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Probing Protein-Sugar Interactions

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ABSTRACT We have investigated the partial specific volumes $\bar{v}_2$ (ml/g), hydration, and cosolvent interactions of rabbit muscle aldolase by equilibrium sedimentation in the analytical ultracentrifuge and by direct density increment $(\partial\rho/\partial c_2)_\mu$ measurements over a range of sugar concentrations and temperature. In a series of sugars increasing in size, glucose, sucrose, raffinose, and α-cyclodextrin, $(\partial\rho/\partial c_2)_\mu$ decreases linearly with the solvent density ρ^0. These sugar cosolvents do not interact with the protein; however, the interaction parameter B_1 (g water/g protein) mildly increases with increasing sugar size. The experimental B_1 values are smaller than values calculated by excluded volume (rolling ball) considerations. B_1 relates to hydration in this and in other instances studied. It decreases with increasing temperature, leading to an increase in $\bar{v}_2$ due to reduced water of hydration electrostriction. The density increments $(\partial\rho/\partial c_2)_\mu$, however, decrease in concave up form in the case of glycerol and in concave down form for trehalose, leading to more complex behavior in the case of carbohydrates playing a biological role as osmolytes and antifreeze agents. A critical discussion, based on the thermodynamics of multicomponent solutions, is presented.

INTRODUCTION

Significant information on protein structure and interactions can be obtained from solution studies in which the concentration of low molecular mass solutes and cosolvents, causing stabilization, destabilization (denaturation), aggregation, crystallization, or no measurable effects, is varied over a broad range of protein concentration, pH, and temperature (cf. Timasheff, 1998, for a recent review). We will limit ourselves in the present study to the analysis of density increments $(\partial\rho/\partial c_2)_\mu$ (ρ is the solution density, c_2 is the protein, component 2, concentration in g/ml of solution, and μ is the chemical potential of components 1, the solvent, and 3, the cosolvent, diffusible through a semipermeable membrane) from equilibrium sedimentation and density increment studies of rabbit muscle aldolase, an enzyme of known sequence (Tolan et al., 1984) and crystal structure at 1.9-Å resolution (Sygusch et al., 1987; Blom and Sygusch, 1997), over a range of sugars and sugar sizes, at various temperatures and concentrations. Various studies of proteins in sugar solutions have been reported and will be referred to (Priev et al., 1996; Bennett and Steitz, 1980; Gekko and Timasheff, 1981; Arakawa and Timasheff, 1982; Lehmann and Zaccai, 1984; Xie and Timasheff, 1997a). A major purpose of our investigation is the critical interpretation of the hydration coefficient B_1, to be defined below, evaluated from the variation in $(\partial\rho/\partial c_2)_\mu$ with solvent density ρ^0. A concise description of protein volumes and hydration, and thermodynamic theory versus a model of solvent and cosolvent interactions, will be presented.

MATERIALS AND METHODS

Crystalline rabbit muscle aldolase suspended in 3.2 M ammonium sulfate (Boehringer Mannheim, Mannheim, Germany) was recrystallized twice by dissolution in 1 M ammonium sulfate, centrifugation, and addition of saturated ammonium sulfate. It was extremely stable in 0.15 M NaCl solution, with and without sugar cosolvents. D-Trehalose dihydrate and α-cyclodextrin were from Fluka (Buchs, Switzerland), and raffinose pentahydrate was from Aldrich (Milwaukee, WI). Solutions for the analytical ultracentrifuge were prepared by dissolving aldolase in 0.15 M NaCl and dialyzing it against this solvent to reach a final A_{280} of about unity. An estimated amount of sugar was added to each 1 ml of sample and subsequently dialyzed against multiple changes of the respective sugar solution in 0.15 M NaCl. The final value of A_{280} was ~0.5. Dialysis was performed with shaking at room temperature, in Pierce (Rockford, IL) Slide-a-lyzer commercial dialyzers, enclosed in small-size Ziplok (DowBrands, Indianapolis, IN) bags. The reduction in size of dialyzing solution is important in the case of expensive sugars, such as cyclodextrin. The density of the dialyzing solvent was determined after the completion of dialysis.

Density measurements

Five-digit precision density measurements of the dialyzing solvent were performed at 20.0°C in an Anton Paar (Graz, Austria) DMA 58 densimeter. High precision six-digit density measurements for density increment determinations were performed in the Paar DMA 60 densimeter equipped with 100 μl cells at (20.00 ± 0.01)°C (cf. Bonneté et al., 1993, for details). For these experiments the protein was dialyzed in 0.32 ml/cm dialysis bags. The protein solubility decreased with increasing sugar content, at protein concentrations exceeding those used in the ultracentrifuge, and precipitates were observed in the dialysis bags. The precipitates separated quite well, and clear solutions were always used for the density measurements. Density measurements, which were performed on three or four protein samples for each sugar concentration, yielded reproducible values for the density increments. Aldolase concentrations, which are not required for the analysis of equilibrium sedimentation data, were determined by use of the extinction coefficient 0.91 for $A_{1\,\mathrm{cm}}^{0.1\%}$ of aldolase at 280 nm.

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Analytical ultracentrifugation

Equilibrium sedimentation runs were performed in the Beckman (Fullerton, CA) XL-A analytical ultracentrifuge, at 8000 rpm at various temperatures, and equilibrium was reached within less than 24 h. Baselines were determined by running the XL-A at 20,000–25,000 rpm for 2–3 h. Double-sector charcoal-filled epon centerpieces were used, with column lengths of ~4 mm. The data were analyzed, assuming a single ideal solute, to obtain the buoyant molar mass $M_2\ (\partial\rho/\partial c_2)_\mu$ (Optima XL-A analysis software, version 2, Beckman), by fitting each scan to Eq. 1b, the integrated form of the differential Eq. 1a (Casassa and Eisenberg, 1964; Fujita, 1994), in the limit of vanishing component 2 concentration:

$$d \ln c_2/\mathrm{d}r^2 = (\omega^2/2RT)M_2(\partial\rho/\partial c_2)_\mu \tag{1a}$$

$$A_\mathrm{r} = \exp(\ln A_0 + (\omega^2/2RT)M_2(\partial\rho/\partial c_2)_\mu(r^2 - r_0^2)) + E, \tag{1b}$$

where A_0 is the absorbance at a reference point at a radial distance r_0, A_r is the absorbance at a given radial position r, ω is the angular speed (in rad/s), R is the gas constant, T is the absolute temperature, and E is a baseline correction term. Residuals were randomly distributed in the single ideal solute data analysis. No improvement was obtained by using the nonideal equation with virial coefficients or nonuniform mass distribution. From the equilibrium sedimentation experiments we derive values $M_2\ (\partial\rho/\partial c_2)_\mu$ for each value of the solvent density ρ^0 in the absence of component 2. If M_2 is known, $(\partial\rho/\partial c_2)_\mu$ is obtained.

PROTEIN VOLUMES AND HYDRATION

There are two ways in which volumes can be defined in biophysical research. A basic definition based on thermodynamic theory, relating to experimental procedures as density measurements and analytical ultracentrifugation, concerns the partial molal or specific volume, $\bar{v}_2$ (ml/g). It represents the increase in volume upon the addition of 1 g of "dry" protein (component 2) to a solution comprising the solvent (component 1), water, and a low-molecular-weight buffer or cosolvent (component 3). Additional components will not be considered in this simplified discussion; however, the basic principles of thermodynamic correctness will be maintained (Casassa and Eisenberg, 1960, 1961, 1964; Cohen and Eisenberg, 1968; Eisenberg, 1976, 1981, 1994, 1999; Reisler and Eisenberg, 1969; Reisler et al., 1977). In the solution process of the dry protein the molality of component 3, w_3 (in units of grams of component 3 per grams of component 1), remains constant. In the process of dissolution the protein is hydrated; however, the volume of the water of hydration does not become part of the protein partial specific volume. Hydration rather likely leads to a decrease in $\bar{v}_2$ after a reduction in solution volume because of electrostriction of the water hydrating the protein. Empty spaces on the surface facing the solvent or the interior of the protein, if they exist, will contribute to $\bar{v}_2$; however, empty spaces in the interior of the protein filled with solvent and cosolvent molecules not interacting with the protein will not contribute to $\bar{v}_2$. Analysis of the protein-solvent interface by x-ray and neutron scattering in H_2O and D_2O solutions, confirming the existence of a hydration volume with an average density ~10% larger than that of the bulk solvent, has recently been reported (Svergun et al., 1998). The calculation of Cohn and Edsall (1943) of $\bar{v}_2$ is based on the addition of the experimentally derived volumes of constituent amino acid residues and yields $\bar{v}_2$ of an unfolded polypeptide chain. Volume changes due to hydration are included for each amino acid residue in the chain. The calculation has been improved recently (Zamyatnin, 1984; Perkins, 1986; Chalikian et al., 1993), with minor changes in $\bar{v}_2$. Iqbal and Verrall (1987) consider a glycine polypeptide chain to which proper side chains were added. In another contribution (Kharakoz, 1997) the effect of ionization on peptide and protein volume has been considered. The volume change transition to the folded protein structure can be estimated, and the calculated results are in approximate agreement with $\bar{v}_2$ values of native proteins reliably determined by densimetry.

The alternative more recent procedure of calculating volumes of native proteins is from structures derived by high-resolution x-ray crystallography by use of Voronoi polyhedra (Harpaz et al., 1994; Gerstein and Chothia, 1996; Richards, 1977; Paci and Velikson, 1997). In these calculations a geometric protein volume is obtained that represents the volume inside the protein surface. Corrections applied include the addition of the so-called thermal volume, corresponding to postulated empty domains around the protein surface, and abstraction of the interaction volume, representing the decrease in the solvent volume deriving from electrostriction in the hydration process. Earlier calculations consisted of the evaluation of 1) the van der Waals volume, namely the volume arising from the molecular components of the polypeptide chain; 2) the molecular volume, defined by rolling a spherical probe of suitable size on the van der Waals surface of the native protein; and 3) the solvent-excluded volume, arising from the limitations of a solvent molecule of a defined size approaching the molecular volume more closely than the solvent effective radius. Uncertainties have been raised with respect to free water in interior protein spaces as interpreted by x-ray (Matthews et al., 1995) and NMR (Ernst et al., 1995) studies.

THERMODYNAMIC THEORY VERSUS MODEL OF SOLVENT AND COSOLVENT INTERACTIONS

The thermodynamic representation of multicomponent systems (Casassa and Eisenberg, 1960, 1961, 1964; Eisenberg, 1976, 1981, 1994, 1999) useful in the interpretation of partial specific volumes and interactions in solvent/protein/cosolvent systems follows thermodynamic and statistical mechanics rigor and points out instances in which molecular models have been introduced for the interpretation and prediction of molecular phenomena. This presentation, based in large part on the pioneering work of Kirkwood, Goldberg, Scatchard, and Stockmayer, is justified in providing clarification of a number of points under current discussion.

When a solution containing components 1, 2, and 3 is dialyzed against the "solvent" containing components 1 and 3 only, dialysis equilibrium is achieved and the chemical potentials μ_1 and μ_3 are equal on both sides of the membrane. The temperature T is maintained; however, the osmotic pressure P, although not determined in a dialysis experiment, is higher in the inner dialysis compartment, containing component 2, which is unable to pass through the semipermeable membrane. As a result of hydration and cosolvent positive or negative (for instance the "Donnan" effect in the case of charged components) interactions, effective concentrations are changed at equilibrium. It is possible to define an interaction parameter $\xi_3 = (\partial g_3/\partial g_2)_\mu$, in units of grams g_3 of component 3 per grams g_2 of component 2, signifying the change in concentration (in gram-molality units $w_3 = g_3/g_1$) of component 3 with the addition of component 2, at constant chemical potentials μ_1 and μ_3. The concentration of component 2 is kept low to avoid macromolecule/macromolecule interactions; however, such interactions can be handled within the thermodynamic framework. The symmetry of the thermodynamic equations yields the simple relationship $\xi_1 = -\xi_3/w_3$, where ξ_1 is the interaction parameter in terms of grams of component 1 per grams of component 2. Note that when ξ_3 is positive ξ_1 is negative, and vice versa. It has been common practice for many years to call ξ_3 and ξ_1 preferential interaction coefficients, and we have, in the earlier stages of our own work (Reisler and Eisenberg, 1969; Reisler et al., 1977), used this term as well. Positive values of ξ_3 and ξ_1 are meant in this practice to indicate enrichment, or inclusion, of the respective component in the close neighborhood of component 2, and negative values indicate deficiency or exclusion, transcending thermodynamic conceptuality. However, ξ_3 and ξ_1 are functions of the concentration of both components 1 and 3, and thermodynamics does not provide a molecular explanation with which the term preferential interaction is associated. When experiments are considered (Eisenberg, 1994) it is seen that, in the case of denatured bovine serum albumin (BSA) in guanidinium hydrochloride, for instance, ξ_1 is negative at lower (but still high and denaturing) values w_3 and increases with increasing concentrations w_3, changing sign and becoming positive in linear extrapolation. Similar results are observed for halophilic malate dehydrogenase (hMDH), the halophilic elongation factor Tu (Ebel et al., 1992), and glyceraldehyde-3-phosphate dehydrogenase from *Haloarcula vallismortis* (Ebel et al., 1995), with increasing NaCl or KCl concentrations. The interaction parameters ξ_3 and ξ_1 should thus not be associated with a single component only. Zero values of either ξ_3 or ξ_1 do not indicate an absence of interactions with either component 3 or 1 or with both components. To reach a valid interpretation, measurements should be performed over a range of component 3 concentrations.

For practical applications a physical model expressing ξ_1 or ξ_3 in terms of two parameters B_1 and B_3, grams of solvent (g_1/g_2), and grams of cosolvent (g_3/g_2) bound per gram of component 2 has been described (Inoue and Timasheff, 1972; Kupke, 1973; Eisenberg, 1976; Reisler et al., 1977). The model postulates that sites occupied by component 1 are excluded to component 3, and vice versa (B_1 may be related to hydration and B_3 may include a positive term deriving from cosolvent binding and a negative term deriving from Donnan exclusion for charged components). Constant values of B_1 and B_3 define an invariant particle model (Tardieu et al., 1981). The simple mathematical expressions resulting from the model are

$$\xi_3 = B_3 - B_1 w_3 \tag{2a}$$

or, equivalently,

$$\xi_1 = B_1 - B_3/w_3. \tag{2b}$$

When a plot of ξ_1 versus w_3^{-1} yields a straight line, B_1 can be obtained from the intercept and B_3 from the slope. This representation has proven useful in many instances (Eisenberg, 1994; cf. footnote 4 in Reisler et al., 1977).

A two-domain model (Record and Anderson, 1995; Record et al., 1998) has recently been proposed for the interpretation of similar results. Additional and alternative rationalizations are not excluded. Thus, rather than considering an invariant particle model, it is possible sometimes to view the solvent/cosolvent interactions in terms of a mutual exchange of weakly attached components, a model extensively analyzed by Schellman (1990, 1994) and Schellman and Gassner (1996). In this analysis, and in terms of the standard notation we have used, B_1 is assumed to decrease as B_3 increases from an initial zero value as water surrounding component 2 is exchanged by component 3 increasing in concentration. Timasheff (1992, 1998) has combined the two concepts by dividing B_1 into a nonexchangeable part, indicating strong hydration, and an exchangeable one, replaced by B_3 in the Schellman scheme. Thus ξ_3 in Eq. 2a will increase from a value of zero as w_3 increases from zero and B_3 exchanges with B_1; it will reach a maximum with B_3 increasing to a steady or constant value, and will then decrease as w_3 increases further, multiplying the nonexchangeable B_1 value. The analytical discrimination of the various components and equilibrium constants is not an easy task.

In molar terms $\Gamma_3 \equiv (M_2/M_3)\xi_3 = (\mu_{23}/\mu_{33})$, where M_3 and M_2 are molar masses and μ_{23} and μ_{33} are the partial derivatives of the chemical potentials μ_2 and μ_3 with molality m_3 of component 3 at constant pressure, temperature, and molality, except for that component indicated in the differentiation (Casassa and Eisenberg, 1964; Eisenberg, 1976). Analysis of the dependence of Γ_3 on processes of "binding" and denaturation (Timasheff, 1992, 1998) allows the analysis of these phenomena in terms of the Wyman "linkage" relation (Wyman, 1964, 1965; Tanford, 1969; Wyman and Gill, 1990). Transfer free energies, complemented by calorimetric studies (Makhatadze and Privalov, 1992), and related thermodynamic parameters can be obtained.

The interaction parameters ξ_3 or ξ_1 can be determined from equilibrium sedimentation (Eq. 1a) or by the direct measurement of density increments $(\partial\rho/\partial c_2)_\mu$ at dialysis equilibrium (Casassa and Eisenberg, 1964; Eisenberg, 1976, 1994):

$$(\partial\rho/\partial c_2)_\mu = (1 - \rho^0\bar{v}_2) + \xi_i(1 - \rho^0\bar{v}_i) \tag{3}$$

$$= (1 + \xi_i) - \rho^0(\bar{v}_2^0 + \xi_i\bar{v}_i), \tag{4}$$

where $i = 1$ or 3. If $(\partial\rho/\partial c_2)_\mu$, ρ^0, and the partial specific volumes are known, the interaction coefficients may be obtained (Cohen and Eisenberg, 1968). Alternative experimental procedures are scattering of light, x-rays, or neutrons, sometimes in constructive combinations (Bonneté et al., 1993; Eisenberg, 1981, 1994, 1999). A serious word of warning should be recalled. Reisler and Eisenberg (1969) concluded that a linear relationship between $(\partial\rho/\partial c_2)_\mu$ and ρ^0, which is often observed, does not mean (consider Eq. 4) that ξ_3 or ξ_1 are necessarily constant. These parameters may vary with w_3 (or ρ^0). However, if the invariant particle model (Tardieu et al., 1981) applies, then we can plot (Eisenberg, 1976; Reisler et al., 1977)

$$(\partial\rho/\partial c_2)_\mu = (1 - \rho^0\bar{v}_2^0) + B_1(1 - \rho^0\bar{v}_1) + B_3(1 - \rho^0\bar{v}_3) \tag{5}$$

$$= (1 + B_1 + B_3) - \rho^0(\bar{v}_2 + B_1\bar{v}_1 + B_3\bar{v}_3), \tag{6}$$

and both B_1 and B_3 can be obtained from the dependence of $(\partial\rho/\partial c_2)_\mu$ on ρ^0. Furthermore, if $B_3 = 0$, then ξ_1 is constant and equal to B_1, and both B_1 and $\bar{v}_2$ can be obtained from Eq. 6.

In studies of nucleosome core particles (Eisenberg and Felsenfeld, 1981; Greulich et al., 1985) different slopes were obtained in linear plots of $M_2(\partial\rho/\partial c_2)_\mu$ versus ρ^0, when small-size sugars (sucrose, raffinose, glycerol) were used in density contrast studies, as distinguished from large-size carbohydrates (dextran, γ-cyclodextrin). The result could be interpreted in terms of fractal probing. The small sugar molecules were able to penetrate into the inner spaces of the core particle and determine hydration, whereas the larger carbohydrates, which did not enter the core particle, only probed the outer surface of the core particle. The correct volume of the core particle could then be evaluated with the compact toroidal sugar octamer γ-cyclodextrin (Greulich et al., 1985), exceeding by far the volume of its nucleic acid and protein components, in good agreement with x-ray crystallographic dimensions (Richmond et al., 1984; Luger et al., 1997). The large, randomly configured dextran probe yielded excessive nucleosome core particle volumes. This may be compared with a study of Bhat and Timasheff (1992) on steric exclusions in proteins in the presence of large multiply-sized polyethylene glycols.

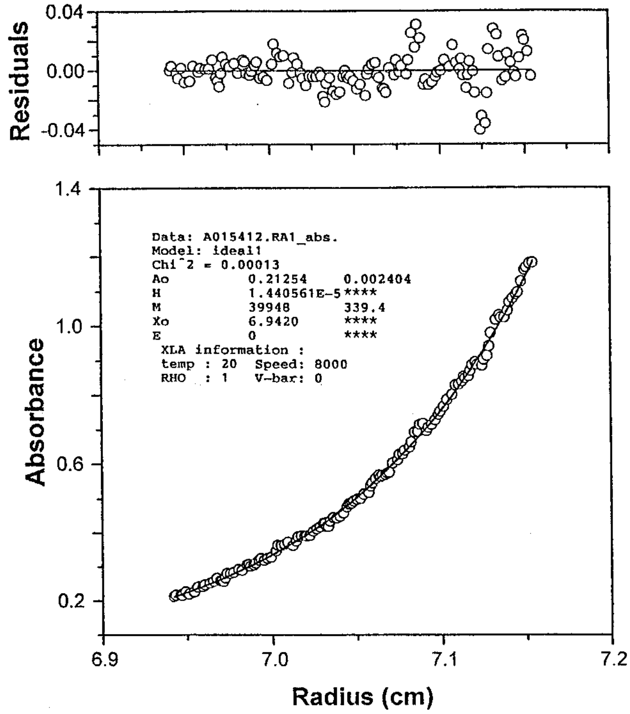
The hydration of DNA, an average of 5 ± 1 molecules of water per nucleotide, determined (Reisler et al., 1977) from density increment experiments with NaCl and CsCl (Cohen and Eisenberg, 1968), was found to be in good agreement with subsequent x-ray diffraction results (Kopka et al., 1983). This is a good example of correspondence between the operational concept of hydration derived from the thermodynamics-based model and x-ray crystallography. In the study of BSA hydration (cf. Eisenberg, 1994), B_1 does not change significantly (within the precision of this experiment) upon guanidinium chloride denaturation, an observation confirming hydration studies of Kuntz (1971). However, B_3 increases substantially, indicating denaturant binding ($\bar{v}_2$ of BSA decreases slightly at 25°C from 0.734 in 0.2 M NaCl to 0.728 in 6 M guanidinium chloride, and $\bar{v}_2$ of aldolase changes from 0.739 to 0.733 under similar denaturating circumstances (Reisler and Eisenberg, 1969)). Qu et al. (1998) have documented the preferred interactions of denaturant (urea) with the protein backbone, as contrasted to the side-chain interactions of protecting osmolytes.

RESULTS AND DISCUSSION

Aldolase is a tetramer of molar mass $M_2 = 156{,}841$ g/mol, the large-mass protein being used to allow ultracentrifugation at a relatively low velocity (8000 r.p.m.), at which concentration redistribution of sugars and pressure effects in general are not significant. The relatively large mass of the protein enhances the effects of volume with respect to surface area.

Partial specific volumes and molar mass

The partial specific volume $\bar{v}_2 = 0.749$ ml/g of the rabbit muscle aldolase tetramer was calculated from the sequence of the enzyme (Tolan et al., 1984) and the recent Kharakoz (1997) protein volume analysis. This does not include the correction due to ionization, which is expected (Kharakoz, 1997) to lower $\bar{v}_2$ by ~3% under close to isoelectric conditions. The $\bar{v}_2$ value calculated by the Voronoi procedure (Harpaz et al., 1994) yielded 0.747, which is quite close to the Kharakoz value. Reisler and Eisenberg (1969) experimentally determined 0.737 ml/g at 20°C and 0.739 ml/g at 25°C. An equilibrium sedimentation run of crystallized aldolase in 0.15 M NaCl is shown in Fig. 1. Using the sequence value $M_2 = 156{,}841$ g/mol, $\rho^0 = 1.00506$, and the average from three equilibrium sedimentation runs, we find $\bar{v}_2 = 0.738$ ml/g, which is quite close to the experimentally determined value. Experimental $\bar{v}_2$ values should thus be preferred to calculations to achieve higher precision in sensitive evaluations.



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M             39948        339.4
Xo            6.9420       ****
E             0            ****
 XLA information :
 temp : 20  Speed: 8000
 RHO  : 1   V-bar: 0
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FIGURE 1 Sedimentation equilibrium profile at 280 nm of aldolase in 0.15 M NaCl at 20.0°C and 8000 rpm ($\rho^0 = 1.00506$). The data have been modeled as a single ideal solute, and the residuals are displayed.

Sugar contrast variation experiments

Fig. 2 summarizes experimental results derived from equilibrium sedimentation at 20°C of aldolase dissolved in 0.15 M NaCl and various concentrations of the sugars glucose, sucrose, raffinose, and α- cyclodextrin, increasing in size in this order. All M_2 $(\partial\rho/\partial c_2)_\mu$ versus ρ^0 plots are linear. Using the sequence value for M_2 and Eq. 6 with the known experimental $\bar{v}_i$ values, B_3 was found to be essentially equal to zero, and therefore ξ_1 essentially equals B_1. B_1 and $\bar{v}_2$ were then calculated (cf. Table 1) from plots of M_2 $(\partial\rho/\partial c_2)_\mu$ versus ρ^0 (Fig. 2), using Eq. 6 with $B_3 = 0$, $\bar{v}_1 = 1.0018$, and the sequence aldolase mass 156,841 g/mol. The aldolase $\bar{v}_2$ values are in good agreement with the experimental value in the absence of sugar cosolvent, confirming the constancy of $\bar{v}_2$ with the sugar cosolvent concentration and the validity of the analysis. The B_1 values increase moderately with increasing sugar size, moving from glucose to sucrose, raffinose, and α-cyclodextrin. The determinations of B_1 for sucrose and raffinose by the density increment procedure (Fig. 3, Table 1) are in reasonable agreement with the ultracentrifuge results.

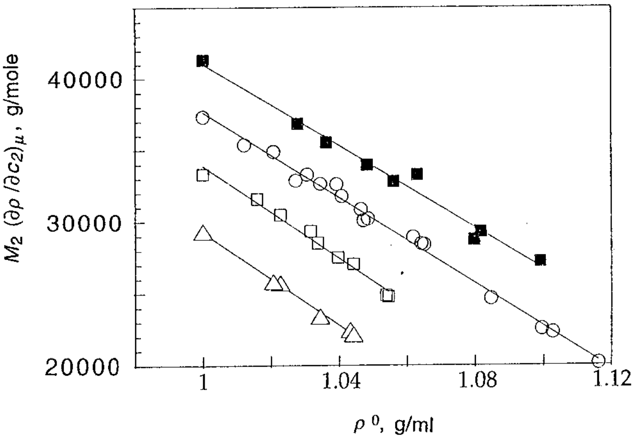

FIGURE 2 Buoyant molecular mass, $M_2(\partial\rho/\partial c_2)_\mu$, of aldolase versus solvent density ρ^0, in glucose (■), sucrose (○), raffinose (□), and α-cyclodextrin (△) solutions in 0.15 M NaCl at 20.0°C, by equilibrium sedimentation. The glucose scale is correct; the sucrose, raffinose, and cyclodextrin data have been shifted by successive decrements of 4000 g/mol for clarity of presentation.

Calculation of the excluded volume

It is possible to calculate the surface of a protein whose crystal structure is known by rolling spherical balls of radius r over the van der Waals surface of the protein (Richards, 1977). An excluded volume for the ball (or sugar) can then be calculated if the surface is multiplied by the radius of the ball. If this is the volume exclusion parameter by which B_1 could be defined, then it should be possible to calculate the variance of B_1 with variable sugar sizes by this calculation. Sugar radii were estimated from the molecular mass and partial specific volumes in spherical approximation. In the case of sucrose and raffinose, radii reflect the molecular asymmetry. In the calculation the area excluded, A_{ex}, increased smoothly for probe radii from zero to 15 Å ($A_{ex} = 0.0066 + 0.1323r + 0.0056r^2$; $R = 0.9999$), pointing to the absence of size-selective accessible clefts. It turns out, however, that the increase in B_1 calculated in this fashion

TABLE 1 Aldolase solvent interactions, in 0.15 M NaCl and sugar cosolvents, by sedimentation equilibrium and density increments measurements, at 20°C

			Rolling ball	
Cosolvent	$\bar{v}_2$ (ml/g)	B_1 (g/g)	r (Å)	B_1 (g/g)
Sedimentation equilibrium				
Water			~1.9	
Glucose	0.738	0.174 ± 0.050	~3.6	~0.56
Sucrose	0.733	0.215 ± 0.021	~5	~0.81
Raffinose	0.732	0.297 ± 0.050	~6	~1
α-Cyclodextrin	0.738	0.288 ± 0.034	~6	~1
No sugar	0.738			
Density increments				
Sucrose	0.742	0.213 ± 0.019		
Raffinose	0.740	0.315 ± 0.075		
Average	0.737			

The molar mass equals 156,841 from the amino acid sequence. Excluded volumes were calculated by the rolling ball procedures (cf. text).

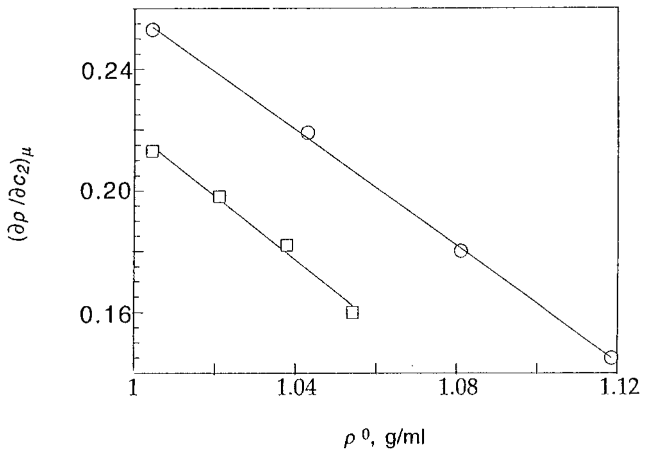

FIGURE 3 Density increments $(\partial\rho/\partial c_2)$ versus solvent density ρ^0 for aldolase in sucrose (○) and raffinose (□) solutions in 0.15 M NaCl at 20.0°C, by density measurements. The sucrose scale is correct; the raffinose data have been shifted by -0.04 units for clarity of presentation.

(Nicholls et al., 1991) considerably exceeds the B_1 values experimentally determined (Table 1) within the observed experimental errors. Thus, for rolling balls of radius 3.6–6 Å, corresponding to the sugars used in our experiments, B_1 calculated in this way increases from ~0.56 to 1.00 g/g in this range.

Temperature dependence of aldolase/raffinose partial specific volume and hydration

Equilibrium sedimentation runs were undertaken for aldolase, 0.15 M NaCl/raffinose solutions over a range of temperatures, achieving equilibrium from 25°C to 5°C in steps of 5°C and returning to 25°C at the end of the 1-week run, to check the stability of the system (Fig. 4). Using the experimental value $\bar{v}_2 = 0.739$ for 25°C, we calculated M_2 to be 160,230 g/mol in this experiment. With this value of M_2, which is slightly higher than the sequence value (156,841 g/mol), $\bar{v}_2$ and B_1 were calculated for each temperature by Eq. 5 with $B_3 = 0$; $\bar{v}_2$ decreases with temperature (at a rate of 4.5×10^{-4} d$\bar{v}_2$/°C) (Fig. 5), in line with an extensive series of data of Chalikian et al. (1996), and B_1 increases slightly (Fig. 5) with decreasing temperature (at a rate of 5×10^{-3} dB_1/°C); however, its absolute value was found, in this set of experiments, to be somewhat higher than the value previously reported (Table 1).

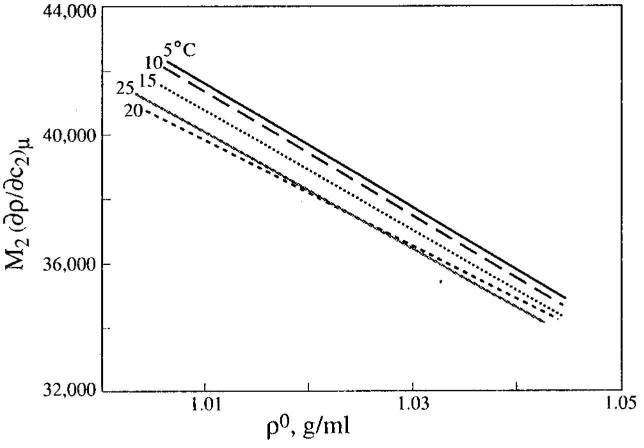

FIGURE 4 Density increments $(\partial\rho/\partial c_2)_\mu$ versus solvent density ρ^0 for aldolase in raffinose solutions in 0.15 M NaCl at 5–25°C, by equilibrium sedimentation.

The temperature dependence behavior of $\bar{v}_2$ we have observed is in agreement with earlier studies (Durchschlag, 1986); however, in recent studies (Xie and Timasheff, 1997a,b) no significant temperature dependence was reported for $\bar{v}_2$ of native and denatured RNase A in trehalose, sorbitol, magnesium chloride, and sulfate solutions between 4°C and 58°C.

Aldolase glycerol and aldolase trehalose contrast variation experiments

In contrast with the studies reported so far in this work we found that glycerol and trehalose (α-D-glucopyranosyl-α-glucopyranoside) did not yield straight lines in plots of $(\partial\rho/\partial c_2)_\mu$ versus ρ^0. In the case of glycerol (Fig. 6) $(\partial\rho/\partial c_2)_\mu$ decreases with increase in ρ^0 in a concave-up fashion, and in the case of trehalose (Fig. 6) it decreases in a concave-down fashion. Both of these cosolvents have been studied before for their biological interest—glycerol (Gekko and Timasheff, 1981; Lehmann and Zaccai, 1984) for its stabilizing role in the native structure of the proteins. A recent study (Priev et al., 1996) discusses the role of glycerol in decreasing the volume and compressibility of the protein interior and raises interesting problems relating to the interactions of sugars with proteins. Glycerol plays a major role in the survival of haloadaptive *Dunaliella* algae

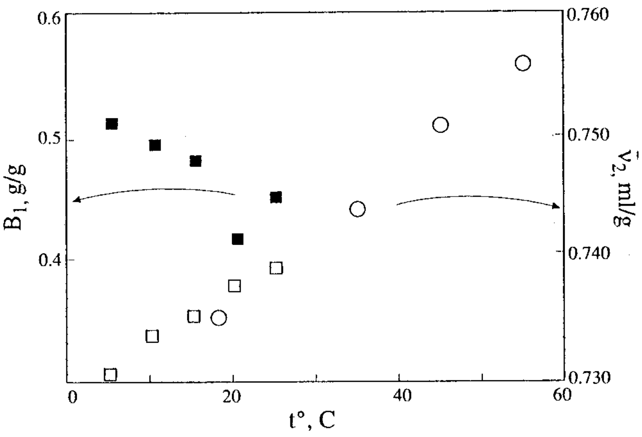

FIGURE 5 Partial specific volume $\bar{v}_2$ (□) and hydration parameter B_1 (■) of aldolase in raffinose solutions in 0.15 M NaCl versus temperature. The BSA (○) data are from Chalikian et al. (1993).

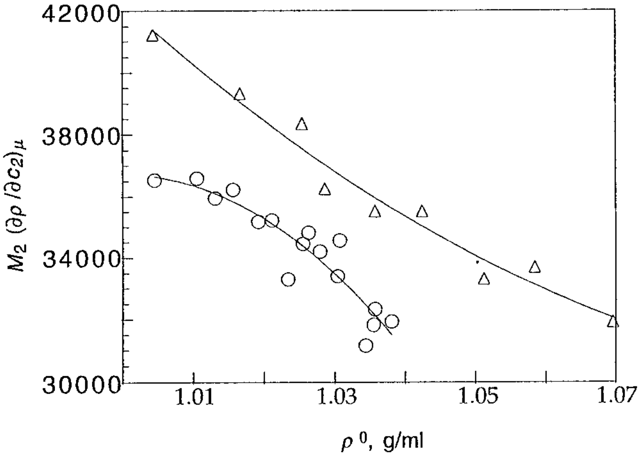

FIGURE 6 Buoyant molecular mass $M_2(\partial\rho/\partial c_2)_\mu$ versus solvent density ρ^0 for aldolase in glycerol (△) and trehalose (○) solutions in 0.15 M NaCl at 20.0°C, determined by equilibrium sedimentation. The trehalose data have been shifted by −4000 g/mol for clarity of presentation.

at high concentrations of salt (Ben Amotz and Avron, 1982; Avron and Ben Amotz, 1992) or in the ability of animals (frogs, for instance) to survive freezing (Storey and Storey, 1990). Trehalose, a nonreducing disaccharide of glucose, plays a role in the ability of organisms to survive stressful environmental conditions. It stabilizes biomaterials during air-drying or freeze-drying (Crowe et al., 1996); however, this may only be due to the ability of the sugar to form a glass. Halophilic phototropic bacteria that thrive in highly saline environments are able to synthesize both nitrogen-containing (betaine, ectoine) and nitrogen-free (trehalose) compatible solutes to retain cytoplasmic water (Galinski and Herzog, 1990). A thermodynamics-based mechanism of protein stabilization by trehalose has recently been presented (Xie and Timasheff, 1997a).

Priev et al. (1996) claim a decrease in $\bar{v}_2$ on the order of ~8% at a glycerol concentration of 30% (v/v) in an extrapolation to large-size proteins, where the effect of the interior outweighs that of the surface. Gekko and Timasheff (1981) report an order of magnitude lower decrease in $\bar{v}_2$ with an increase in glycerol concentration. The strong decrease in $\bar{v}_2$ postulated by Priev et al. (1996) raises uncertainties. For both glycerol and trehalose the stage has been set, yet valid conclusions have not been reached. The extensive data and interpretations presented by Xie and Timasheff (1997a) have not enabled us to achieve convincing, mutually supportive conclusions. Leaving the safe territory of the invariant particle concept is a dangerous pathway in the case where the density dependence of $(\partial\rho/\partial c_2)_\mu$, as observed by us, is not linear.

CONCLUSIONS

B_1 is a hydration parameter. The linear dependence of $(\partial\rho/\partial c_2)_\mu$ versus ρ^0 for aldolase in solutions of the component 3 cosolvents glucose, sucrose, raffinose, and α-cyclodextrin, leading in the analysis by Eqs. 4 and 6 to $B_3 = 0$ and $\xi_1 = B_1$, is summarized in Table 1. B_1 increases with increasing cosolvent size, however, at considerably lower values and rate than predicted by the calculation of rolling balls, the size of the cosolvent molecules, over the enzyme surface, determined by x-ray diffraction. B_1 in this case most likely represents hydration, weakly dependent on the native or denatured state of the protein. Specific changes in water binding occur in the process of protein folding into the native structure; however, the major part of the hydration is determined by summation of contributions due to amino acid residue components (Kuntz, 1971). The protein macromolecular complex is apparently not covered, as assumed in the analysis of hydrodynamic phenomena modeled by increased radii of ellipsoidal bodies (Tanford, 1961), by a uniform layer of hydration specific to the native structure. Water of hydration is apparently better represented by surface and interior patches relating to the protein sequence and structure. The increasing values of B_1 with increasing sugar size indicate moderate excluded volume effect contributions; however, the limits in the accuracy and resolution of the density contrast variation method do not allow the elaboration of more precise statements. Negligible B_3 values indicate uniform w_3 concentration in the domain surrounding the protein (except for spaces covered by B_1 hydration) equal to w_3 in the equilibrium dialysis solution not containing component 2. An increase in protein stabilization or destabilization may still be due to the effect of the sugar on surface tension or solvent activity. Sucrose increases water surface tension, whereas glycerol decreases it (Timasheff, 1992, 1998).

We also observed (Fig. 5) that, confirming most previous reports, $\bar{v}_2$ decreases with decreasing temperature, which is likely due to increasing electrostriction following increasing B_1 values with decreasing temperature. In the temperature range from 25° to 0°C, B_1 increases by 0.125 g/g and $\bar{v}_2$ decreases by 0.0113 ml/g, corresponding to a 9% volume decrease due to electrostriction, similar to the value of 10% found by Svergun et al. (1998). Equilibrium sedimentation yields reasonable values of M_2 and $\bar{v}_2$; however, the values of B_1 are subject to larger uncertainties, particularly in systems involving sugars. The interaction of glycerol and of trehalose with aldolase is more complex and merits further investigation.

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