J. Biosci., Vol. 6, Number 5, December 1984, pp. 589–599. © Printed in India.

Thermodynamics of binding water and solute to myosin*

MADHUSWETA DAS and D. K. CHATTORAJ†
Department of Food Technology and Biochemical Engineering, Jadavpur University, Calcutta 700 032, India

MS received 2 July 1984; revised 18 October 1984

Abstract. From the isopiestic measurements of the extents of adsorption of water vapour by fish myosin at various values of water activities at three different temperatures, the changes in free energy, enthalpy and entropy of dehydration of the protein have been calculated. Extents of excess binding of solvent and solute to myosin have also been determined from isopiestic experiments in the presence of different inorganic salts, sucrose and urea respectively. Mols of water and solute respectively bound in absolute amounts to myosin have been evaluated from these data in limited range of solute concentrations. Free energy changes at different concentrations of these solutes have also been evaluated and their relations with 'salting-in' and 'salting-out' phenomena have been discussed. The order of the values of the standard free energy change for excess binding calculated with respect to an unified thermodynamic scale are found to be consistent with relative reactivity of binding water to myosin in the presence of inorganic salts, sucrose and urea.

Keywords. Hydration of myosin; thermodynamics of binding; Water-solute binding.

Introduction

In the living system, muscle protein myosin in combination with actin remains in the form of biogel and is in constant interaction with water and various types of inorganic and organic solutes present in the aqueous phase. Change in mechanical energy during muscular expansion and contraction involves interaction of water and solute with this protein. The exact mechanism of this process is not yet clear. Myosin constitutes the major protein in animal and fish meat. During preservation of fish and meat with or without addition of salt, the role of water-protein interaction in preventing denaturation and maintaining the muscle texture and taste for long period becomes extremely important (Graham, 1977).

Myosin is insoluble in water but it becomes soluble at relatively high salt concentrations. Possibly for this reason, unlike other globular proteins, the hydration of myosin has not been studied in detail by the conventional physicochemical methods (Kuntz and Kauzmann, 1974). Compared to the globular shape of albumin and haemoglobin, the shape of myosin is highly elongated. The effect of this kind of change in shape on protein hydration needs critical examination.

---

* Part of this work was presented at the 20th Annual Convention of Chemists of the Indian Chemical Society, Cuttack, 26th-30th December 1983.

† To whom correspondence should be addressed.

Abbreviation Used: BSA, bovine serum albumin.

Recently binding of water and various types of solutes to egg albumin (Bull and Breese, 1968, 1970a, b; Chattoraj and Mitra, 1977a, b), serum albumin (Mitra *et al*., 1977), β-lactoglobulin and haemoglobin (Mitra *et al.,* 1978) respectively has been extensively studied using the isopiestic method. These studies of water vapour adsorption are found to be useful in evaluating many thermodynamic parameters related to the binding interactions of water to soluble as well as insoluble proteins. In the present study, thermodynamic aspects of binding water and solute to myosin have been studied on the basis of the data obtained from isopiestic experiments.

Materials and methods

Myosin was isolated from the fresh water fish Rahu (*Labio rohita*) each weighing approximately 250–300 g. After descaling and beheading, the dorsal portion of the fish was isolated. It was then almost deboned, washed and macerated with mortar and pestle. The skin was left and 25 g of macerated muscle was washed three times with water. Following the standard method of isolation of cod myosin (Connell, 1962), the muscle was extracted with 250ml of 0·10μM phosphate buffer at pH 6·4 for 30 min with slow stirring. The buffer also contained 0·47 μM KCl, 0·10 μM pyrophosphate and 15×10^{-3} μM $MgCl_2$. The extract was then centrifuged at 3000 *g* for 15 min, the supernatant solution decanted and diluted 10-fold with water. The precipitated myosin gel was collected by centrifugation at 3000 *g* for 15 min. The precipitate was redissolved in the extractant with slow stirring and the solution diluted 10 times with water. The myosin gel was finally separated by centrifugation at 3000 *g* for 15 min. The extractant and water used in the above process were always ice cold. All the operations were done in cold.

After isolation, the protein was washed with cold distilled water to make it salt free and then lyophilised. Protein content of the lyophilised sample was found to be 100% (dry basis) as measured by the standard Kjeldahl method. The lyophilised sample was also examined for homogenity by the usual procedure of gel electrophoresis using sodium dodecyl sulphate and Polyacrylamide (Weber and Osborn, 1969). Along with the standard bands for myosin, the gel always indicated a faint band corresponding to the presence of actin. Thus, as in the case of cod myosin (Connell, 1962), in the present case also, the isolated myosin always contained a small fraction of actin as impurities. As in the case of cod myosin, the presence of the small fraction of actin has been ignored and it has been assumed that all the material precipitated by dilution is myosin. The lyophilised sample was stored in a desiccator at 4°C and directly used in the isopiestic experiments. All other chemicals were ofanalytical grade and these were used without further purification. Only urea was twice recrystallised from warm ethanol.

For the study of protein hydration in the absence of a solute, the isopiestic measurement was carried out as described in a previous publication (Chattoraj and Mitra, 1977a). Three to four days were sufficient for the attainment of isopiestic equilibrium. At the end of the experiment, mols of water (n_1) adsorbed per kg of the dry protein sample was determined at isopiestic equilibrium from direct weighing. The concentration of sulphuric acid in the reference solution at equilibrium was estimated from which the relative humidity (p/p_0) was known.

In the presence of inorganic salts, sugar and urea, the extent of protein hydration was measured using the same isopiestic technique as described in detail before (Mitra *et al*., 1977). At isopiestic equilibrium, total mols of water (n_1^t) and total mols of solute (n_2^t) associated per kg of myosin (dry basis) were determined from direct weighing and the molality m_2 of the solute in the reference solution was also estimated. It has been shown from thermodynamic considerations (Chattoraj and Mitra, 1977b) that m_2 may also be treated as the molality of the free solute remaining dissolved in the free solvent in contact with myosin in the sample. This involves the assumption that the contribution of protein to vapour compared to that of the solute in the sample bottle is negligible. If X_1 and X_2 are mol fractions of the free solvent and solute respectively in the sample bottle then X_2 /X_1 becomes equal to m_2/55·51. We shall define mols of solvent and solute Γ_1^2 and Γ_2^1, respectively, bound in excess per kg of myosin by the expression (Chattoraj and Mitra, 1977b; Mitra *et al.,* 1977)

$$\Gamma_1^2 = n_1^t - n_2^t \frac{X_1}{X_2} \tag{1}$$

and

$$\Gamma_2^1 = n_2^t - n_1^t \frac{X_2}{X_1}. \tag{2}$$

Values of Γ_1^2 and Γ_2^1 can thus be estimated from the experimental data.

Results and discussion

In figure 1, mols of water vapour (n_1) adsorbed per kg of myosin at temperatures 45°C, 27°C and 10°C have been plotted against relative humidities p/p_0 ranging from zero to unity. These three isotherms are all sigmoid in nature. As in the case of other proteins, here also the sorption isotherms in figure 1 may be divided qualitatively into three classes. Value of n_1 is approximately 2·5 mols of water per kg of myosin at 0·20 relative humidity and is independent of temperature. This amount of water is bound strongly to

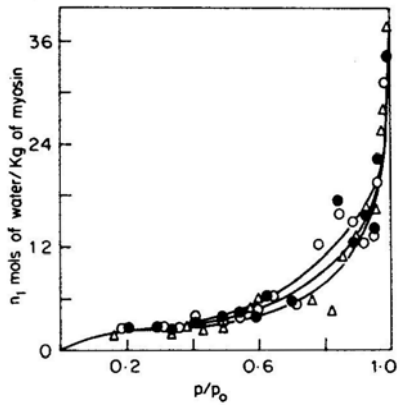

Figure 1. Plot of n_1, *vs.* p/p_0. (O), 45°C; (●), 27°C; (Δ), 10°C

the hydrophilic groups of protein forming a monolayer. According to Pauling (1945), each hydrophilic group is strongly bound to one molecule of water at this state of primary hydration. This strongly bound water behaves in many respects as part of the protein solid (Kuntz and Kauzmann, 1974). For relative humidity ranging between 0·20 and 0·70, water in excess of 2·5 mols per kg of protein are sorbed at new sites or holes and is responsible for the swelling of protein. The steep third part of the curve is probably due to the condensation of randomly oriented water molecules on the hydrated surface of proteins (Kuntz and Kauzmann, 1974).

The extrapolated value of n_1 in figure 1 at p/p_o equal to unity is 40 mols of water per kg of myosin. This value of n_1 (represented by Δn_1^0) is independent of temperature. It may be regarded as the maximum and effective amount of water to be accommodated within the bound phase of protein-water systems thus completing various hydration shells (Chattoraj and Mitra, 1977a).

Average molecular weight, molecular length and molecular diameter of rod-shaped myosin are 5×10^5 dalton, $1{\cdot}5\times10^{-7}$ and 2×10^{-9} meters respectively (Volkenshtein, 1983). From these free surface area A_f of myosin rods are calculated to be $11{\cdot}4 \times 10^{+5}$ square m/kg of the protein. Amount of water (Δn_1^s) accommodated to the maximum extent per square meter of myosin is equal to $\Delta n_1^0/A_f$ or $3{\cdot}5\times10^{-5}$ mol. Values of Δn_1^0 and A_f for globular proteins bovine serum albumin (BSA), haemoglobin and egg albumin have been reported earlier (Chattoraj *et al*., 1977a) so that Δn_1^s of these proteins are calculated to be $2{\cdot}9 \times 10^{-5}$, $3{\cdot}0 \times 10^{-5}$ and $2{\cdot}6 \times 10^{-5}$ mols per square meter respectively. Thus the water content per unit surface area of the globular and rod-shaped proteins are more or less of the same order and magnitude. If one assumes that a molecule of water is able to cover effectively 10×10^{-20} square meters of surface area then $1{\cdot}7 \times 10^{-5}$ mols of water will be needed to form a compact monolayer having one square meter surface area (Chattoraj, 1981). Thus it may appear that water molecules in compact form bound to the smooth protein surface at p/p_0 close to unity tend to form a bilayer of H_2O. Most probably however, bound water molecules are not compact since the energies of interaction of water molecules with different types of hydrophilic and hydrophobic groups forming rough protein boundary are different.

According to the Raoult's law convention, p/p_0 represents the activity (α_1) of water in the system under consideration. As before, we will fix the standard state of reference (Chattoraj and Mitra, 1977a) for the system when α_1 is unity and protein is surrounded by Δn_1^0 mols of water and this in turn remain in equilibrium with free water present in the system. The integral free energy change (ΔG_w) due to the dehydration of protein molecules at activities lower than unity can then be calculated from the relation (Chattoraj and Mitra, 1977a; Chattoraj and Birdi, 1984)

$$-\Delta G_w = RT \int_{a_1=1}^{a_1} \frac{n_1}{a_1} \, da_1, \tag{3}$$

$-\Delta G_w$ at different values of a_1 have been evaluated from the graphical integration using the experimental data. These are shown in figure 2 for various values of p/p_o. The integral free energy changes (ΔG_w^o) of dehydration for bringing water activities from unity to zero at different temperatures are presented in table 1.

From figure 2 as well as from table l, one finds that the free energy change due to the

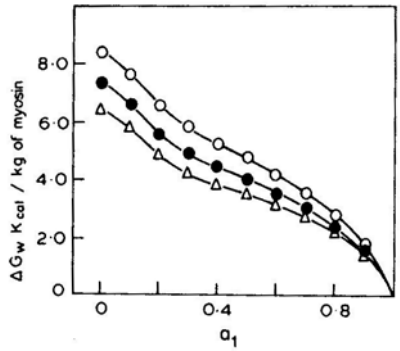

Figure 2. Plot of ΔG_W *vs.* a_1. (O), 45°C; (●), 27°C; (Δ), 10°C.

Table 1. Standard free energy, enthalpy and entropy changes for dehydration of myosin.

Temperature °C	ΔG^o_w Kcal/kg myosin	ΔH^o_w Kcal/kg myosin	$T\Delta S^o_w$ Kcal/kg myosin	ΔS^o_w Cal/degree/kg myosin
10	6·4	−8·0	−14·4	−50
27	7·3	−9·0	−16·3	−54
45	8·3	−9·7	−18·0	−56

dehydration process is positive. Like all other proteins, dehydration of myosin is thus thermodynamically non-spontaneous process. At 27°C ΔG^o_w for BSA, haemoglobin and egg albumin are + 12·3, + 9·4 and + 9·6 Kcals per kg of protein (Chattoraj *et al.*, 1977a), respectively. Compared to this, ΔG^o_w for rod-shaped myosin is + 7·3 Kcals per kg of protein. Since surface area per kg of a protein in rod-shaped form is considerably higher than that in globular form, it seems that the surface free energy per unit area for myosin is considerably low. The structural stability of myosin, on the basis of the surface tensional forces discussed earlier (Chattoraj and Mitra, 1977a, b) is expected to be considerably lower than that of BSA. In agreement with this, the thermal denaturation temperature of myosin in the presence of 0·5 molar salt concentration is lower than 50°C (Chattoraj, D. K., Basu, S. and Das, K. P., unpublished results) whereas that for BSA in water is 65°C and it increases further with addition of neutral salt in the medium (Mitra and Chattoraj, 1978).

In the desorption process considered earlier (Chattoraj and Mitra, 1977a), one mol of hydrated protein in contact with unit mol fraction of free water is dehydrated reversibly until one mol of dry protein has been obtained. ΔG^o_w is the standard free energy change for this desorption process whose value depends significantly on T but negligibly on p/p_0 or n_1 (Denbigh, 1971). The standard enthalpy change ΔH^o_w for the dehydration may be calculated from the thermodynamic relation,

$$\frac{d(\Delta G^o_w/T)}{dT} = -\frac{\Delta H^o_w}{T^2}. \tag{4}$$

From the slope ofthe plot of $\Delta G^o_w/Tvs.T$, values of ΔH^o_w at different temperatures were calculated (vide table 1). Incorporating the values of ΔH^o_w and ΔG^o_w in the equation

$$\Delta G^o_w = \Delta H^o_w - T\Delta S^o_w \tag{5}$$

values of $T\Delta S^o_w$ have been calculated (vide table 1). The adsorption and desorption of water on myosin seem to be significantly entropy controlled processes. Values of entropy change for desorption are included in table 1. It may be pointed out that the contribution of ΔH^o_w in the dehydration process is also not negligible.

In figures 3 to 6, excess binding Γ^1_2 for different solutes have been plotted as functions of X_2/X_1 Γ^1_2 is negative for certain values of X_2/X_1 but at several other values of the mol ratio composition, Γ^1_2 becomes positive. It has been previously shown (Chattoraj

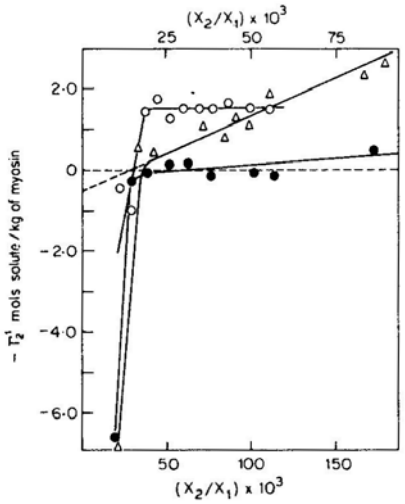

Figure 3. Plot of Γ^1_2 *vs.* X_2/X_1. (Δ), KCl (left-upper scale); (O), NaCl (left-lower scale); (●), NaBr (left-lower scale).

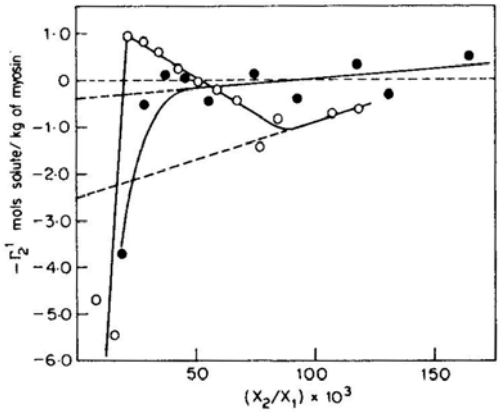

Figure 4. Plot of Γ^1_2 *vs.* X_2/X_1· (O), $CaCl_2$; (●) **KI**

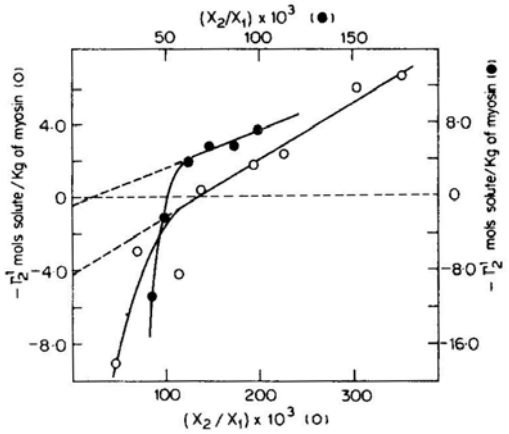

Figure 5. Plot of Γ_2^1 *vs.* X_2/X_1. (O), urea; (●),KCNS.

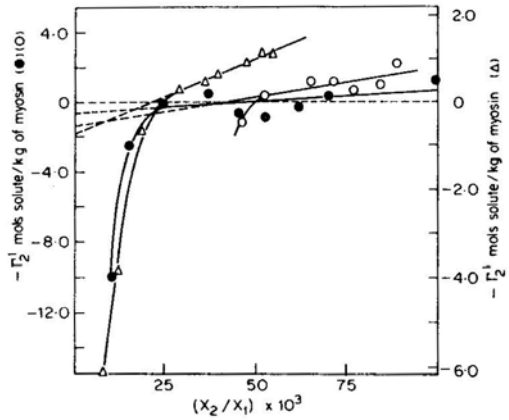

Figure 6. Plot of Γ_2^1 *vs.* X_2/X_1, (O), LiCl; (●), sucrose; (Δ), Na_2SO_4.

and Mitra, 1977b; Chattoraj and Birdi, 1984) that

$$\Gamma_2^1 X_1 + \Gamma_1^2 X_2 = 0. \tag{6}$$

Thus, excess binding of water Γ_1^2 becomes positive when Γ_2^1 is negative and vice versa. We can further set n_1^t and n_2^t equal to $n_1+\Delta n_1$ and $n_2+\Delta n_2$ respectively (Chattoraj and Mitra, 1977b; Chattoraj and Birdi, 1984). Here Δn_1 and Δn_2 represent mols of solvent and solute respectively bound per kg of myosin whereas n_1 and n_2 are respective mols of the components per kg of protein remaining free. Inserting these in eqs (1) and (2), it can be shown that

$$\Gamma_1^2 = \Delta n_1 - \Delta n_2 \frac{X_1}{X_2} \tag{7}$$

and

$$\Gamma_2^1 = \Delta n_2 - \Delta n_1 \frac{X_2}{X_1} \tag{8}$$

As in the case of other proteins (Chattoraj and Mitra, 1977b; Mitra *et al.,* 1978; Sadhukhan and Chattoraj, 1983), Γ_2^1 *vs* X_2/X_1 plots for myosin in the presence of solutes are linear at certain ranges of composition (vide figures 3 to 6) so that Δn_1 and Δn_2 for these systems can be evaluated using eq. (8). These values of Δn_1 and Δn_2 in the presence of various solutes have been presented in table 2 along with standard errors. Values of Δn_2 for $CaCl_2$, Na_2SO_4 and urea may have some significance but for other salts and sucrose, its value may be taken to be negligible in the light of the high error associated with Δn_2. Error in Δn_1 for several solutes and particularly for LiCl, KI, KCNS and sucrose are also high. Such high errors also found in other systems (Bull and Breese, 1970b; Mitra *et al.,* 1978) are possibly associated with insolubility of proteins in the salt solution used.

Table 2. Δn_1 and Δn_2 for myosin in the presence of solutes.

Solute	Δn_1 Mols water/kg myosin	Δn_2 Mols solute/kg myosin
LiCl	34±17	1·4±1·2
KCl	36±7	0·5±0·4
KCNS	80±18	0·8±1·4
KI	4±2	0·4±0·2
NaBr	3±1	0·2±0·1
$CaCl_2$	16±7	2·5±0·7
Urea	31±3	4·2±0·7
Sucrose	13±9	0·7±0·5
Na_2SO_4	34±2	0·7±0·1

Electrolytes and organic solutes stand in the following order in terms of magnitudes of Δn_1

$$KCNS > KCl > LiCl \simeq Na_2SO_4 > \text{urea} > CaCl_2 > \text{sucrose} > KI > NaBr.$$

This order is not in agreement with that expected from the lyotropic series. Lyotropic series in terms of Δn_1 remains qualitatively valid for BSA and egg albumin with reference to many inorganic salts (Bull and Breese, 1970a; Mitra *et al.,* 1977). Except KCNS, values of Δn_1 for myosin with different solutes are always less than Δn_1^o . Thus solutes dehydrate myosin in general and the extent of dehydration is dependent on the nature of the solute.

Deviation of the plots from linearity shown in figures 3 to 6 in certain regions of X_2/X_1 is an indication that Δn_1 or Δn_2 or both vary now with change of X_2/X_1 (Chattoraj and Mitra, 1977b; Chattoraj and Birdi, 1984). For a given solute, values of Γ_2^1 in eq. (8) may become positive when $\Delta n_2/\Delta n_1$ is greater than X_2/X_1 in certain ranges of concentrations whereas in other concentration ranges,Γ_2^1 may be negative when $\Delta n_2/\Delta n_1$ is less than X_2/X_1. Further, for each solute, there exists a definite value of X_2/X_1 (or m_2) when Γ_2^1 becomes zero (vide figures 3 to 6). This is the azeotropic state (Chattoraj and Birdi, 1984) for binding of solvent and solute to myosin when $\Delta n_2/\Delta n_1$ becomes equal to X_2/X_1. Values of m_2 of various solutes at the azeotropic states

presented in table 3 are different from each other. It may be of interest to note that for $CaCl_2$ there exist two azeotropic points. The significance and detailed analysis of the azeotropic states in terms of arrangement of water and solute molecules in the bound phase of a protein need detailed analysis in the future.

Table 3. Azeotropic points and standard free energies of excess hydration of myosin in presence of solute.

Solute	m_2 at azeotropic point	ΔG^a_{ws} Kcal/kg myosin	Γ_1^m Mols water/kg myosin
KCNS	2·83	22	70
NaCl	1·72	14	
$CaCl_2$	1·11	11	43
	2·83		
KCl	0·99	10	25
Na_2SO_4	1·36	5·6	20
LiCl	2·77	4·4	17
Urea	7·54	4·2	15
Sucrose	2·77	−3·4	3
NaBr	3·33		1·5
KI	5·55		1·4

The free energy change ΔG_{ws} in bringing myosin from the surrounding of water to that of a solution of composition X_1 (or X_2) has been calculated from the thermodynamic relation (Chattoraj and Mitra, 1979)

$$\Delta G_{ws} = RT \int_{f_1 X_1 = 1}^{f_1 X_1} \frac{\Gamma_1^2}{f_1 X_1} \mathrm{d}(f_1 X_1) + RT \Gamma_1^2 \ln \frac{f_1 X_1}{0{\cdot}5}. \tag{9}$$

Following the special method given earlier by Chattoraj and Mitra (1979), the integral in eq. (9) has been solved graphically between appropriate limits using values of Γ_2^1 and X_2. ΔG_{ws} is the free energy change when Γ_2^1 mols of solute (or Γ_1^2 mols of solvent) are transferred in excess to the bound phase due to the change of mol fraction of the solvent from unity to 0·5 (or solute mol fraction from 0–0·5).

In figures 7 and 8, values of ΔG_{ws} thus calculated from the experimental data have been plotted (for some solutes) against $X_2/X_1 \cdot \Delta G_{ws}$ is zero when myosin is in contact with pure water. With addition of a solute, ΔG_{ws} becomes at first negative. From the treatment of the Gibbs adsorption equation (Chattoraj and Mitra, 1977b, 1979), this means that the attractive interaction between the protein molecules themselves is reduced and protein-solvent interaction is increased with addition of relatively small amount of solute to the medium. This is in accordance with the salting-in phenomena for protein dissolution (Chattoraj and Mitra, 1979). Myosin is insoluble in pure water but begins to dissolve when the ionic strength of the solution is gradually increased. With increase of solute concentration or X_2/X_1 further, ΔG_{ws} gradually becomes less

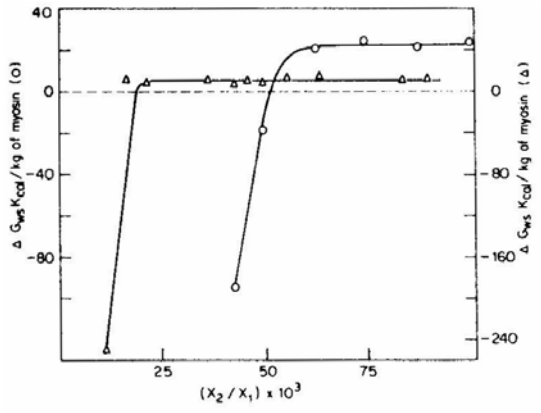

Figure 7. Plot of ΔG_{ws} *vs.* X_2/X_1· (O), KCNS; (Δ), KCl.

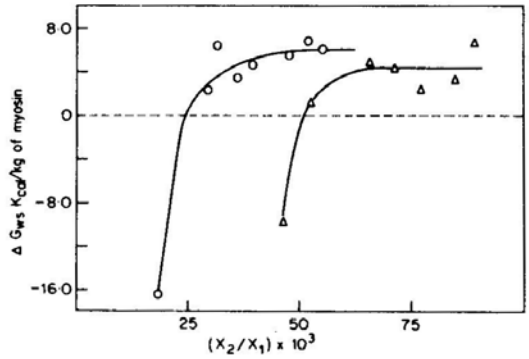

Figure 8. Plot of ΔG_{ws} *vs.* X_2/X_1. (O), Na_2SO_4; (Δ), Licl.

negative, zero and finally it becomes positive (vide figures 7 and 8). In terms of the concept of the Gibbs adsorption equation, this means that with increase of solute concentration to a large extent, protein-solvent interaction decreases considerably so that there is a tendency for the protein molecules to aggregate and precipitate out by attractive interaction. This feature is consistent with the salting out phenomena observed for many proteins (Chattoraj and Mitra, 1979).

At considerably higher values of X_2/X_1, ΔG_{ws} in the presence of each solute approaches a limiting constant value ΔG^o_{ws}. At this state, Γ_1^2 also reach steady constant values Γ_1^m for different solutes. Γ_1^m values for different solutes are presented in table 3. ΔG^o_{ws} may be regarded as the standard free energy change for protein hydration at the state of saturation of the bound phase (Chattoraj and Birdi, 1984; Chattoraj and Mitra, 1979) when mol fraction of the solvent changes from unity to 0·5. ΔG^o_{ws} values thus presented in table 3 are comparable with each other since the thermodynamic scale is a perfectly general one .The order of solutes in terms of Δ^o_{ws} is

KCNS > NaCl > $CaCl_2$ > KCl > Na_2SO_4 > LiCl > urea > sucrose.

This order is consistent with the order of solutes in terms of Γ_1^m representing maximum extent of binding interaction of the proteins with solute and water.

Acknowledgement

One of us (M.D.) is grateful to the Council of Scientific and Industrial Research, New Delhi, for financial assistance.

References

Bull, H. B. and Breese, K. (1968) *Arch. Biochem. Biophys.,* **128,** 488.
Bull, H. B. and Breese, K. (1970a) *Arch. Biochem. Biophys.,* **137,** 299.
Bull, H. B. and Breese, K. (1970b) *Arch. Biochem. Biophys.,* **139,** 93.
Chattoraj, D. K. and Mitra, S. P. (1977a) *Indian J. Biochem. Biophys.,* **14,** 1.
Chattoraj, D. K. and Mitra, S. P. (1977b) *Indian J. Biochem. Biophys.,* **14,** 7.
Chattoraj, D. K. and Mitra, S. P. (1979) *Indian J. Biochem. Biophys.,* **16,** 406.
Chattoraj, D. K. (1981) *Indian J. Chem.,* **20A,** 941.
Chattoraj, D. K. and Birdi, K. S. (1984) *Adsorption and the Gibbs Surface Excess* (New York: Plenum Press).
Connell, J. J. (1962) *J. Sci. Food Agric.,* **13,** 607.
Denbigh, K. (1971) *The Principles of Chemical Equilibrium,* Third Edition (Cambridge: Cambridge University Press).
Graham, H. D. (1977) *Food Colloids,* (West Port: The AVI Publishing Co.).
Kuntz, I. D. and Kauzmann, W. (1974) *Adv. Protein Chem.,* **28,** 239.
Mitra, S. P., Chattoraj, D. K. and Das, M. N. (1977) *Indian J. Biochem. Biophys.,* **14,** 101.
Mitra, S. P., Chattoraj, D. K., Das, M. N. and Sen, A. (1978) *Indian J. Biochem. Biophys.,* **15,** 153.
Mitra, S. P. and Chattoraj, D. K. (1978) *Indian J. Biochem. Biophys.,* **15,** 239.
Pauling, L. (1945) *J. Am. Chem. Soc.,* **67,** 555.
Sadhukhan, B. K. and Chattoraj, D. K. (1983) *Indian J. Biochem. Biophys.,* **20,** 59.
Volkenshtein, M. V. (1983) *Biophysics* (Moscow: MIR Publishers)
Weber, K. and Osborn, M. (1969) *J. Biol. Chem.,* **244,** 4406.