

Thermodynamics of the interaction of some chloro- and fluoro-substituted alcohols with bovine α -lactalbumin

Nand Kishore* and Baby Sabulal

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

The temperature dependence of the excess heat capacity and evaluation of thermodynamic parameters of bovine α -lactalbumin has been studied by high-sensitivity micro differential scanning calorimetry in the presence of 4-chlorobutan-1-ol, *n*-butanol, 3-chloropropan-1-ol, 3-chloropropan-1,2-diol, propan-1,2-diol, *n*-propanol, 2,2,2-trifluoroethanol, and ethanol at neutral pH. The chlorosubstituted alcohols are observed to be more effective destabilisers of α -lactalbumin compared with their normal alcohols in the order: 4-chlorobutan-1-ol > *n*-butanol > 3-chloropropan-1-ol > *n*-propanol > 2,2,2-trifluoroethanol > 3-chloropropan-1,2-diol > ethanol > propan-1,2-diol. A reversible two-state approximation for the unfolding of the protein has been shown in the absence and presence of these alcohols. A second, smaller, endothermic transition, 23 °C beyond the main endotherm was observed only in the presence of 0.125 to 0.250 mol dm⁻³ 3-chloropropan-1-ol. Concentrations higher than 25 mmol dm⁻³ 4-chlorobutan-1-ol resulted in total loss of an observable endotherm. Thermal destabilisation of the protein in these solvent systems is explained on the basis of competing patterns of interactions of the cosolutes with the native *versus* unfolded states of the protein during the native \rightleftharpoons denatured reaction. These results are supported by intrinsic fluorescence, energy transfer and UV difference measurements.

An understanding of the partially folded proteins is very important for the characterization of conformational transitions and folding intermediates in the study of protein folding. For many proteins, partially folded states have been observed which are stable at equilibrium.¹⁻⁷ One approach to generating the partially folded state of proteins is to make use of non-aqueous solvents. Short chain alcohols are known^{3,8-12} to stabilize the structure of partially folded states of small proteins and of biologically active peptides. However, it is not very clear whether such solvents stabilize only native-like structures or whether the stabilization is non-specific. 2,2,2-trifluoroethanol (TFE), which has been considered as structure-stabilizing solvent, has been commonly employed to stabilize peptide conformation in solution.^{3,4} Not much work has been done on the effect of TFE and chloro-substituted alcohols on the conformation of intact proteins.

We have recently reported the thermal denaturation of hen egg-white lysozyme in the presence of several chloro-substituted and normal alcohols at different pH values.¹³ It was observed that all the thermal denaturations are two-state and reversible, except in the presence of 4-chlorobutan-1-ol which induced an unusual intermolecular cooperativity in the protein, especially at pH 4.5. A study of α -lactalbumin and hen egg-white lysozyme becomes very important in this regard because of the similarity in their structures. A stable and compact molten globule state has been observed during the equilibrium denaturation of α -lactalbumin, however, hen egg-white lysozyme undergoes a highly cooperative unfolding transition with only native and highly unfolded states significantly populated at equilibrium.¹⁴

In view of the importance of short-chain alcohols in understanding the partially folded states of proteins, we have looked at the effects of 4-chlorobutan-1-ol, *n*-butanol, 3-chloropropan-1-ol, 3-chloropropan-1,2-diol, propan-1,2-diol, *n*-propanol, 2,2,2-trifluoroethanol and ethanol, on the conformational stability and the heat denaturational behaviour of bovine α -lactalbumin. Our objective is to study the nature of the folding-unfolding process of the protein and to provide an assessment of the thermal destabilisation or stabilization effects in quantitative terms over a wide concentration range

of these alcohols. A study on the effects of alcohols on the conformation of proteins is also important owing to their mixed hydrophilic-hydrophobic character.

Experimental

Materials

α -Lactalbumin (Type I), lyophilized powder was obtained from Sigma Chemical Company. The stock solutions of α -lactalbumin were prepared by extensive dialysis of the protein at 4 °C in 20 mmol dm⁻³ TRIS [tris(hydroxymethyl)aminomethane] buffer obtained from Sigma at pH 7.0 containing 0.1 mol dm⁻³ NaCl with at least four changes. 4-chlorobutan-1-ol, *n*-butanol, 3-chloropropan-1-ol, 3-chloropropan-1,2-diol, propan-1,2-diol, and *n*-propanol were obtained from Fluka, and 2,2,2-trifluoroethanol and ethanol were obtained from Sigma. The pH of the solutions was measured using a standardized Control Dynamics pH meter at room temperature. Protein concentrations were measured spectrophotometrically on a Shimadzu double-beam spectrophotometer UV-160A using absorbance $E^{1\%}_{280} = 20.1$.¹⁵

Calorimetry

All the calorimetric scans were performed with an SETARAM micro differential scanning calorimeter (DSC). The samples were degassed for *ca.* 10 min prior to being scanned at rates of 30 K h⁻¹. The capacity of the sample and the reference cell of the calorimeter is 1 cm³. The volume of the solution containing protein or protein and cosolute was kept constant at 0.85 cm³, and the weights of the solutions in the sample and the reference cells were always matched. The reference solution was buffer when the measurements were made in the buffer, or buffer plus cosolute when the experiments were performed in the presence of the cosolute. All excess power *versus* temperature scans for α -lactalbumin transitions were obtained by subtracting the power input of a thermal scan of solvent *versus* solvent from the power input scan of the solvent *versus* solution. These corrected excess power thermal scans were also corrected for the thermal lag of the DSC by the Tian

equation, and were then converted to excess heat capacity *versus* temperature scan by dividing by the scan rate. The reversibility of the DSC scans was checked by heating the sample to a little over the transition maximum, cooling immediately and then re-heating.

Calorimetry data analysis

The corrected DSC data were analysed by the EXAM program of Kirchhoff,¹⁶ which gives least-squares fits of the data to a two-state model including the base lines

$$C_p(T) = (1 - \alpha)[B_a + B'_a(T - T_m)] + N[\Delta H(T)^2/RT^2]F(\alpha) + \alpha[B_b + B'_b(T - T_m)] \quad (1)$$

following the procedures described by Schwarz and Kirchhoff.¹⁷ In this expression, N is the number of moles of cooperative units per mole of the protein in the calorimetric cell and can be varied as one of the parameters of the fit or held fixed at some predetermined value. The pre-transition asymptotic base line, $B_a + B'_a(T - T_m)$ and post-transition base line, $B_b + B'_b(T - T_m)$ are determined independently with linear least-square fits of low-temperature and high-temperature data, respectively. The transition parameters of the endotherm $\Delta_{\text{cal}}H$, $t_{1/2}$, N and ΔC_p are determined by an iterative non-linear fit of the above equation using analytical derivatives and holding the base-line parameters fixed. The deviation of N from unity provides information on the cooperativity of the denaturation transition. For a reaction with unit stoichiometry in which the number of moles of the denatured species is the same as the number of moles of the native protein, $F(\alpha)$ is given by $(1 - \alpha)$ where α is the molar fraction of the reaction product and is related to the equilibrium constant by

$$\Delta G^\circ = -RT \ln K = -RT \ln[\alpha/(1 - \alpha)] \quad (2)$$

The temperature dependence of ΔH was expressed as a power series in T about $T_{1/2}$ ($T_{1/2} = 273.15 + t_{1/2}$), the midpoint of the transition where the area under the transition profile is one half the total area

$$\Delta H(T) = \Delta H(T_{1/2}) + \Delta C_p(T - T_{1/2}) \quad (3)$$

The temperature dependence of the Gibbs energy change required to bring the system to standard state was obtained by substituting the above equation into the Gibbs-Helmholtz equation and integrating between T and $T_{1/2}$ to give

$$\Delta G^\circ/RT = \Delta H^\circ(T_{1/2} - T)/(RT_{1/2}) + \Delta C_p/R \ln(T_{1/2}/T) + \Delta C_p(T - T_{1/2})/(RT) \quad (4)$$

where the standard-state temperature $T_{1/2}$ is the temperature at which the equilibrium constant is unity and the number of moles of native and denatured protein are equal, and ΔH° is the standard enthalpy change at that temperature.

The van't Hoff enthalpies were calculated according to the following expression,

$$\Delta_{\text{vH}}H = (ART_{1/2}^2 C_{\text{ex}, 1/2})/\Delta_{\text{cal}}H \quad (5)$$

where $C_{\text{ex}, 1/2}$ is the excess over the base line value of apparent specific heat at $T_{1/2}$, R is the gas constant and $A = 4.0$ for a two-state process involving neither association nor dissociation. If the process is strictly two state, which is a reversible process following the van't Hoff equation

$$d \ln K/dT = \Delta_{\text{vH}}H/RT^2$$

the ratio of the van't Hoff to calorimetric enthalpy, hereafter designated by the parameter β , is unity. It has frequently been observed that β may differ significantly from unity. Several possible causes for such a deviation include some degree of aggregation in either the initial or the final state or both, and

variation in the domain interactions during unfolding of multi-domain proteins.¹⁸

UV-VIS spectrophotometry

The UV difference spectroscopy was performed on a Shimadzu UV-160 A spectrophotometer at room temperature. The protein concentration in the sample and reference cells was kept identical and the effect of a varied amount of the alcohol in the sample cell was studied. As a result, the final signal is due to the effect of added alcohol.

Fluorescence spectroscopy

Fluorescence measurements were carried out on a SPEX-DM 1B spectrofluorimeter at room temperature. For the intrinsic fluorescence study, the protein sample was excited at 295 nm to probe the conformational change around the tryptophan residues and the emission wavelength was set at 315 nm in all cases. The emission spectra obtained for the sample was always compared with the corresponding reference buffer, or cosolute in buffer. In all the intrinsic fluorescence measurements, the protein concentration was kept at 0.1 mg ml^{-1} and the concentration of the alcohol was varied. ANS (1-anilino-naphthalene-8-sulfonate) binding and fluorescence energy transfer experiments were performed with the respective excitation wavelengths at 365 and 295 nm. Emission spectra of solutions of $5.8 \times 10^{-5} \text{ mol dm}^{-3}$ in ANS and $30 \mu\text{mol dm}^{-3}$ in α -lactalbumin were thus recorded as a function of alcohol concentration. The reference spectra were recorded with corresponding amounts of ANS and alcohol in buffer solution and subtracted from the original scan.

Results and Discussion

The heat capacity of holo- α -lactalbumin measured at pH 7.0 in 20 mmol dm^{-3} TRIS is shown in Fig. 1. The transition is fully reversible, as observed by repeated scans of the same sample. The thermal unfolding of α -lactalbumin is characterized by a single peak centred at 64.5°C , and analysis of the excess heat capacity obtained after base-line subtraction yields a calorimetric enthalpy of $277 \pm 6 \text{ kJ mol}^{-1}$. The ratio of van't Hoff enthalpy, calculated by eqn. (5), to the measured calorimetric enthalpy, the parameter β , is equal to 1.00 ± 0.02 , in good agreement with the two-state unfolding nature at neutral pH and at different concentrations of TRIS as reported by Griko *et al.*¹⁹

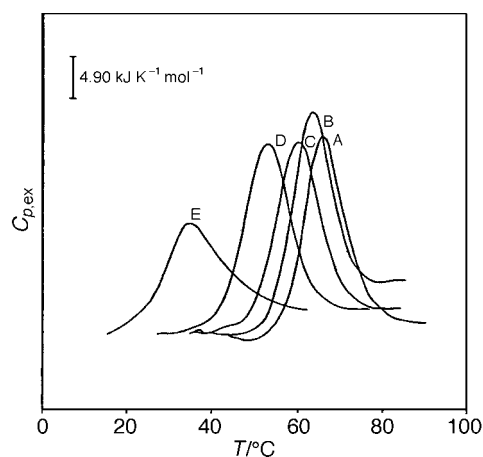


Fig. 1 DSC scans of the thermal denaturation of $0.34 \text{ mmol dm}^{-3}$ α -lactalbumin in the presence of 2,2,2-trifluoroethanol in 20 mmol dm^{-3} TRIS at pH 7.0 and a heating rate of 30 K h^{-1} : A, 0.0; B, 0.25; C, 0.50; D, 1.0 and E, 2.0 mol dm^{-3}

The thermodynamic parameters, transition temperature ($t_{1/2}$), heat capacity of denaturation (ΔC_p), calorimetric enthalpy of unfolding ($\Delta_{cal}H$), and the cooperativity ratio β have been deposited as supplementary data.† With increasing concentration of 4-chlorobutan-1-ol, *n*-butanol, 3-chloropropan-1-ol, 3-chloropropan-1,2-diol, propan-1,2-diol, *n*-propanol, 2,2,2-trifluoroethanol and ethanol, the thermal stability of α -lactalbumin decreases. Fig. 1 shows the representative endotherms from which the instrumental baseline determined with buffer or buffer and cosolute in each cell has been subtracted, for α -lactalbumin concentration of 0.359 mol dm⁻³ at pH 7.0 in the presence of varying concentrations of 2,2,2-trifluoroethanol. The decrease in the transition temperature ($\Delta t_{1/2}$) upon addition of alcohols to α -lactalbumin is shown in Fig. 2 and obeys the following order:

4-chlorobutan-1-ol > *n*-butanol > 3-chloropropan-1-ol > *n*-propanol > 2,2,2-trifluoroethanol > 3-chloropropan-1,2-diol > ethanol > propan-1,2-diol.

In general, the chloro- or fluoro-substituted alcohols provide greater thermal destabilisation to α -lactalbumin than the normal alcohols.

From DSC scans, we find that the thermal unfolding of α -lactalbumin in the presence of 3-chloropropan-1,2-diol, propan-1,2-diol, *n*-propanol, 2,2,2-trifluoroethanol, ethanol and *n*-butanol is reversible and two state over the whole concentration range of alcohols studied. The unfolding and refolding processes over this range are apparently unchanged. This permits the application of equilibrium thermodynamics to the unfolding data.

A comparison of the thermodynamic data associated with the thermal unfolding of α -lactalbumin in 3-chloropropan-1-ol, 3-chloropropan-1,2-diol, propan-1,2-diol and *n*-propanol, all with the same number of carbon atoms, indicates that 3-chloropropan-1-ol reduces the transition temperature of the protein the most, and propan-1,2-diol the least. Thus (1) the alcohol with the same number of carbon atoms but with chlorine replacing hydrogen is more effective in decreasing $t_{1/2}$; (2) the alcohol with the higher number of carbon atoms is a more effective destabiliser of α -lactalbumin than its lower member; (3) the presence of an additional —OH group in the alcohol reduces its effect in lowering the $t_{1/2}$ value of α -lactalbumin.

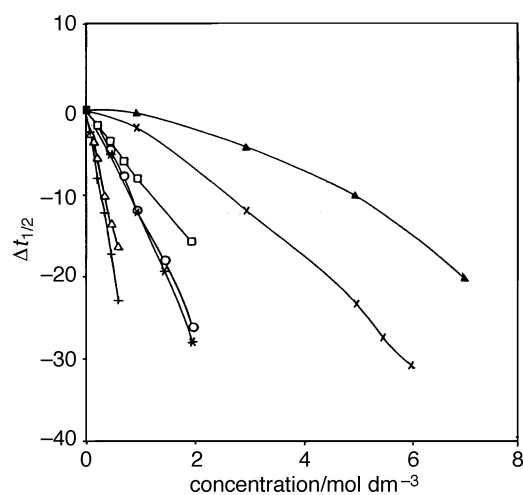


Fig. 2 Relative fall in the transition temperature ($\Delta t_{1/2}$) of α -lactalbumin as a function of alcohol concentration in 20 mmol dm⁻³ TRIS at pH 7.0: (+) *n*-butanol; (Δ) 3-chloropropan-1-ol; (\square) 3-chloropropan-1,2-diol; (\blacktriangle) propan-1,2-diol; (*) *n*-propanol; (\circ) 2,2,2-trifluoroethanol; (\times) ethanol

† Available as supplementary material (SUP 57349; 6 pp.) deposited with the British Library. Details are available from the editorial office.

The effect of cosolvents on protein stability is defined by a balance between their preferential interactions with the two end-states of the protein.^{20–22} During this $N \rightleftharpoons D$ reaction, new extensive non-polar hydrophobic and polar peptide groups become exposed to the solvent and the distance between the charges increases greatly. It is clear, from the deposited data,† that the hydrophobic effects of alcohols are larger on the unfolded state of α -lactalbumin because of a larger exposure of the constituent groups of the protein exposed to the solvent than in the native state. All the alcohols studied with partly hydrophobic properties interact favourably with these hydrophobic side chains exposed on the protein, thus leading to the stabilization of the denatured state. The organization of water in the presence of alcohols^{23,24} as well as the direct binding of alcohols to the non-polar groups of the proteins²⁵ has been reported. A reduction in the effect of the hydrophobic groups of the alcohol on the organization of the solvent because of this competitive binding to the hydrophobic groups exposed on protein denaturation leads to an increase in the net entropy of denaturation. This shifts the $N \rightleftharpoons D$ equilibrium toward the denatured state and manifests in the lowering of the $t_{1/2}$ values. The larger fall in the $t_{1/2}$ values by the alcohol with the greater number of —CH₂— groups is explained by the increased hydrophobic effect.

Relative to *n*-propanol, 3-chloropropan-1-ol acts as the stronger stabiliser of the denatured state. This can be attributed to a higher electronegativity of chlorine, compared to that of carbon and hydrogen, which enhances preferential binding of this group through electrostatic interactions with the polar sites exposed to the solvent during the unfolding of the protein. 3-Chloropropan-1,2-diol is also observed to reduce the thermal stability of α -lactalbumin at all the concentrations studied. The transition temperature falls with an increase in the concentration of the diol. As observed earlier with the protein lysozyme,¹³ 3-chloropropan-1,2-diol is a less potent destabiliser of the protein than 3-chloropropan-1-ol and 1-propanol. Another similar observation is that the effectiveness of 3-chloropropan-1-ol in reducing the transition temperature of α -lactalbumin is larger than that by 3-chloropropan-1,2-diol at higher concentration. As an example, $\Delta\Delta t_{1/2}$ [$\Delta t_{1/2}$ (3-chloropropan-1,2-diol) — $\Delta t_{1/2}$ (3-chloropropan-1-ol)] is 3.9 at 0.25 mol dm⁻³ and 10.0 at 0.5 mol dm⁻³. This $\Delta\Delta t_{1/2}$ observed with the protein lysozyme was 5.3 at 0.5 mol dm⁻³ 3-chloropropan-1,2-diol. This comparison indicates the stronger destabilising effect of these alcohols on α -lactalbumin than on lysozyme.

It has been observed that ethylene glycol stabilizes certain proteins.^{26,27} This effect has been explained²⁷ on the basis of the preferential hydration of the protein with increasing number of hydroxy groups on the cosolvent molecules. The preferential hydration favours the compact native state and disfavours an increase in the surface area of the protein. In DSC experiments, this situation should be reflected by an increase in the transition temperature or a reduction in the effectiveness of the diol as destabiliser compared with the monohydric alcohol having the same number of carbon atoms. This is observed in the $\Delta\Delta t_{1/2}$ values given above obtained with the interaction of 3-chloropropan-1-ol and 3-chloropropan-1,2-diol with the protein.

Comparing the results for 2,2,2-trifluoroethanol and ethanol, it is observed that the former is a stronger destabiliser of α -lactalbumin (Fig. 2). The shift of the $N \rightleftharpoons D$ equilibrium towards the denatured state in the presence of TFE reflects weaker internal protein hydrophobic interactions with increasing concentration of TFE. Again, the value for β shows that the denaturation process under this concentration range of TFE is two-state reversible.

In the presence of 0.125, 0.187 and 0.250 mol dm⁻³ 3-chloropropan-1-ol, the thermal denaturation of α -lactalbumin

resulted in a second small endothermic transition beyond the first major peak at a difference of nearly 23 °C (Fig. 3). Deconvolution of these DSC traces, by excluding the second peak, gave a β value close to unity. If the first scan is stopped just past the first transition, cooled and then re-heated, both the peaks are found at their same positions with the same areas under their respective endotherms. Rescanning the sample after heating beyond the second peak resulted in an unstable signal without endotherm, and the solution was clear without any turbidity. Below and above the concentration range 0.125–0.250 mol dm⁻³ 3-chloropropan-1-ol, thermal denaturation of α -lactalbumin resulted in only one endotherm with a calorimetric reversibility of more than 90%. From the DSC studies on holo α -lactalbumin at low pH, and apo- α -lactalbumin in 500 mmol dm⁻³ TRIS at pH 8, Griko *et al.*¹⁹ observed a highly cooperative transition followed by a gradual increase in the heat capacity. They reported that acid or heat denatured α -lactalbumin does not unfold completely and that the molten globule state is accompanied by a gradual increase in heat capacity. The transition of the compact denatured state to the unfolded state was reported to be non-cooperative.

Following the DSC scan from 30 to 90 °C, UV difference spectroscopy of α -lactalbumin in the presence of 0.125 0.187 and 0.250 mol dm⁻³ 3-chloropropan-1-ol was performed after transferral of the sample from the DSC cell to the UV sample cuvette. An equal amount of the protein was maintained in the buffer of the reference cuvette. The UV difference spectrum recorded in the range 240–320 nm showed a small peak in the range 303–305 nm. This indicates that the second small endotherm, and the absence of the characteristic DSC main endotherm upon rescanning after heating to 90 °C, is due to unknown post-major endotherm effects leading to irreversibility. The reversibility of the DSC endotherms in the certain concentration range of 3-chloropropan-1-ol indicates that the alcohol does not arrest the refolding of α -lactalbumin from the denatured state upon cooling.

The DSC scans of α -lactalbumin in the presence of varying concentrations of *n*-butanol were two-state reversible with the transition temperature decreasing with increase in the concentration of the alcohol. The thermal denaturation of α -lactalbumin in the presence of 5 mmol dm⁻³ 4-chlorobutan-1-ol resulted in an endotherm with a mid-point of transition at 64.8 °C with instability in the DSC signal above this temperature. The value is same for the unfolding of α -lactalbumin without alcohol. At 25 mmol dm⁻³ 4-chlorobutan-1-ol, the area under the endotherm was very small, again with instability in the DSC output after the mid point of transition. In both cases, re-heating resulted in a

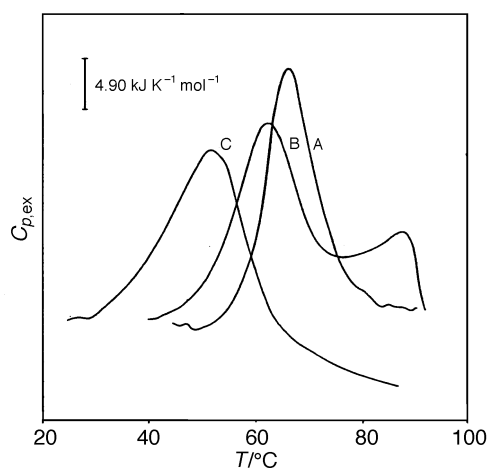


Fig. 3 DSC scan of α -lactalbumin at pH 7.0 in 20 mmol dm⁻³ TRIS in the presence of 3-chloropropan-1-ol: A, 0.0; B, 0.125 (showing a second endotherm) and C, 0.500 mol dm⁻³

signal without any endotherm. No endotherm was observed in the presence of 4-chlorobutan-1-ol at 63 mmol dm⁻³ and higher concentrations up to 500 mmol dm⁻³. At concentrations higher than 500 mmol dm⁻³ of the alcohol, precipitation was observed in the solution.

In order to have more insight into the interactions, intrinsic fluorescence studies were carried out on α -lactalbumin in the presence of 5 to 500 mmol dm⁻³ 4-chlorobutan-1-ol. The concentration of α -lactalbumin in all the fluorescence experiments was kept at 0.1 mg ml⁻¹. α -Lactalbumin contains four tryptophans at positions 26, 60, 104 and 118 and, at neutral pH, all the tryptophans are buried in the interior of the protein.²⁸ Excitation at 295 nm of the tryptophanyl residues results in maximal emission at 336.5 in 20 mmol dm⁻³ TRIS buffer at pH 7.0, consistent with reported values in the literature.^{29,30} The emission maxima in the absence and presence of 5 mmol dm⁻³ 4-chlorobutan-1-ol were both at 337.5 nm. A similar match was observed in $t_{1/2}$ and $\Delta_{cal}H$ values, agreeing with this observation. In the presence of 20–500 mmol dm⁻³ 4-chlorobutan-1-ol, the emission maxima were observed in the range 354–357 nm, close to that observed (358 nm) in the presence of 6 mol dm⁻³ guanidinium chloride (GuHCl) indicating a larger degree of exposure of previously buried non-polar residues to the solvent.

ANS is believed to bind to the hydrophobic region in the proteins.⁴ In the presence of 5 mmol dm⁻³ 4-chlorobutan-1-ol, the emission maxima was observed at 547.5 nm which is nearly the same value as for pure ANS in 20 mmol dm⁻³ TRIS at pH 7.0. At higher than 20 mmol dm⁻³ levels of 4-chlorobutan-1-ol, the α -lactalbumin emission maxima and intensity increased somewhat as shown in Fig. 4, indicating increased binding of ANS to the protein as a result of alcohol induced exposure of hydrophobic groups from within the protein. Energy transfer studies at an excitation wavelength of 295 nm showed an increase in the values of emission maxima from 482 to 487 nm (red shift), and no appreciable change in intensities with increasing amounts of the alcohol (Fig. 5). The result obtained in the presence of 5 mmol dm⁻³ 4-chlorobutan-1-ol indicates that a major fraction of α -lactalbumin is in the native state. The shift in the emission maximum from 336.5 obtained for α -lactalbumin in TRIS buffer, to 482 to 487 nm in 20 to 500 mmol dm⁻³ 4-chlorobutan-1-ol, indicates a high degree of exposure of the hydrophobic groups on the protein and resultant energy transfer.

In UV-difference measurements, the output signal for α -lactalbumin in the presence of 5 mmol dm⁻³ 4-chlorobutan-1-ol was essentially the same as in the absence of alcohol in the range 240–320 nm. At higher concentrations of 4-

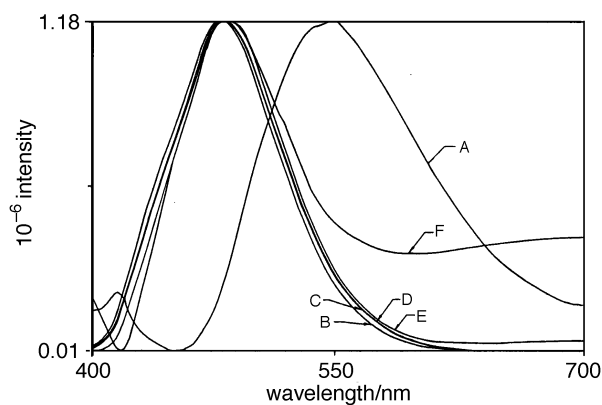


Fig. 4 Fluorescence emission spectra obtained from ANS (5.8×10^{-5} mol dm⁻³) binding studies on 30 μ mol dm⁻³ α -lactalbumin at pH 7.0 in 20 mmol dm⁻³ TRIS with increasing concentrations of 4-chlorobutan-1-ol: A, 0.005; B, 0.020; C, 0.063; D, 0.125; E, 0.250; F, 0.500 mol dm⁻³

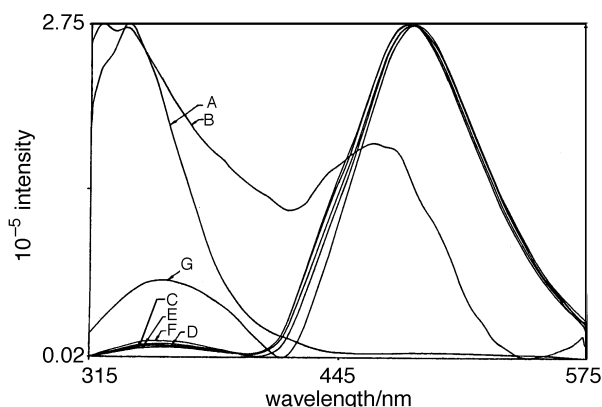


Fig. 5 Fluorescence emission spectra obtained from ANS ($5.8 \times 10^{-5} \text{ mol dm}^{-3}$) energy transfer studies on $30 \mu\text{mol dm}^{-3}$ α -lactalbumin at pH 7.0 in 20 mmol dm^{-3} TRIS with increasing amounts of 4-chlorobutan-1-ol: A, 0.0; B, 0.005; C, 0.020; D, 0.063; E, 0.125; F, 0.250 and G, 0.500 mol dm^{-3}

chlorobutan-1-ol, a signal of two peaks was observed as shown in Fig. 6 in the range 282–297 nm, one centred at 285 nm and the other at 293 nm. An increase in the signal intensity with increasing concentration of alcohol from 20 to 500 mmol dm^{-3} indicates the larger exposure of the constituent groups of the protein to the solvent. The UV-difference spectra of α -lactalbumin in 20 mmol dm^{-3} TRIS at pH 7.0 in the presence of 6 mol dm^{-3} GuHCl resulted in a similar signal at the same wavelength. The peak around 293 nm indicates the exposure of tryptophan residues in proteins.³¹ Solvent perturbation studies of globular proteins in the presence of urea, methanol, ethylene glycol, and 2-chloroethanol solutions have shown that aromatic residues in the interior of the protein are largely exposed and accessible to the solvent.^{31–33}

The results of the DSC, fluorescence, and UV difference spectroscopy measurements indicate that α -lactalbumin at pH 7.0 in the presence of 63 mmol dm^{-3} and higher concentrations of 4-chlorobutan-1-ol is in the unfolded state at room temperature. The absence of a DSC endotherm, intrinsic fluorescence emission maxima, ANS binding and energy transfer studies for α -lactalbumin in the presence of 63 to 500 mmol dm^{-3} 4-chlorobutan-1-ol indicate an exposure of the interior

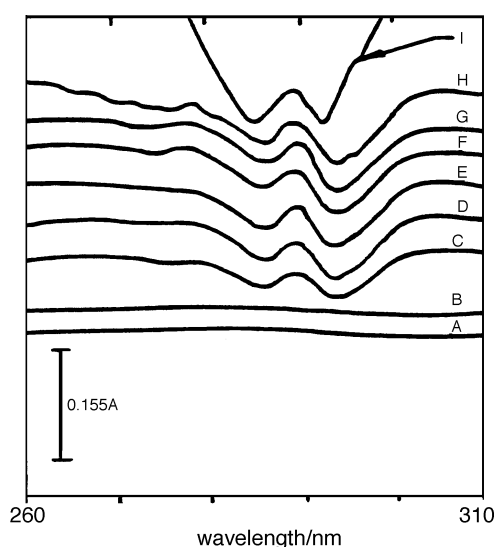
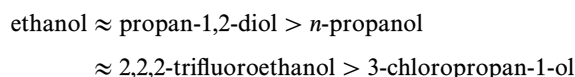


Fig. 6 UV difference spectrum of α -lactalbumin at pH 7.0 in 20 mmol dm^{-3} TRIS with increasing amounts of 4-chlorobutan-1-ol and 6 mol dm^{-3} guanidinium chloride: A, 0.0; B, 0.005; C, 0.020; D, 0.063; E, 0.125; F, 0.187; G, 0.250; H, 0.500 and I, 6 mol dm^{-3} guanidinium chloride

hydrophobicity of the protein comparable to that achieved by 6 to 8 mol dm^{-3} urea and GuHCl, respectively. The strong denaturing tendency of 4-chlorobutan-1-ol is due to the long hydrocarbon chain, and the chlorine terminal causing a dipole on the alcohol end also adds to the shift of the $\text{N} \rightleftharpoons \text{D}$ equilibrium to the right.

The calorimetric enthalpy showed a complex dependence on the concentration of the alcohols in the protein solution, except in the presence of 3-chloropropan-1,2-diol (Fig. 7). The calorimetric enthalpy, $\Delta_{\text{cal}}H$ first increased with increasing concentration of the alcohol then decreased. The maximum in the values of $\Delta_{\text{cal}}H$ occurred in the following order:



Similar observations were made by Sturtevant *et al.*³⁴ and Fujita *et al.*³⁵ with hen egg-white lysozyme in the presence of methanol, ethanol, *n*-propanol and *n*-butanol. Two important points are to be noted here, (i) the maximum in the values of $\Delta_{\text{cal}}H$ observed decreases with the increasing length of the alkyl chain and (ii) this order of variation in the maxima of $\Delta_{\text{cal}}H$ values with concentration of alcohol is nearly opposite to that observed in the $\Delta t_{1/2}$ values.

As discussed earlier, the hydrophobic part of the alcohol can interact selectively with those groups exposed on the protein upon denaturation, leading to weakening of the hydrophobic interactions between the non-polar groups of the protein. It has been claimed³⁶ that breaking of the hydrophobic bond in aqueous solution is an exothermic process rather than an endothermic one. Hence, the maxima observed in the plot of $\Delta_{\text{cal}}H$ versus concentration of the alcohol can be attributed to the reduction in exothermic contribution of the hydrophobic bond breakage to the total experimentally observed calorimetric enthalpy of unfolding. Non-aqueous solvents, particularly alcohols, have also been shown to act as secondary structure stabilisers^{1,3,4} upon addition to aqueous protein solutions. A well defined state of ubiquitin in aqueous methanol that contains elements of the native structure has been reported.³⁷ TFE, in particular, has been used to generate structured states of peptides and to stabilize helical regions.^{3,4,12} It has also been shown that a high degree of stable helical structure is formed after addition of TFE to lysozyme.³ Addition of TFE to bovine, human and guinea pig α -lactalbumin at pH 2.0 has been found, in each case, to induce a conformational transition in the molten globule state of α -lactalbumin. It has been suggested that the TFE stabilizes secondary structure by favouring hydrogen bonds within the

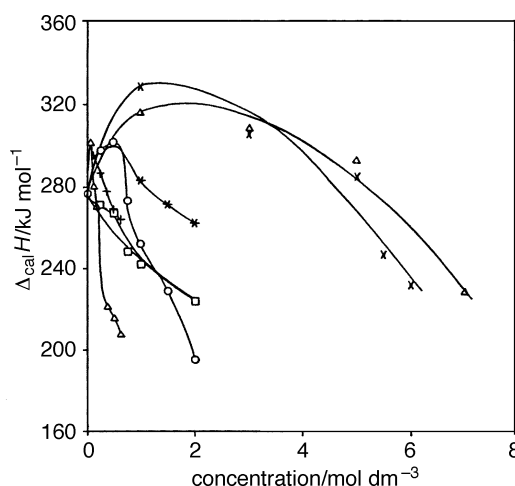


Fig. 7 Dependence of $\Delta_{\text{cal}}H$ of α -lactalbumin on the concentration of alcohols: (+) *n*-butanol; (Δ) 3-chloropropan-1-ol; (\square) 3-chloropropan-1,2-diol; (\blacktriangle) propan-1,2-diol; (*) *n*-propanol; (O) 2,2,2-trifluoroethanol; (x) ethanol

protein chain at the expense of intermolecular hydrogen bonds between the protein and the cosolvent.^{12,38} If it is assumed that the interactions between the polar groups of the protein are responsible for this structure stabilizing propensity, then this factor will also add to the endothermic contribution to the total calorimetric enthalpy. At still higher concentration of the alcohol, the hydrophobic interactions within the protein are weakened to a large extent, leading to a sharp decrease in the values of $\Delta_{\text{cal}}H$. The molten globule state of α -lactalbumin, which has a poorly defined tertiary structure but a secondary structure similar to the native state, has been shown to unfold with lower $\Delta_{\text{cal}}H$ than the holofrom of the protein.¹⁴

The change in the heat capacity, ΔC_p , of the protein upon denaturation, is one of the most important parameters because it essentially controls the variation with temperature of the enthalpy, entropy, and Gibbs energy changes accompanying the process. It has been usually observed³⁹ that ΔC_p obtained from individual scans shows a large scatter from scan to scan, and also that ΔC_p is a function of the circumstances under which the variation of $\Delta_{\text{cal}}H$ with respect to $t_{1/2}$, is observed. Experiments reported by Sontoro *et al.*⁴⁰ show that a varying value for ΔC_p can also result if the temperature of unfolding is changed by means other than change in pH. Large scatter was observed in the plot of $\Delta_{\text{cal}}H$ as a function of $t_{1/2}$, possibly due to the complex concentration dependence of the calorimetric enthalpy on a certain range of the alcohol concentration. If the data is fitted to a straight line, a value of $2.24 \text{ kJ K}^{-1} \text{ mol}^{-1}$ is obtained for ΔC_p with a standard deviation of $0.53 \text{ kJ K}^{-1} \text{ mol}^{-1}$. This value of $2.24 \text{ kJ K}^{-1} \text{ mol}^{-1}$ is close to most of the values deposited as supplementary data,[†] and was used to calculate the thermodynamic parameters at temperatures other than at $t_{1/2}$.

The Gibbs–Helmholtz equation given in eqn. (4) provides a means for determining the change in the Gibbs energy of stabilization or destabilization caused by the presence of alcohols. In the absence of alcohols, the Gibbs energy of unfolding change for α -lactalbumin at the transition temperature ($T = 337.8 \text{ K}$) is zero, whereas the equilibrium constant for a two-state process is unity. In the presence of alcohol/diol, the transition temperature and the calorimetric enthalpy of unfolding at the transition temperature can be combined with the average heat capacity of unfolding to calculate the stability of the protein at 337.8 K . The Gibbs energy change of unfolding for α -lactalbumin varies from zero in the absence of alcohols to as high as $-15.55 \text{ kJ mol}^{-1}$ in the presence of 500 mmol dm^{-3} *n*-butanol. These values, at the same concentration of alcohol, are $-9.69 \text{ kJ mol}^{-1}$ in 3-chloropropan-1-ol, $-2.92 \text{ kJ mol}^{-1}$ in 3-chloropropan-1,2-diol, $-4.80 \text{ kJ mol}^{-1}$ in *n*-propanol, and $-4.34 \text{ kJ mol}^{-1}$ in the presence of 2,2,2-trifluoroethanol. Taking into account one standard deviation in $\Delta_{\text{cal}}H$ and ΔC_p values, the errors in ΔG° values are *ca.* 8%. It is obvious that the accuracy of the values for ΔG° at temperatures far removed from $t_{1/2}$ suffer from uncertainties in ΔC_p . The data indicate that, at 25°C , the protein is destabilised about six-fold more severely by 3-chloropropan-1-ol than by *n*-propanol, a difference caused by the replacement of $-\text{H}$ by $-\text{Cl}$ in the destabilising alcohol. The presence of another hydroxy group in the same alcohol makes it a *ca.* two-fold lesser destabiliser compared with *n*-propanol.

Stability of bovine α -lactalbumin and hen egg-white lysozyme, comparison of thermodynamic and structural data

Bovine α -lactalbumin and hen egg-white lysozyme are structurally homologous proteins diverged from a common ancestral protein with different biological functions. This has stimulated many comparative studies on these proteins. The primary sequence of α -lactalbumin and hen egg-white lysozyme have been shown to be strikingly similar^{28,41} with

regard to both residue identity and position of disulfide bridges. Overall, the main polypeptide fold of α -lactalbumin molecule is held together by four disulfide bridges maintaining a similar conformation of hen egg-white lysozyme with significant changes in the loop regions and at the C-terminus. The C-terminus is reported to be rather flexible in α -lactalbumin compared to lysozyme. We have recently reported DSC studies on the interactions of chloro-substituted alcohols with hen egg-white lysozyme.¹³ The $t_{1/2}$ observed for this protein in 20 mmol dm^{-3} phosphate buffer at pH 2.4 is 64.7°C and that of α -lactalbumin observed in this work at pH 7.0 is 64.6°C . The thermal stabilities of these two proteins under these conditions are the same, as indicated by the stability index $t_{1/2}$. Hence, we will compare the effect of chloro-substituted and normal alcohols on these two proteins under these conditions.

The deposited data[†] gives a comparison of the effect of alcohols on $t_{1/2}$ and $\Delta_{\text{cal}}H$ for α -lactalbumin and hen egg-white lysozyme. The reduction in the thermal transition temperature of α -lactalbumin is always greater than that for hen egg-white lysozyme. For example, in the presence of 0.5 mol dm^{-3} butan-1-ol, the reduction in $t_{1/2}$ in case of α -lactalbumin is nearly double the value for lysozyme. This comparison supports the hypothesis that α -lactalbumin has a less stable and relatively flexible structure than hen egg-white lysozyme.

Acharya *et al.*²⁸ have observed from crystallographic studies that the three-dimensional native conformations of α -lactalbumin have a relatively loose structure compared with the C-type lysozymes. The total solvent accessible area for α -lactalbumin (7241 \AA^2) is greater than that of hen egg-white lysozyme (6844 \AA^2). In α -lactalbumin, 76% of the hydrophobicity is reported to be fully exposed or partly exposed to the media whereas, in hen egg-white lysozyme, the corresponding figure is 66%.³⁶ Again, the C-terminus was found to be more flexible in α -lactalbumin than in lysozyme and the regions between residues 104 and 111 were found to be highly fluctuating.^{41,42} The partial heat capacities of native α -lactalbumin and hen egg-white lysozyme are 1.40 ± 0.05 and $1.30 \pm 0.05 \text{ J K}^{-1} \text{ g}^{-1}$, respectively, at a similar temperature.¹⁹ This difference between the heat capacities of these two structurally homologous proteins is also a reflection of the somewhat looser structure of α -lactalbumin. These observations are consistent with the thermodynamic data reported here.

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