two independent endothermic transitions were obtained as shown in Fig. 1. There is a major peak centered at 58.5 °C and a minor peak centered at 73.0 °C. The first major endotherm was observed to be calorimetrically reversible. This is based upon the observation that when the sample was heated only up to a temperature just past the first endotherm, cooled and then re-heated, the major endotherm re-appeared. However, when the sample in the DSC cell was scanned through both the peaks, cooled and rescanned, none of the two transitions appeared back. This shows that irreversibility in thermal transition at this alcohol concentration is due to the process occurring at the temperature corresponding to the second transition. No precipitates were observed in the DSC cell even after the third scan. The deconvolution of this DSC trace including only the first peak gives a calorimetric enthalpy of 332 kJ mol^{-1} and β value of 0.72 which shows a significant departure from the simple two-state unfolding behavior. When cooperativity ratio (β) is less than one, it may be concluded that one or more states intermediate between the native and denatured states are significantly populated [18]. The calorimetric transitions were completely irreversible when the concentration of the alcohol was between 50 mM and 250 mM in solution. When the concentration of the alcohol was further increased (250–400 mM) in the protein solution, sharpness in the transition decreased and the reversibility in the thermal transitions reappeared.

3.1.1. Analysis of the reversible thermal transitions

The reversibility and two-state character of the thermal unfolding of ribonuclease A up to 50 mM 4-chlorobutan-1-ol as additive has been demonstrated by the equality of calorimetric and van't Hoff enthalpy data cited above. This permits application of equilibrium thermodynamics for the evaluation of thermodynamic parameters as a function of temperature and alcohol concentration. The heat capacity of denaturation (ΔC_p) of ribunuclease A in the absence of alcohol is 7.0 ± 0.5 kJ mol^{-1}. The average ΔC_p of denaturation in the presence of 4-chlorobutan-1-ol is 5.9 ± 0.8 kJ mol^{-1} which is slightly lower than the value in buffer only. The decreased value of ΔC_p of denaturation can be attributed to altered water structure in the presence of alcohol. Enhanced organization of water upon addition of alcohols has been reported in literature [19,20]. Since the difference in heat capacity of denaturation in the absence and presence of 4-chlorobutan-1-ol is only small but negative, this indicates that the interaction between the hydrophobic part of 4-chlorobutan-1-ol and those exposed on protein upon unfolding leads to lesser ordering of water structure around apolar residues.

The data on the reversible thermal transitions from differential scanning microcalorimetry has been used for practical calculations of the denaturational change in preferential solvation of the protein ($\Delta\Gamma_{23}$) by 4-chlorobutan-1-ol at the transition temperature using the method described by Kovrigin and Potekhin [21]. Due to low concentration of the alcohol, unit activity coefficients have been assumed throughout. It is seen in Fig. 2 that the denaturational change in preferential solvation of the protein increases with increase in the concentration of 4-chlorobutan-1-ol, which is due to interaction between the hydrophobic groups of the protein upon unfolding and that of the alcohol. Fig. 2 also compares $\Delta\Gamma_{23}$ values corresponding to actual transition temperature observed and isothermal $\Delta\Gamma_{23}$ calculated at 303.15, 313.15, and 323.15 K. It is also seen in this figure that the temperature contribution to preferential solvation is less at lower concentration but increases at higher concentration of the alcohol.

A convenient measure of the change produced by an additive in the apparent stability of a protein is the quantity:

$$\Delta\Delta G^{\circ}_{\mathrm{d}} = \Delta G^{\circ}_{\mathrm{d}}\,(\text{in additive}) - \Delta G^{\circ}_{\mathrm{d}}\,(\text{in buffer}).$$

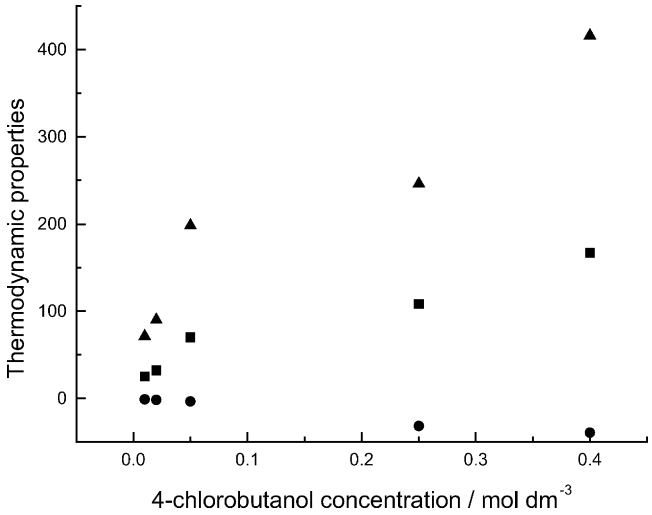

Fig. 3. Plot of $\Delta\Delta H_d^\circ$/ kJ mol^{-1} (■), $\Delta\Delta G_d^\circ$/ kJ mol^{-1} (●), $\Delta\Delta S_d^\circ$/J K^{-1} mol^{-1} (▲) as a function of 4-chlorobutan-1-ol concentration.

This is difference in the standard Gibbs energy of unfolding reaction evaluated at $t_{1/2}$ in buffer by means of Gibbs-Helmholtz equation [22]. The $\Delta\Delta G_d^\circ$, $\Delta\Delta H_d^\circ$, and $\Delta\Delta S_d^\circ$ values are shown in Fig. 3. It is obvious that in the application of the above equation, the term, $\Delta\Delta G_d^\circ$, (in buffer) = 0 so that the destabilization of the native state or stabilization of the denatured state is indicated by a negative value of $\Delta\Delta G_d^\circ$. It is also seen in this figure that the entropic contribution to the free energy of unfolding increases with increase in the concentration of the alcohol. It is to be noted that $\Delta\Delta G_d^\circ$, presented in Fig. 3 is isothermal at the transition temperature of the protein in buffer only. However, $\Delta\Delta H_d^\circ$ and $\Delta\Delta S_d^\circ$ values are plotted at the transition temperature of the protein in presence of alcohol.

The effect of additives on the conformational stability of a protein is defined by a balance between their preferential interactions with the native and denatured states of the protein [23–25]. The results on denaturational increase in the preferential solvation ($\Delta\Gamma_{23}$) and change in apparent stability ($\Delta\Delta G_d^\circ$) of the protein quantitatively indicated that the hydrophobic effects of 4-chlorobutan-1-ol are greater on the unfolded state of ribonuclease A due to a larger exposure of the constituent groups of the protein to the solvent compared to that in the native state which leads to reduction in the thermal denaturation temperature of the protein in the presence of the alcohol.

3.1.2. Analysis of the irreversible thermal transitions

Since the thermal transitions of ribonuclease A were irreversible in the concentration range 50 mM to 200 mM of 4-chlorobutan-1-ol, scan rate dependence was studied in the presence of 175 mM alcohol, where the sharpness in the DSC transition was maximum. The DSC traces were highly dependent on the scan rate even after correction for the instrumental response time as shown in Fig. 1 (inset). Therefore, it can be concluded that the thermal denaturation of ribonuclease A in this concentration range of the alcohol is kinetically controlled. It is seen that the irreversible step takes place during the time the protein spends in the transition region. This information is based upon the observation that when the first scan is performed only until 25% of the transition is complete, cooled and re-scanned, the transition was obtained centered at 5.5 °C lower than that obtained in the first complete scan. Also, the ΔH_{cal} associated with this transition was lesser compared to that in the first complete scan.

The irreversible thermal denaturation of a protein is believed to involve minimum of two steps: (i) reversible unfolding of native protein (N) to an unfolded state (D), and (ii) irreversible alteration in the unfolded state (D) to arrive at a final state (F) that is unable to fold back to the native state. The well known Lumry–Eyring model [26] represents such a scheme that takes into account the two-step irreversible denaturation reaction, i.e. N ⇌ D → F. We have analyzed our data as described by Sanchez-Ruiz [27] in the analysis of irreversibly denatured proteins studied by DSC. It has been shown that the concentration of the unfolded states remains very low when the process responsible for the irreversible step is very fast [28–31]. Under such circumstances, the only significantly populated states during denaturation are the native and the final states. The mathematical elaboration of the two-state kinetic model $N \xrightarrow{k} F$ leads to the following methods of calculating the activation energy of the kinetic process [27]. The rate constant of the two-state irreversible process following the first order kinetics can be obtained [27] using

$$k = \nu C_p^{ex} (Q_t - Q) \quad (1)$$

where ν, C_p^{ex}, Q_t, and Q represent the scan rate, excess heat capacity, total heat of the process, and heat evolved at a given temperature T, respectively. A plot of ln k versus $1/T$ at different scan rates is shown in Fig. 4 (inset). From the linear fit of the data, activation energy of 176±10 kJ mol^{-1} is calculated. It has also been shown [27] that the two-state kinetic model predicts the variation of the temperature corresponding to the maximum heat capacity (T_m) with scan rate (ν) according to

$$\frac{\nu}{T_m^2} = \left(\frac{AR}{E}\right) e^{-E/RT_m} \quad (2)$$

where A, R, and E are pre-exponential factor of the Arrhenius equation, gas constant, and activation energy, respectively. The activation energy of the process can be calculated from the plot of $\ln(\nu/T_m^2)$ versus $1/T_m$. The linear fit of the data shown in Fig. 4 gives an activation energy of 160 ± 2 kJ mol^{-1}. A close agreement between the calculated activation energies using both the methods justifies the chosen $N \xrightarrow{k} F$ two-state kinetic model.

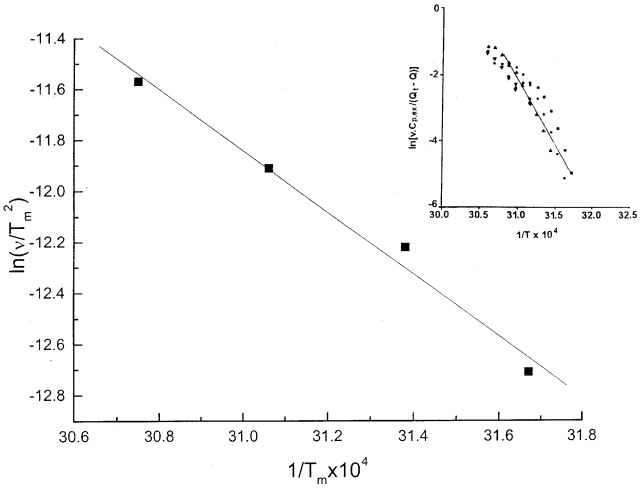

Fig. 4. Plot of ln (ν/T_m^2) versus $1/T_m$ for thermal denaturation of 0.32 mM ribonuclease A at pH 5.5 in presence of 175 mM 4-chlorobutan-1-ol. Inset: plot of $\ln[\nu C_{p,ex}/(Q_t - Q)]$ versus $1/T$ for thermal denaturation of 0.32 mM ribonuclease A at pH 5.5 in the presence of 175 mM 4-chlorobutan-1-ol heated at scan rates of 0.3 (■), 0.5 (●), 0.7 (▲), and 1.0 (▼) K min^{-1}.

3.2. Conformational analysis

The UV absorbance spectra of 13 μM ribonuclease A in 20 mM acetate buffer shows two bands, one centered at 215 nm and the other at 277 nm. The UV absorbance spectra of the protein was recorded in the presence of 50 mM alcohol concentration at room temperature, heated to 65.0 °C where the first transition ends, and heated to 80.0 °C, a temperature past the second minor endotherm. In all the three cases, the absorbance spectra of the solution was similar to the native protein and no abrupt change in the spectra was observed in the range 340–400 nm where no absorbing species of the protein are present. This negates aggregation of the protein particles at the second transition in the presence of 50 mM alcohol. At 100 mM 4-chlorobutan-1-ol concentration, where the DSC transitions were completely irreversible, the effect of variation of the protein concentration from 0.07 to 0.44 mM on the transition temperature was studied, the results of which are presented in Table 2. It is observed that in this protein concentration range, the $t_{1/2}$ gradually increases from 51.6 °C for 0.07 mM protein concentration to 55.6 °C for 0.44 mM protein concentration.

Table 2
Thermodynamic parameters of thermal unfolding of ribonuclease A in presence of 100 mM 4-chlorobutan-1-ol at pH 5.5 (scan rate 0.5 K min^{-1})

Protein (mM)	$t_{1/2}$ (°C)	ΔH_{cal} (kJ mol^{-1})
0.00	71.6	360.3
0.07	51.3 ± 0.3	284 ± 7
0.15	52.8 ± 0.2	301 ± 3
0.22	53.4 ± 0.1	311 ± 4
0.29	54.6 ± 0.2	322 ± 2
0.37	55.4 ± 0.3	341 ± 6
0.44	56.6 ± 0.0	327 ± 3

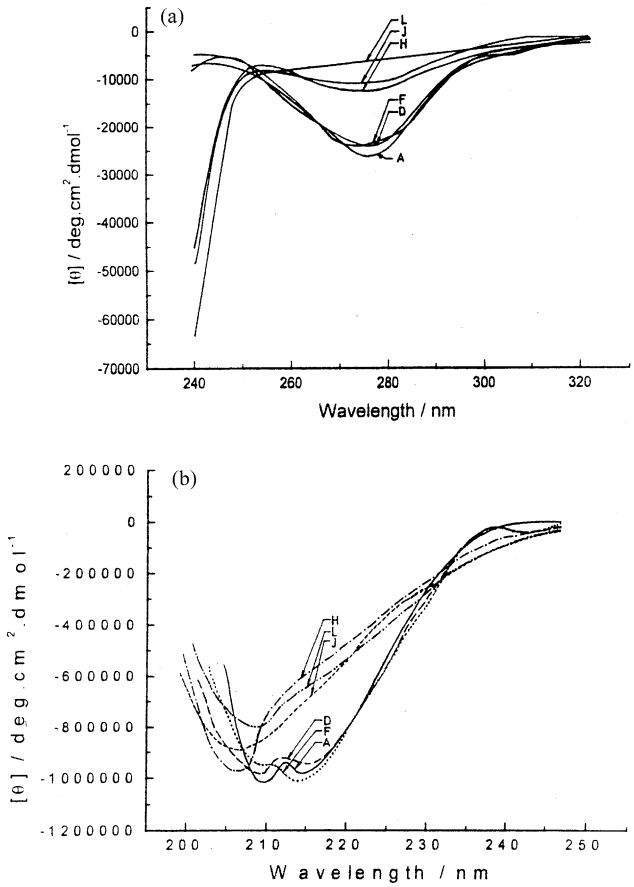

Fig. 5. Near-UV CD spectra (a) and far-UV CD spectra (b) of 15 μM ribonuclease A at pH 5.5: buffer (A), 50 mM (D), 50 mM at 64.0 °C (F), 50 mM at 83.0 °C (H), 175 mM (J), and 400 mM (L) 4-chlorobutan-1-ol.

However, the change of $T_{1/2}$ ($T_{1/2}/\mathrm{K} = t_{1/2}/°\mathrm{C} + 273.15$) with concentration of protein is calculated to be 14 K mM^{-1} from the linear fit of the plot of $T_{1/2}$ versus protein concentration. This can be interpreted as small change in the extent of aggregation accompanying the unfolding of the protein. It is observed that the ΔH_{cal} gradually increases with protein concentration and after reaching a certain maximum around 337 and 341 kJ mol^{-1} for 0.32 and 0.37 mM protein concentration, again starts decreasing.

3.2.1. Circular dichroism of ribonuclease A in presence of 4-chlorobutan-1-ol at pH 5.5

CD spectra of ribonuclease A in the presence of 4-chlorobutan-1-ol at pH 5.5 in the near-UV region (Fig. 5a) shows that there is not much change for ribonuclease A in the presence of 50 mM 4-chlorobutan-1-ol at ambient temperature and at 64.0 °C. However, there is a considerable loss of tertiary structure of ribonuclease A in presence of 50 mM 4-chlorobutan-1-ol at 83.0 °C, except for the negative band at 275 nm which is still observed. Higher concentrations of 4-chlorobutan-1-ol, i.e. 175 mM and 400 mM lead to a significant loss of the tertiary structure in ribonuclease A at pH 5.5. There is considerable amount of secondary

structure in native ribonuclease A and in presence of 50 mM 4-chlorobutan-1-ol at pH 5.5 at ambient temperature as can be observed from the far-UV CD spectra obtained under these conditions (Fig. 5b). On heating the protein in presence of 50 mM 4-chlorobutan-1-ol to 64.0 °C, there is no marked change in the CD signal. While on heating the protein sample in presence of 50 mM 4-chlorobutan-1-ol to 83.0 °C, there is a loss of secondary structure around 220 nm while the secondary structure around 210 nm still remains. Similar spectra are obtained at higher concentrations of 4-chlorobutan-1-ol, i.e. 175 and 400 mM, though the intensity of the band at 210 nm gradually goes on decreasing with rise in the alcohol concentration.

The CD studies very clearly support the DSC results, wherein minor conformational change is observed for ribonuclease A in presence of 50 mM 4-chlorobutan-1-ol at 64.0 °C. But in the presence of 50 mM alcohol at 83.0 °C, drastic conformational changes both in the far and near-UV spectra are observed. This is the temperature where the second DSC transition ends leading to irreversibility in the thermal unfolding.

3.2.2. Fluorescence studies of ribonuclease A in presence of 4-chlorobutan-1-ol at pH 5.5

Intrinsic fluorescence of ribonuclease A under similar conditions of alcohol employed as discussed above shows that with increasing concentration of 4-chlorobutan-1-ol from 10 to 400 mM at pH 5.5, the emission intensity goes on increasing (Fig. 6). This suggests an enhanced exposure of tyrosine residues upon addition of these concentrations of 4-chlorobutan-1-ol. Also, there is a gradual increase in the intrinsic fluorescence of ribonuclease A in the presence of 50 mM 4-chlorobutan-1-ol at ambient temperature, 64.0 °C, and 83.0 °C (Fig. 6 inset), which is due to an increased exposure of the buried hydrophobic residues to the solvent.

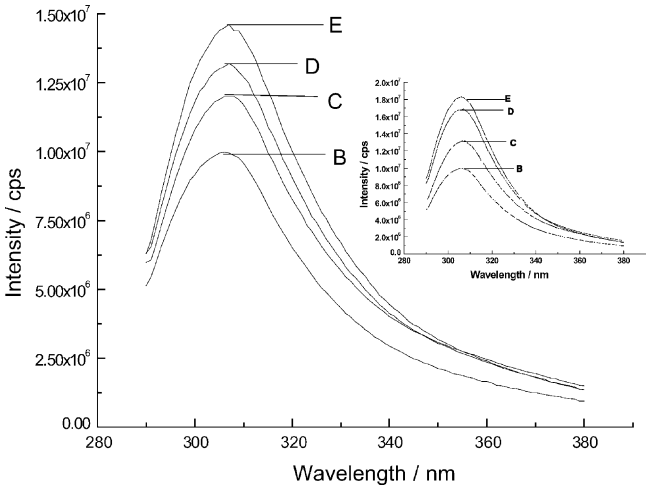

Fig. 6. Fluorescence emission spectra of 7 μM ribonuclease A at pH 5.5 in (B) buffer; and in the presence of varying concentrations of 4-chlorobutan-1-ol: (C) 10 mM; (D) 50 mM; (E) 100 mM. Inset: fluorescence emission spectra of 7 μM ribonuclease A at pH 5.5 in (B) buffer; and in the presence of varying concentrations of 4-chlorobutan-1-ol: (C) 50 mM; (D) 50 mM at 64.0 °C; and (E) 50 mM at 83.0 °C.

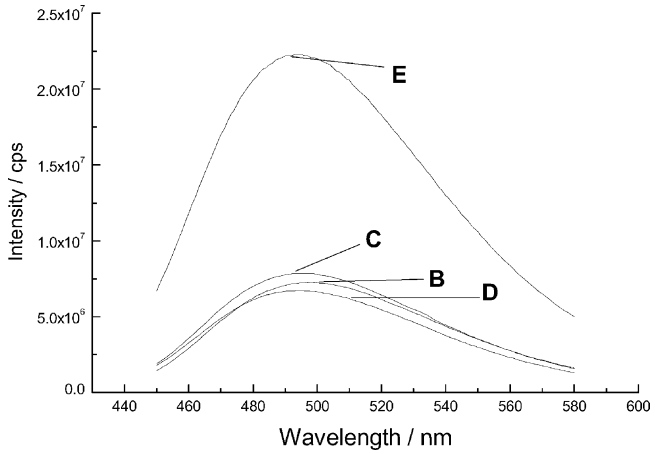

Fig. 7. Fluorescence emission spectra obtained from ANS (5.8×10^{-5} M) binding studies of 7 μM ribonuclease A at pH 5.5: buffer (B), and in the presence of 4-chlorobutan-1-ol: 50 mM (C), 50 mM at 64.0 °C (D), 50 mM at 83.0 °C (E).

It has been shown earlier that ANS, a hydrophobic probe, has a stronger affinity for the molten globule intermediate state of the protein that for the native or fully unfolded state [28]. Therefore ANS binding studies to ribonuclease A were carried out in the presence and absence of 4-chlorobutan-1-ol. In the presence of 50 mM 4-chlorobutan-1-ol at 83.0 °C, the fluorescence intensity is very high although no shift in λ_{max} is observed (Fig. 7) compared to that in the presence of the alcohol at ambient temperature and heated to 64 °C. This very well supports the DSC results, wherein reversibility is observed for ribonuclease A in presence of 50 mM 4-chlorobutan-1-ol on heating upto 64.0 °C at pH 5.5 and irreversibility is observed on heating up to 83.0 °C. This also correlates well with the CD data, wherein there is almost no change in the secondary and tertiary structure after heating ribonuclease A in presence of 50 mM 4-chlorobutan-1-ol upto 64.0 °C. While for ribonuclease A in presence of 50 mM 4-chlorobutan-1-ol at 83.0 °C, significant amounts of secondary structure is present but the tertiary structure is poorly defined.

3.3. Interaction of ribonuclease A with 4-chlorobutan-1-ol at pH 3.9

Similar sets of calorimetric and spectroscopic experiments were carried out on ribonuclease A in presence of 4-chlorobutanol at pH 3.9. The results (given as Supporting Information) were found similar to those obtained at pH 5.5 except that two thermal transitions were observed in the presence of 50 mM as well as 75 mM alcohol. In order to check whether the two endotherms observed at this pH were due to aggregation, the DSC studies in 50 mM alcohol concentration were carried out in the presence of low concentrations of urea as it has been used to reversibly denature

proteins and inhibit aggregation [32]. It is observed that on increasing the concentration of urea from 0.5 to 2.5 M in the protein solution containing 50 mM 4-chlorobutan-1-ol, the ΔH_{cal} accompanying the second transition went on decreasing and finally vanished in presence of 2.5 M urea (Fig. S7). These results clearly demonstrate that the second transition in the thermal denaturation of ribonuclease A in the presence of 50 mM 4-chlorobutan-1-ol is due to another conformational state of the protein.

3.4. Mechanism of thermal denaturation

The thermal transitions of ribonuclease A at pH 5.5 are irreversible under a certain concentration range (50–200 mM) of 4-chlorobutan-1-ol, and reversible above and below this concentration. In the presence of 50 mM 4-chlorobutan-1-ol, two distinct endotherms are observed. The irreversible thermal transitions are strongly scan rate dependent suggesting that the denaturation in this concentration range (50 mM $< c <$ 250 mM) of the alcohol is under kinetic control. The following mechanism can be proposed for the thermal unfolding of ribonuclease A in presence of 4-chlorobutan-1-ol at pH 5.5:

(a) Below 50 mM 4-chlorobutan-1-ol, ribonuclease A at pH 5.5 follows the two-state reversible model, $N \rightleftharpoons D$ where N and D refer to the native and reversibly denatured states of the protein, respectively.
(b) In the presence of 50 mM 4-chlorobutan-1-ol, ribonuclease A at pH 5.5 follows the mechanism: $N \rightleftharpoons D \rightarrow F$ where the transition $N \rightleftharpoons D$ occurs at 58.6 °C, and $D \rightarrow F$ occurs at 73.0 °C. Here F refers to the final unfolded conformational state of the protein.
(c) Between 50 and 250 mM 4-chlorobutan-1-ol concentrations, the data corresponding to the irreversible thermal denaturation conform to the two-state irreversible model, $N \rightarrow F$, yielding an activation energy of $168 \pm 11\ kJ\ mol^{-1}$.
(d) At and above 250 mM 4-chlorobutan-1-ol concentration, the thermal denaturation of ribonuclease A at pH 5.5 is reversible following the two-state behavior, $N \rightleftharpoons D$.

Similar mechanisms are observed for ribonuclease A in presence of 4-chlorobutan-1-ol at pH 3.9, wherein the thermal transitions are partially reversible between 25 and 100 mM, completely irreversible between 100 and 150 mM, and reversible between 150 and 300 mM alcohol concentration.

These DSC results are well supported by the CD and fluorescence data. CD studies on ribonuclease A in presence of 50 mM 4-chlorobutan-1-ol at 83.0 °C at both pH 5.5 and 3.9 show the presence of significant amounts of secondary structure and loss of the tertiary structure of the protein. These results support the formation of an intermediate structure in the protein in the presence of 50 mM 4-chlorobutan-1-ol at temperature corresponding to second transition at both pH 5.5 and 3.9. The results also demonstrate high denaturing capacity of 4-chlorobutan-1-ol. For example, the presence of just 400 mM alcohol brings down the transition temperature of the protein by 35.0 °C. At low concentration of the alcohol in solution, the two-state reversible thermal unfolding of ribonuclease A at lower temperature compared to that in the absence of alcohol could be explained by preferential interaction arguments discussed above. In the intermediate concentration range (50 mM $< c <$ 250 mM) of the alcohol, it appears from the DSC data on the concentration dependence of the transition temperature in the presence of 100 mM alcohol that the small change in the extent of aggregation upon unfolding in the above-mentioned concentration range of the alcohol is responsible for the irreversibility in the thermal transitions. However, in the presence of 250 mM and higher concentration of the alcohol, the re-appearance of the reversibility in the thermal transitions could be due to the absence of aggregation in this concentration range where the alcohol can dissolve protein aggregates by weakening hydrophobic interactions responsible for associations among protein molecules. Dissolution of peptide aggregates by alcohols such as HFIP and ethanol has also been reported in literature [33].

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References

[1] Shiraki K, Nishikawa K, Goto Y. J Mol Biol 1995;245:18.
[2] Nelson J, Kallenbach NR. Biochemistry 1989;28:5256.
[3] Buck M, Schwalbe H, Dobson CM. Biochemistry 1995;34:13219.
[4] Hamada D, Kuroda Y, Tanaka T, Goto Y. J Mol Biol 1995;254:737.
[5] Chitra R, Smith PE. J Chem Phys 2001;114:426.
[6] Mchaourab HS, Hyde JS, Feix JB. Biochemistry 1993;32:11895.
[7] Sirangelo I, Piaz FD, Malmo C, Cassilo M, Birolo L, Pucci P, et al. Biochemistry 2003;42:312.
[8] Hirota N, Mizuno K, Goto Y. Protein Sci 1997;6:416.
[9] Gasset M, Baldwin MA, Lloyd DH, Gabriel JM, Hotzman DM, Cohen F, et al. Proc Natl Acad Sci USA 1992;89:10940.
[10] Zhang H, Kaneko K, Nguyen JT, Livshits TL, Baldwin MA, Cohen FE, et al. J Mol Biol 1995;250:514.
[11] Barrow CJ, Yasuda A, Kenny PT, Zagorski MG. J Mol Biol 1992;225:1075.
[12] Snyder SW, Ladror US, Wade WS, Wang GT, Barrett LW, Matayoshi ED, et al. Biophys J 1994;67:1216.
[13] Wood SJ, Maleef B, Hart T, Wetzel R. J Mol Biol 1996;256:870.
[14] Tsong TY, Hearn RP, Wrathall DP, Sturtevant JM. Biochemistry 1970;9:2666.
[15] Sabulal B, Kishore N. J Chem Soc Faraday Trans 1995;91:2101.
[16] Bismuto E, Gratton E, Sirangelo I, Irace G. Eur J Biochem 1993;218:213.
[17] Privalov PL, Khechinashvili NN. J Mol Biol 1974;86:665.
[18] Sturtevant JM. Annu Rev Phys Chem 1987;38:463.

[19] Franks F, Eagland D. CRC Crit Rev Biochem 1975;3:165.
[20] Franks F, Ives DJG. Q Rev Chem Soc 1965;20:1.
[21] Kovrigin EL, Potekhin SA. Biochemistry 1997;36:9195.
[22] Kitamura S, Sturtevant JM. Biochemistry 1989;28:3788.
[23] Kita Y, Arakawa T, Lin TY, Timasheff SN. Biochemistry 1994; 33:15178.
[24] Lin TY, Timasheff SN. Biochemistry 1994;33:12695.
[25] Timasheff SN. Biochemistry 1992;31:9857.
[26] Lumry R, Eyring H. J Phys Chem 1954;58:110.
[27] Sanchez-Ruiz JM, Lopez JL, Cortijo M, Mateo PL. Biochemistry 1988;27:1648.
[28] Galisteo ML, Mateo PL, Sanchez-Ruiz JM. Biochemistry 1991; 30:2061.
[29] Tomazic SJ, Kilbanov AM. J Biol Chem 1998;263:3086.
[30] Hernandez-Arana A, Rojo-Dominguez A, Altamirano MM, Calcagno ML. Biochemistry 1993;3:3644.
[31] Semisotnov GV, Rodionova NA, Razgulyaev OI, Uversky VN, Gripas AF, Gilmanshin RI. Biopolymers 1991;31:119.
[32] Buck M, Schwalbe H, Dobson CM. Biochemistry 1995;34: 13219.
[33] Sabaté R, Estelrich J. Int J Biol Macromol 2003;32:10.