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Differential scanning calorimetric studies on the effect of ammonium and tetraalkylammonium halides on the stability of lysozyme

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Abstract

The thermal denaturation of lysozyme was studied at pH 2.50 and 6.00 in aqueous solutions of ammonium (NH_4Cl and NH_4Br) and tetraalkylammonium halides (Me_4NCl, Me_4NBr, Et_4NBr, Pr_4NBr, Bu_4NBr) using high-sensitivity differential scanning calorimetry. The transition temperature, heat capacity, enthalpy, entropy and free energy of denaturation have been determined by a least-squares fit of the excess heat capacity data to the two-state model. Ammonium and tetraalkylammonium halides (except Me_4NCl and NH_4Cl at high concentrations at pH 6.00) are found to destabilize lysozyme and the destabilization increases with increasing concentration and alkyl chain length. However, NH_4Cl and Me_4NCl act as stabilizers at high concentrations at pH 6.00. Results are discussed in terms of electrostatic and hydrophobic interactions. The stabilization of lysozyme by NH_4Cl and Me_4NCl can be attributed to the charge on the quaternary nitrogen atom while destabilization in tetraalkylammonium halides solution is due to the interaction between hydrophobic molecules of the medium and the hydrophobic parts of the protein.

Keywords: Protein stability; Hydrophobic effect; Thermodynamics; Calorimetry; Lysozyme; Tetraalkylammonium halides

1. Introduction

The interactions between the proteins and the surrounding solvent play a crucial role in determining the native structure of proteins. Various substances stabilize or destabilize the native structure of proteins when added to their aqueous solutions [1–4]. The stabilizing or destabilizing effect of additives on proteins has been explained in terms of either direct binding or indirectly through solvent mediated effects. Solvent additives such as urea, guanidinium hydrochloride, monohydric alcohols and some salts such as NaSCN and $MgCl_2$ destabilize proteins by binding which weakens the hydrophobic interactions between non-polar residues of proteins [5–12]. The solvent additives such as polyhydric alcohols, sugars, lower homologues of amino acids, methylamines and salts such as Na_2SO_4 and $MgSO_4$ stabilize proteins by preferential hydration which strengthens the hydrophobic interactions between the non-polar residues of proteins [10,13–17].

The effect of salts on the stability of proteins has

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been correlated with their preferential interactions with proteins [18]. It was found [10,19] that the preferential hydration increased in the order $Cl^- < CH_3COO^- < SO_4^{2-}$, regardless of the cationic species used in agreement with the anionic Hofmeister series [20] and the same parameter exhibited a tendency to increase in the order Mn^{2+}, $Ni^{2+} < Ca^{2+}$, $Ba^{2+} < Mg^{2+} < Na^+$. The stabilizing and destabilizing effectivenesses of the salts were interpreted in terms of the observed preferential interactions.

Tetraalkylammonium salts can give better insight into the effect of electrostatic and hydrophobic interactions on the stability of proteins as these salts are expected to influence macromolecular conformation by weakening attractive or repulsive inter- and intra-chain charge–charge interactions and by affecting hydrophobic interactions through the side-chains of the alkyl groups.

The effect of tetraalkylammonium salts on the ribonuclease transition has been investigated by Von Hippel and Wong [2], but direct calorimetric studies of such systems are not available. Differential scanning calorimetry (DSC) can be used to obtain a quantitative estimate of the degree of stabilization [21]. Lysozyme was chosen as the protein for study because it is a typical globular protein and its thermal unfolding has been shown to be a two-state process in aqueous solution [22]. In the present study, transition temperature, T_d (at which 50% of the protein is denatured), and thermodynamic functions of denaturation like enthalpy, $\Delta_N^D H$, entropy, $\Delta_N^D S$, and heat capacity, $\Delta_N^D C_p$, have been determined in aqueous solutions at pH 2.50 and 6.00 in the presence of ammonium and tetraalkylammonium salts.

2. Materials and methods

Hen egg lysozyme was obtained from Sigma and used after exhaustive dialysis against distilled and deionized water at 4°C for over 24 h and lyophilization. NH_4Cl and NH_4Br (> 99%) were BDH products and recrystallized from deionized water.

Me_4NBr and Me_4NCl (> 98%) procured from BDH and SRL, were recrystallized from dry methanol and precipitated twice from methanol by addition of dry, peroxide-free ether. Et_4NBr (> 98%), Bu_4NBr (> 99%) procured from SRL and Pr_4NBr (> 99%) from Merck were precipitated three times from saturated solution in chloroform by the addition of petroleum ether followed by cooling [23]. The precipitate was washed with petroleum ether two to three times before filtration. The recrystallized salts were dried in vacuum at 60–80°C and kept in a vacuum desiccator over P_2O_5 after purification.

The experiments were carried out in either 0.02 mol dm^{-3} NaCl–tetraalkylammonium salts mixture adjusted to the desired pH 6.00 or in 0.05 mol dm^{-3} glycine–tetraalkylammonium salts mixture adjusted to pH 2.50 with HCl or NaOH after calibration with standard buffers of pH 7.00 and 4.00. The molar mass of lysozyme was close to 14.3 kDa. Protein concentrations were measured spectrophotometrically on a Shimadzu UV 1201 spectrophotometer by using an absorbance value of 2.635 in 0.2 mol dm^{-3} Na_2HPO_4–NaH_2PO_4 buffered at pH 7.00 [24].

Calorimetric measurements were performed on a SETARAM micro-DSC apparatus at a scan rate of 0.6 K min^{-1}. The concentration of lysozyme was about 2.5 mg cm^{-3} in buffer solution of about 0.85 g in a vessel of 1 ml capacity. To analyze a DSC scan, the instrumental baseline determined with buffer in both cells was subtracted from the results obtained with the sample. All the excess power thermal scans were converted to excess heat capacity vs. temperature scans, following the procedure described by Schwarz and Kirchoff [25]. Thermodynamic functions of protein denaturation, transition temperature, T_d, heat capacity, $\Delta_N^D C_p$ and heat of denaturation, $\Delta_N^D H$, of lysozyme in aqueous solutions of tetraalkylammonium salts at pH 2.50 and 6.00 were determined from the least-squares fit of the excess heat capacity data to the two-state model.

The two-state model provides a functional dependence of the measured heat capacity on temperature of the following form:

$$C_{p,s} = a + b(T - T_d) + \alpha[\Delta a + \Delta b(T - T_d)] \quad (1)$$

where $a + b(T - T_d)$ is the linear fit of the pre-transition baseline to T, and $\Delta a + \Delta b(T - T_d)$ is the difference between extrapolation of the pre- and post-transitional baselines at T. $C_{p,s}$ is the sigmoidal baseline extrapolated under the transition curve and α is the functional area under the transition curve at T. The transition enthalpy $\Delta_N^D H$ is determined from

the area under the transition curve and from the number of moles of protein in the cell.

3. Results and discussion

A single reversible endothermic peak was obtained for lysozyme under all conditions employed except in few cases, where the precipitation of solution was observed. Thermodynamic parameters obtained from the analysis of DSC scans of lysozyme in presence and absence of ammonium and tetraalkylammonium salts at pH 2.50 and 6.00 are summarized in Tables 1 and 2 and plotted in Figs. 1 and 2, respectively. The transition temperature T_d decreases with increasing salt concentrations, while the peak area becomes smaller and the peak width slightly larger. Each value represents an average of three or four experiments. The T_d values have a maximum experimental error of ± 0.5 K and $\Delta_N^D H$ values have a maximum expected error of ± 20 kJ mol^{-1}, including errors in sample preparation, calibration constant and reproducibility. $\Delta_N^D H$ values for lysozyme are 437 and 495 kJ mol^{-1} at pH 2.50 and 6.00, respec-

Table 1
Thermodynamic parameters for denaturation of lysozyme in the presence of tetraalkylammonium salts at pH 2.50

Molality (mol kg^{-1})	T_d (K)	ΔT_d (K)	$\Delta_N^D H$ (kJ mol^{-1})	$\Delta_N^D C_p$ (kJ K^{-1} mol^{-1})	η
0.0	336.2	–	437 ± 10.7	6.7 ± 1.1	1.010
Tetramethylammonium bromide					
0.5	330.8	−5.4	406 ± 10.2	6.1 ± 1.5	0.972
1.0	327.8	−8.4	375 ± 10.7	4.1 ± 1.2	0.941
2.0	323.2	−13.0	354 ± 9.3	4.1 ± 1.0	0.911
Tetraethylammonium bromide					
0.5	329.4	−6.8	390 ± 11.0	6.4 ± 1.0	0.963
1.0	326.9	−9.3	368 ± 8.5	4.3 ± 0.2	0.978
2.0	321.8	−14.4	346 ± 4.7	2.0 ± 0.6	0.986
Tetrapropylammonium bromide					
0.5	324.5	−11.7	385 ± 8.5	6.3 ± 0.7	1.020
1.0	318.2	−18.0	348 ± 6.4	4.4 ± 0.7	1.040
Tetrabutylammonium bromide					
0.2	328.7	−7.5	391 ± 8.8	5.5 ± 1.3	0.970
0.5	320.5	−15.7	348 ± 5.9	3.5 ± 1.5	0.997
Tetramethylammonium chloride					
0.5	331.6	−4.6	410 ± 7.8	5.6 ± 1.9	0.977
1.0	332.2	−4.0	415 ± 8.5	4.7 ± 1.2	0.990
2.0	333.7	−2.5	423 ± 10.3	6.8 ± 2.0	0.985
4.0	334.7	−1.5	pptd.	–	–

[lysozyme] = 0.171 mM. Scan rate = 0.6 K min^{-1}. Buffer 0.05 M Gly·HCl.

Table 2
Thermodynamic parameters for denaturation of lysozyme in the presence of ammonium and tetraalkylammonium salts at pH 6.00

Molality (mol kg^{-1})	T_d (K)	ΔT_d (K)	$\Delta_N^D H$ (kJ mol^{-1})	$\Delta_N^D C_p$ (kJ K^{-1} mol^{-1})	η
0.0	348.3	–	495 ± 9.5	6.4 ± 1.1	1.110
Ammonium bromide					
0.5	346.8	−1.5	467 ± 9.2	3.2 ± 1.1	0.998
1.0	345.5	−2.8	440 ± 5.1	1.9 ± 0.3	0.995
2.0	345.1	−3.2	pptd.	–	–
Tetramethylammonium bromide					
0.5	345.3	−3.0	448 ± 13.2	6.0 ± 0.9	0.975
1.0	342.9	−5.4	427 ± 5.2	4.1 ± 1.3	0.994
2.0	339.4	−8.9	409 ± 14.8	4.0 ± 0.4	0.990
Tetraethylammonium bromide					
0.5	342.7	−5.6	435 ± 4.6	6.5 ± 0.5	0.992
1.0	340.1	−8.2	421 ± 9.2	5.5 ± 1.0	0.996
2.0	336.6	−11.7	399 ± 17.5	3.2 ± 1.4	0.987
Tetrapropylammonium bromide					
0.5	337.5	−10.8	417 ± 15.8	5.7 ± 1.9	1.040
0.82	333.8	−14.5	394 ± 18.6	4.6 ± 1.5	0.972
1.24	329.5	−18.8	371 ± 11.1	2.7 ± 1.6	0.985
2.0	321.1	−27.2	350 ± 9.2	2.1 ± 1.2	1.050
Tetrabutylammonium bromide					
0.2	337.1	−11.2	424 ± 9.8	5.3 ± 0.9	0.990
0.5	329.0	−19.3	391 ± 9.2	3.4 ± 1.5	1.010
1.0	316.0	−32.3	337 ± 4.4	2.5 ± 1.4	0.981
Ammonium chloride					
0.5	347.3	−1.0	478 ± 5.8	5.0 ± 1.9	0.955
1.0	347.4	−0.9	485 ± 9.9	4.1 ± 0.8	0.995
2.0	348.2	−0.1	486 ± 5.8	4.5 ± 0.7	1.009
4.0	348.5	0.2	pptd.	–	–
Tetramethylammonium chloride					
0.5	345.6	−2.7	425 ± 7.7	5.7 ± 1.6	0.958
1.0	346.0	−2.3	436 ± 10.2	5.8 ± 0.4	0.962
2.0	346.3	−2.0	457 ± 11.2	5.3 ± 1.5	0.984
4.0	348.8	0.5	496 ± 5.8	5.1 ± 1.6	0.983
6.0	351.0	2.7	527 ± 8.3	5.1 ± 1.5	0.992

[lysozyme] = 0.171 mM. Scan rate = 0.6 K min^{-1}. Buffer 0.02 M NaCl–NaOH.

tively, which are close to 428 and 505 kJ mol^{-1} reported by Schwarz [24]. Thermal denaturation of lysozyme in the presence of tetraalkylammonium salts is a two-state transition as the mean value of the cooperativity index, $\eta(=\Delta_N^D H/\Delta H_{VH}) = 0.990 \pm 0.021$. Further reversibility for the denaturation was checked by reheating the protein solution after rapidly cooling from the first scan. On reheating, no significant variation in the position and area of the transition peak was observed in presence of Me_4NBr, Et_4NBr or Pr_4NBr indicating that the transitions are reversible. This is not so with NH_4Br and NH_4Cl (at pH 6.00), Me_4NCl and Bu_4NBr (at pH 2.50 and 6.00), possibly due to decreased solubility of the denatured protein as observed by turbidity after the first scans. DSC endotherms could not be obtained for NH_4Br and NH_4Cl at pH 2.50 as the protein precipitated before denaturation.

Ammonium and tetraalkylammonium salts (except Me_4NCl at higher concentrations at pH 6.00) lower T_d of lysozyme indicating that these salts act as destabilizers. Lowering of T_d and hence destabilization increases with concentration, which becomes more pronounced with higher alkyl chain lengths as shown in Figs. 1 and 2. In fact, tetrabutylammonium bromide which lowers T_d by about 32°C mol^{-1} at pH 6.00 is among the most effective destabilizers.

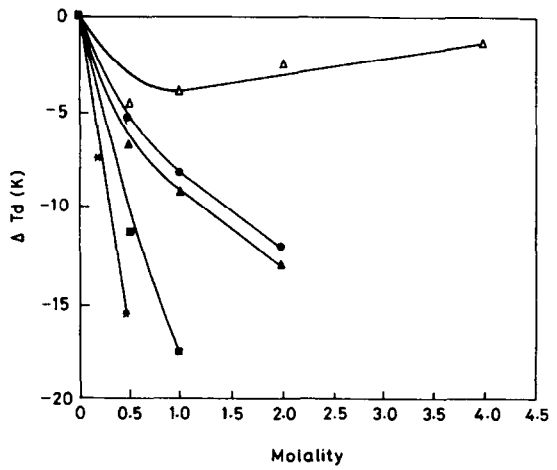

Fig. 1. ΔT_d of lysozyme as a function of tetraalkylammonium halides concentration at pH 2.50. (△) Me_4NCl; (●) Me_4NBr; (▲) Et_4NBr; (■) Pr_4NBr; (*) Bu_4NBr.

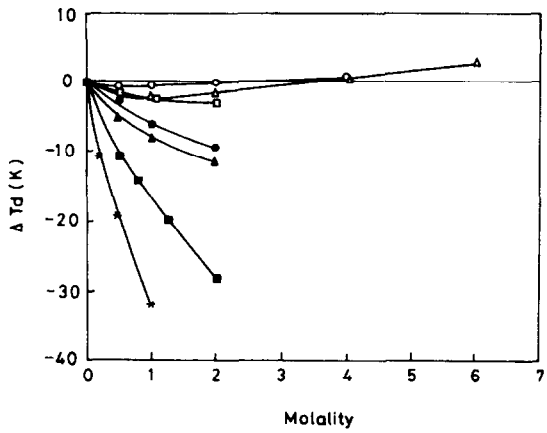

Fig. 2. ΔT_d of lysozyme as a function of ammonium and tetraalkylammonium halides concentration at pH 6.00. (○) NH_4Cl; (△) Me_4NCl; (□) NH_4Br; (●)Me_4NBr; (▲) Et_4NBr; (■) Pr_4NBr; (*) Bu_4NBr.

These salts lower T_d of lysozyme in the following order:

$$Me_4NCl < NH_4Cl < NH_4Br < Me_4NBr < Et_4NBr < Pr_4NBr < Bu_4NBr$$

Similarly, heat of denaturation, $\Delta_N^D H$, decreases in the presence of ammonium and tetraalkylammonium salts. The decrease in $\Delta_N^D H$ with salt concentration (Figs. 3 and 4) is not due to the direct effect of salt concentration but is rather due to the lowering of T_d of lysozyme in the presence of salt. Using $\Delta_N^D C_p$ of lysozyme at pH 2.50 and 6.00 as 6.7 and 6.4 kJ mol^{-1} K^{-1}, the enthalpy of denaturation in the presence of salt can be accounted for in terms of the temperature dependence of $\Delta_N^D H$; e.g. the calculations show that in 0.5 m Bu_4NBr at pH 2.5, T_d is lowered by as much as 15.7 K, and the enthalpy of denaturation would decrease by about 105 kJ mol^{-1}, which indeed is in agreement with the observed value of 89 kJ mol^{-1} within the experimental error.

The tetraalkylammonium halides destabilize lysozyme 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, $\Delta_N^D H$ decreases in the presence of tetraalkylammonium halides.

A better estimate of the effect of tetraalkylammo-

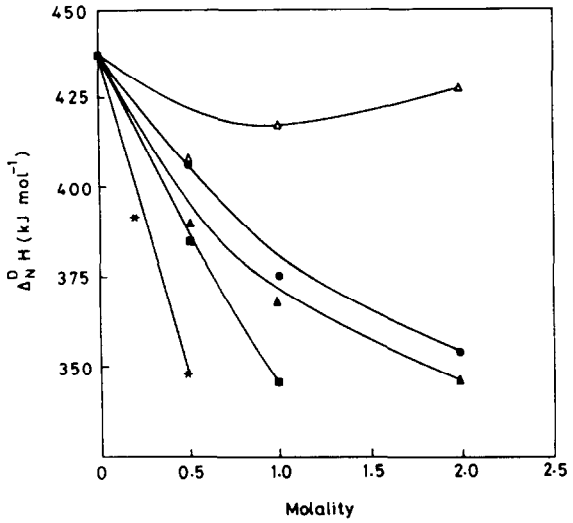

Fig. 3. $\Delta_N^D H$ of lysozyme as a function of tetraalkylammonium halides concentrations at pH 2.50. (△) Me_4NCl; (●) Me_4NBr; (▲) Et_4NBr; (■) Pr_4NBr; (*) Bu_4NBr.

nium salts on protein stability may be given by the comparison of the free energy of stabilization, $\Delta_N^D G(T)$, which can be calculated using the following equation:

$$\Delta_N^D G(T) = (T_d - T/T_d)\Delta_N^D H - (T_d - T)\Delta_N^D C_p + T\Delta_N^D C_p(\ln T_d/T) \quad (2)$$

The experimental values of $\Delta_N^D C_p$ of lysozyme in presence of tetraalkylammonium salts at higher concentrations have a high degree of uncertainty as the dependability of the values for $\Delta_N^D C_p$ is primarily limited by the uncertainty in establishing the pre-transition base lines at lower T_d. Further, the independence of $\Delta_N^D C_p$ on salt concentration has been shown above from the fact that at the same temperature $\Delta_N^D H$ values in the presence and absence of salts are the same. It has been asserted by Privalov and Khechinashivili [22] that denaturational changes of heat capacity of proteins do not display any dependence on the transition temperature. Therefore, the values of $\Delta_N^D G(T)$ were calculated using the value of $\Delta_N^D C_p$ lysozyme in water. The variation of $\Delta_N^D G(T)$ with concentration and alkyl chain length of salts at 298.15 K at pH 6.00 is illustrated in Fig. 4. The variation of $\Delta_N^D G(T)$ with temperature (283–368 K) is shown in Fig. 5. The errors in the $\Delta_N^D G(T)$ values are on the order of 10%. As concentration and alkyl chain length increases, $\Delta_N^D G(T)$ becomes more negative, leading to enhanced destabilization. The destabilization is further enhanced with temperature as shown in Fig. 5.

The tetraalkylammonium salts destabilize the proteins by weakening hydrophobic interactions between non-polar residues as well as by perturbing the characteristic water structure around the protein

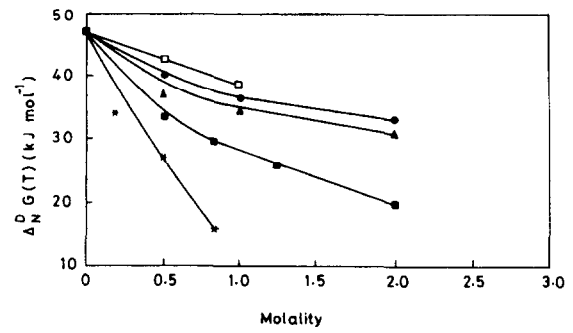

Fig. 4. $\Delta_N^D G(T)$ of lysozyme as a function of ammonium and tetraalkylammonium bromide concentration at 298.15 K and pH 6.00. (□) NH_4Br; (●) Me_4NBr; (▲) Et_4NBr; (■) Pr_4NBr; (*) Bu_4NBr.

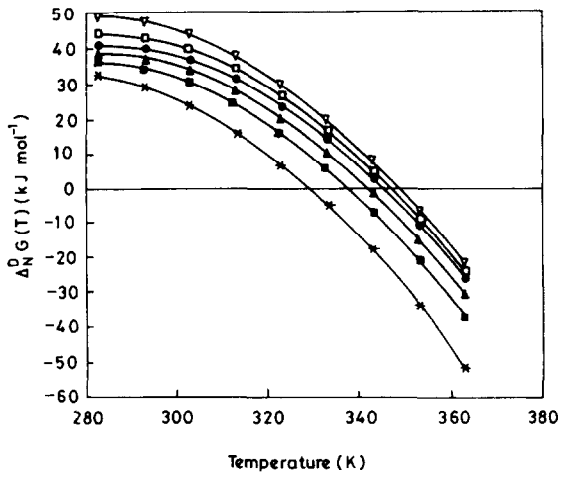

Fig. 5. Variation of $\Delta_N^D G(T)$ of lysozyme with temperature in the presense of 0.5 mol kg^{-1} ammonium and tetraalkylammonium bromide at pH 6.00. (▽)Lysozyme; (□) NH_4Br; (●) Me_4NBr; (▲) Et_4NBr; (■) Pr_4NBr; (*)Bu_4NBr.

molecule. This behaviour can be explained in terms of the overlap of the hydration cosphere of the salts and the non-polar groups of protein exposed upon denaturation. The region occupied by the solvent that is markedly affected by the presence of solute molecules is termed as cosphere. According to the cosphere overlap model [26,27], properties of water molecules in the hydration cosphere depend on the nature of solute species. When two solute particles come close together so that their cospheres overlap, some of the cosphere material is displaced and this is accompanied by a change in the thermodynamic parameters [28]. As the chain length of the tetraalkylammonium salts increases, the hydrophobic effect dominates over the electrostatic effects. The overlap of the cosphere of the non-polar groups of the protein with those of the salts releases water molecules from their hydrophobic hydration cosphere resulting in enhanced hydrophobic interactions, thus favouring the denatured state. This effect is greater in Bu_4NBr due to the presence of longer alkyl chain resulting in maximum destabilization (Figs. 1 and 2).

The above explanation of the denaturing action of the tetraalkylammonium halides with the hydrophobic parts of the protein is also consistent with the view of Timasheff and coworkers [29–31] that protein unfolding by denaturing cosolvents is due to the binding of the denaturant molecules to the denatured state of the protein, which is stronger than the exclusion of cosolvent from the protein surface.

Further, the effect of the number of methylene units on the change in denaturation temperature, ΔT_d of lysozyme is shown in Fig. 6. T_d and $\Delta^D_N G(T)$ decrease with the number of methylene units but the destabilization effect denoted by ΔT_d increases in a non-linear way. The results suggest that the methyl/methylene groups adjacent to the nitrogen atom are not as effective in their hydrophobicity as the subsequent methylene groups in higher homologues [32].

The decrease in T_d with concentration is almost linear in all cases except in NH_4Cl and Me_4NCl. In fact, Me_4NCl stabilizes lysozyme at higher concentrations. This behaviour of Me_4NCl has also been observed in the case of ribonuclease [2]. Bouquiere and coworkers, in their neutron diffraction study of lysozyme [33], have also observed the stabilizing influence of Me_4NCl. They suggested that Me_4NCl

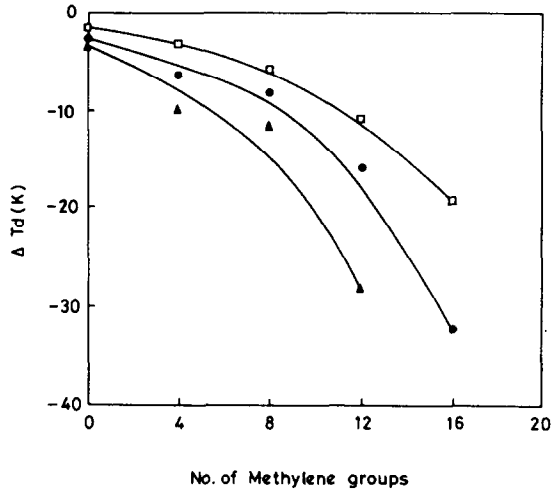

Fig. 6. ΔT_d of lysozyme as a function of number of methylene units at different concentrations of tetraalkylammonium salts at pH 6.00. (□) 0.5 mol kg^{-1}; (●) 1.0 mol kg^{-1}; (▲) 2.0 mol kg^{-1}.

tends to bind predominantly to polar sites on the protein surface although the molecular surface is made up of non-polar methyl groups. This could relate to the charge on the quaternary nitrogen, and/or polarization effects induced by the polar groups in the near environment.

A similar dependence of T_d on the concentration and alkyl chain length of the tetraalkylammonium salts has been reported for thermal denaturation of ribonuclease at pH 6.00 spectrophotometrically [2].

ΔT_d values in Tables 1 and 2 also give some useful information about the ion effect. Comparison of chlorides and bromides in Figs. 1 and 2 shows that Br^- is more destabilizing than Cl^-, in agreement with the Hofmeister series [10,34]. ΔT_d values at pH 2.50 are more negative than those at pH 6.00 for lower members as the protein molecule is highly charged at low pH [35]. This can be explained on the basis of greater accessibility of the unfolded conformation to the salt due to expansion of the molecule resulting from repulsive electrostatic interactions among the positive charges [36]. As the alkyl chain length increases, the hydrophobic interaction dominates over the electrostatic forces leading to enhanced lowering of T_d at pH 6.00 in the case of tetrabutylammonium bromide.

4. Conclusions

The effect of ammonium and tetraalkylammonium salts on the thermal denaturation of lysozyme has been observed calorimetrically and results have been explained in terms of balance of electrostatic and hydrophobic interactions. All tetraalkylammonium salts destabilize the protein at all concentrations studied, except Me_4NCl, which stabilizes at high concentrations. This has been attributed to the charge on the quaternary nitrogen and induced polarization. These results conform well with that observed in the case of monohydric alcohols.

References

[1] W. Kauzmann, Adv. Protein Chem., 14 (1959) 1.
[2] P.H. von Hippel and K.-Y. Wong, J. Biol. Chem., 240 (1965) 3909.
[3] C. Tanford, Adv. Protein Chem., 23 (1968) 121.
[4] D. Eagland, in F. Franks (Ed.), Water: A Comprehensive Treatise, Vol. 4, Plenum Press, New York, 1975.
[5] R.F. Greene, Jr. and C.N. Pace, J. Biol. Chem., 249 (1974) 5388.
[6] J.A. Schellman, Biopolymers, 17 (1978) 1305.
[7] J.A. Schellman, Biopolymers, 26 (1987) 549.
[8] J.A. Schellman, Annu. Rev. Biophys. Biophys. Chem., 16 (1987) 115.
[9] G. Velicelebi and J.M. Sturtevant, Biochemistry, 18 (1979) 1180.
[10] T. Arakawa and S.N. Timasheff, Biochemistry, 23 (1984) 5912.
[11] T.E. Creighton, Curr. Opin. Struct. Biol., 1 (1991) 5.
[12] G.I. Makhatadze and P.L. Privalov, J. Mol. Biol., 226 (1992) 491.
[13] G.Y. Gerlsma, J. Biol. Chem., 243 (1968) 957.
[14] H. Uedaira and H. Uedaira, Bull. Chem. Soc. Jpn., 53 (1980) 2451.
[15] T. Arakawa and S.N. Timasheff, Biochemistry, 21 (1982) 6536.
[16] S. Gopal and J.C. Ahluwalia, J. Chem. Soc. Faraday Trans., 89 (1993) 2769.
[17] S. Gopal and J.C. Ahluwalia, Pure Appl. Chem., 66 (1994) 473.
[18] T. Arakawa and S.N. Timasheff, Biochemistry, 21 (1982) 6545.
[19] T. Arakawa and S.N. Timasheff, Arch. Biochem. Biophys., 224 (1983) 169.
[20] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus and G.N. Somero, Science, 217 (1982) 1214.
[21] P.L. Privalov, Adv. Protein Chem., 33 (1979) 167.
[22] P.L. Privalov and N.N. Khechinashivili, J. Mol. Biol., 86 (1974) 665; see also W.Pfeil and P.L. Privalov, Biophys. Chem., 4 (1976) 23 and 33.
[23] A.K.R. Unni, L. Elias and H.I. Schiff, J. Phys. Chem., 67 (1962) 1216.
[24] F.P. Schwarz, Thermochim. Acta, 147 (1989) 71.
[25] F.P. Schwarz and W.H. Kirchoff, Thermochim. Acta, 128 (1988) 267.
[26] H.S. Franks and M.W. Evans, J. Chem. Phys., 13 (1945) 507.
[27] R.W. Gurney, Ionic Process in Solution, McGraw-Hill, New York, 1953.
[28] T.S. Sarma and J.C. Ahluwalia, Chem. Soc. Rev., 2 (1973) 203.
[29] H. Inoue and S.N. Timasheff, Biopolymers, 11 (1972) 737.
[30] S.N. Timasheff, in R.B. Gregory (Ed.), Protein Solvent Interactions, Marcel Dekker, New York, 1995.
[31] T. Arakawa, S.N. Timasheff, R. Gilles, E.K. Hoffman and L. Bolis (Eds.), Volumes and Osmolytes Control in Animal Cells, Vol. 9, Springer-Verlag, Berlin, 1991.
[32] C. Tanford, The Hydrophobic Effect, Wiley, New York, 1973.
[33] J.P. Bouquiere, J.L. Finney and M.S. Lehmann, J. Chem. Soc. Faraday Trans., 89 (1973) 2701.
[34] P.H. von Hippel and T. Schleich, in S.N. Timasheff and G.D. Fasman (Eds.), Structure and Stability of Biological Macromolecules, Marcel Dekker, New York, 1973.
[35] Y. Goto and A.L. Fink, Biochemistry, 28 (1973) 945.
[36] C.N. Pace, D.V. Laurents and J.A. Thomson, Biochemistry, 29 (1973) 2564.