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A mechanistic analysis of the increase in the thermal stability of proteins in aqueous carboxylic acid salt solutions

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Abstract

The stability of proteins is known to be affected significantly in the presence of high concentration of salts and is highly pH dependent. Extensive studies have been carried out on the stability of proteins in the presence of simple electrolytes and evaluated in terms of preferential interactions and increase in the surface tension of the medium. We have carried out an in-depth study of the effects of a series of carboxylic acid salts: ethylene diamine tetra acetate, butane tetra carboxylate, propane tricarballylate, citrate, succinate, tartarate, malonate, and gluconate on the thermal stability of five different proteins that vary in their physico-chemical properties: RNase A, cytochrome *c*, trypsin inhibitor, myoglobin, and lysozyme. Surface tension measurements of aqueous solutions of the salts indicate an increase in the surface tension of the medium that is very strongly correlated with the increase in the thermal stability of proteins. There is also a linear correlation of the increase in thermal stability with the number of carboxylic groups in the salt. Thermal stability has been found to increase by as much as 22 °C at 1 M concentration of salt. Such a high thermal stability at identical concentrations has not been reported before. The differences in the heat capacities of denaturation, ΔC_p for RNase A, deduced from the transition curves obtained in the presence of varying concentrations of GdmCl and that of carboxylic acid salts as a function of pH, indicate that the nature of the solvent medium and its interactions with the two end states of the protein control the thermodynamics of protein denaturation. Among the physico-chemical properties of proteins, there seems to be an interplay of the hydrophobic and electrostatic interactions that lead to an overall stabilizing effect. Increase in surface free energy of the solvent medium upon addition of the carboxylic acid salts appears to be the dominant factor in governing the thermal stability of proteins.

Keywords: carboxylic acid salts; preferential hydration; protein stability; surface tension; transition temperature

Salts have been known to affect the physico-chemical properties of proteins like their solubility (Green, 1932; Carbonnaux et al., 1995), stability (von Hippel & Schleich, 1969), and pK_a (Abe et al., 1995). Perturbation of these properties by salts depends upon the chemical nature of the salts as well as that of the proteins. Salts affect mainly electrostatic and hydrophobic interactions in the protein molecules (Melander & Horvath, 1977). Based on their analysis, Matthews and Richards (1982) suggested that about half of the free energy of stabilization of RNase A originates from electrostatic contributions. The effect of salts on the hydrophobic interactions is most obvious from the phenomenon of decreased solubility and aggregation of proteins at high salt concentrations (von Hippel & Schleich, 1969). The effect of salts on proteins can be defined in terms of two opposing forces: salting-in of proteins in the salt solutions assigned to Debye–Hückel screening of surface charges at low concentrations; and salting-out, which usually takes place at higher salt concentrations (≥1.0 ionic strengths). Apparently it is observed that salting-in agents work as good denaturants and salting-out agents as good stabilizers of protein structure (Arakawa et al., 1990a).

Proteins have been known to have specific ion binding sites (Pace & Grimsley, 1988). Some ions bind to proteins at very low concentrations and affect their stability (Makhatadze et al., 1998), while other salts affect the stability in a general manner without having any specificity toward proteins (von Hippel & Schleich,

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Abbreviations: α-CTgen, α-chymotrypsinogen; A, absorbance; ASA, accessible surface area; BTC, butane tetracarboxylic acid; cyt *c*, cytochrome *c*; *D*, denatured state; EDTA, ethylene diamine tetraacetic acid; GdmCl, guanidinium chloride; HEW, hen egg white; MOPS, 3-[N-morpholino]propane-sulfonic acid; *N*, native state; PTC, propane tricarboxylic acid; RNase A, ribonuclease *A*; T_m, transition temperature; Trp-Inh, trypsin inhibitor; UV, ultraviolet; VIS, visible.

1969; Busby et al., 1981; Arakawa et al., 1990b; Bonnette et al., 1994; Jensen et al., 1995). The differential stability provided by salts of different combinations indicates the contribution of both the cations and anions for providing stability to the protein to an extent depending on their position in the Hofmeister series (Hofmeister, 1888; Baldwin, 1996). In salt systems the effect of an individual ion has been found to be essentially additive (Arakawa & Timasheff, 1991).

Salts that do not have specific binding to proteins increase their thermal stability essentially through their effect on water structure manifested by an increase in its surface tension (Sinanoglu & Abdulnur, 1965; Lin & Timasheff, 1996). It is suggested that protein stability and solubility are linked to this phenomenon. All structure stabilizing, precipitating, or self-association inducing agents are preferentially excluded from the protein surface while all structure destabilizing agents increase protein solubility and lead to the preferential binding to proteins as determined by multicomponent thermodynamic analysis using equilibrium dialysis and densimetry techniques (Timasheff, 1993). Interfacial energy at the protein-solvent boundary plays an important role in the protein-solvent preferential interactions. A change in the surface tension of the medium upon the addition of solutes would also govern the mode of interaction of the cosolvent with the protein. Simple electrolytes which increase the thermal stability of proteins have been found to essentially act by increasing the surface tension of the solvent medium (Arakawa & Timasheff, 1982; Arakawa et al., 1990b).

Among carboxylic acid salts, acetate and citrate have long been known to act as protein stabilizers (von Hippel & Schleich, 1969). Studies carried out on the effect of acetate on the interactions with lysozyme indicate that the salt induces preferential hydration to the protein at high concentrations, while at low concentrations does not have any appreciable interaction with the protein (Arakawa & Timasheff, 1982). The stabilizing effect of the carboxylic acid salts, like acetate, may be essentially mediated via solvent water. A series of carboxylic acid salts have also been used to increase the thermal stability of antithrombin III (Busby et al., 1981; Busby & Ingham, 1984). However, unlike inorganic salts, a comprehensive study to investigate their mechanism of action is lacking in the literature. In this paper we report the results of our studies on the thermal stability of five different globular proteins: ribonuclease A (RNase A), cytochrome *c* (cyt *c*), trypsin inhibitor (Trp-Inh), myoglobin, and lysozyme at several pH values in the presence of sodium salts of various carboxylic acids. The carboxylic acid salts studied were ethylene diamine tetraacetic acid (EDTA), butane tetracarboxylic acid (BTC), propane tricarboxylic acid (PTC), citric acid, succinic acid, tartaric acid, malonic acid, and gluconic acid, which vary in their carbon chain length and the number of carboxylic groups. The proteins varying in their physico-chemical properties were selected to enable us to elucidate the role of their hydrophilicity, hydrophobicity, and net charge in the salt mediated thermal stability of proteins. This paper also presents an analysis of the role of surface tension of the solvent medium and its correlation with the increase in the thermal stability of proteins.

Results

Thermal denaturation studies

Figures 1 and 2 show the thermal denaturation profiles of several proteins in the presence of carboxylic acid salts. The effect of carboxylic acid salts, which vary in the number of carbon atoms in their chain and the total number of carboxylate groups, was studied on RNase A, trp-inh, lysozyme, and cyt *c* at pH 5.0, 7.0, and 9.2. Thermal denaturation studies on cyt *c* at pH 9.2 were not possible due to its precipitation at higher temperatures. Sodium tartarate and sodium succinate were not soluble to 1 M concentration at pH 5.0. EDTA also has very low solubility at pH 5.0 and was found to denature and precipitate cyt *c* at all the pH values. This could be due to the chelating action of EDTA on the heme Fe^{3+} group. Figure 1A–H shows the thermal unfolding behavior of RNase A at pH 5.0, 7.0, and 9.2 in 0.5 and 1.0 M salt concentrations and of myoglobin at pH 7 in the presence of 1.0 M salts.

All the proteins studied lead to an irreversible thermal denaturation at alkaline pH, particularly at pH 9.2. In the absence of any salts, the proteins showed some reversibility up to pH 9.2, except for lysozyme. Trp-inh and RNase A showed diminished reversibility as seen on reheating and comparing the thermodynamic parameters obtained with that of the first heating. Thermal denaturation temperatures (T_m) on second heating were found to be within $\pm 0.5\,^{\circ}C$ of that of the first heating but the enthalpy of denaturation (ΔH_m) was found to be decreased by 20–25%. The observed hysteresis is mainly because of irreversible denaturation, which is more prominent at alkaline pH (Zale & Klibanov, 1986). The denaturation profiles, which were highly irreversible, have not been subjected to thermodynamic analysis, while RNase A unfolding, which was reversible up to 80%, was subjected to thermodynamic analysis. It has been observed that even in such cases the obtained parameters can throw some light on the protein–solvent interactions (Edge et al., 1988; Hernandez-Arana & Rojo-Dominguez, 1993). It was observed that in the presence of carboxylic acid salts many of the proteins undergo aggregation at higher temperatures. To avoid aggregation and to bring down the T_m to manageable limits, 1.5 M or 1.0 M GdmCl was always added in the solutions. It was observed that the effect of GdmCl was additive within the experimental errors as observed earlier by other authors (Arakawa & Timasheff, 1985).

Figure 3 shows the effect of various concentrations of GdmCl on the thermal denaturation of RNase A at pH 7.0. The T_m of RNase A linearly decreases with GdmCl concentration, with a slope of $\sim 10\,^{\circ}C$/(mol of GdmCl). Addition of 0.5 M carboxylic salts like BTC, PTC, and citrate increase the T_m of RNase by 8–10 °C in the presence of varying concentrations of GdmCl. The slopes of plots between T_m and solvent-concentration for GdmCl and GdmCl with carboxylic salts differ slightly, indicating that these salts are more effective under denaturing conditions.

Figure 4 shows the effect of concentration of various carboxylic acid salts on the thermal stability of RNase A at pH 5.0 and 7.0 in the presence of 1.5 M GdmCl. ΔT_m, the difference in T_m of RNase in the presence and absence of salts, increases linearly with the salt concentrations in the case of EDTA, malonate, tartarate, and gluconate. However, the effect of BTC, PTC, citrate, and succinate is not linear. The ΔT_m values in the presence of salts are in the order: BTC $>$ PTC $\sim$ citrate $\sim$ succinate $>$ EDTA $>$ tartarate $>$ malonate $>$ gluconate. BTC increases the T_m of RNase A at pH 7.0 by as much as 18 °C at 0.6 M concentration, while PTC increases it by 23.8 °C at 1.2 M concentration. Such a significant increase in the thermal stability of proteins at these concentrations, with any cosolvent additives, had not been observed earlier.

The effectiveness of these salts is consistent with the position of -COOH group in the anionic lyotropic series, which is based on the effectiveness of different ions on the salting-out of proteins (Hofmeister, 1888; von Hippel & Schleich, 1969). The effective-

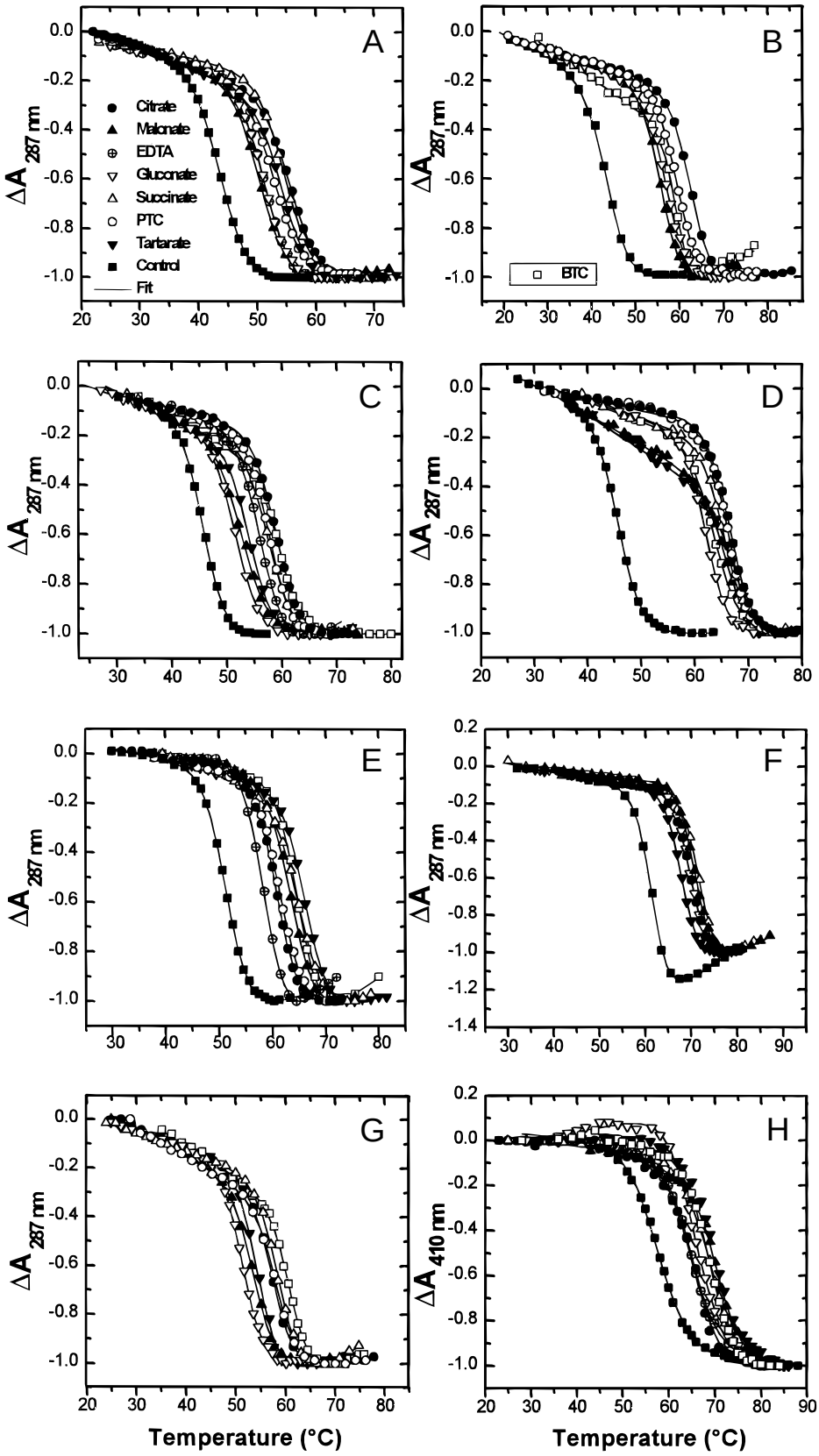

Fig. 1. Thermal denaturation profiles of RNase A in the presence of carboxylic acid salts at (**A**) pH 5.0, 1.5 M GdmCl, and 0.5 M salts; (**B**) pH 5.0, 1.5 M GdmCl, and 1.0 M salts; (**C**) pH 7.0, 1.5 M GdmCl, and 0.5 M salts; (**D**) pH 7.0, 1.5 M GdmCl, and 1.0 M salts; (**E**) pH 7.0, 1.0 M GdmCl, and 1.0 M salts; (**F**) pH 7.0, no GdmCl; 0.5 M tartarate, 0.7 M citrate, 1 M each of gluconate, succinate, and malonate; (**G**) pH 9.2, 1.5 M GdmCl, and 0.5 M salts; and (**H**) myoglobin at pH 7.0, 1.0 M GdmCl, and 1 M salts.

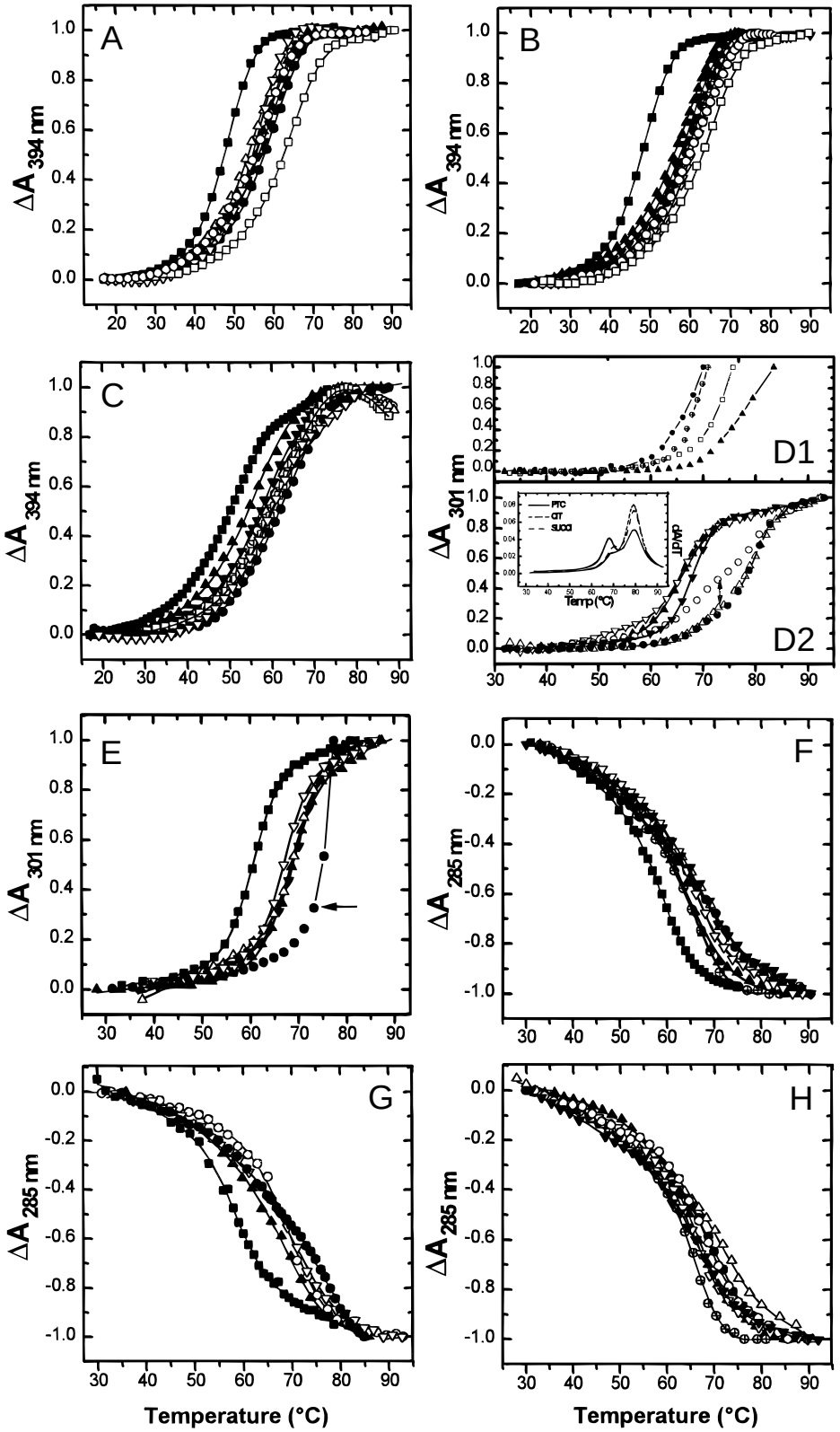

Fig. 2. Thermal denaturation profiles, in the presence of various carboxylic acid salts, of cyt *c* at (**A**) pH 5.0, 1.5 M GdmCl, and 0.5 M salts; (**B**) pH 5.0, 1.5 M GdmCl, and 1.0 M salts; **(C)** pH 7.0, 1.5 M GdmCl, and 0.5 M salts; lysozyme at (**D1**) pH 5.0, 1.5 M GdmCl, and 1.0 M salts; (**D2**) pH 5.0, 1.5 M GdmCl, and 0.5 M salts (inset shows the first derivative spectra of lysozyme denaturation in the presence of 0.5 M citrate, PTC, and succinate, the first peak indicates the aggregation of lysozyme just at the completion of the transition, whereas the second peak indicates the saturation of the aggregation phenomenon at the peak temperature); (**E**) pH 7.0, 1.5 M GdmCl, and 0.5 M salts (the arrow points to the onset of aggregation of the protein); Trp-Inh at (**F**) pH 5.0, 1.5 M GdmCl, and 0.5 M salts; (**G**) pH 5.0, 1.5 M GdmCl, and 1.0 M salts; and (**H**) pH 7.0, 1.5 M GdmCl, and 0.5 M salts. Symbols shown are the same as in Figures 1A and 1B. In (**A**) and (**B**) BTC concentration was 0.6 M.

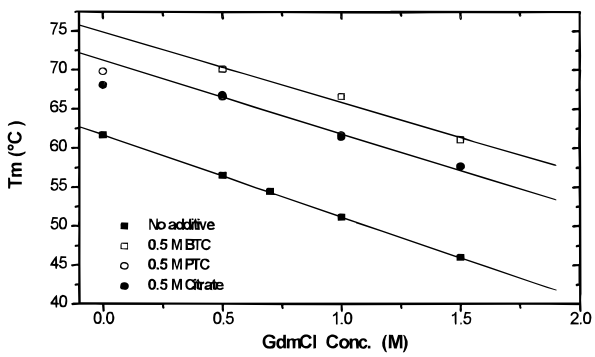

Fig. 3. Thermal stability of RNase A in the presence of varying concentrations of GdmCl and 0.5 M carboxylic acid salts at pH 7.0. At 0.5, 1.0, and 1.5 M GdmCl concentration, the open and filled circles are overlapping.

ness of these salts in stabilizing RNase A is very much consistent with their efficacy in providing thermal stability to antithrombin III (Busby & Ingham, 1984). In the case of RNase A, it is found that the effectiveness of carboxylic salts can be correlated with the number of COOH groups in the acid. The greater the number of COOH groups in the acid chain, the higher its effectiveness in providing stability.

Figure 5 shows the effect of carboxylic acid salts on thermal stability of cyt *c*. The trend in the effect of different salts on cyt *c* has been found to be similar to that on RNase A. This suggests that there should be some common forces operating in the stabilization of different proteins. These salts stabilize different proteins to different extents, perhaps reflecting variations in protein–cosolvent interactions depending upon the physico-chemical properties of proteins.

Surface tension measurement and analysis

Surface tension measurement for all the carboxylic acid salts in water were carried out at 25 °C as a function of their concentration. It has been observed that surface tension of all the aqueous salt solutions increases linearly with an increase in their concentration in the studied range (Fig. 6). Surface tension data for the aqueous carboxylic acid salt solution at various concentrations at 25 °C are in Table 1. The effectiveness of different carboxylic acid salts in raising the surface tension of water is in the order of BTC ~ EDTA > PTC > citrate > succinate > tartarate > malonate > gluconate. The order is almost the same as observed in the case of effectiveness of these salts in increasing the thermal stability of proteins, suggesting the possible role of increased surface tension of the solvent in the stabilization. This has been observed in amino acids, simple inorganic salts (Arakawa & Timasheff, 1983, 1984), and sugars (Lee & Timasheff, 1981; Kita et al., 1994; Lin & Timasheff, 1996).

Figure 7 shows the correlation of the increase in protein thermal stability (ΔT_m) with the increase in the surface tension of water ($\Delta\sigma$) in the presence of carboxylic acid salts. ΔT_m values for RNase A and cyt *c* have been calculated on a per molar basis of salt concentration by taking the slopes of the lines from Figures 4 and 5, respectively. In cases where T_m is not the linear function of salt concentration, the slopes from the tangents drawn at 0.5 M salt concentration were used. For BTC the slope of the tangent at 0.25 M salt concentration was taken, whereas for PTC and citrate, for which the curve is almost linear at lower concentration, a tangent at 0.5 M was taken to determine the values of $\Delta T_m/\Delta C$. $\Delta\sigma/\Delta C$ values for the salts were calculated from Figure 6. Figure 7 shows a nonlinear increase in $\Delta T_m/\Delta C$ with respect to $\Delta\sigma/\Delta C$. Both the quantities T_m and σ have been observed to increase linearly with an increase in the salt concentration. The observation clearly indicates the strong dependence of protein thermal stability on the surface tension of the solvent. But the nonlinear behavior of the curves (Fig. 7A,B) suggests that surface tension is not the sole factor leading to the increase in the thermal stability of RNase A and cyt *c*.

Figures 8A and 8B show a correlation of the increase in the T_m of RNase A and cyt *c* due to the addition of salts with the number of -COOH groups, C_N present in these molecules. For both the proteins, the magnitude of the slope of the plot has been found to be equal to 7.3 °C per -COOH group. This suggests the common and additive effect of carboxylic groups in providing stability to these proteins. However, the slope of the plot in the case of Trp-Inh

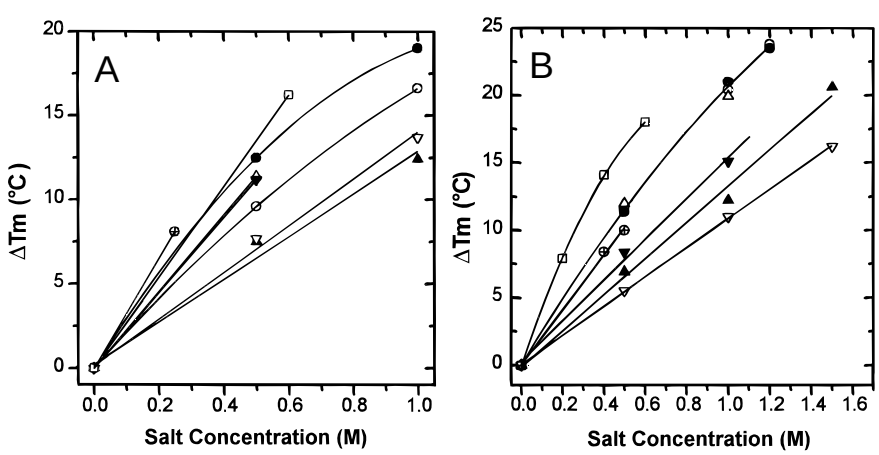

Fig. 4. Increase in the thermal stability of RNase A, ΔT_m, in varying salt concentrations at (**A**) pH 5.0 and (**B**) pH 7.0. Symbols shown are the same as that in Figures 1A and 1B.

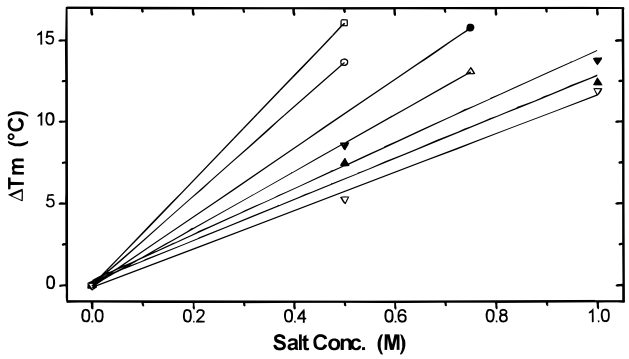

Fig. 5. Thermal stability of cyt *c* as a function of salt concentration at pH 7.0. Symbols shown are the same as that in Figures 1A and 1B.

is different (Fig. 8C), though the trends are quite similar in all three cases. Interestingly, the plots are similar to that obtained for the dependence of $\Delta\sigma/\Delta C$ with respect to C_N (Fig. 8D).

Thermal stability as a function of pH

Thermal denaturation studies on proteins have been carried out at several pH values to determine the role of net charges on protein surfaces in modulating the protein–solvent interactions. Figure 9 shows the effect of pH on the stability provided by these salts in the case of RNase A. The ΔT_m values in the presence of BTC, PTC, succinate, and malonate decrease linearly with the increase in net charges on proteins, i.e., these cosolvents provide greater stability at higher pH values. On the other hand, citrate, tartarate, and gluconate show a break in the linear trend below pH 7.0. This anomalous effect may be arising due to the presence of hydroxyl groups in these cosolvent molecules. BTC, PTC, succinate, and malonate have a backbone to which only -COOH groups are attached while in the case of citrate, tartarate, and gluconate, hydrogens of the methylene groups in the backbone of these molecules are substituted by hydroxyl groups. These extra hydroxyl groups may somehow be affecting the protein–solvent interactions depending on the charge status of the protein or the COOH groups in these molecules, or both. Studies on polyols as additives show that

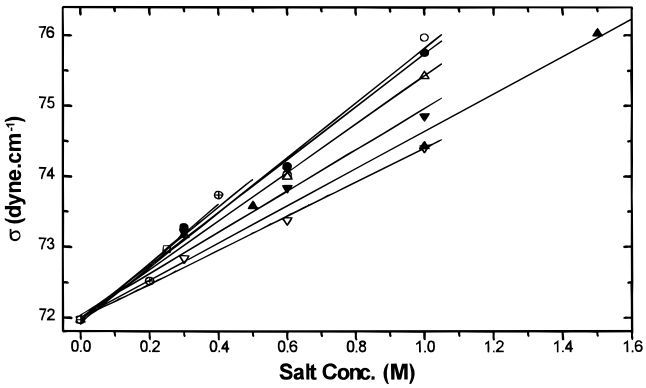

Fig. 6. Surface tension σ of aqueous solutions of carboxylic acid salts as a function of salt molarity. Symbols are as shown in Figures 1A and 1B.

Table 1. *Surface tension values of aqueous solutions of carboxylic acid salts*[a] *at different concentrations*[b] *at 25 °C*

	Surface tension (dyne cm^{-1})		
Cosolvent additive	0.3 M	0.6 M	1.0 M
EDTA	72.52 ± 0.22 (0.2 M)	73.76 ± 0.07 (0.4 M)	—
BTC	72.97 ± 0.09 (0.25 M)	73.72 ± 0.06 (0.5 M)	—
PTC	—	74.03 ± 0.21	75.97 ± 0.02
Citrate	73.28 ± 0.18	74.14 ± 0.15	75.76 ± 0.04
Succinate	73.17 ± 0.03	74.00 ± 0.05	75.43 ± 0.12
Tartarate	73.21 ± 0.05	73.84 ± 0.07	74.87 ± 0.06
Malonate	73.58 ± 0.02 (0.5 M)	74.43 ± 0.01 (1.0 M)	76.03 ± 0.01 (1.5 M)
Gluconate	72.84 ± 0.07	73.38 ± 0.2	74.41 ± 0.07

Note: Surface tension of water was taken as 71.97 dyne cm^{-1} at 25 °C.
[a]pH of the aqueous salt solutions was not adjusted and was usually 7–9 except EDTA, which had pH ∼ >11.
[b]Concentrations of EDTA, BTC, and malonate have been shown in parentheses.

they are more effective in providing stability at low pH values and their effectiveness increases linearly with a decrease in the pH of the solvent (Kaushik & Bhat, 1998). Hydroxyl groups in the carboxylic acid salts may be acting in the same fashion.

Heat capacity of proteins

We have obtained a value of ΔC_p of 2.14 ± 0.07 kcal mol^{-1} K^{-1} for RNase A denaturation evaluated by varying the T_m using different concentrations of GdmCl. Figure 10A shows the plot of ΔH_m vs. T_m for RNase A. The fitting errors are very low whereas the experimental errors are within ±2–4%. The ΔC_p value obtained by us (shown as the slope of the plot in Fig. 10A) for RNase A unfolding is very close to 2.2 ± 0.3 kcal mol^{-1} K^{-1} obtained spectroscopically by varying the T_m by the addition of urea (Pace & Laurents, 1989). Liu and Sturtevant (1996) have reported a ΔC_p of 2.10 ± 0.18 kcal mol^{-1} K^{-1} in the presence of 1.0 M GdmCl and found that ΔC_p varies depending on the concentration of GdmCl used. In the absence of GdmCl, they have reported a ΔC_p value of 1.74 ± 0.02 kcal mol^{-1} K^{-1} for RNase A. We have observed different slopes for ΔH_m vs. T_m plots in the case of RNase A in the presence of carboxylic acid salts at pH 5.0 and 7.0 (Fig. 10B). ΔC_p at pH 7.0 was found to be higher than that at pH 5.0 in the presence of salts, but both the values are much lower than that obtained in the presence of GdmCl (Fig. 10A).

Enthalpy–entropy compensation

Figure 11 shows the compensation between enthalpy and entropy in the case of protein stability provided by carboxylic acid salts. $\Delta\Delta H°$ and $\Delta\Delta S°$ values are the differences in enthalpy and entropy for proteins calculated at the T_m of the control (in the absence of salts) in the absence and presence of carboxylic acid salts. A value of 276.3 °C as the temperature of compensation, T_c, for RNase A

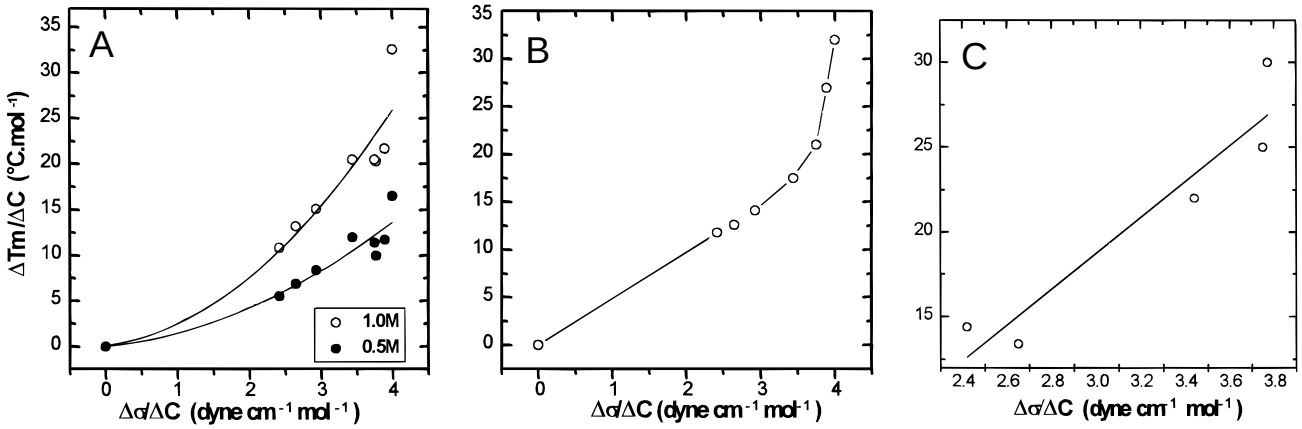

Fig. 7. The contribution of surface tension of the solvent in providing thermal stability to (**A**) RNase A at pH 7.0. Open circles data have been calculated by taking the slope of the tangent at 0.5 M salt concentration, while the filled circles are the actual experimental data obtained at 0.5 M salt concentration, (**B**) cyt *c* at pH 5.0, and (**C**) Trp-Inh at pH 5.0. ΔT_m values have been calculated as the slope of the tangent at 0.5 M salt concentration. The surface tension data have been calculated as the slope of the linear curve fitted to the experimental data. The standard deviation in ΔT_m values is within $\pm 0.3\,^{\circ}$C and in $\Delta\sigma$ is within ± 0.2 dyne cm^{-1}.

has been obtained signifying the role of water in imparting stability to the protein (Lumry & Rajender, 1970; Eftink et al., 1983). A T_c much lower than the temperature midpoint of transition, T_m, at which $\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$ were evaluated suggests the overwhelming entropic contribution to the net free energy of stabilization provided by the carboxylic acid salts.

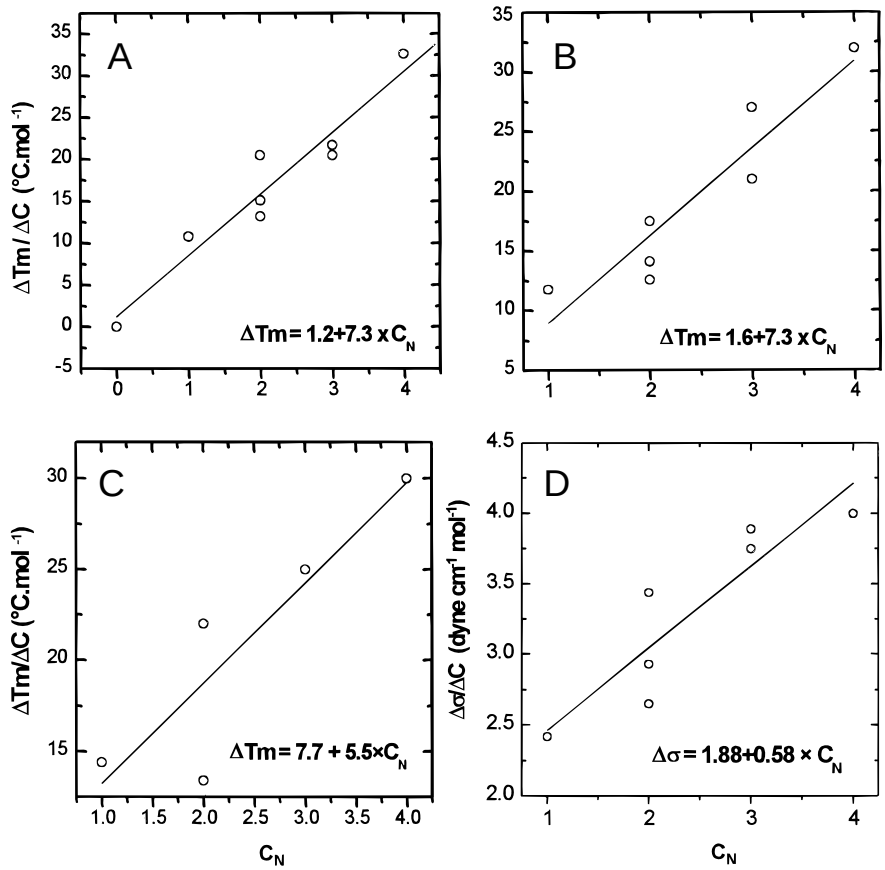

Fig. 8. Plots showing dependence of thermal stability of (**A**) RNase A, (**B**) cyt *c*, (**C**) Trp-Inh, and (**D**) surface tension of the aqueous solution of carboxylic acid salts on the number of -COOH- groups, C_N present in the cosolvent molecules. The standard deviation in ΔT_m and $\Delta\sigma$ values are the same as for Figure 7.

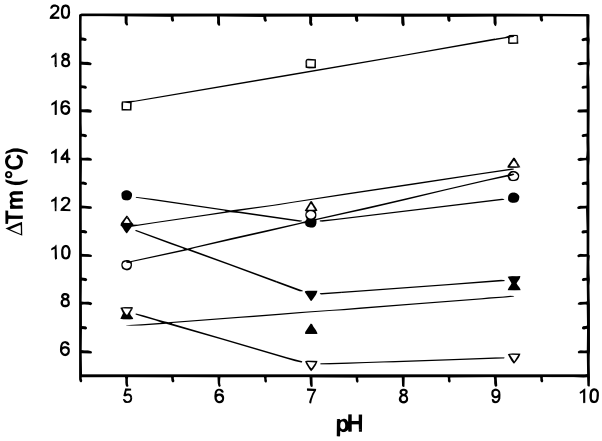

Fig. 9. Thermal stability of RNase A in the presence of various carboxylic acid salts as a function of pH of the solution. Symbols used are the same as in Figures 1A and 1B.

Role of physico-chemical properties of proteins

To elucidate the role of net charges and net hydrophobicity ($\Delta ASA_{NP} - \Delta ASA_P$) of proteins, where ΔASA_{NP} is the buried nonpolar accessible surface area (ASA) which gets exposed upon denaturation of the native state, and ΔASA_P is the buried polar ASA getting exposed upon protein unfolding, plots between these parameters and the increase in T_m were calculated (data not shown). The values of ΔASA_{NP} for polar and nonpolar surface areas are from Myers et al. (1995). It was observed that there was no direct correlation between the two parameters and the ΔT_m for proteins. There was also no direct correlation observed between the total change in ΔASA_{NP} and the ΔT_m of proteins. This indicates that neither the net charges, irrespective of the protein type, nor the net hydrophobicity, is a dominant factor governing protein thermal stability in the presence of carboxylic acid salts.

Discussion

Role of surface tension

In Figure 7, surface tension is the major force in governing protein–solvent interactions, providing thermal stability to the protein molecules in the presence of carboxylic acid salts. These salts vary in the number of carboxylic and hydroxyl groups that affect the surface tension of water to varying extents, increasing the thermal stability of proteins in the same order as the increase in the surface free energy of water. Increased thermal stability in a solvent of high surface free energy has been explained on the basis of two operative forces (1) an increased energy required for cavity formation (Sinanoglu & Abdulnur, 1964, 1965; Lee & Timasheff, 1981; Arakawa & Timasheff, 1983; Lin & Timasheff, 1996) and (2) preferential hydration of proteins (Gekko & Morikawa, 1981; Arakawa et al., 1990b).

Our results show that the magnitude of the increase in the surface tension of the solvent water due to the added cosolvents depends upon the number of -CH_2, -COOH, and -OH groups present in them. We have found that the increase in surface tension of the solvent correlates well with the increase in the thermal stability of globular proteins studied (Fig. 7). However, nonlinearity in the curves between $\Delta T_m/\Delta C$ and $\Delta\sigma/\Delta C$ suggests that surface tension is not the sole factor responsible for the thermal stability of proteins. The stabilization in general involves a fine balance between two opposing forces, adsorption or binding and exclusion. The tendency of an ion to interact with a surface has a direct binding part and a solvation-sphere part (Timasheff, 1993). The net free energy of stabilization depends on the positive free energy of cavity formation in a solvent of given surface free energy and the free energy contribution from protein-cosolvent interaction ($\Delta G^\circ_{solvation}$) (Breslow & Guo, 1990), i.e.,

$$\delta(\Delta G^\circ)_{stab} = \delta(\Delta G^\circ)_{cavitation} + \delta(\Delta G^\circ)_{solvation}. \quad (1)$$

The contribution due to electrostatic interactions of the charged cosolvent molecules including that of the Na^+ ions are likely to be

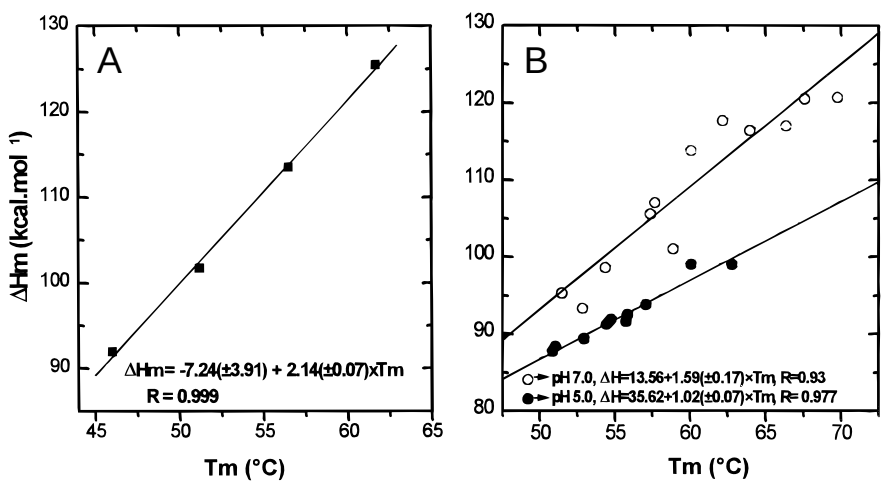

Fig. 10. Calculation of heat capacity of denaturation, ΔC_p of RNase A from ΔH_m vs. T_m plots: (**A**) in the presence of 0–1.5 M GdmCl in the buffer at pH 7.0; (**B**) in the presence of various carboxylic acids at pH 5.0 and 7.0. At pH 5.0, 1.5 M GdmCl was always present in the solution whereas at pH 7.0 GdmCl was present from 0–1.5 M concentration along with various salts.

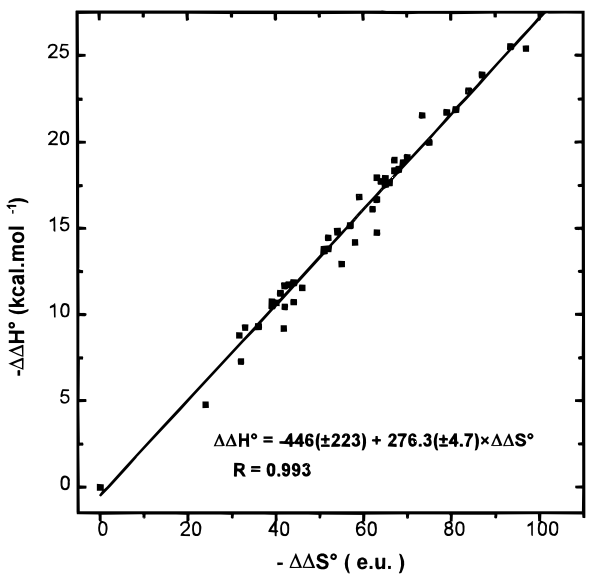

Fig. 11. Enthalpy–entropy compensation plots for RNase A. Data plotted are for different pH conditions in the presence of several carboxylic acid salts.

negligible as compared to the surface tension effect. Acetate has been found to lead to large preferential hydration of lysozyme at 0.5 and 1.0 M concentrations (Arakawa & Timasheff, 1982). At 20 mM concentration it has been found to have no net interactions with lysozyme and BSA at pH 4.5. Although these interactions would be highly pH dependent, we expect their contribution, if any, to be marginal at very high cosolvent concentrations. The carboxylic acid salts used by us have the basic acetate moiety in them and are therefore expected to act similar to acetate. A possible origin of the nonlinear trend of $\Delta T_m/\Delta C$ vs. $\Delta\sigma/\Delta C$ may be due to the extrapolation of the properties of the solvent system determined at 25 °C to higher temperatures, i.e., T_m of proteins. It is quite possible that the nature and magnitude of these properties may change at higher temperatures in a nonlinear fashion depending on the solute–solvent interactions. Further, it must be pointed out that the surface tension studies represent measurements for the flat air–water interface, which is quite different from the complex curved protein–solvent interface, even though the effect of the curvature on the surface tension can be accounted for by geometric considerations (Nicholls et al., 1991; Sharp et al., 1991). Nonetheless, in the case of carboxylic acid salt induced stability of proteins, surface tension seems to be at least a dominant, if not the sole factor.

Competition between stabilizers and denaturants

A cocktail of lyotropic and chaotropic salts as cosolvents provides a very complex situation at the protein–solvent interface. As a result of the structure making action of the lyotropes, water molecules will try to occupy the protein surface to avoid the contact of lyotropes with the protein surface, while the chaotropes like GdmCl will compete with water for site occupancy on the protein surface (Timasheff, 1992, 1993). The variation in the slopes of curves between T_m and GdmCl concentration and T_m and (GdmCl + 0.5 M carboxylic acids) (Fig. 3) indicates that carboxylic acid salts are more effective under denaturing conditions, i.e., ΔT_m in their presence increases with an increase in the GdmCl concentration. The variation in slopes could be due to small changes in the denaturation enthalpy of the protein under the different conditions used and points to the possible competition between GdmCl and carboxylic acid salts resulting in a shift in the equilibrium between the two end states for thermodynamic stability of the system.

Solvent effect on heat capacities of proteins

It has been found that solvents can drastically affect the heat capacity of denaturation of proteins, depending on the composition of the solution and on the method employed to change the temperature of unfolding (Liu & Sturtevant, 1996). Small errors in ΔC_p may not affect the estimation of ΔG° over a small range of temperature away from T_m, although these errors may affect the estimation of $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ appreciably. We have found that carboxylic acid salts decrease the ΔC_p of RNase A in the absence and presence of GdmCl (Fig. 10). Liu and Sturtevant (1996) observed an increase in the ΔC_p value for RNase A in the presence of sucrose. This could be due to the aggregation of the denatured state as observed by them. Santoro et al. (1992) reported a $\Delta C_p = 0$ for RNase in the presence of sarcosine. Lysozyme also showed similar behavior in high concentration of osmolytes (Santoro et al., 1992). Woolfson et al. (1993) have reported a decrease in ΔC_p for ubiquitin with increasing methanol concentration. The positive value of ΔC_p of denaturation is considered to be essentially due to the exposure of hydrophobic groups to the solvent medium (water), which can form clathrate hydrates around the nonpolar residues. When a cosolute is added to water, the ΔC_p would depend on the additional interactions the buried groups would have with water in the presence of the cosolute or with the cosolute itself. The lowering in the ΔC_p value in the presence of carboxylic salts implies that the solvation of the exposed nonpolar groups is considerably diminished. The variation in the ΔC_p values obtained by us at pH 5.0 and 7.0 indicate the varied ionization states of the carboxylate ions at different pHs that lead to different magnitudes of interactions between the nonpolar groups of proteins and charged carboxylate ions.

Role of protein in protein–solvent interactions

At high concentration, carboxylic acid salts act as strong salting-out agents. This is due to unfavorable interactions between the highly polar cosolvent molecules and the nonpolar residues in proteins. Carboxylic acids have been observed to alter the properties of water due to their kosmotropic nature which leads to the strengthening of cohesive forces among the water molecules. According to Breslow and Guo (1990), the effectiveness of chaotropic or kosmotropic agents is governed by a fine balance between the free energy of cavitation, which is positive for solvents with increased surface tension due to the presence of cosolvents, and free energy of solvation which depends upon the nature of solute–solvent interactions. The free energy of solvation should be dependent upon the favorable interactions between polar solvent and polar protein atoms, and the unfavorable polar–nonpolar interactions between cosolvent and protein molecules. Protein denaturation involves a large change in the surface area, which is essentially due to the buried hydrophobic residues that are exposed to solvent upon denaturation of the protein molecule. The data on ΔASA for several globular proteins elucidated by Myers et al. (1995) and

Makhatadze and Privalov (1995) show that the value of ΔASA_{NP} is more than twice that for ΔASA_P. Therefore the net interaction between the exposed surfaces of the denatured protein and the carboxylic acid salt molecules should be of repulsive nature. Strong repulsion from solvent should force the nonpolar residues to get buried. The stronger the repulsion, the stronger the hydrophobic interactions in the core of the protein. The net positive contribution of both the free energy of cavity formation and the free energy of solvation should lead to a large free energy of stabilization. Recently Liu and Bolen (1995) and Wang and Bolen (1997), based on the free energy of transfer studies of amino acids and a model peptide from water to osmolyte solutions, have demonstrated that the stabilizing effect of osmolytes for proteins is governed by unfavorable peptide backbone interactions with the cosolvents and not the amino acid side chains. Similar studies in the case of carboxylic acid salts would perhaps throw further light on the nature of interactions between various protein groups and these salts and their role in protein stabilization.

The compensation plot between $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ for the studied proteins indicates the role of water in imparting thermal stability to proteins. T_c between 250 and 315 K has been proposed to be due to the altered state of water in protein–water interactions (Lumry & Rajender, 1970; Eftink et al., 1983), which in the present case could result from the increase in the surface tension of the medium in the presence of carboxylic acid salts. Nicholls et al. (1991) and Sharp et al. (1991) have proposed that surface tension can be used as a measure of the hydrophobic stabilization of proteins. Tanford (1979) has argued for the relationship between interfacial free energy and the hydrophobic interactions. Our data also support the above proposition since we have observed that the higher the surface tension of the aqueous cosolvent medium, the greater its effectiveness in raising the thermal stability of globular proteins. However, the lack of correlation between the net hydrophobicity and net charges with ΔT_m for a wide variety of proteins indicates that in addition to the predominant role of hydrophobic interactions, the interaction of charged residues in proteins with the charged carboxylic salt ions, either by direct weak binding or through long range interactions mediated by water, leads to the overall stability effect.

In conclusion, there is a significant increase in the thermal stability of proteins in the presence of carboxylic acid salts, being as high as 22 °C at 1.0 M concentration, compared to several of the cosolvent additives studied by us or used by others. Increase in thermal stability also seems to correlate well with the increase in the number of carboxyl groups of the salt. Several physicochemical properties of proteins like net hydrophobicity and charges, irrespective of the protein used, seem to be playing a compensatory role, leading to the overall thermal stability effect in the presence of these salts. The studies carried out on a wide variety of proteins and carboxylic acid salts indicate that the predominant force behind carboxylic acid salt induced stabilization of proteins is the increase in the surface free energy of the solvent medium.

Materials and methods

Materials

Ribonuclease A (RNase A), lysozyme (hen egg white (HEW)), α-chymotrypsinogen (α-CTgen), cytochrome *c* (cyt *c*), myoglobin and trypsin inhibitor from HEW (Trp-Inh) were procured from Sigma Chemical Company (St. Louis, Missouri). They were dialyzed against distilled de-ionized water and lyophilized. The lyophilized samples were dried over P_2O_5. PTC and gluconic acid were purchased from Sigma Chemical Company. BTC was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Trisodium citrate and disodium tartarate were obtained from E. Merck (Mumbai, India). Disodium succinate hexahydrate and disodium malonate were obtained from Sisco Research Laboratories (Mumbai, India). Tetrasodium EDTA and GdmCl were procured from Amresco (Solon, Ohio). Glass double distilled water was used to make the solutions. The pH of the solutions was adjusted on a Consort or Radiometer PHM84 research pH meter. A 40 mM acetate buffer (E. Merck) at pH 5.0, 20 mM phosphate (E. Merck) or MOPS (Sigma Chemical Co.) buffers at pH 7.0, and 20 mM glycine NaOH buffer (E. Merck) at pH 9.2 were used from their stock solutions.

Methods

Thermal denaturation experiments

Thermal denaturation experiments were carried out using a Cecil 599 or Shimadzu 160A model UV/VIS spectrophotometer to which a linear temperature programmer CE-247 (Cecil) was attached. The concentrations of the protein solutions were ~0.5 mg/mL, except for cyt *c* and myoglobin where 0.1 mg/mL of protein concentration was used. The protein solutions were heated in a 0.5 mL masked and Teflon stoppered quartz cuvette (Hellma, Mülheim/Baden, Germany). The temperature of the protein solution was raised linearly at a constant scan rate of 1 °C/min in all experiments. The wavelengths for monitoring the conformational changes were 287 nm for RNase A, 293 nm for α-CTgen, 301 nm for lysozyme, 285 nm for trypsin inhibitor, 394 nm for cyt *c* and 410 nm for myoglobin based on the difference spectra of the respective proteins. The reversibility of the denaturation curves were determined by reheating the protein solutions. In all the cases, except at pH 9.2 and wherein proteins became aggregated during the denaturation reaction, the thermal transitions were reversible and amenable to thermodynamic analysis.

Surface tension measurements

Surface tension of aqueous carboxylic acid salts solutions was determined by drop weight method (Kita et al., 1994). The rate of flow through the capillary was controlled to 5–6 drops per minute. The temperature of the stalagmometer was maintained at 25 ± 0.1 °C by circulating water in the glass jacket around it from a circulator bath, which was stopped during the collection of drops to avoid any vibrations. In each experiment, the weight of 50 drops was measured in a Precision Scientific weighing balance immediately after the collection of drops.

Analysis of data

The evaluation of thermodynamic parameters obtained from spectroscopic technique is based on the equilibrium constant *K*, for $N \Leftrightarrow D$ conversion in a two-state reversible transition, where *N* and *D* represent the native and the denatured states, respectively. Equilibrium constant was deduced from the equation:

$$K = [\text{Unfolded}]/[\text{Native}] = (A_N - A_O)/(A_O - A_D) \qquad (2)$$

where A_N is the absorbance of the pure native state in the transition zone at any temperature T and extrapolated from the pre-transition region, A_D is the corresponding absorbance of the pure denatured state, and A_O is the observed absorbance at temperature T in the transition zone. Equation 2 can be rewritten as

$$A_O = (A_N + K \cdot A_D)/(1 + K) \tag{3}$$

and

$$K = \exp(-\Delta G^\circ/RT) \tag{4}$$

where ΔG° is the standard free energy change, R is the gas constant, and T is the temperature in kelvin. Substituting the value of K in Equation 3, we deduce

$$A_O = [(A_N + A_D \cdot \exp(-\Delta G^\circ/RT))/(1 + \exp(-\Delta G^\circ/RT)]. \tag{5}$$

The Gibbs–Helmholtz equation can be written as

$$\Delta G^\circ(T) = \Delta H_m(1 - T/T_m) - \Delta C_p(T_m - T + T \cdot \ln(T/T_m)) \tag{6}$$

where ΔH_m is the enthalpy of denaturation evaluated at the T_m, ΔC_p is the heat capacity change for denaturation, T_m is the temperature at the midpoint of the transition, and T refers to any temperature where $\Delta G^\circ(T)$ is calculated.

Substituting the value of $\Delta G^\circ(T)$ obtained from Equation 6 in Equation 5, we deduce

$$A_O = \frac{[A_N + A_D \cdot \exp(-1/R(\Delta H_m(1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + \ln(T/T_m)))]}{[1 + \exp(-1/R(\Delta H_m(1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + \ln(T/T_m)))]}. \tag{7}$$

Since A_N and A_D have been found to be a linear functions of temperature, Equation 7 can be rewritten as

$$A_O = \frac{[a_N + m_N \cdot T + (a_D + m_D \cdot T) \cdot \exp(-1/R(\Delta H_m(1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + \ln(T/T_m)))]}{[1 + \exp(-1/R(\Delta H_m(1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + \ln(T/T_m)))]} \tag{8}$$

where a_N and a_D are the intercepts of A_N and A_D in the absorbance vs. temperature plots and m_N and m_D are the corresponding slopes. Equation 8 has been used to fit to the thermal denaturation data by using nonlinear least-squares fit and successive iterations using Marquardt–Levenberg routine as provided in the Origin™ software (Microcal Inc., Northampton, Massachusetts). A minimum of 50 iterations or more were performed until the fractional change in χ^2 value was obtained within the tolerance limit set to 0.0005. Usually a value of the order of 10^{-5}–10^{-4} of fractional χ^2 change was obtained in the final iterations. Parameters a_N, a_D, m_N, m_D, T_m, and ΔH_m were floated freely so as to fit them simultaneously to Equation 8.

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