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Insights into the energetics and mechanism underlying the interaction of tetraethylammonium bromide with proteins

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Abstract

Calorimetry has been employed to investigate the quantitative energetic aspects and mechanism underlying protein–tetraethylammonium bromide (TEAB) interactions. Differential scanning calorimetry and UV–Visible spectroscopy have been used to study the thermal unfolding of three proteins of different structure and function (bovine serum albumin, α-lactalbumin, and bovine pancreatic ribonuclease A). The mode of interaction has been studied by using isothermal titration calorimetry, which demonstrates the absence of appreciable specific binding of TEAB to the protein. This suggests the involvement of solvent mediated effects and, possibly weak non-specific binding. The thermal unfolding transitions were found to be calorimetrically reversible for α-lactalbumin and bovine pancreatic ribonuclease A and partially reversible in the case of bovine serum albumin. The results indicate protein destabilization promoted by the TEAB interaction. The preferential interaction parameters of TEAB with α-lactalbumin and ribonuclease A confirm that an increased interaction of the hydrophobic groups of the TEAB with that of the protein upon denaturation is responsible for the reduced thermal stability of the protein. The decrease in the thermal stability of proteins in the presence of TEAB is well supported by a red shift in the intrinsic fluorescence of these proteins leading to conformational change thereby shifting the native ⇌ denatured equilibrium towards right. The forces responsible for the thermal denaturation of the proteins of different structure and function in the presence of TEAB are discussed.

Keywords: Tetraethylammonium bromide; Calorimetry; Fluorescence; Binding; Preferential interaction

1. Introduction

The interaction of proteins with the surrounding solvent environment plays an important role in their conformational characteristics. Studies of these interactions provide insights into the stability and unfolding behaviour of the globular proteins.

Various co-solutes/co-solvents such as guanidine hydrochloride, sodium thiocyanate, magnesium chloride, urea, and alcohols affect proteins in different ways acting as effective probes of their conformations in solutions [1–6]. Investigations of these conformational changes provide valuable information on the role of the solvent in maintaining the native, intermediate, and denatured states of the proteins. The stabilizing or destabilizing effect of the additives on proteins can either be due to direct binding or indirectly through solvent mediated effects that needs to be experimentally investigated.

Salts have long been known to exert strong influence on protein stability [7,8]. At high concentrations, many salts are found to stabilize proteins [9,10] with some notable exceptions such as that of potassium thiocyanate (KSCN) [11,12]. Reported studies indicate that salts promote conformational conversion of the recombinant prion protein into a PrP^{Sc}-like form which is associated with prion diseases [13–15]. The effect of salts on the stability of proteins has been correlated with their preferential interactions with the latter [16–18]. In order to get better insights into the combined effects of electrostatic and hydrophobic interactions on the

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stability of proteins, tetraethylammonium bromide is an appropriate co-solute. This co-solute is expected to influence macromolecular conformation by weakening the attractive or repulsive inter- and intra-chain charge–charge interactions and by affecting hydrophobic interactions through the side chains of the ethyl group. Tetraalkylammonim salts are bulky in nature and are known to orient water molecules around them depending on their alkyl chain [19]. These salts undergo hydrophobic hydration in water that is usually understood as the formation of more ordered and rigid structure of solvent surrounding the solute molecules. Hydrophobic interactions between protein and bulky alkyl groups on tetraalkylammonium ions are believed to play an essential role in inhibiting aggregation properties of phycocyanin [20,21]. Tetraalkylammonium salts are known to act as inhibitors of cholinesterases [22]. They are also known to reduce the temperature required for conversion of the DNA double-strand helix to the coil. This has been explained as being due to their preferential binding to the coil of DNA during heating and preferential hydrophobic interactions with the adenine–thymine bases [23]. The TEAB has been reported to be a ganglion blocking agent [24]. In fact, dosages of TEAB of 20 $mg \cdot kg^{-1}$ result in complete cardiovascular block [25]. It is also known to produce considerable lowering of the blood pressure in animals [26].

In this study, we used a combination of calorimetry and fluorescence spectroscopy to explore the thermal unfolding of bovine serum albumin, α-lactalbumin, and bovine pancreatic ribonuclease A in the presence of TEAB quantitatively. Also, isothermal titration calorimetry has been exploited to understand the mode of interaction of TEAB with proteins. The forces possibly responsible for the thermal denaturation of the proteins of different structure and function in the presence of TEAB are discussed.

2. Materials and methods

2.1. Materials

Bovine serum albumin (fatty acid free), α-lactalbumin (from bovine milk, Type I), and bovine pancreatic ribonuclease A (type IIA) were procured from Sigma–Aldrich Company, USA. Tetraethylammonium bromide (TEAB) was of extra-pure analytical reagent grade purchased from Sisco Research Laboratories India. For thermal denaturation experiments, bovine serum albumin was dialyzed extensively against $20 \cdot 10^{-3}\ mol \cdot dm^{-3}$ potassium phosphate at pH 7.0, α-lactalbumin against $20 \cdot 10^{-3}\ mol \cdot dm^{-3}$ TRIS–HCl at pH 7.0, and ribonuclease A against $20 \cdot 10^{-3}\ mol \cdot dm^{-3}$ sodium acetate at pH 5.5 and $T = 277$ K. The concentration of the protein was determined on a Shimadzu UV-260 double-beam spectrophotometer using the absorbance $A^{1\%}_{1\ cm} = 6.8$ and 20.1 for BSA [27] and α-LA [28], respectively. The concentration of RNase A was determined using a molar absorption coefficient of $98 \cdot 10^{3}\ mol^{-1} \cdot dm^{2}$ [29].

2.2. Differential scanning calorimetric measurements

2.2.1. Thermal denaturation of bovine serum albumin and α-lactalbumin

The thermal denaturation experiments were performed with a SETARAM micro differential scanning calorimeter (DSC) equipped with removable Hastelloy C-276 fluid tight batch cells of 1 cm^3 capacity. The scan rate in all the experiments was kept at $0.5\ K \cdot min^{-1}$. The protein concentration in these experiments was $0.12 \cdot 10^{-3}\ mol \cdot dm^{-3}$ for bovine serum albumin and $0.281 \cdot 10^{-3}\ mol \cdot dm^{-3}$ for α- lactalbumin. The excess power thermal scans were also corrected for the thermal lag of the calorimeter and then converted to excess heat capacity versus temperature scan by dividing by the scan rate. The corrected DSC data were analyzed by the EXAM program of Kirchoff [30]. The calorimetric reversibility of the thermal transitions was determined by heating the sample to a temperature that is little above the transition maximum, cooling immediately, and then reheating. The calibration of the DSC was done by using the Joule Calibration device supplied with the instrument.

2.3. UV–Visible experiments

2.3.1. Thermal denaturation of ribonuclease A

The thermal denaturation of ribonuclease A in the presence of TEAB at pH 5.5 was carried out by employing a Shimadzu UV-260 double-beam spectrophotometer to which a Cole Parmer constant-temperature circulator was attached. The temperature of the solutions in the cuvettes was stable to within ±0.1 K. The individual absorbance measurements were done at each fixed temperature to obtain the complete thermal denaturation profile. The concentration of ribonuclease A in all the experiments was kept at $0.145 \cdot 10^{-4}\ mol \cdot dm^{-3}$, while the concentration of TEAB was varied. The reference solution was buffer when the measurements were made in buffer, or (buffer + salt) when the experiments were performed in the presence of the salt. The conformational changes accompanying the thermal denaturation were monitored at 287 nm [31] which is based on the difference in absorbance at the wavelength of maximum change. To check the reversibility of thermal denaturation, the sample in the first run was heated to a temperature slightly above the complete denaturation temperature, cooled immediately, and then reheated. The thermal transitions were reversible in all cases and hence amenable to equilibrium thermodynamic analysis. The evaluation of thermodynamic parameters accompanying the thermal denaturation monitored by using spectroscopic techniques is based on the equilibrium constant for the reversible two-state native = denatured transition. The absorbance as a function of temperature was fed to the EXAM program, and thermodynamic parameters were calculated.

2.4. Isothermal titration calorimetry (ITC)

The ITC measurements were done at $T = 298.15$ K using a VP-ITC titration calorimeter (MicroCal Northampton, MA, USA). All solutions were thoroughly degassed on a Thermovac unit provided with the instrument. The reference cell was filled with the degassed buffer. The protein was kept in the sample cell and aqueous TEAB solution was filled in the syringe of volume 0.250 cm^3. The TEAB solution was added sequentially in 0.010 cm^3 aliquots (for a total of 25 injections, 20 s duration each) at 4 min intervals. The values of heats of dilution were determined with similar parameters by injecting (i) TEAB solution in buffer at the respective pH values, (ii) buffer into $0.058 \cdot 10^{-3}$ mol · dm^{-3} BSA, $0.282 \cdot 10^{-3}$ mol · dm^{-3} α-lactalbumin and $0.292 \cdot 10^{-3}$ mol · dm^{-3} ribonuclease A solutions separately, and (iii) by injecting buffer into buffer solution. The control heats of dilution were subtracted from the measured heats to obtain the net enthalpy change for the interaction of TEAB with proteins. The calibration of the ITC was measured by using pulse control as suggested by the supplier.

2.5. Fluorescence experiments

The fluorescence experiments were done at $T = 298$ K on a Perkin–Elmer LS-55 spectrofluorimeter. The concentration in all the experiments was kept at $0.5 \cdot 10^{-6}$ mol · dm^{-3} for bovine serum albumin, $7 \cdot 10^{-6}$ mol · dm^{-3} for α-lactalbumin and $21 \cdot 10^{-6}$ mol · dm^{-3} for ribonuclease A. Both the excitation and emission slit-widths were fixed at 5 nm. The excitation wavelength was 295 nm to selectively excite the tryptophan residues in the case of bovine serum albumin and α-lactalbumin. The emission spectra were recorded. Since ribonuclease A does not contain tryptophan, 280 nm was used to excite the tyrosines present. All the fluorescence experiments were repeated at least three times. The maximum error in the intensity measurements was within 1% in the repeated experiments.

3. Results and discussion

3.1. Thermal unfolding of proteins in the presence of TEAB

3.1.1. Bovine serum albumin

Representative differential scanning calorimetric profiles of thermal unfolding of BSA in the absence and presence of TEAB are presented in figure 1 and the corresponding thermodynamic parameters: temperature at half denaturation ($T_{1/2}$), and calorimetric enthalpy (ΔH_{cal}) accompanying the denaturation process are reported in table 1. The calorimetric transitions in the absence and presence of TEAB were found to be partially reversible. Therefore, equilibrium thermodynamics could not be applied to calculate the values of the van't Hoff enthalpy. However, the calorimetric transitions were found to be more cooperative in the presence of TEAB in solution.

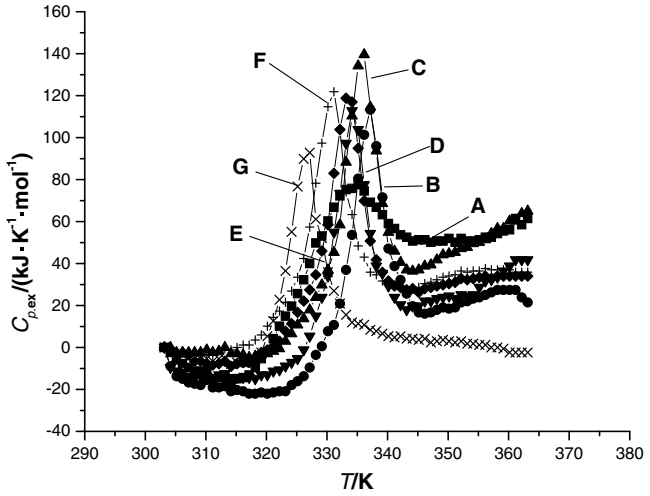

FIGURE 1. Plot of heat capacity against temperature to show the thermal transitions of $0.12 \cdot 10^{-3}$ mol · dm^{-3} bovine serum albumin at pH 7 in the presence of varying concentration of TEAB: 0 (A, ■), 0.25 (B, ●), 0.5 (C, ▲), 0.75 (D, ◆), 1.0 (E, ▼), 1.5 (F, +), and (G, ×) 2.5 mol · dm^{-3}.

TABLE 1
Thermodynamic parameters[a] accompanying the thermal unfolding of $0.12 \cdot 10^{-3}$ mol · dm^{-3} bovine serum albumin at pH 7.0 in the absence and presence of TEAB at scan rate of 0.5 K · min^{-1}

[TEAB]/(mol · dm^{-3})	$T_{1/2}$/K	ΔH_{cal}/(kJ · mol^{-1})
0	335.2	797
0.25	336.7	664
0.50	335.8	668
0.75	334.2	640
1.00	331.5	617
1.50	330.7	607
2.50	325.7	540

[a] Incorporating errors in sample preparation, reproducibility, and sample impurities, the errors in the values of $T_{1/2}$ and ΔH_{cal} are ±0.1 K and 2%, respectively.

The values of $T_{1/2}$ and ΔH_{cal} for BSA are 335.2 K and 797 kJ · mol^{-1}, respectively, at pH 7 in the absence of additive which are in good agreement with those reported in the literature [32]. In the presence of 0.25 mol · dm^{-3} to 0.75 mol · dm^{-3} TEAB, there is almost no change in the transition temperature beyond which it gradually decreases with increase in the concentration of the salt. The results on thermal denaturation of BSA in the presence of TEAB can be explained on the basis of competing patterns of interactions of the co-solute with the native versus unfolded state of the protein during the native ⇌ denatured reaction. Since between 0.25 mol · dm^{-3} and 0.75 mol · dm^{-3}, TEAB there is almost no change in the thermal stability of the protein, it indicates a balance of hydrophilic and hydrophobic interactions. Beyond 0.75 mol · dm^{-3}, both the values of transition temperature and the calorimetric enthalpy decrease with an increase in the concentration of TEAB. This can be attributed to the stronger interaction of the TEAB molecules with the denatured state of the protein compared to the native state under these conditions.

3.1.2. α-Lactalbumin

The representative differential scanning calorimetric profiles of thermal denaturation of α-lactalbumin in the absence and presence of varying concentrations of TEAB at pH 7.0 are shown in figure 2, and the corresponding thermodynamic parameters accompanying the thermal denaturation process in the absence and presence of TEAB are reported in table 2. In this table, $T_{1/2}$ is the transition temperature where the area under the transition curve is half of the total area. The ΔH_{cal}, ΔC_p, and ΔS^o are the calorimetric molar enthalpy, molar heat capacity, and molar entropy of denaturation, respectively, β is the ratio of the van't Hoff to the molar calorimetric enthalpy. A well behaved two-state transition centred at $T = 336.2$ K characterized by a calorimetric enthalpy of 255 kJ mol^{-1} and $\beta = 0.99$ is observed for $20 \cdot 10^{-3}$ mol · dm^{-3} TRIS buffer at pH 7.0. The value of β calculated for all the experiments is (0.99 ± 0.02). Therefore, the thermal denaturation of α-lactalbumin under the conditions studied is two state and reversible. With an increase in the concentration of TEAB, the thermal stability of the protein was found to decrease as seen by the decrease in the denaturation temperature.

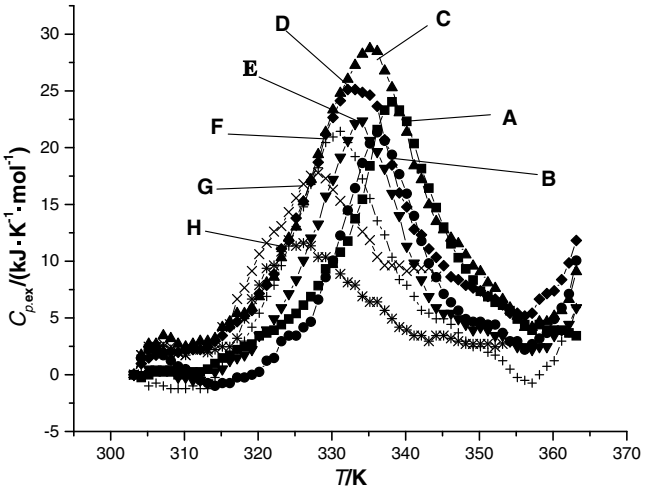

FIGURE 2. Plot of heat capacity against temperature to show the thermal transitions of $0.12 \cdot 10^{-3}$ mol · dm^{-3} α-lactalbumin at pH 7 in the presence of TEAB: 0 (A, ■), 0.25 (B, ●), 0.5 (C, ▲), 0.75 (D, ◆), 1.0 (E, ▼), 1.5 (F, +), 2.5 (G, ×), and (H, ∗) 3 mol · dm^{-3}.

The calorimetric enthalpy remains almost the same up to 1.5 mol · dm^{-3}, beyond which it decreases. This permits application of equilibrium thermodynamics for the calculation of thermodynamic parameters as a function of temperature and concentration of TEAB. The results in table 2 suggest that the hydrophobic effects of TEAB are greater in the unfolded state of α-lactalbumin because of a larger exposure of the constituent groups of this protein to the solvent than in the native state under the TEAB concentration range employed. This is manifested in a lowering of the thermal denaturation temperature.

Large and positive values of ΔC_p for protein denaturation are largely due to the exposure of the non-polar groups, previously buried in the native structure, to the surrounding solvent. This exposure leads to the ordering of the solvent molecules around them. However, it has been shown [33,34] that a negative contribution from the exposure of the polar groups is expected to partially offset the positive contribution. The contribution to the heat capacity from the polar and apolar groups are opposite in sign [$\Delta C_{pol} < 0$; $\Delta C_{apolar} > 0$]. The overall denaturational heat capacity is given by

$$\Delta C_p = \Delta C_{ap} \Delta ASA_{ap} + \Delta C_{pol} \Delta ASA_{pol}. \quad (1)$$

Here, ΔASA_{ap} and ΔASA_{pol} are the denaturational changes in apolar (ap) and polar (pol) accessible surface area. The ΔC_{ap} and ΔC_{pol} are the heat capacity changes accompanying the exposure of 1 mol A^2 of apolar and polar ASA, respectively.

In the absence of TEAB, ΔC_p for α-lactalbumin is (4.6 ± 0.4) kJ · K^{-1} · mol^{-1}, which indicates a significant contribution from the exposure of apolar groups to the buffer medium. The average value of ΔC_p in the presence of TEAB is (2.9 ± 0.6) kJ K^{-1} mol^{-1}. Assuming that both polar and apolar accessible surface area do not change significantly, then reduction in the overall value of ΔC_p in the presence of TEAB may be attributed to the reduction in the contribution of ΔC_p accompanying the exposure of apolar accessible surface area. Therefore, the extent of reordering of the water structure around the exposed non-polar groups upon unfolding is expected to be lower in the presence of TEAB. This is due to the interaction of the exposed hydrophobic groups of the protein upon denaturation with the ethyl group of TEAB. The tetraethylammonium bromide

TABLE 2
Thermodynamic parameters accompanying the thermal unfolding of $0.281 \cdot 10^{-3}$ mol · dm^{-3}α-lactalbumin at pH 7 in the presence of TEAB at scan rate of 0.5 K · min^{-1}

[TEAB]/(mol · dm^{-3})	$T_{1/2}$/K	ΔH_{cal}/(kJ · mol^{-1})	β	ΔC_p/(kJ · K^{-1} · mol^{-1})	ΔS/(kJ · K^{-1} · mol^{-1})
0.00	336.2	255	0.99	4.6	7.58
0.25	333.6	269	1.00	2.6	8.01
0.50	335.4	266	0.97	2.1	7.97
0.75	333.7	267	1.00	2.3	7.99
1.00	331.9	252	1.02	2.8	7.55
1.50	329.7	246	1.02	3.8	7.47
2.50	323.2	214	0.99	3.6	6.61
3.00	322.9	195	1.04	3.6	6.08

destabilizes α-lactalbumin by interacting with the exposed hydrophobic groups of the denatured state and simultaneously weakening the hydrophobic interactions between the non-polar groups of the protein. Since the rupture of the hydrophobic interaction is exothermic, the value of ΔH decreases in the presence of tetraethylammonium bromide.

3.1.3. Ribonuclease A

Figure 3 shows the thermal denaturation profiles of $0.0145 \cdot 10^{-3}$ mol · dm^{-3} ribonuclease A in acetate buffer at pH 5.5 in the absence and presence of TEAB obtained from the change in absorbance at 287 nm as a function of temperature. The results obtained in the absence and presence of TEAB are given in table 3.

The thermal denaturation of ribonuclease A under these conditions was observed to be reversible as deduced from the recovery of the absorbance against temperature profile upon reheating.

The enthalpy of denaturation increased with addition of TEAB up to 0.25 mol · dm^{-3} beyond which its value decreased. It is widely accepted that tetra-alkyl ammonium salts, with the exception of $(CH_3)_4NX$, are "structure formers" in aqueous solution, the extent of structure promotion increasing with cation size. On this basis, it is likely that, at low concentrations, these salts will act as selective hydrophobic bond breakers [35], while at higher concentrations (>0.2 mol · dm^{-3}) when most hydrophobic stabilizing interactions are broken, the polar nature of the salts become dominant, causing both the transition temperature and the denaturation enthalpy to decrease.

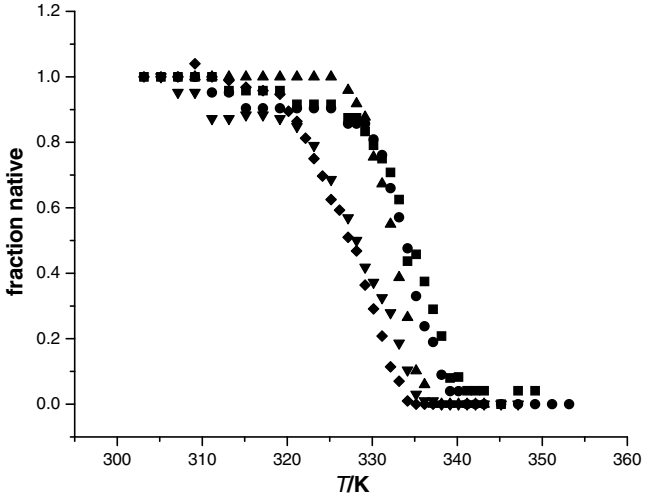

FIGURE 3. Plot of the fraction of native material against temperature to show thermal denaturation profiles of ribonuclease A at pH 5.5 in the presence of TEAB: 0 (■), 0.1 (●), 0.25 (▲), 0.5 (▼), and 0.75 (◆) mol · dm^{-3}.

TABLE 3
Thermodynamic parameters accompanying the thermal unfolding of $0.145 \cdot 10^{-4}$ mol · dm^{-3} ribonuclease A at pH 5.5 in the presence of TEAB

[TEAB]/ (mol · dm^{-3})	$T_{1/2}$/ K	ΔH_{VH}/ (kJ · mol^{-1})	ΔS/ (kJ · K^{-1} · mol^{-1})
0.00	335.2	410	1.22
0.10	333.9	504	1.51
0.25	331.9	511	1.54
0.50	329.3	393	1.19
0.75	327.9	359	1.09

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 When the native protein denatures, its surface changes its character. Hence, the preferential solvation of the macromolecule by the surrounding solvent also changes. Data on reversible thermal transitions obtained from differential scanning microcalorimetry have been used to calculate the denaturational change in preferential solvation of the protein ($\Delta\Gamma_{23}$) by TEAB at the transition temperature using the method described by Kovrigin and Potekhin [36]:

$$\Delta\Gamma_{23} = \Gamma_{D3} - \Gamma_{N3} = -\frac{\Delta H \left(\frac{\partial T_{1/2}}{\partial x_3}\right)_{pH}}{RT_{1/2}^2 \left(\frac{\partial \ln a_3}{\partial x_3}\right)_{T_{1/2}}}. \quad (2)$$

Here, Γ_{D3} and Γ_{N3} are the preferential solvation of the protein by TEAB in the denatured and native state, respectively. Here, x is the mole fraction and a is the activity of TEAB in solution.

The values of activity coefficient were determined by using the Debye–Huckel equation [37]. Figure 4 shows the variation in the values of denaturational change in preferential interaction of TEAB with change in its mole fraction or activity.

The values of $\Delta\Gamma_{23}$ for both ribonuclease A and α-lactalbumin were found to be positive (table 4) indicating that unfolding at these temperatures leads to a state with a larger affinity to component 3 (TEAB) than that of the native

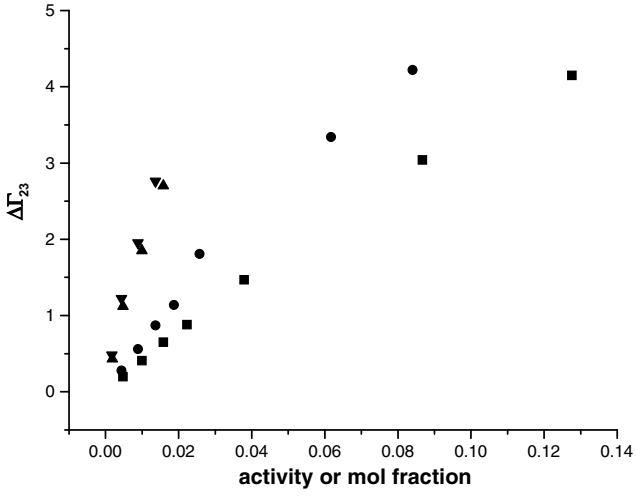

FIGURE 4. Plot of denaturational change in preferential solvation against activity (●), (▼), and mole fraction (■), (▲) of TEAB for α-lactalbumin and ribonuclease A, respectively.

TABLE 4
Denaturational change in the preferential solvation parameter ($\Delta\Gamma_{23}$), accompanying the thermal unfolding of $0.282 \cdot 10^{-3}$ mol · dm^{-3} α-lactalbumin in the presence of TEAB at pH 7.0

[TEAB]/(mol · dm^{-3})	Mol fraction	Activity	$\Delta\Gamma_{23}(\chi_3)$	$\Delta\Gamma_{23}$ (a)
		α-Lactalbumin		
0.25	0.00473	0.00436	0.20	0.28
0.50	0.00997	0.00887	0.41	0.56
0.75	0.01579	0.01363	0.65	0.87
1.0	0.02230	0.01872	0.88	1.14
1.5	0.0379	0.03017	1.47	1.81
2.5	0.08668	0.06170	3.04	3.34
3.0	0.12760	0.08400	4.15	4.22
Ribonuclease A				
0.10	0.00184	0.00175	0.43	0.48
0.25	0.00473	0.00436	1.12	1.22
0.50	0.00997	0.00887	1.85	1.95
0.75	0.01579	0.01363	2.70	2.76

$\Delta\Gamma_{23}(\chi_3)$ is based on mole fraction.
$\Delta\Gamma_{23}$(a) is based on activity.

state. This is most likely due to the interaction between the hydrophobic groups of the protein upon unfolding and the TEAB. Within the concentration range studied the variation is observed to be linear.

Figure 4 also shows that inter ionic interactions between TEAB molecules in solution are significant because the plot of $\Delta\Gamma_{23}$ against mole fraction and activity are not overlapping in the case of α-lactalbumin. However, the non-overlap is not that prominent in the case of ribonuclease A. The extent of non-overlap increases with an increase in the mole fraction of TEAB in solution. Figure 4 also suggests that the interaction of the TEAB molecule with denatured state is greater for ribonuclease A than for α-lactalbumin. Inter ionic interactions between the TEAB molecules affect their interaction with α-lactalbumin more than with ribonuclease A.

The various thermodynamic properties accompanying the thermal denaturation of BSA, α-lactalbumin, ribonuclease A, and lysozyme [38] are shown in table 5. These pH values were selected to be those where the thermal stability index (transition temperature) of the protein was found to be the same. It is seen that TEAB is a stronger denaturant of ribonuclease and lysozyme than BSA and α-lactalbumin. This can be attributed to the lower pH in the case of ribonuclease A and lysozyme. Hence there is greater accessibility of partly unfolded conformation of the protein to the salt due to expansion of the molecule resulting from the repulsive electrostatic interactions among the positive charges.

3.2. Fluorescence results on interaction of TEAB with proteins

3.2.1. Bovine serum albumin

Fluorescence experiments were performed to examine the conformational variations around tryptophan residues. Figure 5 shows the intrinsic fluorescence of BSA in the presence of TEAB at pH 7.0. There is a concentration dependent decrease in the fluorescence intensity with the increase in the concentration of TEAB.

There is a slight red shift from 345 nm to 347 nm in the presence of 0.75 mol · dm^{-3} TEAB thereby indicating a reduced hydrophobicity of the tryptophan microenvironments. This small red shift is consistent with only a slight fall in transition temperature of BSA upon addition of TEAB at the same concentration level. The observed decrease in the fluorescence intensity with shift of energy maximum towards longer wavelength is suggestive of unfolding of the protein in the presence of TEAB.

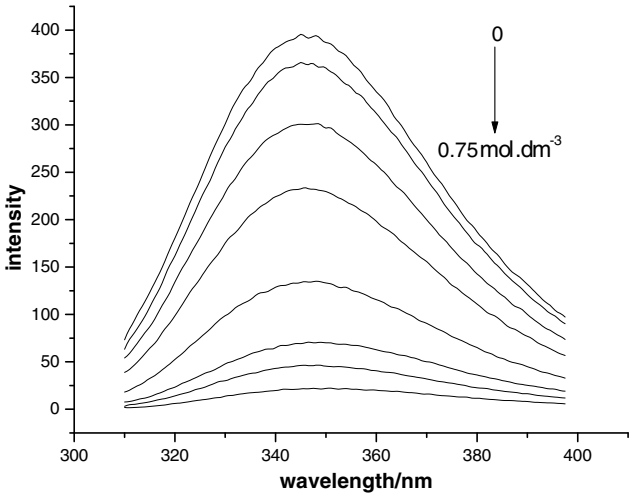

FIGURE 5. Plot of intensity against wavelength to show the emission spectra of $0.5 \cdot 10^{-6}$ mol · dm^{-3} BSA at pH 7 in the presence of TEAB: 0, 0.025, 0.05, 0.10, 0.25, 0.40, 0.50, and 0.75 mol · dm^{-3}.

TABLE 5
Comparison of the thermodynamic parameters for the denaturation of the proteins having the same thermal stability index in the presence of TEAB

Proteins	pH	$T_{1/2}$/K	[TEAB]/(mol · dm^{-3})	$\Delta\Delta T_{1/2}$/K	$\Delta\Delta H^{\circ}$/(kJ · mol^{-1})
BSA	7.0	335.2	0.5	0.6	−129
			1.0	−3.7	−180
Ribonuclease A	5.5	335.2	0.5	−6.4	−9
α-Lactalbumin	7.0	336.2	0.5	−0.8	11
			1.0	−4.3	−1
Lysozyme[38]	2.4	336.2	0.5	−6.8	−47
			1.0	−9.3	−69

The extent of fluorescence quenching Q can be calculated using the following equation:

$$Q = (F_0 - F)/F_0. \quad (3)$$

Here, F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, and Q_{max} is the maximum quenched fluorescence. It follows from the mass law considerations that

$$Q = (Q_{max} \cdot [TFE]/(K_D + [TFE]), \quad (4)$$

where K_D is the average dissociation constant and [TFE] is the concentration of TFE.

Shown in figure 6 is a plot of the fluorescence quenching of BSA as a function of the concentration of TEAB at pH 7. A least squares fit of equation (4) to the data yields $Q_{max} = (0.92 \pm 0.05)$ and $K_D = (0.22 \pm 0.02)$ mol · dm^{-3} which indicate that both the tryptophans at position Trp 134 and Trp 212 are effectively quenched in the presence of TEAB.

3.2.2. α-Lactalbumin

The intrinsic fluorescence of α-lactalbumin in the absence and presence of varying concentrations of TEAB is shown in figure 7. Here, also, the fluorescence emission intensity is observed to decrease with an increase in the concentration of TEAB. Above 1.0 mol · dm^{-3}, TEAB there is a red shift from 331 nm to 337 nm with an increase in the concentration of TEAB up to 2.5 mol · dm^{-3}. Figure 8 shows a plot of the fluorescence quenching of α-lactalbumin against concentration of TEAB at pH 7. With an increase in TEAB concentration in solution from (1.0 to 2.5) mol · dm^{-3}, there is a red shift of 6 nm indicating partial exposure of tryptophan residues to the environment. This correlates well with the fall in the transition temperature by 4.3 K and 12.8 K in the presence of 1.0 mol · dm^{-3} and 2.5 mol · dm^{-3} TEAB, respectively. The values of $Q_{max} = (0.93 \pm 0.04)$ and $K_D = (0.92 \pm 0.04)$ mol · dm^{-3}

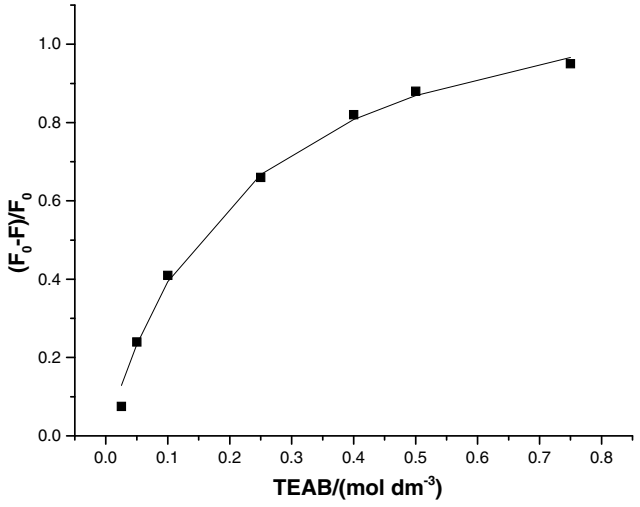

FIGURE 6. Plot of the extent of fluorescence against concentration TEAB to show the quenching of $0.5 \cdot 10^{-6}$ mol · dm^{-3} of BSA at pH 7.

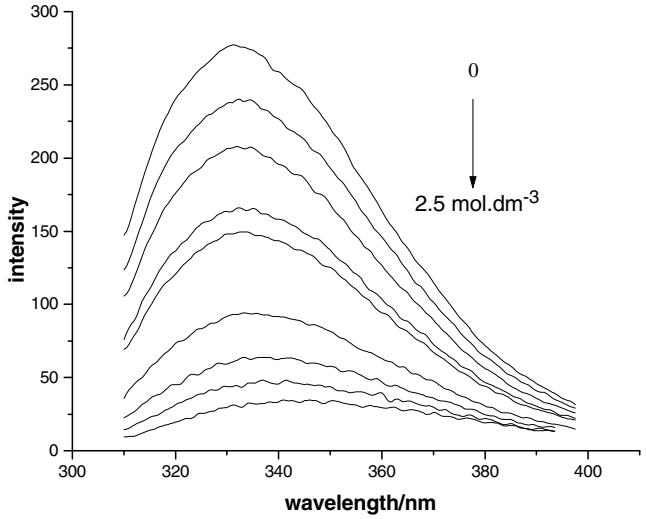

FIGURE 7. Plot of intensity against wavelength to show the emission spectra of $7 \cdot 10^{-6}$mol · dm^{-3} α-lactalbumin at pH 7 in the presence of TEAB: 0, 0.10, 0.25, 0.40, 0.50, 1.0, 1.5, 2.0, and 2.5 mol · dm^{-3}.

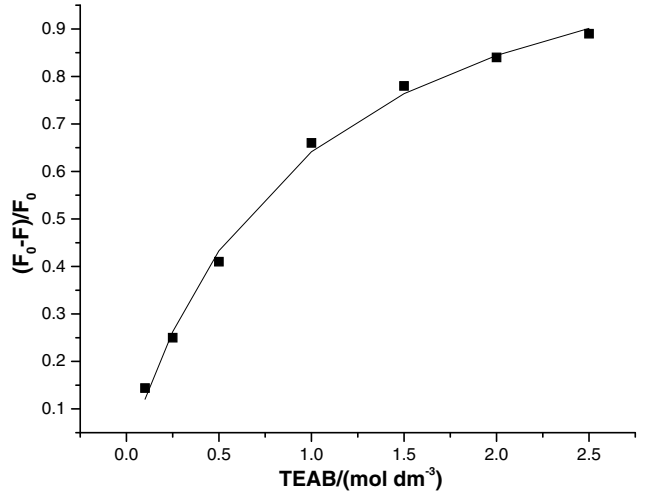

FIGURE 8. Plot of the extent of fluorescence against concentration TEAB to show the quenching of $7 \cdot 10^{-6}$ mol · dm^{-3} of α-lactalbumin at pH 7 at different concentrations of TEAB.

have been obtained by using equation (4). The α-lactalbumin has four tryptophans at positions 26, 60, 104, and 118. The value of $Q_{max} = (0.93 \pm 0.04)$ indicates that all the four tryptophans are effectively quenched in the presence of TEAB. For BSA the value of K_D {(0.22 ± 0.02) mol dm^{-3}} is smaller than that of α-lactalbumin K_D {(0.92 ± 0.04) mol dm^{-3}}. This could be attributable to enhanced hydrophobic interactions between the fluorophor and TEAB in the former.

3.2.3. Ribonuclease A

The results for the intrinsic fluorescence of ribonuclease at pH 5.5 show that with an increase in the concentration of TEAB in solution from (0.01 to 0.5) mol · dm^{-3}, the

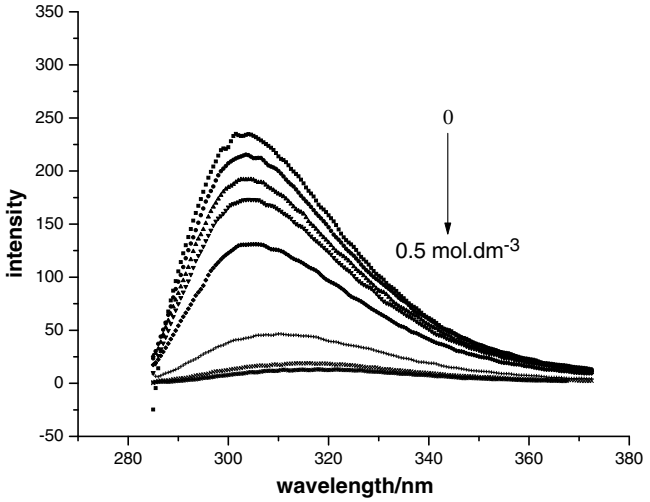

FIGURE 9. Plot of intensity against wavelength to show the emission spectra of $21 \cdot 10^{-6}$mol $\cdot$ dm^{-3} ribonuclease A at pH 5.5 in the presence of TEAB: 0, 0.01, 0.025, 0.05, 0.075, 0.1, 0.4, and 0.5 mol $\cdot$ dm^{-3}.

emission intensity goes on decreasing (figure 9). Here, also in the presence of 0.5 mol $\cdot$ dm^{-3} TEAB, there is a red shift of 8 nm. The use of equation (4) yields $Q_{max} = (0.94 \pm 0.04)$ and $K_D = (0.16 \pm 0.01)$ mol $\cdot$ dm^{-3} indicating enhanced hydrophobic interactions between the tyrosine and TEAB. A fall in the transition temperature of ribonuclease A by 6.4 K in the presence of 0.5 mol $\cdot$ dm^{-3} TEAB is consistent with a red shift of 8 nm in intrinsic fluorescence.

3.3. Isothermal titration calorimetric results of interaction of TEAB with proteins

The isothermal titration calorimetric (ITC) profile for the titration of TEAB with BSA at pH 7 showed small negative deviations from the stable baseline upon addition of TEAB indicating that the process is slightly exothermic. No variation in the heat evolved was observed with increase in the molar ratio of [TEAB]/[BSA] in solution. Hence the average of the heat evolved at each injection was taken as the enthalpy of interaction. The enthalpy of interaction of TEAB with BSA at pH 7 is (-326 ± 96) J $\cdot$ mol^{-1}. The values of the molar enthalpy of interaction (ΔH) of TEAB with α-lactalbumin and ribonuclease A at pH 7 and at pH 5.5 are (-100 ± 42) J $\cdot$ mol^{-1} and (-389 ± 25) J $\cdot$ mol^{-1}, respectively. The fall in transition temperature, fluorescence quenching, and isothermal titration calorimetric results correlate with each other. All the results obtained from these three independent approaches indicate an increased interaction of TEAB with the denatured state of the protein thereby decreasing its thermal stability. Since the ITC profile does not indicate a specific binding pattern, the mode of interaction of TEAB with α-lactalbumin, ribonuclease A and BSA is most likely operating through solvent mediated effects where the characteristic water structure around the protein is perturbed.

4. Mode of interaction of TEAB with proteins

The mechanism by which TEAB interacts with the proteins is not clearly understood, and needs further experimental investigation. Previous studies [20,21,38] invariably presume the binding of TEAB with the proteins, though the experimental proof is not clearly given. Chen and Berns [20] reported a binding constant of (12 ± 3) M^{-1} for the binding of TEAB with the trimer of phycocyanin. This value is too small to be interpreted in terms of significant binding. Stojan *et al.* [22] have reported that tetraethyl ammonium ion competes with the catalytic substrate at the peripheral site, inhibiting the substrate hydrolysis at the catalytic site. The denaturing action of the tetraalkylammonium halides on proteins has been attributed by Timasheff and co-workers [16–18] to the binding of the denaturant molecules to the denatured state of the protein which is stronger than the exclusion of co-solvent from the protein surface. Based upon enthalpy and entropy data obtained for the protein–quaternary bromides, it has been postulated [20] that the direct contact region between the protein and hydrophobic solute is mainly on the hydrophobic area of the protein, and that the bulky alkyl groups of the hydrophobic solutes interact principally by means of hydrophobic forces instead of a direct contact between charged ions and polar groups of the protein favoured by electrostatic interactions. Studies on the interaction of lysozyme with tetramethylammonium chloride (Me_4NCl) indicated that the stabilizing influence of Me_4NCl is due predominantly to binding of Me_4NCl on the polar sites of the protein surface [39]. The titration of TEAB with three proteins of different structure and function do not follow a typical binding pattern in the present study. The interaction of TEAB with proteins can occur either directly by binding to its various functional groups or, alternatively, indirectly via interaction between TEAB and solvent. The TEAB has an alkyl chain with eight carbon atoms which can induce reorganization in water to ''icebergs''. It may lower the thermal stability of the protein by weakening hydrophobic interactions that can be achieved by decreasing the availability of water molecule for ''iceberg'' formation about the exposed polar side chains by providing competitive centres of water structure organization. The TEAB can compete with the protein in its unfolded form in directing the local distribution or arrangement of water molecules. Based on ITC results on the interaction of TEAB with three proteins of different structure and function, it is clear that the binding component in the interaction of the salt with the protein is very weak, and the interactions operate mainly via solvent mediated effects.

5. Conclusions

The differential scanning calorimetry and UV–visible spectroscopy on the interaction of TEAB with α-lactalbumin and ribonuclease A shows that the thermal denaturation of the protein follows a two-state reversible process as

reflected by the equality of the van't Hoff and calorimetric enthalpies. Quantitative thermodynamic parameters, transition temperature, enthalpy, and entropy, accompanying the thermal denaturation of α-lactalbumin and ribonuclease A in the absence and presence of TEAB have been measured. The preferential interaction parameters of TEAB with α-lactalbumin and ribonuclease A confirm that an increased interaction of the hydrophobic groups of the TEAB with that of the protein upon denaturation is responsible for the reduced thermal stability of the protein in the presence of the TEAB. Whereas in case of bovine serum albumin the ratio of van't Hoff to calorimetric enthalpy was observed to be less than unity in the absence and presence of TEAB indicating deviation from the two-state native to denatured unfolding mechanism. Isothermal titration calorimetric results indicate an absence of appreciable specific binding of TEAB with all the above studied proteins. Addition of TEAB cause quenching of the intrinsic fluorescence accompanied with a red shift in BSA and α-lactalbumin and tyrosine fluorescence in ribonuclease A. The results obtained from three independent approaches indicate an increased interaction of TEAB with the denatured state of the protein thereby decreasing its thermal stability. Finally, our results demonstrate that the binding component in the interaction of TEAB with proteins is very weak and operates mainly via solvent mediated effects.

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