USE OF ULTRACENTRIFUGE TO STUDY PREFERENTIAL INTERACTION OF SOLVENT COMPONENTS WITH PROTEINS IN MIXED SOLVENT SYSTEMS

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ABSTRACT

Ultracentrifuge technique has been applied to evaluate preferential interaction of bovine serum albumin, lysozyme, insulin, and β-lactoglobulin with one of the solvent components of water-2-chloroethanol mixtures. The results agree very well with the published data for the same systems, strengthening the validity of the present approach for a study of the conformational transition in proteins and synthetic polypeptides.

INTRODUCTION

PREFERENTIAL interaction of proteins and synthetic polymers has been studied[1] and several experimental techniques[2-10] have been employed and theories[11-15] proposed to understand the phenomenon of preferential interaction of macromolecules in mixed solvent systems. The most widely used experimental techniques are light scattering, densimetry, sedimentation equilibria and membrane equilibria[16-25]. The coefficient of preferential interaction can be measured[17] either by using partial specific volume or refractive index increment, both at constant composition of the mixture and at constant chemical potential, together with the refractive increments and density measurements of solvent components in the absence of the macromolecule. A modified version of the ultracentrifugal technique has been used to assess the magnitude of the thermodynamic interaction parameters of polystyrene samples in mixed binary solvent systems. In the present study, we have employed the same approach to study the preferential binding of four proteins to one of the solvent components of the water-2-chloroethanol system. This procedure avoids dialysis of the protein samples en route (which normally takes longer time) and yields precise thermodynamic information.

THEORETICAL

In treating these three-component systems, we have chosen the notation used earlier[11,12] in which water is component 1, the organic solvent component 2 and the macromolecular solute component 3. The preferential interaction of component 2 to the macromolecule is measured directly by the difference between the refractive index increments under conditions in which the chemical potential of the macromolecule and its molality are kept identical in the solvent and in solution. In practice, however, it is more convenient to measure the concentration in g/ml of the solution. Thus, preferential binding is usually expressed by $(\partial C_3/\partial C_2)_{T,P,\mu_3}$ and is given by equation (1):

$$\Psi = (\partial C_3/\partial C_2)_{T,P,\mu_3} = [(\partial n/\partial C_2)_{T,P,\mu_3} - (\partial n/\partial C_2)_{T,P,C_3}]/(\partial n/\partial C_3)_{T,P,C_2} \quad (1)$$

where the C's and μ's represent respectively, the concentrations in g/ml and chemical potentials of the indicated components; n, the refractive index of the solution and P and T have their usual meanings. The use of this equation requires the independent measrements of three quantities, *viz.*, the refractive index increment of the macromolecule at constant chemical potential, $(\partial n/\partial C_2)_{T,P,\mu_3}$, that at constant composition of the mixture, $(\partial n/\partial C_2)_{T,P,C_3}$ and the refractive index increments of solvent components in the absence of the macromolecular species, $(\partial n/\partial C_3)_{T,P,C_2}$. The latter two quantities can be obtained by using a differential refractometer according to the method described earlier[18]. In the present study, however, $(\partial n/\partial C_2)_{T,P,\mu_3}$ has been obtained by an earlier ultracentrifugal technique[2].

EXPERIMENTAL

All the four proteins used in this work *viz.* bovine serum albumin, lysozyme, insulin and β-lactoglobulin were from Sigma Chemicals (USA) and were recrystallised before use to remove metallic contaminants.

The molecular weight of β-lactoglobulin, as measured by the light scattering technique was 18,100. The values thus obtained for lysozyme, bovine serum albumin and insulin were 14,100, 66,000 and 12,000 respectively. The protein solutions were prepared in the concentration range 0·005 to 0·01 g/ml, in standard phosphate buffer (pH=7), with a salt

concentration of 20 millimolar and different volume fractions of 2-chloroethanol. The solutions and the buffers were stored at 0° C.

Refractive index measurements

Refractive index increments at constant composition of the buffer and protein and 2-chloroethanol were measured using a Brice-Phoenix differential refractometer (Model BP-2000-V) equipped with a special glass cell R-101-4. All measurements were made using the mercury green line (546 nm). Density measurements were carried out on a DMA 02C digital precision densitymeter (A. Paar K. G., Austria). Refractive index increments of the buffer +2-chloroethanol solvent mixtures were calculated by measuring the refractive indices of water, 2-chloroethanol and their mixtures using a Bausch and Lomb Precision Refractometer, equipped with sodium, as well as mercury light sources, with a precision of $\pm 3 + 10^{-5}$ units of refractive index. The initial calibration of the instrument was made at 20° C using a standard glass test piece and calibration checks were made with distilled water and the glass test piece at intervals. All refractive index measurements were made at 25° C.

Ultracentrifuge experiments

For the measurement of refractive index increments at constant chemical potentials, a synthetic boundary experiment in an analytical ultracentrifuge (Spinco Model E) was adopted, which serves to eliminate dialysis. In the ultracentrifugal experiment, the heavy rotor ANJ (with a capillary type double sector synthetic boundary cell and quartz windows) was used at 25°C. For each experiment, the cell was filled according to standard experimental procedure[19], with 0·14 ml of the protein solution (0·005–0·01 g/ml) in one sector and 0·44 ml of the appropriate solvent mixture in the other sector. The rotor was accelerated fast to complete the transport of solvent through the capillary in the shortest possible time (6–8 min). The rotor speed was maintained at 4908 rpm to minimise sedimentation of the protein. Both schlieren (figure 1) and interference photographs (figure 2) of the cell were taken at regular intervals for about two hours, using mercury green light. The baseline correction was made by removing the rotor from the chamber, shaking it several times to destroy concentration gradient and accelerating again to 4908 rpm. The present experiments were not found to be affected by convection inside the cell.

The interference photographs from the ultracentrifuge experiments were evaluated using a Nikon Profile Projector Model 6C by means of a fringe count, correction being made for the baseline.

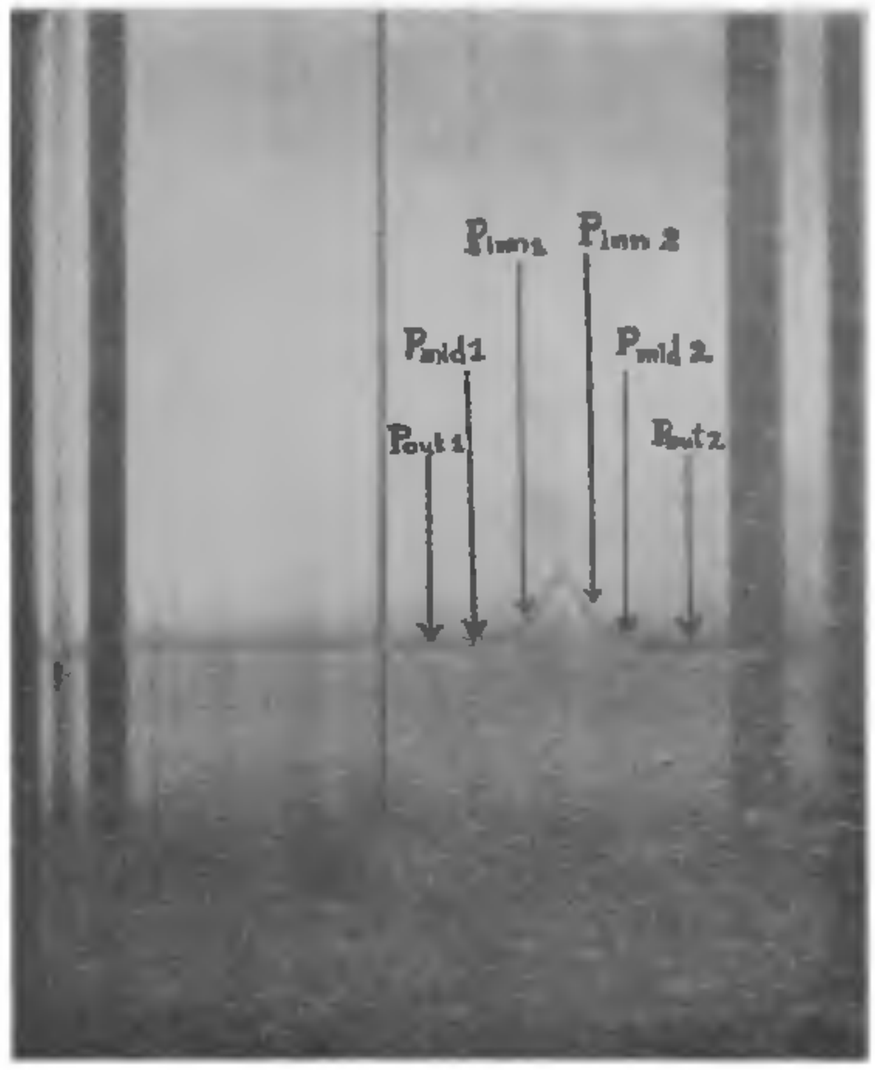

Figure 1. Schlieren photograph of the centrifuge cell for lysozyme in 50% of 2-chloroethanol in the mixture after 30 min from the start of the experiment (speed 4908 rpm; $C_2 = 0{\cdot}007$ g/ml).

Figure 2. Interference photograph of the centrifuge cell for lysozyme in 50% of 2-chlorethanol after 35 min from the start of the experiment (speed 4908 rpm; $C_2 = 0{\cdot}007$ g/ml).

The fringe count Δf is related to the refractive index difference Δn at the two selected points by the relation:

$$\Delta f = h\,(\Delta n/\lambda) \qquad (2)$$

where h represents the thickness of the cell (12 mm) and λ, is the wavelength of the light used (546 nm). The purpose of the measurement is to find that part of Δf, which corresponds to the solvated macromolecule at the time when the boundary was formed. The fringe count obtained from the interference photographs corresponds to the superposition of the polymer boundary and the solvent boundary at some later time. Due to the process of diffusion, the boundary spreads out so that the concentration in the cell becomes essentially homogeneous.

For a diffusing species for which the diffusion constant D is independent of concentration within the concentration range covered in the cell, the profile of concentration is a function of the boundary conditions (length of the sector-shaped cell), the initial conditions (position and size of the sharp boundary) and the product Dt, where t is the time. In the present approach, the initial position of the boundary is estimated from the schlieren photographs. The experimental concentration profile (the profile of refractive index) is sampled at six points more or less symmetrically placed around the initial boundary (figure 1). The outer points, $P_{out\ 1}$ and $P_{out\ 2}$ are well removed from the peak, while the inner points, $P_{inn\ 1}$ and $P_{inn\ 2}$ are directly on the peak and $P_{mid\ 1}$ and $P_{mid\ 2}$, close to the peak. In each interference photograph (figure 2), the fringe count is measured for each pair of points. The diffusion of a unit-sized boundary is simulated in an IBM computer, using a Fortran Program CZERO-6 for identical conditions and the concentration profile is computed as a function of Dt and sampled for each of the three pairs of points. Thus, for each pair (subscript j), the function $F_j(Dt)$ is obtained in the form of a table. The experimental fringe count Δf_j is now expressed as:

$$\Delta f_j = \Delta f_p^{\circ}\, F_j\left[D_p(t-\Delta t)\right] + \Delta f_s^{\circ}\, F_j\left[D_s(t-\Delta t)\right] \quad (3)$$

where the subscripts p and s refer to the protein and solvent boundaries respectively; the superscript zero refers to the original sharp boundary; t represents the time from the start of the run and Δt takes into account the period required for the original formation of the boundary as well as the original disturbance of the boundary. A nonlinear least squares procedure was then applied to all three sets of experimental values simultaneously. This procedure yielded five adjustable parameters, *viz.*, Δf_p° Δf_s°, D_p D_s and Δt. The Δt value was usually quite close to the observed time for the initial formation of the boundary. D was usually close to the value obtained in an analogous experiment in the absence of the protein. The D_s value from the latter experiment was used in the actual evaluation to secure better precision. The values of $(\partial n/\partial C_2)_{T.P.\mu_3}$ were then calculated as $\Delta n / C_2$, where Δn was obtained equation (2) using the Δf°_p values and C_2 was the concentration known from the sample preparation.

Results and Discussion

The preferential interaction parameters for all the proteins were calculated using equation (1) and these results are presented in tables 1–4, which also contain the experimental values of refractive index increments, together with literature data.

As seen from table 1, the parameter Ψ for the β-lactoglobulin-water-2-chloroethanol system, is found to increase up to 40% by volume of 2-chloroethanol in the mixture. At higher concentrations of the same, a negative adsorption is indicated suggesting preferential hydratioin. This variation in the amount of bound organic solvent parallels almost exactly the conformational transition[23] in the protein, indicating that 2-chloroethanol enhances the helical content of the protein by creating hydrophobic domains, which favour the formation of α-helical conformations. A comparison with the less extensive data[24] shows excellent agreement for this system.

TABLE 1

Preferential interaction coefficient for β-lactoglobulin in water -2-chloroethanol solvent mixture at 25° C

ϕ_2	$(\partial n/\partial C_2)_{T,\mu_1,\mu_3}$	$(\partial n/\partial C_2)_{T,P,C_3}$	$(\partial n/\partial C_3)_{T,P,C_2}$	Ψ	
				Present work	Data from Ref. 24
0·1	0·209	0·188	0·104	0·202	0·171
0·2	0·225	0·186	0·100	0·390	0·359
0·3	0·241	0·184	0·095	0·600	0·643
0·4	0·244	0·182	0·093	0·667	0·718
0·5	0·237	0·180	0·090	0·633	0·711
0·6	0·219	0·179	0·089	0·449	0·366
0·7	0·180	0·178	0·087	0·023	—
0·8	0·118	0·176	0·083	−0·699	−0·848
0·9	0·105	0·175	0·080	−0·875	—

*volume fraction of the second component in the mixture is designated by the symbol ϕ_2, in all the tables.

Table 2 shows the dependence of Ψ on volume fraction of 2-chloroethanol for the lysozyme-water-2-chloroethanol system. A similar behaviour is again observed with the highest preferential binding being observed at 40% of the 2-chloroethanol in the mixture. Here again, an inversion in Ψ is observed after about 60% of the organic component. Tables 3

TABLE 2
Preferential interaction coefficient for lysozyme in water -2-chloroethanol solvent mixture at 25° C

ϕ_2	$(\partial n/\partial C_2)_{T,\mu_1,\mu_3}$	$(\partial n/\partial C_2)_{T,P,C_3}$	Ψ Present work	Ψ Data from Ref. 24
0·1	0·194	0·173	0·202	—
0·2	0·201	0·171	0·300	0·347
0·3	0·200	0·164	0·379	—
0·4	0·192	0·157	0·376	0·431
0·5	0·178	0·151	0·300	—
0·6	0·148	0·143	0·056	−0·028
0·7	0·114	0·136	−0·253	—
0·8	0·093	0·129	−0·434	−0·485
0·9	0·083	0·122	−0·488	—

TABLE 3
Preferential interaction coefficient for bovine serum albumin in water -2-chloethanol solvent mixture at 25° C

ϕ_2	$(\partial n/\partial C_2)_{T,\mu_1,\mu_3}$	$(\partial n/\partial C_2)_{T,P,C_3}$	Ψ Present work	Ψ Data from Ref. 24
0·1	0·201	0·175	0·250	—
0·2	0·209	0·170	0·390	0·433
0·3	0·208	0·164	0·463	—
0·4	0·206	0·160	0·495	0·619
0·5	0·192	0·155	0·410	—
0·6	0·155	0·150	0·056	−0·113
0·7	0·110	0·145	−0·402	—
0·8	0·083	0·141	−0·699	−0·787
0·9	0·071	0·137	−0·825	—

and 4 present data for the systems bovine serum albumin-water-2-chloroethanol and insulin-water-2-chloroethanol respectively and show the same general trend as in the other two systems.

In general, it is seen that the preferential interaction of the 2-chloroethanol for the proteins passes through a miximum at about 30–40 volume percent of this component, then decreases and becomes negative as preferential hydration takes place. The data of Inoue *et al*[24] agree to within 2–3% for all systems.

This study suggests a competition between water and 2-chloroethanol for the protein with its hydrophilic and hydrophobic features. 2-chloroethanol lowers the activity of water, thus weakening its ability to destroy the intermolecular

TABLE 4
Preferential interaction coefficient for insulin in water -2-chloroethanol solvent mixture at 25° C

ϕ_2	$(\partial n/\partial C_2)_{T,\mu_1,\mu_3}$	$(\partial n/\partial C_2)_{T,P,C_3}$	Ψ Present work	Ψ Data from Ref.24
0·1	0·199	0·177	0·212	—
0·2	0·208	0·169	0·390	0·359
0·3	0·211	0·159	0·547	—
0·4	0·207	0·150	0·613	0·664
0·5	0·192	0·143	0·544	—
0·6	0·164	0·136	0·315	0·253
0·7	0·130	0·129	0·012	—
0·8	0·098	0·123	−0·301	−0·424
0·9	0·086	0·118	−0·400	—

non-covalent interactions, which stabilise the α-helix when its concentration is increased, the water of the solvent mixture is adsorbed at the hydrophilic sites of the protein. This is clearly indicated by the changing-over of the preferential interaction with the organic solvent to preferential hydration, which is consistent with Inoue and Izumi's observations[25]; when the concentration of 2-chloroethanol increases, preferential hydration increases as well.

CONCLUSIONS

The preferential interaction of β-lactoglobulin lysozyme, bovine serum albumin and insulin with one of the solvent components in water-2-chloroethanol mixtures has been investigated by an ultracentrifugal technique. The 2-chloroethanol which is a structure-enhancing denaturant interacts with the proteins in a rather complicated manner. At low concentrations of 2-chloroethanol, it is preferentially bound to the proteins in all the cases studied, whereas in mixtures rich in 2-chloroethanol, preferential water-binding occurs. No close correlation was found between the conformational transition and the variation of preferential interaction. The present approach is quite promising especially in the study of proteins and polypeptides undergoing helix-coil transition and thus represents a powerful technique in the study of other biochemcial systems, such as the *lac* operator-repressor interactions currently in progress.

The technique also gives the refractive index increment values at constant chemical potential without the actual dialysis of the sample, thus saving considerable amount of time. These values have been obtained only after dialysis in the earlier efforts.

The present refined measurements confirmed the

published data for the same systems obtained by other experimental techniques and strengthen the validity of the present approach to the study of proteins in mixed solvent systems.

ACKNOWLEDGEMENTS

IMA is grateful to the Karanatak University, Dharwar, for study leave to carry out post-doctoral research. This work represents a joint research project between the Clarkson College of Technology, Potsdam, and the Karnatak University. Part of this research work forms a Ph. D. thesis of BRP.

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