ELSEVIER

Thermochimica Acta 302 (1997) 17–24

thermochimica acta

Synergetic effect of polyols with tetrabutylammonium bromide and urea on the thermal stability of lysozyme

S. Jain*, J.C. Ahluwalia

Department of Chemistry, Indian Institute of Technology, Hauz Khas, New Delhi 110 016, India

Received 25 July 1996; accepted 8 January 1997

Abstract

The thermal denaturation of lysozyme was studied in 2.0 molal aqueous solutions of polyols at pH 2.50 and varying concentration of glycerol at pH 2.50 and 6.00 using differential scanning calorimetry (DSC). The transition temperature, heat capacity, enthalpy, entropy and free energy of stabilization have been determined by a least square fit of the excess heat capacity data to the two-state model. Polyols are found to stabilize lysozyme and the stabilization increases with an increase in the number of hydroxyl groups. The stabilization increases with an increasing concentration of glycerol. The stabilization has been explained in terms of preferential hydration or due to the strengthening of the water structure which in turn intensify the hydrophobic interactions of the protein.

The calorimetric studies have been done on the thermal denaturation of lysozyme in the presence of 0.5 m tetrabutylammonium bromide (Bu_4NBr) + 2.0 m polyols at pH 2.50 and 0.5 m Bu_4NBr or urea + varying concentration of glycerol (0.0–10.0 m) at pH 2.50 and 6.00. The effect is found to be nearly additive in the case of Bu_4NBr and polyols. The results on the combined systems of Bu_4NBr and varying concentration of glycerol shows that effect is additive at low pH but not at high pH. This has been explained in terms of predominance of the enthalpic contribution of glycerol in comparison to the entropic contribution of Bu_4NBr. The comparison of the combined effect of 0.5 m urea + glycerol and 0.5 m Bu_4NBr + glycerol indicates that Bu_4NBr is a stronger destabilizer than urea. © 1997 Elsevier Science B.V.

Keywords: Calorimetry; Lysozyme; Polyols; Protein-stability; Tetrabutylammonium bromide; Urea

1. Introduction

Soluble globular proteins are known to be stabilized or destabilized by a number of salts and other compounds [1–7]. In our previous paper [8], the effect of tetraalkylammonium bromides on the thermal denaturation of lysozyme has been reported. These salts destabilize the protein at all concentrations and the destabilization increases with an increase in the alkyl chain length, that is, they increase the hydrophobic character of the solvent. The destabilization effect of these salts has been explained in terms of the binding of denaturant molecules to the hydrophobic residues of the protein as well as perturbing the solvent structure.

It is now known that glycerol and other polyolic cosolvents are preferentially excluded from the surfaces of globular proteins [2,3], that is, their con-

*Corresponding author.

PII S0040-6031(97)00149-4

centration in the domain of a protein molecule is lower than that in the bulk solvent, which is apparent as preferential hydration of the protein. These kinds of cosolvents enhance protein stability. Polyols are common cell osmolytes and occur in many unicellular algae, certain salt-tolerant plants and many insects exposed to freezing temperatures [9]. These organic osmolytes found in nature share a common ability to provide environments 'compatible' for macromolecular structure and function. There have been many investigations which show that polyols [10–13] stabilize the native conformation of proteins by enhancing the water structure in their immediate neighbourhood. However, the manner in which polyols reduce the extent of denaturation by other reagents is not completely understood.

Urea is an important product of nitrogen metabolism and is accumulated by some species as the major blood and intracellular osmolyte [9]. As urea is known to be a destabilizer [4,14,15], to cope with its destabilization effect, various small molecular weight compounds like sugars, polyhydric alcohols, amino acids and methylamines are present in marine organisms. One of such combination of urea and methylamines has been studied extensively [7,16,17]. Simpson and Kauzmann [18] observed that the extent of denaturation of ovalbumin in urea solutions was reduced in the presence of glycerol or sucrose. The influence of polyhydric alcohols and sugars on the rate of subunit dissociation of tetrameric *L*-asparaginase in the presence of urea was investigated by Shifrin and Parrott [19].

Thus, based on above studies, the combined effects of stabilizers and destabilizers have been investigated in the present work to see the mechanism of action of the above cosolutes on protein stability, which has been the subject of extensive studies over the years. The calorimetric experiments have been carried out on the thermal denaturation of lysozyme in the presence of urea + glycerol and Bu_4NBr + polyols in order to understand the counteraction of urea or Bu_4NBr and polyols. The reversible nature of lysozyme has been well established by Privalov [20,21] and Schwarz [22]. In this paper, we report the transition temperature T_d, and the thermodynamic parameters ΔH_d, ΔS_d, ΔG_d and $\Delta C_{p,d}$ of denaturation of lysozyme in the presence of polyols, 0.5 m urea + glycerol and 0.5 m Bu_4NBr + polyols.

2. Experimental

Lysozyme (Approx. 95% protein)) were procured from Sigma Chemical Co. These proteins were dialysed against distilled and deionized water at 277 K for over 24 h and lyophilized. Glycerol procured from Glaxo, India was of analytical grade. Erythritol, adonitol, arabitol and urea were procured from Sigma Chemical Co. and used as such. Bu_4NBr (>99%) obtained from Sisco Research Laboratories (SRL), India was purified from the saturated solution in chloroform by the addition of petroleum ether followed by cooling. The recrystallized salts were dried in vacuum at 60–80°C and kept in a vacuum desiccator over P_2O_5 after purification.

The DSC experiments were carried out in either 0.05 M MOPS (2-(*N*-Morpholino) propane-sulphonic acid) or Glycine buffer adjusted with HCl to the desired pH of 6.00 and 2.50, respectively. Protein concentrations were determined spectrophotometrically on a Perkin Elmer Lambda 3B UV/VIS spectrophotometer. The extinction coefficients were taken to be 2.635 at 280 nm [22] for 1 mg cm^{-3} aqueous solutions of lysozyme of molar mass 14.3 kDa.

Calorimetric measurements were performed on a SETARAM micro-DSC Batch and flow calorimeter at a scan rate of 0.6 K min^{-1} and 25 μV amplification full scale deflection (f.s.d.). The concentration of the protein used was about 2.5 mg cm^{-3} in buffer solution of about 0.850 g in a vessel of 1 ml capacity. The transition temperature T_d, enthalpy ΔH_d, entropy ΔS_d, heat capacity $\Delta C_{p,d}$ and free energy ΔG_d of denaturation were determined from the DSC curves as described in our previous paper [8].

3. Results and discussion

Thermal denaturation of lysozyme in the aqueous solutions of glycerol, erythritol, arabitol, adonitol, urea and Bu_4NBr is a two state transition with a stoichiometry of one as the values of the cooperativity index, $\eta(=\Delta H_d/\Delta H^{vH})$ are found to be close to 1.0 (within experimental errors). Lysozyme shows reversibility as there is no change in the position of the peaks on re-scanning of the already scanned protein solution.

Table 1
Thermodynamic parameters obtained from DSC scans on lysozyme in the presence of 2.0 molal polyols at pH 2.50

T_d (K)	ΔT_d (K)	ΔH_d (kJ mol^{-1})	$\Delta C_{p,d}$ (kJ K^{-1} mol^{-1})	η
336.2 ± 0.1	—	437 ± 11	6.7 ± 1.1	1.010
Glycerol				
338.5 ± 0.2	2.3	466 ± 9	6.8 ± 0.4	1.055
i-Erythritol				
339.8 ± 0.1	3.6	490 ± 6	6.3 ± 0.5	1.034
Adonitol				
341.7 ± 0.1	5.5	510 ± 10	7.3 ± 0.9	1.042
Arabitol				
341.2 ± 0.1	5.0	492 ± 8	6.0 ± 1.1	1.045

Table 2
Thermodynamic parameters obtained from DSC scans on lysozyme in the presence of solvent system: 0.5 m Bu_4NBr + polyols at pH 2.50

Concentration (m)		T_d (K)	ΔT_d (K)	ΔH_d (kJ mol^{-1})	$\Delta C_{p,d}$ (kJ K^{-1} mol^{-1})
Bu_4NBr	Polyols				
0.0	0.0	336.2±0.1	—	437±11	6.7±1.1
Bu_4NBr					
0.5	0.0	320.5±0.1	−15.7	348±6	3.5±1.5
Bu_4NBr + Glycerol					
0.5	2.0	322.9 ± 0.4	−13.3	375 ± 10	4.0 ± 1.3
Bu_4NBr + Erythritol					
0.5	2.0	324.9 ± 0.2	−11.3	389 ± 6	2.7 ± 0.3
Bu_4NBr + Arabitol					
0.5	2.0	326.2 ± 0.2	−10.0	394 ± 8	3.9 ± 0.2
Bu_4NBr + Adonitol					
0.5	2.0	326.1 ± 0.3	−10.1	398 ± 12	5.9 ± 1.5

Effect of polyols and Bu_4NBr: The thermodynamic parameters obtained from the analysis of DSC scans of lysozyme in the presence of polyols (2.0 m) and 0.5 m Bu_4NBr + polyols (2.0 m) at pH 2.50 are recorded in Tables 1 and 2 with uncertainty given as the standard error of the mean.

The observed values of T_d, 336.2 ± 0.1 K and ΔH_d of pure lysozyme, 437 ± 11 kJ mol^{-1} at pH 2.50 are close to the values reported in the literature [21,22]. T_d is enhanced with the addition of polyols as shown in Table 1. ΔT_d increases from 2.3 to 5.5 K in 2 molal aqueous solutions of polyols, as the number of hydroxyl groups increase from 3 for glycerol to 5 for adonitol. This indicates that protein is stabilized in the presence of polyols and the stabilization increases with an increase in the number of hydroxyl groups. The order of increase in the stabilization by polyols is:

Adonitol ≈ *Arabitol* (5 – OH) > *i* – Erythritol (4 – OH) > Glycerol (3 – OH)

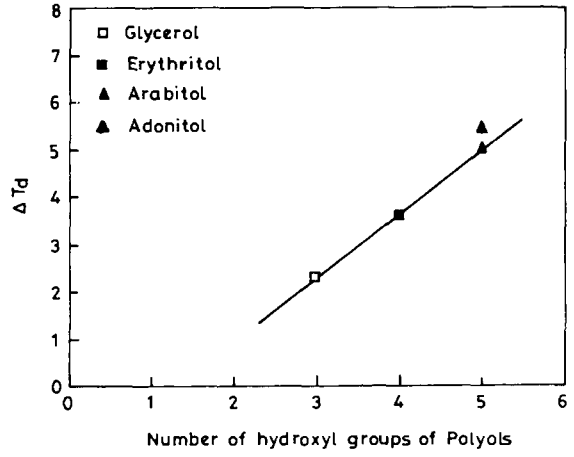

Fig. 1. ΔT_d of lysozyme as a function of number of hydroxyl groups of polyols at pH 2.50.

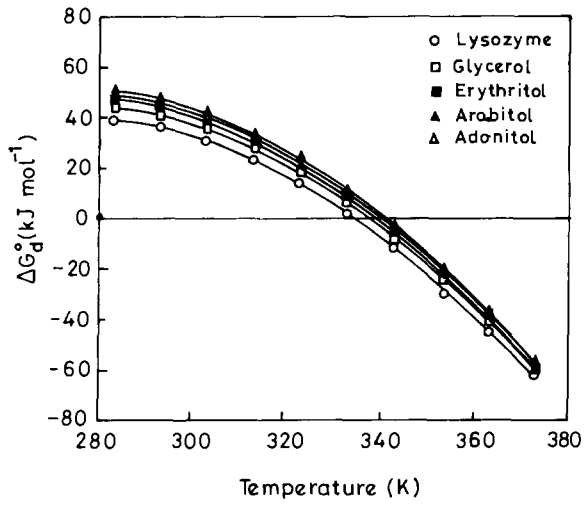

Fig. 2. Variation of ΔG_d^0 of lysozyme with temperature in the presence of 2.0 m aqueous.

Adonitol and arabitol increase T_d to the same extent as shown in Fig. 1. This is due to the presence of same number of hydroxyl groups. T_d is increased by 1.35 K per hydroxyl group as determined by the slope of the graph in Fig. 1 which is in agreement with the value of 1.4 K per hydroxyl group, reported by Fujita et al. [10]. An increase in T_d of 0.8°C per hydroxyl group of sugars and polyhydric alcohols has been reported earlier by Uedaira and Uedaira [12] and Gerlsma and Sturr [13]. It has been suggested [13] that there is no direct molecular interaction between proteins and polyhydric alcohols. The hydrophillic effect of polyols on the stability of proteins appears mainly to be of an indirect kind, namely, either through strengthening of the polar interactions by a lowering of the dielectric constant, and/or an enhancement of the hydrophobic interaction of the protein by altering the water structure.

The stabilization of protein in the presence of polyols has been reported by Timasheff and coworkers [2,3,11] to be the direct consequence of the preferential interactions of the polyols with the proteins, due to which polyols are excluded preferentially from the surface of the protein which creates a tendency of the protein to minimize its surface without inducing conformational changes.

The stabilization of lysozyme in the presence of polyols has been shown by standard free energy of denaturation, ΔG_d^0 which is given by

$$\Delta G_d^0(T) = \Delta H_d^0(T) - T\Delta S_d^0(T) \quad (1)$$

where

$$\Delta H_d^0(T) = \Delta H_d - \Delta C_{p,d}(T_d - T) \quad (2)$$

$$\Delta S_d^0(T) = \Delta S_d - \Delta C_{p,d} \ln\left(\frac{T}{T_d}\right) \quad (3)$$

Heat capacity of denaturation, $\Delta C_{p,d}$ for each solvent composition determined experimentally has high degree of uncertainty, therefore, $\Delta C_{p,d}$ values of pure proteins are used for the calculation of the thermodynamic functions of denaturation.

The graph between ΔG_d^0 versus temperature in the presence and absence of lysozyme has been shown in Fig. 2. The change in ΔG_d^0 with respect to pur lysozyme is positive indicating enhanced stability of lysozyme in the presence of polyols. The ΔG_d^0 is further enhanced with an increase in the number of hydroxyl groups and the enhancement is the same order as for ΔT_d.

When 0.5 m Bu_4NBr is added to an aqueous mixtures of protein and polyols, the structure stabilizing

influence of polyols and structure destabilizing influence of Bu_4NBr on the thermal denaturation of lysozyme is found to be nearly additive (Table 2) in all the cases, for example, the observed ΔT_d of lysozyme in the aqueous mixtures of 0.5 m Bu_4NBr + 2.0 m polyols, −13.3 K for glycerol, −11.3 K for erythritol, −10.0 K for arabitol, −10.1 K for adonitol is indeed in agreement (within experimental errors) with the calculated values of −13.4, −12.1, −10.7, −10.2 K as obtained by summation of their individual effects. The stabilization effect induced by increasing number of polyols is not sufficient enough to counteract the destabilization effect of Bu_4NBr as unfolding of protein by denaturing cosolvents is due to the binding of the denatured molecules to the denatured state of the protein which is stronger than the exclusion of the polyols from the protein surface.

Effect of concentration of glycerol and 0.5 m Bu_4NBr or urea: To study the effect of the concentration of polyols in the combined mixtures of protein and Bu_4NBr, we have investigated the thermal denaturation of lysozyme at varying concentrations of glycerol (0–10 m) and 0.5 m Bu_4NBr. The addition of glycerol to an aqueous solution of lysozyme induces an increase in denaturation temperature and enthalpy of denaturation as shown in Table 3. The transition temperature of lysozyme increases with an increase in the concentration of glycerol at pH 2.50 and 6.00, indicating stabilization of the proteins. Glycerol is essentially a small and hydophilic compound capable of occupying a part of the solvation sheath around a protein with a concomitant stabilization of the solvent structure around the protein. Lysozyme 'bind' glycerol essentially in direct proportion to glycerol concentration through the interaction of glycerol with the water of hydration around polar groups. This binding may result in the strengthening of the polar interactions which in turn intensify the hydrophobic interactions [23].

The enthalpies of denaturation, ΔH_d of lysozyme in aqueous solution at pH 6.00 are 495 ± 10 kJ mol^{-1}, which are close to the literature values [21,22]. ΔH_d increases with an increase in the concentration of glycerol as shown in Table 3. In fact, ΔH_d increases by 93 kJ mol^{-1} for lysozyme in 10 molal glycerol solution, which cannot be solely explained as due to an increase in temperature, for example, calculations show that T_d of lysozyme increases by 5.9 K and using $\Delta C_{p,d}$ of 6.4 kJ K^{-1} mol^{-1}, the enthalpy of denaturation would increase by 38 kJ mol^{-1}, which is smaller than the observed ΔH_d (93 kJ mol^{-1}). The increase in ΔH_d can be accounted for by an increase in the electrostatic interactions, as addition of glycerol lowers the dielectric constant of water [24]. The standard free energy, ΔG_d^0 entropy, ΔS_d^0 and enthalpy, ΔH_d^0 for the denaturation of lysozyme as a function of glycerol concentration has been calculated at denaturation temperature of pure lysozyme (348.3 K) using Eq. (1) to Eq. (3). The increase in these thermodynamic parameters as shown in Fig. 3 suggests that stabilization of protein by glycerol is dominantly produced by an enthalpic rather than an entropic effect.

When 0.5 m Bu_4NBr is added to the aqueous mixtures of protein and glycerol, it has been observed that

Table 3
Thermodynamic parameters obtained from DSC scans on lysozyme in the presence of glycerol

Conc. (m)	T_d (K)	ΔT_d (K)	ΔH_d (kJ mol^{-1})	$\Delta C_{p,d}$ (kJ K^{-1} mol^{-1})	η
pH 6.00					
0.0	348.3 ± 0.2	—	495 ± 10	6.4 ± 1.1	1.110
2.0	349.5 ± 0.1	1.2	519 ± 6	5.7 ± 0.7	1.058
6.0	352.0 ± 0.1	3.7	558 ± 13	5.8 ± 0.2	1.055
10.0	354.2 ± 0.2	5.9	588 ± 6	4.8 ± 0.6	1.060
pH 2.50					
0.0	336.2 ± 0.1	—	437 ± 11	6.7 ± 1.1	1.010
2.0	338.5 ± 0.2	2.3	466 ± 9	6.8 ± 0.4	1.055
6.0	340.5 ± 0.1	4.3	495 ± 7	6.5 ± 1.2	1.028
10.0	342.7 ± 0.1	6.5	560 ± 4	6.8 ± 1.9	1.070

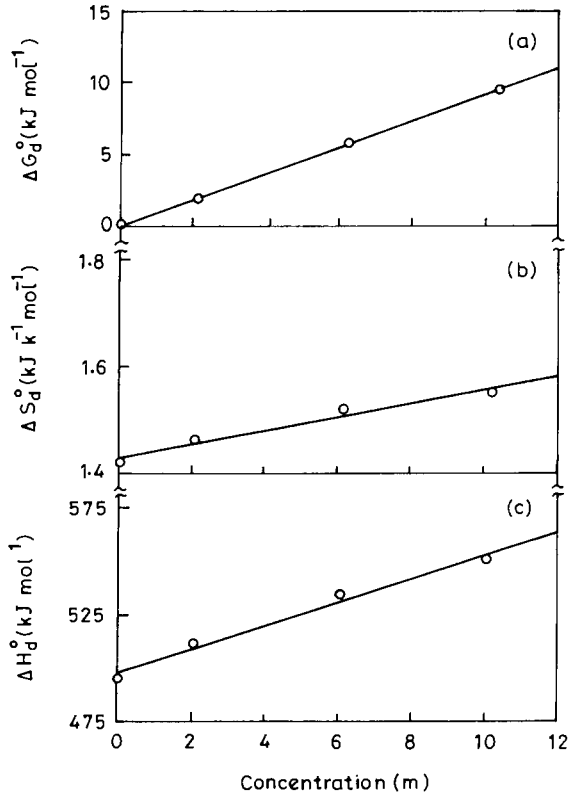

Fig. 3. Variation of standard thermodynamic parameters of the denaturation of lysozyme with glycerol concentration at 348.3 K at pH 6.00.

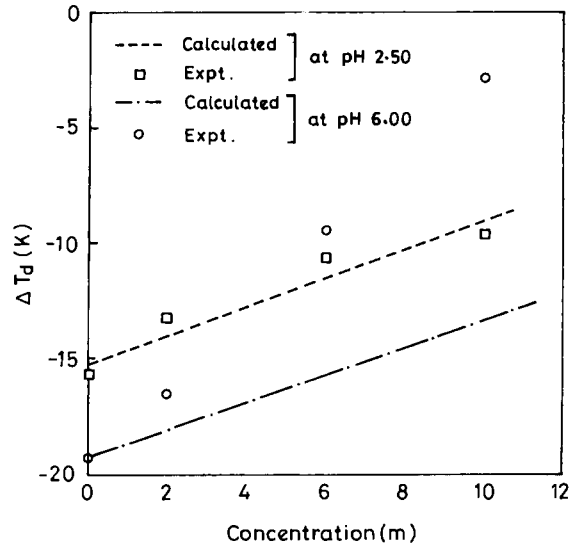

Fig. 4. ΔT_d of lysozyme as a function of glycerol concentration at pH 2.50 and 6.00.

on varying the glycerol concentration, the effect is additive only at low pH (low temperature), not at high pH (high temperature) as shown in Table 4 and Fig. 4. T_d of lysozyme, −10.7 K, as observed in 0.5 m Bu_4NBr + 6 m glycerol solution is very close to the calculated value of −11.4 K at pH 2.5, but at pH 6.00, the observed value of ΔT_d is −9.5 K, while the calculated value is −15.6 K. Similar situation has been observed in 0.5 m Bu_4NBr + 10 m glycerol. In fact, the stabilization by glycerol is more than that of

Table 4
Thermodynamic parameters obtained from DSC scans on lysozyme in the presence of solvent system: 0.5 m Bu_4NBr + glycerol

Concentration (m) of		T_d (K)	ΔT_d (K)	ΔH_d (kJ mol^{-1})	$\Delta C_{p,d}$ (kJ K^{-1} mol^{-1})
Bu_4NBr	glycerol				
pH 6.00					
0.0	0.0	348.3 ± 0.2	—	495 ± 10	6.4 ± 1.1
0.5	0.0	329.0 ± 0.1	−19.3	391 ± 9	3.4 ± 1.5
0.5	2.0	331.8 ± 0.2	−16.5	437 ± 7	5.3 ± 2.1
0.5	6.0	338.8 ± 0.3	−9.5	462 ± 8	5.6 ± 1.9
0.5	10.0	345.4 ± 0.2	−2.9	481 ± 10	5.4 ± 0.9
pH 2.50					
0.0	0.0	336.2 ± 0.1	—	437 ± 11	6.7 ± 1.1
0.5	0.0	320.5 ± 0.1	−15.7	348.6 ± 6	3.5 ± 1.5
0.5	2.0	322.9 ± 0.4	−13.3	375 ± 10	4.0 ± 1.3
0.5	6.0	325.5 ± 0.1	−10.7	403 ± 6	6.7 ± 0.1
0.5	10.0	326.5 ± 0.2	−9.7	435 ± 5	6.9 ± 1.8

Table 5
Thermodynamic parameters obtained from DSC scans on lysozyme in the presence of solvent system: 0.5 m Urea + glycerol at pH 6.00

Concentration (m)		T_d (K)	ΔT_d (K)	ΔH_d (kJ mol^{-1})	$\Delta C_{p,d}$ (kJ K^{-1} mol^{-1})
Urea	Glycerol				
0.0	0.0	348.3 ± 0.1	—	495 ± 10	6.4 ± 1.1
0.5	0.0	345.8 ± 0.2	−2.5	478 ± 9	5.9 ± 0.1
0.5	2.0	347.5 ± 0.1	−0.8	514 ± 30	7.5 ± 0.6
0.5	6.0	349.8 ± 0.2	1.5	540 ± 20	5.4 ± 0.9

the destabilization by Bu_4NBr at pH 6.00. The hydrophobic interactions increase in strength at high temperatures [25,26] because of the dominance of the enthalpy contribution compared to that of entropy. On a similar basis, the greater stabilization by glycerol at high pH in the combined system can be explained as due to the predominance of the enthalpic effect of glycerol over the entropic effect of Bu_4NBr. This may result in the intensification of the hydrophobic interactions of the protein in the presence of glycerol and consequently, the reduction in the surface of contact between protein and Bu_4NBr.

To further differentiate between the mechanism of Bu_4NBr and urea on the stability of protein in the combined mixtures of stabilizers and destabilizers, we have studied the combined systems of 0.5 m urea and glycerol at varying concentrations as shown in Table 5. The transition temperature T_d, of lysozyme decreases in the presence of urea. A decrease of 2.5 K in T_d and 17 kJ mol^{-1} in ΔH_d of lysozyme is observed in 0.5 mol kg^{-1} of urea. Therefore, urea also act as destabilizers. There is ample proof [4,14,15,27] that urea acts as a destabilizer. Recently, Makhatadze and Privalov [15] have shown from a simple binding model that the urea molecule, having four proton-donor and one acceptor groups interacts mainly with the polar groups of the protein and there is polyfunctional hydrogen bonding between the peptide groups and denaturant molecules and, therefore, urea is a hydrogen bond rupturing agent.

When 0.5 m urea is added to an aqueous mixture of protein and glycerol at pH 6.00, the structure stabilizing influence of glycerol and the structure destabilizing influence of urea on the thermal denaturation of lysozyme is found to be nearly additive (Table 5). The observed ΔT_d, −0.8 K, of lysozyme in aqueous solution of 0.5 m urea +2 m glycerol is close to the calculated value, −1.3 K (adding the individual effects of both urea and glycerol). Similarly, in aqueous solution of 0.5 m urea +6 m glycerol, the observed 1.5 K is proximal to the calculated 1.2 K. The counteracting stabilization effect induced by glycerol against denaturation by urea can be explained by assuming a decrease in the hydrogen bond rupturing capacity of the medium. These results indicate that destabilization of lysozyme by Bu_4NBr is not fully compensated by glycerol, whereas destabilization by urea is fully compensated with the addition of glycerol. This is because of the fact that Bu_4NBr is a stronger destabilizer than urea.

References

[1] A. Ben-Naim, in: R.B. Gregory (Ed.), Protein-Solvent interactions, Marcel Dekker Inc., New York, 1995, p. 387.
[2] S.N. Timasheff, in: R.B. Gregory (Ed.), Protein-Solvent interactions, Marcel Dekker Inc., New York, 1995, p. 445.
[3] T. Arakawa and S.N. Timasheff, in: R. Gilles, E.K. Hoffman and L. Bolis (Eds.),Volumes and Osmolytes Control in Animal Cells, Vol 9, Springer-Verlag, Berlin, 1991, p. 226.
[4] J.F. Brandts, in: S.N. Timasheff and G.D. Fasman (Eds.), Structure and Stability of Biological Macromolecules, Marcel Dekker, New York, 1969, p. 213.
[5] T. Arakawa, R. Bhat and S.N. Timasheff, Biochem., 29 (1990) 1924.
[6] S. Gopal and J.C. Ahluwalia, Pure and Appl. Chem., 66 (1994) 473.
[7] S. Gopal and J.C. Ahluwalia, J Chem. Soc. Faraday Trans., 89 (1993) 2769.
[8] Sandhya Jain and J.C. Ahluwalia, Biophysical Chem., 59 (171) 1996.
[9] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus and G.N. Somero, Science, 217 (1982) 1214.
[10] Y. Fujita, Y. Iwasa and Y. Noda, Bull. Chem. Soc. Jpn., 55 (1982) 1896.
[11] K. Gekko, J. Biochem., 91 (1982) 1197.
[12] H. Uedaira and H. Uedaira, Bull. Chem. Soc. Jpn., 53 (1980) 2451.

[13] S.Y. Gerlsma and E.R. Sturr, Int. J. Peptide Protein Res., 4 (1972) 377.
[14] C.N. Pace, D.V. Laurents and J.A. Thomson, Biochem., 29 (1990) 2564.
[15] G.I. Makhatadze and P.L. Privalov, J. Mol. Biol., 226 (1992) 491.
[16] M.M. Santoro, Y. Liu and S.M.A. Khan, Li-Xiang Iou and D. W. Bolen, Biochem., 31 (1992) 5278.
[17] T.Y. Lin and S.N. Timasheff, Biochem., 33 (1994) 12695.
[18] R.B. Simpson and W. Kauzmann, J. Am. Chem. Soc., 75 (1953) 5139.
[19] S. Shifrin and C.L. Parrott, Arch. Biochem. Biophys., 166 (1975) 426.
[20] P.L. Privalov, Advan. Protein Chem., 33 (1979) 167.
[21] P.L. Privalov and N.N. Khechinashivili, J. Mol. Biol., 86 (1974) 665.
[22] F.P. Schwarz, Thermochim. acta., 147 (1989) 71.
[23] K. Gekko and S.N. Timasheff, Biochem., 20 (1981) 4667.
[24] G. Akerlof, J. Am. Chem. Soc., 54 (1932) 4125.
[25] G. Nemethy and H.A. Scherega, J. Phys. Chem., 66 (1962) 1773.
[26] P.L. Privalov in L.P. Kayushin (Ed.), Water in Biological Systems, Consultants Bureau, New York, 1969, p. 38.
[27] E. Liepinsh and G. Otting, J. Am. Chem. Soc., 116 (1994) 9670.