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Interactions of Glycine Betaine with Proteins: Insights from Volume and Compressibility Measurements

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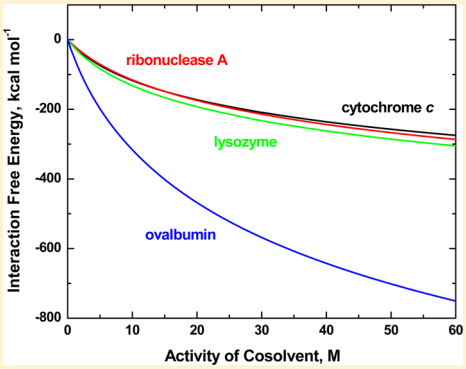
Supporting Information

ABSTRACT: We report the first application of volume and compressibility measurements to characterization of interactions between cosolvents (osmolytes) and globular proteins. Specifically, we measure the partial molar volumes and adiabatic compressibilities of cytochrome *c*, ribonuclease A, lysozyme, and ovalbumin in aqueous solutions of the stabilizing osmolyte glycine betaine (GB) at concentrations between 0 and 4 M. The fact that globular proteins do not undergo any conformational transitions in the presence of GB provides an opportunity to study the interactions of GB with proteins in their native states within the entire range of experimentally accessible GB concentrations. We analyze our resulting volumetric data within the framework of a statistical thermodynamic model in which each instance of GB interaction with a protein is viewed as a binding reaction that is accompanied by release of four water molecules. From this analysis, we calculate the association constants, *k*, as well as changes in volume, ΔV_0, and adiabatic compressibility, ΔK_{S0}, accompanying each GB−protein association event in an ideal solution. By comparing these parameters with similar characteristics determined for low-molecular weight analogues of proteins, we conclude that there are no significant cooperative effects involved in interactions of GB with any of the proteins studied in this work. We also evaluate the free energies of direct GB−protein interactions. The energetic properties of GB−protein association appear to scale with the size of the protein. For all proteins, the highly favorable change in free energy associated with direct protein−cosolvent interactions is nearly compensated by an unfavorable free energy of cavity formation (excluded volume effect), yielding a modestly unfavorable free energy for the transfer of a protein from water to a GB/water mixture.



Water-soluble cosolvents may exert a profound influence on protein stability by selectively shifting the conformational equilibrium between the protein species toward the native or denatured state.[1−4] In many instances, cosolvent-induced modulation of protein stability is biologically relevant by facilitating survival of cells under extreme conditions.[5−8] The mode of action of water-soluble cosolvents on the stability and biological activity of proteins remains a much debated topic in biophysical chemistry, although new insights are increasingly emerging from a combination of experimental and theoretical studies.[1,3,4,9−15] In one approach, the net free energy of interactions between a cosolvent and a protein is analyzed as the sum of the contribution arising from direct cosolvent−protein interactions and the contribution reflecting the differential free energy of cavity formation in pure water and a water/colsolvent mixture (excluded volume effect).[9,16] While the differential effect of cavity formation can be evaluated, in principle, within the framework of scaled particle theory (SPT) or some other more rigorous computational approach, it is a challenge to detect and quantify thermodynamically direct protein−cosolvent interactions. To address this deficiency, we have begun a program in which volumetric measurements are systematically employed in conjunction with a statistical thermodynamic algorithm to characterize interactions of various cosolvents with proteins and individual protein groups.[17−20]

In a recent paper, we have used volumetric measurements to study the interactions of the stabilizing osmolyte glycine betaine (GB) with low-molecular weight compound modeling proteins.[20] We have determined the association constants, *k*, and changes in volume, ΔV_0, and adiabatic compressibility, ΔK_{S0}, accompanying the binding of GB to various atomic groups in low-molecular weight model compounds.[20] In the work presented here, we extend the volumetric approach to characterization of the interactions of GB with globular proteins. The fact that globular proteins do not undergo any conformational transitions in the presence of GB provides one with the opportunity to study the interactions of GB with proteins in their native states within the entire range of experimentally accessible GB concentrations.

One objective of this work is to understand if there are sizable cooperative (nonadditive) effects involved in the interactions of GB with globular proteins that are absent in

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small molecules. This is a question of both fundamental and practical importance. The practical implications stem from the fact that low-molecular weight compound data have been used extensively to develop additive models for evaluating the stabilizing and destabilizing effects of cosolvents on the conformational stability of proteins.[14,21−23]

To assess the cooperativity of interactions of GB with proteins, we measure the partial molar volumes, $V°$, and adiabatic compressibilities, $K°_S$, of four typical globular proteins differing in size and structural properties at GB concentrations ranging from 0 to 4 M. Specifically, the proteins are cytochrome *c* (12.4 kDa), ribonuclease A (13.6 kDa), hen egg lysozyme (14.3 kDa), and ovalbumin (46.0 kDa). We analyze the resulting volumetric data within the framework of a statistical thermodynamic formalism and determine the average values of k, ΔV_0, and ΔK_{S0}. Comparison of these data with similar data previously obtained for small molecules allows one to judge the presence of nonadditive effects in GB−protein interactions. Further, we use our association constants, k, to evaluate the free energy contributions of direct GB−protein interactions. These results provide insights into the balance of forces governing the mode of action of individual cosolvents.

MATERIALS AND METHODS

Materials. GB and the proteins hen egg lysozyme, ribonuclease A from bovine pancreas, ferricytochrome *c* from horse heart, and ovalbumin from chicken egg white were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, ON). All the reagents were of the highest purity commercially available. GB was used without further purification. To eliminate salts and any other low-molecular weight compounds that may be present in the samples, the proteins were exhaustively dialyzed against distilled water and lyophilized.

Solution Preparation. Aqueous solutions of GB with concentrations between 0 and 4 M were prepared by weighing 10−50 g of GB and adding pre-estimated amounts of water to achieve the desired molalities, m. The molar concentrations, C, of a GB solution were determined from the molal values, m, using the relationship $C = [1/(m\rho_W) + \phi V/1000]^{-1}$, where ρ_W is the density of water and ϕV is the apparent molar volume of GB.

GB solutions were used as solvents for the proteins. Protein solutions were prepared by dissolving 5−10 mg of a solute material in a known amount of solvent (GB solution).

Spectroscopic Measurements. The concentrations of the proteins at each experimental GB concentration were determined spectrophotometrically at 25 °C with a Cary 300 Bio spectrophotometer (Varian Canada, Inc., Mississauga, ON). The extinction coefficients for the proteins were determined individually for each GB concentration by dry-weight analysis. Table S1 of the Supporting Information lists our determined extinction coefficients for cytochrome *c*, ribonuclease A, lysozyme, and ovalbumin at 0, 1, 2, 3, and 4 M GB.

Near- and far-UV circular dichroism (CD) spectra of the proteins in the presence and absence of GB were recorded at 25 °C in a 1 mm path length cuvette using an Aviv model 62 DS spectropolarimeter (Aviv Associates, Lakewood, NJ). The proteins' concentrations were ∼0.25 and ∼1 mg/mL for the far- and near-UV CD measurements, respectively.

Determination of the Partial Molar Volumes and Adiabatic Compressibilities of the Proteins. Solution densities were measured at 25 °C with a precision of $\pm 1.5 \times 10^{-4}$ % using a vibrating tube densimeter (DMA-5000, Anton Paar, Gratz, Austria). The apparent molar volumes, ϕV, of the proteins were evaluated from the relationship

$$\phi V = M/\rho_0 - (\rho - \rho_0)/(\rho_0 C) \quad (1)$$

where M is the molecular weight of the solute, C is the molar concentration of the solute, and ρ and ρ_0 are the densities of the solution and the solvent (water or a GB solution), respectively.

Solution sound velocities, U, were measured at 25 °C at a frequency of 7.2 MHz using the resonator method and a previously described differential technique.[24−27] The analysis of the frequency characteristics of the ultrasonic resonator cells required for sound velocity measurements was conducted with a Hewlett-Packard (Mississauga, ON) model E5100A network/spectrum analyzer. For the type of ultrasonic resonators used in this work, the precision of sound velocity measurements is on the order of $\pm 1 \times 10^{-4}$ %.[25,28,29] The key acoustic characteristic of a solute that can be derived directly from ultrasonic measurements is the relative molar sound velocity increment: $[U] = (U - U_0)/(U_0 C)$, where C is the molar concentration of a solute and U and U_0 are the sound velocities in the solution and the solvent, respectively.

The values of $[U]$ were combined with ϕV to calculate the apparent molar adiabatic compressibility, ϕK_S:[30,31]

$$\phi K_S = \beta_{S0}(2\phi V - 2[U] - M/\rho_0) \quad (2)$$

where β_{S0} $(=\rho_0^{-1}U_0^{-2})$ is the coefficient of adiabatic compressibility of the solvent. The values of ρ_0, U_0, and β_{S0} for GB solutions have been measured and reported previously.[20] For each evaluation of ϕV or ϕK_S, three to five independent measurements were taken within a protein concentration range of 1−2 mg/mL. Our reported values of ϕV and ϕK_S represent the averages of these measurements, while the errors were calculated as standard deviations.

Given the small protein concentrations used in our study and the weak concentration dependencies of the apparent molar volumes and adiabatic compressibilities of proteins,[32,33] we do not discriminate below between the apparent and partial molar volumetric characteristics of the proteins.

RESULTS

CD Measurements. To ensure that, in the presence of GB, the proteins do not undergo any structural alterations, we recorded the near-UV (250−330 nm) and far-UV (200−250 nm) CD spectra of the proteins in the presence and absence of GB (data not shown). No significant GB-induced changes in the CD spectra of any of the proteins were observed. We conclude that the proteins retain their native structures over the entire range of GB concentrations (0−4 M) used in this work. Consequently, we assume that our measured GB-dependent changes in the volumetric properties of the proteins can be ascribed predominantly to changes in their solvation.

Volumetric Measurements. Tables 1 and 2 list the partial molar volumes, $V°$, and partial molar adiabatic compressibilities, $K°_S$, respectively, of the proteins at various GB concentrations. The relative molar sound velocity increments, $[U]$, are included in Table S2 of the Supporting Information. Figures 1 and 2 graphically illustrate the data listed in Tables 1 and 2, respectively. Figure 1 presents the partial molar volumes of cytochrome *c* (panel a), ribonuclease A (panel b), lysozyme (panel c), and ovalbumin (panel d) plotted versus the activity

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Table 1. Partial Molar Volumes [$V°$ (cm^3 mol^{-1})] of the Proteins at Various GB Concentrations

[GB] (M)	cytochrome *c*	ribonuclease A	lysozyme	ovalbumin
0	9080 ± 10	9620 ± 10	10171 ± 20	33390 ± 50
0.5	9100 ± 20	9640 ± 10	10180 ± 20	33440 ± 20
1.0	9110 ± 10	9670 ± 20	10200 ± 20	33500 ± 20
1.5	9130 ± 20	9680 ± 20	10210 ± 10	33550 ± 20
2.0	9130 ± 10	9690 ± 10	10230 ± 10	33580 ± 20
2.5	9150 ± 30	9700 ± 20	10240 ± 20	33640 ± 50
3.0	9160 ± 10	9710 ± 20	10250 ± 20	33670 ± 10
3.5	9160 ± 10	9720 ± 10	10250 ± 40	33690 ± 40
4.0	9170 ± 10	9720 ± 10	10250 ± 40	33710 ± 10

Table 2. Partial Molar Adiabatic Compressibilities [$K_S°$ (10^{-4} cm^3 mol^{-1} bar^{-1})] of the Proteins at Various GB Concentrations

[GB] (M)	cytochrome *c*	ribonuclease A	lysozyme	ovalbumin
0	260 ± 20	20 ± 20	400 ± 30	2790 ± 80
0.5	360 ± 30	100 ± 20	500 ± 30	3000 ± 70
1.0	440 ± 10	230 ± 60	620 ± 50	3230 ± 50
1.5	520 ± 30	340 ± 20	720 ± 10	3510 ± 60
2.0	580 ± 40	440 ± 10	800 ± 50	3720 ± 40
2.5	664 ± 40	550 ± 80	900 ± 50	3930 ± 80
3.0	730 ± 10	590 ± 40	970 ± 40	4120 ± 40
3.5	770 ± 50	630 ± 60	1000 ± 50	4210 ± 70
4.0	800 ± 40	650 ± 50	1030 ± 60	4240 ± 40

of GB. The activities of GB, a_3, for the concentration range of 0–4 M were taken from the literature.[10] Inspection of Figure 1 reveals that, for all proteins studied in this work, the partial molar volumes increase hyperbolically with an increase in GB activity and level off at ~20 M.

Figure 2 presents the GB dependencies of the partial molar adiabatic compressibilities of cytochrome *c* (panel a), ribonuclease A (panel b), lysozyme (panel c), and ovalbumin (panel d). Note that, analogous to volume, the partial molar adiabatic compressibilities of the proteins increase hyperbolically with an increase in the GB activity and level off at ~20 M.

Analysis of Partial Molar Volume Data. We analyzed the experimental data in Figures 1 and 2 within the framework of a statistical thermodynamic model in which the association of a cosolvent with a hydrated solute is accompanied by release of *r* water molecules from the hydration shell of the solute to the bulk.[17,18,20] Four water molecules are released to the bulk per GB–protein association event ($r = 4$).[20] If a solute has *n* binding sites for water, n/r is the maximal number of cosolvent binding sites. Assuming n/r identical and independent cosolvent binding sites, a change in volume associated with the transfer of a solute from water to a concentrated cosolvent solution has been expressed via the relationship[18]

$$\Delta V° = \Delta V_C - \gamma_1 n \Delta V°_1 + \Delta V(n/r)(a_3/a_1^r)k / [1 + (a_3/a_1^r)k] \quad (3)$$

where a_1 and a_3 are the mole fraction activity of water and the molar activity of GB, respectively, ΔV_C is the differential volume of the cavity enclosing a solute in a concentrated cosolvent solution and water (V_C in a cosolvent solution minus V_C in water), k is the effective equilibrium constant for the reaction in which a cosolvent molecule replaces *r* water molecules by binding to each of the n/r binding sites (SW_r + C ↔ SC + rW, where S, C, and W stand for the binding site, cosolvent, and water, respectively), $\Delta V = \Delta V_0 + \gamma_1 r \Delta V°_1 - \gamma_3 \Delta V°_3$ is the change in volume associated with replacement of water with cosolvent normalized per binding site in a concentrated cosolvent solution, ΔV_0 is the solvent exchange volume in an ideal solution, $\Delta V°_1$ and $\Delta V°_3$ are the excess partial molar volumes of water and cosolvent in a concentrated solution, respectively, and γ_1 and γ_3 are the correction factors reflecting the influence of the bulk solvent on the properties of the solvating water and cosolvent, respectively.

Activities a_1 and a_3 have been determined as a function of GB concentration by Felitsky and Record.[10] The values of γ_1 and γ_3 may change from 0 (the properties of the solvation shell change in parallel with those of the bulk) to 1 (the properties of the solvation shell are independent of those of the bulk).

We used eq 3 to approximate the experimental data shown in Figure 1. We have reported the excess partial molar volume of water, $\Delta V°_1$, as a function of GB concentration.[20] The value of *n* was calculated for each protein as the ratio of its solvent accessible surface area, S_A, to 9 $Å^2$, the effective cross section of a water molecule.

Correction factors γ_1 and γ_3 in eq 3 can be evaluated for each protein on the basis of the following considerations.[17,18] Water molecules solvating charged atomic groups interact with the latter via strong charge–dipole interactions, thereby becoming highly compressed and partially immobilized. Given the small size of water molecules (and, hence, essentially localized interactions), waters influenced by charged groups should be relatively insensitive to the properties of water in the bulk. Thus, we have proposed that, for charged groups, γ_1 can be approximated by 1.[17,18] On a similar note, at low to moderate temperatures, waters solvating nonpolar groups become highly oriented in an attempt to maximize their mutual hydrogen bonds within a restricted configurational space. It is, therefore, reasonable to assume that the structural and thermodynamic properties of such waters should also be relatively insensitive to changes in the properties of bulk water. Thus, we have proposed that nonpolar groups are also characterized by a γ_1 close to 1. In contrast, waters hydrating polar (but uncharged) groups form continuous networks of hydrogen bonds extending from solute to water in the bulk and, therefore, should be significantly influenced by the latter. Consequently, we posit that, for polar groups, $\gamma_1 \approx 0$.[17,18]

GB, like most water-soluble organic cosolvents, is bulkier than water and can form numerous hydrogen bonding and electrostatic interactions with its neighboring solvent and cosolvent molecules. Consequently, despite its being engaged in solute–solvent interactions, GB can still develop numerous interactions with solvent in the bulk, thereby being influenced significantly by the latter. We, therefore, use in our analysis an approximation of $\gamma_3 \approx 0$.[17,18]

On the basis of the discussion presented above, correction factor γ_1 is roughly equal to the fraction of nonpolar and charged groups in the solvent accessible surface area of a solute $[(S_n + S_C)/S_A]$, while γ_3 is effectively 0. The solvent accessible surface area of nonpolar, S_n, and charged, S_C, groups and the total solvent accessible surface area, S_A, were taken from a previous work.[34]

Our SPT-based computations has revealed that, for low-molecular weight compounds (with hard sphere diameters on the order of or less than 10 Å), the cavity contribution, ΔV_C, is small and does not exceed ~4 cm^3 mol^{-1} at 4 M GB.[19] However, for solutes as large as proteins, ΔV_C in eq 3 is not

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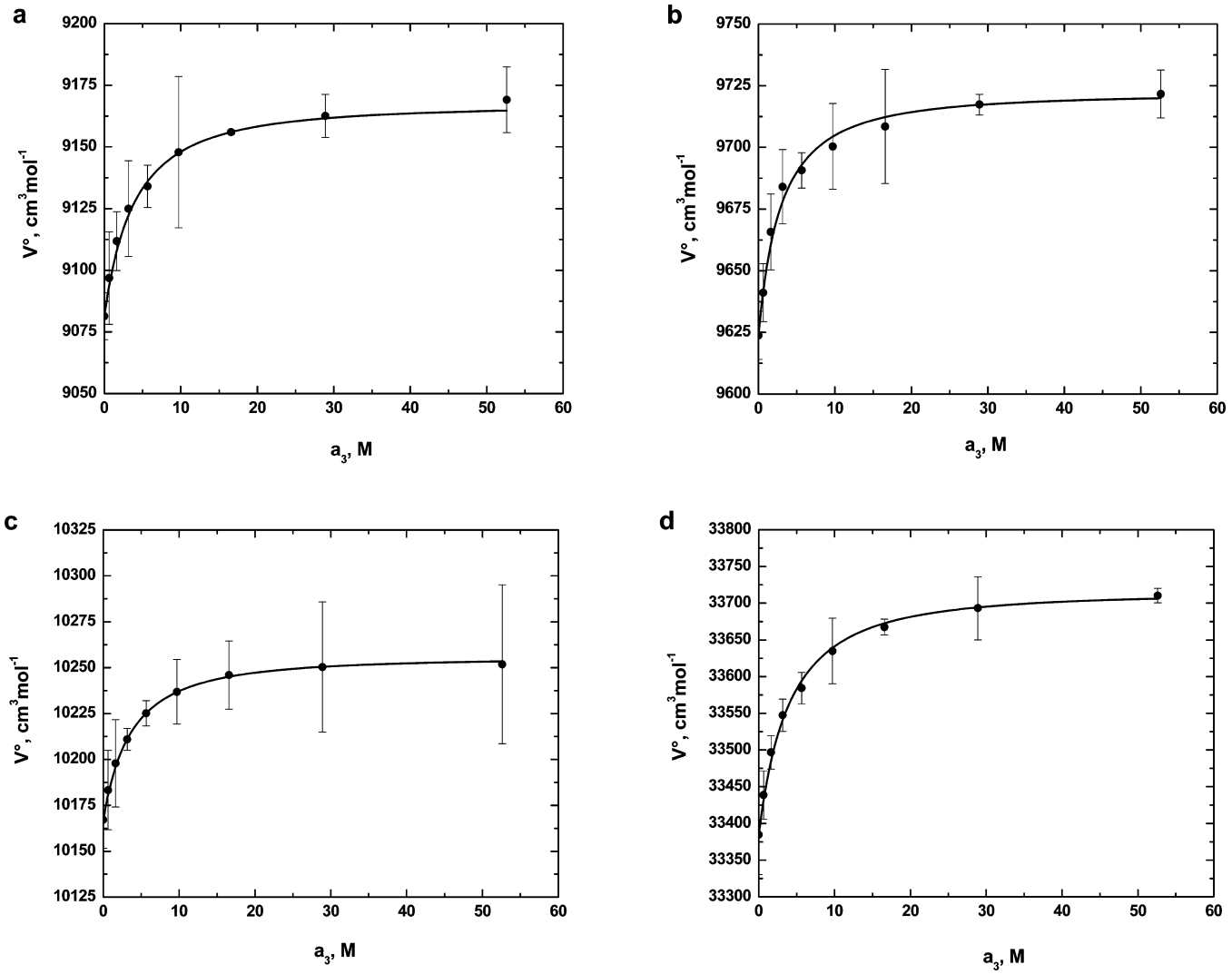

Figure 1. Partial molar volumes of cytochrome *c* (a), ribonuclease A (b), lysozyme (c), and ovalbumin (d) as a function of GB activity.

negligible. In principle, the values of ΔV_C can be calculated as a function of GB concentration using SPT.[35,36] Our computations (data not shown) suggest that, for a solute with a diameter of 30 Å (which corresponds to a protein of the size of lysozyme), the dependence of ΔV_C on the concentration of GB is nearly parabolic; it decreases with an increase in GB concentration from 0 to ~1.2 M, reaching a minimum of −8 $cm^3\ mol^{-1}$, and then increases upon a further increase in GB concentration, passing zero at ~2.4 M and reaching a value of 35 $cm^3\ mol^{-1}$ at 4 M GB. It should be noted, however, that the results of SPT-based calculations critically depend on the choice of the hard sphere diameters of the primary solvent and cosolvent molecules as well as on the precise values of their concentrations.[37] In general, the problem of approximation of a complex molecular shape by a hard sphere does not have any universal solution, with different approaches yielding significantly different estimates for the effective hard sphere diameters of solvent and cosolvent molecules.[37] Ambiguities exist not only with respect to solutes and water-miscible cosolvents, such as glycine betaine, but also with respect to the size of the water molecule itself.[38] The impact of these effects on the validity of SPT-based calculations grows as the size of a solute increases, becoming very significant for solutes approaching the size of proteins.

There is one more consideration that may limit the usefulness of SPT-based calculations for accurate volumetric analyses. SPT assumes a similar distribution of solvent and cosolvent molecules in the vicinity of the solute and in the bulk. However, depending on the nature of the cosolvent, the microenvironment of a solute, as reflected in the distribution of solvent and cosolvent molecules in its vicinity, may significantly differ from that in the bulk. The disparity between the local and bulk solvent properties may cause an additional deviation in the SPT-calculated characteristics of a solute.

Considering these complexities and the errors associated with them, we do not attempt to evaluate the ΔV_C term in eq 3 Instead, we fit the experimental data shown in Figure 1 to eq 3 without explicitly taking into account the ΔV_C term. In such a treatment, the cosolvent-induced change in the cavity volume, ΔV_C, will appear as an added contribution to the values of ΔV_0 determined from the fit.

Analysis of Partial Molar Compressibility Data. A change in isothermal compressibility accompanying the water-to-cosolvent transfer of a solute is described by the relationship[18]

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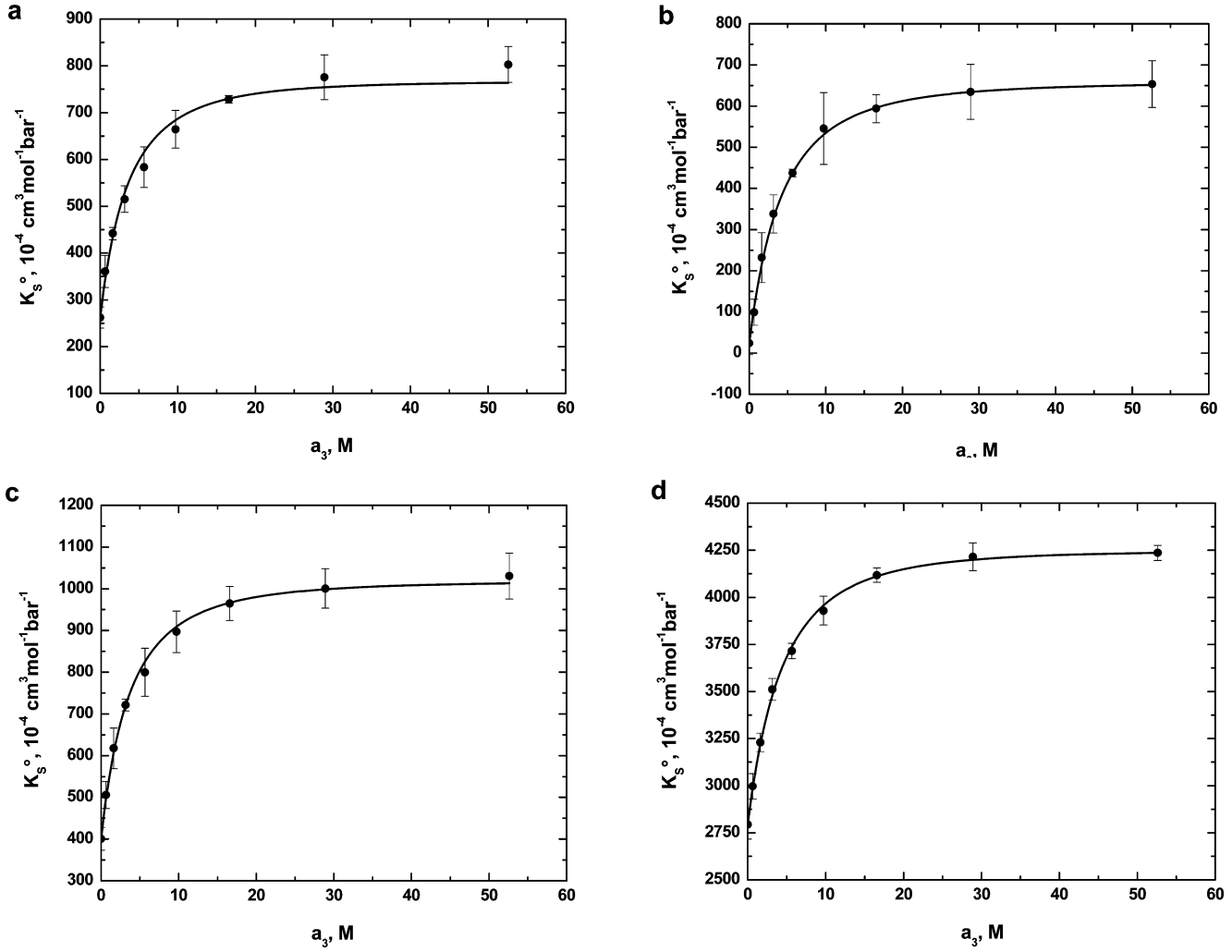

Figure 2. Partial molar adiabatic compressibilities of cytochrome *c* (a), ribonuclease A (b), lysozyme (c), and ovalbumin (d) as a function of GB activity.

Table 3. Protein Solvent Accessible Surface Areas [S_A (Å^2)], Correction γ_1, Changes in Volume [ΔV_0 (cm^3 mol^{-1})], and Adiabatic Compressibility [ΔK_{S0} (10^{-4} cm^3 mol^{-1} bar^{-1})] for GB−Protein Association in an Ideal Solution and Equilibrium Constants Determined from Volume [k(vol) (M^{-1})] and Compressibility [k(comp) (M^{-1})] Data

protein	S_A	γ_1	ΔV_0	ΔK_{S0}	k(vol)	k(comp)
cytochrome *c*	6115	0.75	0.509 ± 0.007	2.97 ± 0.04	0.26 ± 0.02	0.24 ± 0.02
ribonuclease A	6790	0.63	0.523 ± 0.006	3.37 ± 0.04	0.35 ± 0.03	0.20 ± 0.01
lysozyme	6685	0.62	0.481 ± 0.006	3.34 ± 0.04	0.28 ± 0.01	0.24 ± 0.01
ovalbumin	16938	0.69	0.709 ± 0.008	3.10 ± 0.02	0.25 ± 0.02	0.19 ± 0.01

$$\Delta K°_T = \Delta K_{TC} - \gamma_1 n \Delta K°_{T1} + \Delta K_T (n/r)(a_3/a_1{}^r)k / [1 + (a_3/a_1{}^r)k] + \Delta V^2 (n/r)(a_3/a_1{}^r)k / RT[1 + (a_3/a_1{}^r)k]^2 \quad (4)$$

where $\Delta K_{TC} = -(\partial \Delta V_C/\partial P)_T$ is the differential compressibility of the cavity enclosing a solute in water and a concentrated cosolvent solution, $\Delta K_T = \Delta K_{T0} + \gamma_1 r \Delta K°_{T1} - \gamma_3 \Delta K°_{T3}$ is the change in compressibility associated with replacement of water with cosolvent normalized per binding site in a concentrated cosolvent solution, $\Delta K°_{T1}$ and $\Delta K°_{T3}$ are the excess partial molar isothermal compressibilities of water and cosolvent in a concentrated cosolvent solution, respectively, and $\Delta K_{T0} = -(\partial \Delta V_0/\partial P)_T$ is the change in compressibility associated with solvent replacement in an ideal solution.

Given the large heat capacity and small expansibility of water-based solutions, the difference between the partial molar adiabatic and isothermal compressibilities of a solute is small and, therefore, can be ignored. Thus, we use eq 4 to fit the experimental data shown in Figure 2.[39] Consequently, instead of the excess partial molar isothermal compressibility of water, $\Delta K°_{T1}$, we use, in eq 4, the excess partial molar adiabatic compressibility, $\Delta K°_{S1}$, that has been measured as a function of GB concentration.[20]

As with volume, when analyzing our compressibility results, we ignore the differential cavity contribution ΔK_{TC} in eq 4. It should be emphasized that, in semiempirical analyses, the cavity contribution, K_{TC} or K_{SC}, is approximated reasonably well by

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the intrinsic compressibility, K_M, of a solute.[40−43] As discussed above, our CD spectral measurements did not reveal any significant GB-induced structural changes for the four proteins studied in this work. By extension, their intrinsic compressibilities should not be affected significantly by GB.

DISCUSSION

Properties of GB−Protein Association. Table 3 presents the values of ΔV_0 and ΔK_{S0} and the association constants, k, obtained from fitting our volume (Figure 1) and compressibility (Figure 2) data to eqs 3 and 4, respectively. Inspection of data in Table 3 reveals that, with the exception of ribonuclease A, there is good agreement between the equilibrium constants, k, derived from the volume and compressibility data. The disparity between the volume- and compressibility-based evaluations of the affinity constant, k, for ribonuclease A may reflect the large relative error of its individual partial molar volume data (see Figure 1c). In addition, the effect of unaccounted cavity volume, ΔV_C, on the value of k for ribonuclease A may be larger than that for the other proteins because of some structural and surface features of the former. In fact, because the relative error of the volume experimental points is generally larger than that of the compressibility points (see Figures 1 and 2), we use below the values of k obtained from our compressibility data.

It is instructive to compare the properties listed in Table 3, which were determined for proteins, with those that can be additively calculated on the basis of the data for small molecules modeling proteins.[20] Our previously determined equilibrium constants, k, for the association of GB with various amino acid side chains in *N*-acetyl amino acid amides and the glycyl unit (-CH_2CONH-) in oligoglycines range between 0.02 M^{-1} (for the alanine side chain) and 0.32 M^{-1} (for the phenylalanine side chain) with no pronounced correlation with the type of the constituent atomic groups.[20] One can expect that, in the absence of any significant cooperativity, the effective equilibrium constant for the GB−protein interactions should be on the order of 0.17 ± 0.09 M^{-1}, the average value of k evaluated for small protein analogues.[20] It should be noted that the affinity of GB for individual atomic groups does not generally follow their polar versus nonpolar pattern but is rather consistent with the scheme reflecting interactions between donors and acceptors of hydrogen bonding, cation−π-electron attraction, and other short-range interactions.[11,23] The most unfavorable interactions are those between GB, lacking hydrogen bond donors, and amide and carboxylate oxygens, while the most favorable interactions are formed between GB and aromatic carbons and amide and cationic nitrogens.[23]

There is a large difference between the volumetric changes (ΔV_0 and ΔK_{S0}) accompanying the binding of GB to polar and nonpolar atomic groups in low-molecular weight model compounds.[20] The values of both ΔV_0 and ΔK_{S0} are smaller for nonpolar side chains than for their polar counterparts.[20] In fact, the values of ΔV_0 are negative for nonpolar groups and positive for polar ones.[20] The average values of ΔV_0 are equal to −0.72 ± 0.25 and 1.13 ± 0.53 cm^3 mol^{-1} for nonpolar and polar (and charged) moieties, respectively, while the average values of ΔK_{S0} for nonpolar and polar (and charged) groups are equal to (1.82 ± 0.71) × 10^{-4} and (4.37 ± 1.80) × 10^{-4} cm^3 mol^{-1} bar^{-1}, respectively.[20] The estimates of ΔV_0 and ΔK_{S0} for a protein with no cooperativity in its interactions with GB can be made on the basis of the relationships $\Delta V_0 = \alpha_n \Delta V_{np} + (1 - \alpha_n)\Delta V_p$ and $\Delta K_{S0} = \alpha_n \Delta K_{Snp} + (1 - \alpha_n)\Delta K_{Sp}$, respectively, where ΔV_{np} and ΔV_p are the average ΔV_0 contributions of nonpolar and polar groups, respectively, ΔK_{Snp} and ΔK_{Sp} are the average ΔK_{S0} contributions of nonpolar and polar groups, respectively, and α_n is the nonpolar fraction of the solvent accessible surface area of a protein. Table 4 presents the small molecule-based estimates of ΔV_0 and ΔK_{S0} for the four proteins studied in this work.

Table 4. Changes in Volume [ΔV_0 (cm^3 mol^{-1})] and Adiabatic Compressibility [ΔK_{S0} (10^{-4} cm^3 mol^{-1} bar^{-1})] for GB−Protein Association in an Ideal Solution Estimated on the Basis of Low-Molecular Weight Model Compound Data

protein	ΔV_0(est.)	ΔK_{S0}(est.)
cytochrome *c*	0.128	3.01
ribonuclease A	0.228	2.95
lysozyme	0.282	3.06
ovalbumin	0.231	3.17

Inspection of data in Tables 3 and 4 reveals a number of important observations. First, the experimentally evaluated association constants, k, range compactly between 0.19 and 0.24 M^{-1} and are in good agreement with the estimate of 0.17 ± 0.09 M^{-1} made on the basis of small molecule data. The observed similarity between the measured and predicted association constants suggests that the interactions of GB with native globular proteins do not involve any significant cooperativity. Second, the measured changes in compressibility, ΔK_{S0}, accompanying the binding of GB to proteins, ranging from 2.97 × 10^{-4} to 3.37 × 10^{-4} cm^3 mol^{-1} bar^{-1}, agree well with the estimated values of 2.95 × 10^{-4} to 3.17 × 10^{-4} cm^3 mol^{-1} bar^{-1}. This observation is also consistent with limited or no cooperativity in GB−protein interactions. On the other hand, the measured changes in volume, ΔV_0, ranging between 0.481 and 0.709 cm^3 mol^{-1}, are significantly larger than the predicted values of 0.128−0.282 cm^3 mol^{-1}. The discrepancy is not unexpected and, most probably, originates from the unaccounted cavity contribution to volume and the fact that the ΔV_0 effects of polar and nonpolar atomic groups are opposite in sign. The predicted values of ΔV_0, thus, represent a small difference between large numbers and, consequently, are prone to significant errors, being critically sensitive to the values of α_n used in the calculations.

Taken together, our results are consistent with the picture in which the association of GB with native proteins does not involve any noticeable cooperative effects related to the size of the protein and/or the geometry and chemical nature of the microenvironment of amino acid residues on its surface. The properties of binding of GB to a protein appear to scale with the size of the latter. These results lend support to studies in which the interactions of GB and other cosolvents with proteins have been modeled by small molecules mimicking protein groups.[21,22,44−46]

Energetic Considerations. The energetics of transfer of a protein from water to a cosolvent solution is linked to the stabilizing and destabilizing effect of protecting and denaturing cosolvents. In general, stabilizing cosolvents are preferentially excluded from proteins in their both folded and unfolded states, while destabilizing cosolvents are preferentially bound to these states.[1,3] The preferential exclusion of protecting cosolvents facilitates protein folding because the native state is characterized by a lower level of exposure of surface groups. In contrast, the preferential binding of denaturing cosolvents

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shifts the conformational equilibrium toward the unfolded state that has a greater level of surface group exposure. The transfer of a protein from water to a protecting osmolyte is accompanied by a positive change in free energy, while the transfer to a denaturing cosolvent leads to a decrease in free energy. Thus, the sign and magnitude of the water-to-cosolvent transfer free energy for a specific protein are quantitative measures of the protecting or denaturing influence of a particular cosolvent.

Our determined equilibrium constants, k, can be used to gain insights into the energetics of interactions of GB with proteins. A change in free energy accompanying the transfer of a solute from water to a solvent/cosolvent mixture is given by the sum

$$\Delta G_{tr} = \Delta\Delta G_C + \Delta\Delta G_I \quad (5)$$

where $\Delta\Delta G_C$ is the differential free energy of cavity formation in a cosolvent solution and water and $\Delta\Delta G_I$ is the differential free energy of solute−solvent interactions.[9,16,18]

For a low-concentration solute with n/r identical and independent cosolvent binding sites, ΔG_I can be calculated from the relationship[20]

$$\Delta G_I = -(n/r)RT \ln(a_1^r + ka_3) \quad (6)$$

We have approximated the experimental data on the basis of a_1 and a_3 values presented by Felitsky and Record[10] by the exponential function $a_1 = 0.76 + 0.24 \exp(-a_3/24.11)$.[20] We use this relationship in conjunction with our values of k to calculate the interaction free energies, ΔG_I, of the proteins as a function of a_3 from eq 6. The results of the calculation are graphically presented in Figure 3.

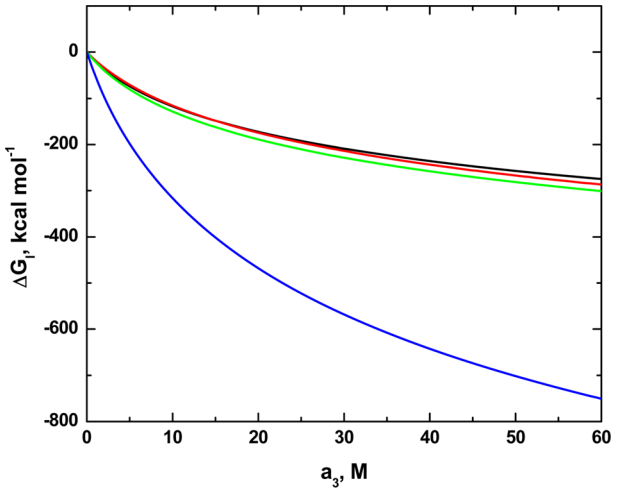

Figure 3. Free energies of direct GB−protein interactions for cytochrome c (black), ribonuclease A (red), lysozyme (green), and ovalbumin (blue) as a function of GB activity.

Inspection of Figure 3 reveals a very significant decrease in the interaction free energy, ΔG_I, upon an increase in GB activity for all the proteins studied here. This observation signifies highly favorable interactions between GB and protein groups. Note that the changes in the interaction free energy, ΔG_I, appear to roughly scale with the size of the protein. A favorable change in ΔG_I must be compensated by an unfavorable change in the free energy of cavity formation, ΔG_C, to produce a net positive change in the water-to-GB transfer free energies, ΔG_{tr}. For example, the free energy of the transfer of lysozyme from water to 2 M GB is on the order ∼10 kcal mol^{-1} as can be estimated from the values of $(\partial\mu_2/\partial m_3)_{m2}$ presented by Arakawa and Timasheff.[47]

The values of $\Delta\Delta G_C$ may be calculated, in principle, on the basis of the concepts of SPT.[35,36] Our SPT-based calculations (data not shown) reveal that the values of $\Delta\Delta G_C$ are on the same order of magnitude but opposite in sign as the values of $\Delta\Delta G_I$. However, as discussed above, such calculations are not of sufficient accuracy to allow reliable evaluation of the water-to-GB transfer free energies, ΔG_{tr}, for the proteins studied here.

One can judge the balance of forces governing the stabilizing action of GB by comparing the relative values of ΔG_{tr}, $\Delta\Delta G_I$, and $\Delta\Delta G_C$. The positive transfer free energy, ΔG_{tr}, for lysozyme on the order of ∼10 kcal mol^{-1} originates from the compensation between the interaction, $\Delta\Delta G_I$, and cavity, $\Delta\Delta G_C$, terms each on the order of ∼200 kcal mol^{-1}. By extension, a similar fine balance of thermodynamic forces governs the water-to-GB transfer free energies of the other proteins. Thus, we conclude that the mode of action of a specific cosolvent depends on the sign of a small difference (∼5%) between two large numbers: the interaction free energy and the free energy of cavity formation. A qualitatively similar inference has been drawn on the basis of the data for low-molecular weight model compounds mimicking proteins.[20]

CONCLUSION

Water-soluble cosolvents may exert a stabilizing or destabilizing influence on proteins, thereby modulating their conformational equilibria. However, the balance of thermodynamic forces governing the mode of action of an individual cosolvent is still poorly understood. In this work, we apply the volumetric measurements to characterizing the interactions of GB with four typical globular proteins differing in size and structural properties (cytochrome c, ribonuclease A, lysozyme, and ovalbumin). Comparison of the parameters of association of GB with proteins with similar data obtained for small analogues of proteins suggests an absence of cooperative effects involved in GB−protein interactions. This result lends credence to studies in which protein−cosolvent interactions are modeled on the basis of low-molecular weight compounds.

We used the equilibrium constants for GB−protein association to calculate the free energy of direct GB−protein interactions, ΔG_I. For all the proteins studied here, direct GB−protein interactions are highly favorable and characterized by large negative values of $\Delta\Delta G_I$. Comparison of the value of $\Delta\Delta G_I$ determined for lysozyme with the estimate of its differential free energy of cavity formation, $\Delta\Delta G_C$, and the water-to-GB transfer free energy, ΔG_{tr}, suggests that the stabilizing action of GB is determined by a fine balance (∼5%) between the $\Delta\Delta G_I$ and $\Delta\Delta G_C$ contributions. By extension, we propose that the mode of action of a specific cosolvent is also determined by the compensation between the $\Delta\Delta G_I$ and $\Delta\Delta G_C$ free energy components, two large quantities producing a relatively small difference on the order of ≤5%.

ASSOCIATED CONTENT

Supporting Information

Tables listing the extinction coefficients (Table S1) and the relative molar sound velocities (Table S2) of the proteins at various GB concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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