Articles

Glycerol Decreases the Volume and Compressibility of Protein Interior†

A. Priev, A. Almagor, S. Yedgar,* and B. Gavish

Biochemistry Department, The Hebrew University−Hadassah Medical School, Jerusalem 91010, Israel

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ABSTRACT: The addition of hydrogen-bonded cosolvents to aqueous solutions of proteins is known to modify both thermodynamic and dynamic properties of the proteins in a variety of ways. Previous studies suggest that glycerol reduces the free volume and compressibility of proteins. However, there is no directly measured evidence for that. We have measured the apparent specific volume (V) and adiabatic compressibility (K) of a number of proteins, sugars, and amino acids in water and in 30% glycerol at pH 7.4 and 30 °C. The values of V and K in water and their changes induced by glycerol were extrapolated to the limit of infinite solute size. The main results were the following: (a) glycerol decreases V and K of proteins, but increases it for amino acids; (b) the V and K values of the protein interior in water were found to be 0.784 ± 0.026 mL/g and $(12.8 \pm 2.5) \times 10^{-6}$ mL/g·atm, where the glycerol reduces these values by 8 and 32%, respectively; (c) the coefficient of adiabatic compressibility of the structural component of proteins affected by the glycerol is estimated to be $(50 \pm 10) \times 10^{-6}$ atm^{-1}, which is comparable to that of water. We propose that the glycerol induces a release of the so-called "lubricant" water, which maintains conformational flexibility by keeping apart neighboring segments of the polypeptide chain. This is expected to lead to the collapsing of the voids containing this water as well as to increase intramolecular bonding, which explains the observed effect.

Glycerol and other polyolic cosolvents have been shown to affect both dynamic and thermodynamic properties of protein solutions. The kinetic coefficients of protein reactions performed in viscous aqueous solutions are found to depend on the bulk solvent viscosity (Gavish & Werber, 1979; Beece et al., 1980; Yedgar et al., 1987; Gavish & Yedgar, 1995).

Solvent viscosity has been suggested to modify dissipative processes at the protein interior (Gavish, 1980). This has been demonstrated by measurements of the ultrasonic absorption of bovine serum albumin and has been attributed to an effect on the water associated with the internal protein friction (Almagor et al., 1992), termed "lubricating water" (Zak & Klibanov, 1988).

These kinds of cosolvents enhance protein stability. It has been proposed that the mechanism of this phenomenon is due to "preferential hydration" of the protein, i.e. exclusion of the cosolvent molecules from the protein surface, which creates a tendency of the protein to minimize its surface (Reisler et al., 1969; Timasheff et al., 1976; Gekko & Timasheff, 1981) without inducing conformational changes (Timasheff, 1993). The same mechanism was proposed for the decrease in the flexibility of monomeric proteins, as probed by the kinetics of tryptophan phosphorescence decay, induced by either glycerol or hydrostatic pressure (Cioni & Strambini, 1994). This was suggested to lead to a reduction in the size and number of voids at the protein structures which accommodate water. It has been hypothesized that glycerol and pressure act similarly on decreasing internal free volume and increasing protein rigidity.

Other studies, using Rayleigh scattering of Mossbauer radiation in concentrated proteins solutions (Goldanskii & Krupyanskii, 1995) or pressure-induced denaturation of DNA-binding proteins (Oliveira et al., 1994), concluded that glycerol dehydrates proteins by means of displacing some hydration water and with preferential hydration.

However, the above studies could not probe directly reduction of the protein volume or compressibility claimed to be affected by the glycerol. In contrast, Gekko and Timasheff (1981), using volumetric measurement of protein−glycerol solution, attributed the glycerol effect on the apparent specific volume of proteins to the modification of the hydration layer, with no effect on the protein interior.

Thus, the studies summarized above present disparate views on the effect of glycerol on the protein interior. Yet, studies mentioned above suggested that glycerol modifies the protein "interior" in a way which leads to a decrease in the protein volume and compressibility.

The present study was undertaken to determine if glycerol affects the protein interior. For this purpose, we have measured the apparent specific volume and the adiabatic compressibility of a series of proteins, sugars, and amino acids, which differ in surface to volume ratio. By extrapolation of these measures to infinite ratio of solute volume to surface, we determined the extent of the obtained glycerol effect on the internal volume and the adiabatic compressibility of proteins.

BASIC CONCEPTS

A volumetric description of a protein in solution is illustrated schematically in Figure 1. Following the current

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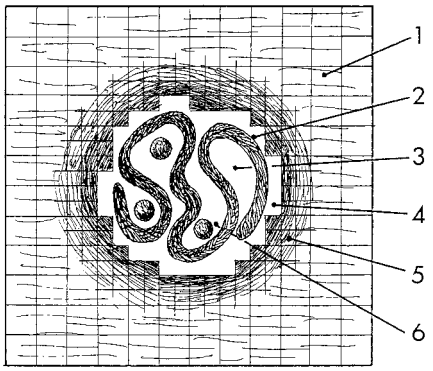

FIGURE 1: Domains in a solvated protein. These domains are illustrated highly schematically. Notations that appear in the text are marked. The domains include (1) the bulk solvent; (2) the constitutive volume of the polypeptide chain itself (m); (3) voids or cavities formed by the polypeptide chain itself; (4) voids located at the interface between the protein and the solvents; (5) solvent at the protein−solvent interface with properties different from that of the bulk; (6) solvent molecules, which interact with the polypeptide chain only and are isolated from the solvent. Using this classification, the protein "*core*" (c) consists of regions 2 + 3 + 4, and the "*solvation region*" (s) includes 5 + 6. However, the "internal" part of the protein can be identified with 2 + 3 + 6 while its "surface" with 4 + 5.

view (Kauzmann, 1959; Zamyatnin, 1972; Gavish et al., 1983; Gekko & Hasegawa, 1986; Kharakoz & Sarvazyan, 1993), the partial specific volume of a solute, in units of mL/g of dried solute mass, can be expressed by eq 1

$$V = V_m + V_v + \Delta V_s \quad (1)$$

where V_m is the volume of the protein constitutive atoms and groups, as determined from their van der Waals dimensions (region 2 in Figure 1), and V_v is the total free volume, including voids or cavities formed by the folding of the polypeptide chain (region 3), as well as packing defects at the protein−solvent interface originated by the solvent molecules (region 4). $V_m + V_v$ form the protein "*core*", which is free of solvent molecules. ΔV_s is the volume *change* of the solution caused by the protein "*solvation*", including "*hydration*". ΔV_s can be attributed to solvent at the protein−solvent interface (region 5) with properties different from those of the bulk (region 1), as well as solvent molecules, which interact with the polypeptide chain only and are isolated from the solvent as "trapped" water molecules (region 6).

Using these definitions the "*internal*" part of the protein can be identified with regions 2 + 3 + 6. On the other hand, the "*surface*" includes regions 4 + 5. The same definitions can be used for the other solutes investigated in this study.

Effect of Cosolvents. Since cosolvents do not affect the van der Waals volume of the solute itself (V_m), the change in apparent specific volume V, δV, observed after the addition of cosolvents to the original buffer solution can be expressed by eq 2. Hereby, we will use the notation δ to mark changes

$$\delta V = V(\text{cosolvent}) - V(\text{water}) = \delta V_v + \delta \Delta V_s \quad (2)$$

in variables observed after the addition of the cosolvent.

Adiabatic Compressibility. The *apparent adiabatic compressibility*, K, of the *solute* is defined by $K = \partial V/\partial P|s$, where P is the pressure applied at constant entropy (S). With this definition, we obtain from eq 1

$$K = K_m + K_v + \Delta K_s \quad (3)$$

where K_m, K_v, and ΔK_s correspond, respectively, to the change of the volumes V_m, V_v, and ΔV_s with pressure.

Since the cosolvents do not affect the compressibility K_m of the solute constitutive atoms and groups, the changes in the various compressibilities caused by the addition of the cosolvents are expressed by eq 4.

$$\delta K = K(\text{cosolvent}) - K(\text{water}) = \delta K_v + \delta \Delta K_s \quad (4)$$

Dependence of the Apparent Solute Volume (V) and Compressibility (K) on Solute Molecular Weight (M). Equation 1 can be rewritten as $V = (V_m + V_v)[(1 + \Delta V_s(V_m + V_v)]$. For a *globular* solute of average radius R, $(V_m + V_v) \propto R^3$. As a surface effect, ΔV_s is proportional to a *volume of a surface layer*, which is proportional to R^2 at large enough R values. Thus, we expect $\Delta V_s/(V_m + V_v) \propto 1/R \propto M^{-1/3}$ for *large enough M* values. Similar arguments can be applied to K (eq 3), δV (eq 2), and δK (eq 4). We may conclude that the *extrapolation to zero* of the $M^{-1/3}$ dependence of these quantities provides their *value for the solute interior, free of surface contributions*. At small M values, surface contributions are maximal, which does not mean that contribution of the solute core is small, unless justified.

Following these considerations, cosolvent effects on the solute can be investigated at two extremes: (I) *large solute limit*, in which the cosolvent effect on the solute *interior* is *dominant*, and (II) *small solute size*, in which the cosolvent effect on the solute−solvent *interface* is *maximal*. Thus, by plotting δV or δK as functions of the solute size, one can find the effect of the cosolvent on the solute interior by extrapolating the results to the large-solute-size limit. This procedure was originally suggested by Sarvazyan et al. (1988) and by Kharakoz and Sarvazyan (1993) for globular solutes. Our objective in the present study is to investigate the cosolvent effect on proteins at the large-size limit and on amino acids, taken as small-size solutes, among which glycine ($M = 75$ and $M^{-1/3} = 0.24$) is the smallest.

MATERIALS AND METHODS

Solutes. We have used as solutes bovine serum albumin, hemoglobin, β-lactoglobulin, myoglobin, lysozyme, lactose, proline, and glycine (Sigma, St. Louis, MO), the molecular weights of which are listed in Table 1. Proteins were deionized by exhaustive dialysis against distilled water at 4 °C and lyophilized before use. To bridge the large gap between the molecular weights of proteins and amino acids, we have studied lactose and dextran-10. The fact that dextran is not globular was taken into account in the analysis (see Table 1).

Solutions. Stock solutions of 3.2% (w/v) solute concentration in Tris buffer (pH = 7.4) and 60% (v/v) glycerol (Frutarom, Israel) in Tris buffer were prepared. The buffer and the glycerol stock solutions were then mixed to obtain solute concentrations of 0, 0.4, 0.8, or 1.6% (w/v) in pure buffer or in 30% (v/v) glycerol. Solutions were degassed in vacuum for at least 24 h at 4 °C before performing volumetric or ultrasonic measurements. Final solute concentrations were determined by the "dry weight" method, which we found to agree well with optical density measurements. The experimental error in the determination of the solute concentration was about 1%.

Table 1: Experimental Data Obtained for Proteins and Amino Acids at 30 °C and pH 7.4 in Tris and with 30% Added Glycerol[a]

	M	$M^{-1/3}$	V_t (mL/g)	K_t (10^{-6} mL/g·atm)	V_g (mL/g)	K_g (10^{-6} mL/g·atm)	δV (mL/g)	δK (10^{-6} mL/g·atm)
hemoglobin	68000	0.0245	0.754	8.97	0.702	5.26	−0.52	−3.71
BSA	68000	0.0245	0.737	8.55	0.690	5.42	−0.047	−3.13
β-lactoglobulin	37000	0.0300	0.753	7.11	0.701	4.05	−0.052	−3.06
myoglobin	17300	0.0387	0.744	7.81	0.697	4.87	−0.047	−2.94
lysozyme	14700	0.0408	0.722	4.77	0.679	2.95	−0.043	−1.82
dextran-10[b]	10500	0.084[b]	0.614	−3.25	0.588	−3.55	−0.026	−0.30
lactose	362	0.1403	0.615	−5.80	0.616	−1.51	0.001	4.29
proline	115	0.2056	0.720	−18.72	0.747	−5.46	0.027	13.26
glycine	75	0.2371	0.580	−33.63	0.616	−13.45	0.036	20.18

[a] M is the molecular weight of the solute; V and K are, respectively, the solute apparent specific volume and apparent adiabatic compressibility. $\delta V = V_g - V_T$ and $\delta K = K_g - K_T$ correspond to the value of these quantities with glycerol (g) minus that measured in Tris (T). Experimental error is denoted in footnote *c*. [b] This molecule has an oval-ellipsoid shape of axial ratio 10:1 (Nordmeir, 1993). For the purpose of calculation, the size parameter $M^{1/3}$ was defined by the mass of a spherical molecule with a surface to volume ratio equal to that of the dextran and a typical protein density of 1.35 g/mL. [c] Experimental errors for V_T, K_T, V_g, and K_g are 0.002, 0.2, 0.006, and 0.6 in the corresponding units.

Volumetric Measurements. The densities, $\rho(c)$, of solutions at the selected solute concentration (c, in grams of dried solute per 1 mL of solution) at 30 °C were determined using the vibrating tube densitometer DMA-60/DMA-601 (Anton Paar, Gratz, Austria), with a precision of $\pm 3 \times 10^{-6}$ g/mL. The apparent specific volume of the solute, V, is defined by the change in the solution volume resulting from dissolving 1 g of the solute at infinite dilution. It has been shown by Zamyatnin (1972) that

$$V = 1/\rho(0) - [\rho] \quad (5)$$

where $[\rho] = \lim_{c\to 0}[(\rho(c) - \rho(0))/\rho(0)c]$. The error in the determination of V was about 1% in the presence of viscous cosolvents.

Compressibility Measurements. It is convenient experimentally to measure the *apparent adiabatic compressibility* K using its relation to the *coefficient of adiabatic compressibility* $\beta = K/V$ defined by $-(1/V)\partial V/\partial P|_s$. Note that in contrast to K (measured in mL/g·atm), β (measured in atm^{-1}) is independent of the apparent solute volume. β can be calculated from the measured density ρ and the sound velocity U using the relation $\beta = 1/\rho U^2$. It has been shown (Sarvazyan, 1991) that

$$K = \beta(0)(2V - 2[U] - 1/\rho(0)) \quad (6)$$

where $[U] = \lim_{c\to 0}[(U(c) - U(0))/U(0)c]$. $(U(c) - U(0))/U(0)$ was measured at 7.5 MHz with a precision of about 1 ppm, using the "resonator method" (Eggers & Funk, 1973) in its differential version (Sarvazyan, 1982). The ultrasonic cells contained 0.7 mL sample volume and were thermostated within ±0.01°. The experimental error in determining [U] was about 0.1%.

Data Analysis. Following eqs 5 and 6, the apparent values of V and K were obtained from the slopes of the regression lines obtained from plots of $[\rho]$ and $[U]$ versus solute concentration. An example of raw data is given in Figure 2. Calculations and analysis of error were done using SYSTAT software (Evanston, IL). A good linearity of the dependence of ρ and U on c assures that the contribution of protein−protein interaction to V and K is insignificant.

RESULTS

The values of apparent volume V and compressibility K in water and in 30% glycerol at pH = 7.4 and 30 °C for the tested proteins, sugars, and amino acids are given in Table 1, along with the calculated differences in these measures between glycerol and water. Using the definition given by eqs 2 and 4, $\delta V = V$(glycerol) − V(Tris) and $\delta K = K$(glycerol) − K(Tris).

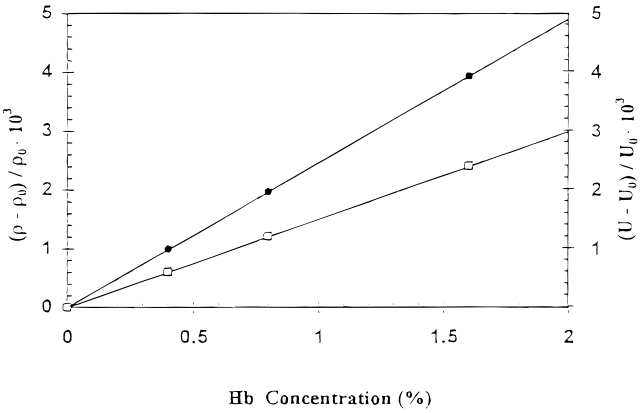

FIGURE 2: Volumetric and ultrasonic measurements. The graph depicts an example of raw data of the relative changes in solution density $(\rho - \rho_0)/\rho_0$ (filled circles) and in sound velocity $(U - U_0)/U_0$ (empty circles) obtained at different Hb concentrations, with respect to the same solvent with no protein (Tris buffer at 30 °C and pH 7.4). Regression analysis gives a correlation coefficient >0.9999, which ensures the linearity of the plot.

Figure 3 depicts the dependence of δV and of δK on $M^{-1/3}$. It shows that glycerol decreases the apparent specific volume and compressibility of the *proteins*, but *increases* them for *amino acids*. The sugars display intermediate values, which bridge between those of proteins and amino acids. Using the notation $x = M^{-1/3}$, the expression $\delta V(x) = \delta V(0) + Ax$ was best-fitted to the data by linear regression, giving $\delta V(0) = -0.061 \pm 0.002$ mL/g and $A = 0.422 \pm 0.013$ with a correlation coefficient of 0.996. The dependence of the apparent compressibility on x was best-fitted to the expression $\delta K(x) = \delta K(0) + A[\exp(-Bx) - 1]$ by nonlinear regression, giving $\delta K(0) = -4.04 \pm 0.27 \times 10^{-6}$ mL/g·atm, $A = (3.36 \pm 0.76) \times 10^{-6}$ mL/g·atm, and $B = 8.86 \pm 0.84$ with a correlation coefficient of 0.999. The large-size limit of $V(x)$ and $K(x)$ in water (given in Table 1) has been obtained by the $x \to 0$ limit of the regression curves. Since the values of $V(x)$ and $K(x)$ for the low molecular weight solutes are too scattered and thus outweigh the values for proteins, we have applied linear regression for the protein data only, which for water gave $V(0) = 0.784 \pm 0.026$ mL/g and $K(0) = (12.8 \pm 2.5) \times 10^{-6}$ mL/g·atm. This yields $\beta(0) = K(0)/V(0) = (16.3 \pm 3.2 \times 10^{-6}$ atm^{-1}, which agrees

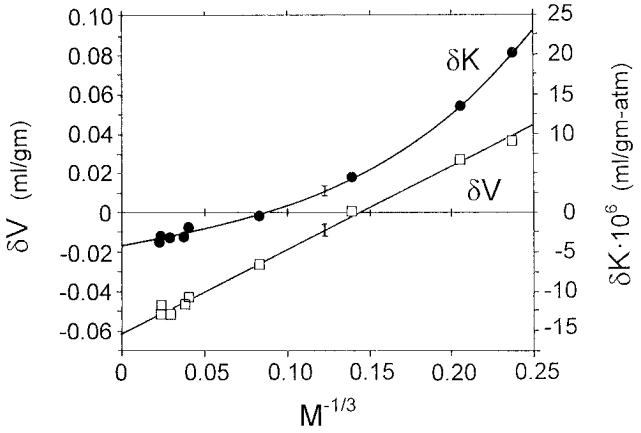

FIGURE 3: The effect of glycerol on the apparent specific volume (V) and adiabatic compressibility (K) of proteins, amino acids, and sugars. The differences $\delta V = V$(glycerol) − V(Tris) and $\delta K = K$(glycerol) − K(Tris) are plotted versus $x = M^{-1/3}$ in the bottom and top curves, respectively. The data obtained at 30 °C and pH 7.4 were taken from Table 1. The experimental error is marked. The smooth curves are best-fitted phenomenological expressions, using the large-size limits $\delta V(0)$ and $\delta K(0)$, and the M values for which δV and δK change sign were obtained from best-fitted phenomenological expressions (smooth curves) given in Results. The data show that V(glycerol) < V(Tris) and K(glycerol) < K(Tris) for proteins, but V(glycerol) > V(Tris) and K(glycerol) > K(Tris) for amino acids.

with the previously reported value of $(13 \pm 3) \times 10^{-6}$ atm^{-1} obtained at 25 °C (Kharakoz & Sarvazyan, 1993).

Using the observed values $\delta V(0) = -0.061$ mL/g ($=-0.078V(0)$) and $\delta K(0) = -4.04 \times 10^{-6}$ mL/g·atm ($=-0.0316K(0)$), it is found that for glycerol $V(0) = 0.723 \pm 0.026$ mL/g, $K(0) = (8.8 \pm 2.5) \times 10^{-6}$ mL/g·atm, and thus $\beta(0) = (12.1 \pm 3.2) \times 10^{-6}$ atm^{-1}. This shows that *the addition of glycerol decreases the apparent specific volume of the protein core by almost 8% and its apparent adiabatic compressibility by about 32%*. It is of interest compare these values of $V(0)$ and $\beta(0)$ with those obtained by the extrapolation of the values of V and β given by Gekko and Hasegawa (1986) for 25 proteins in water. Substituting our $V(0)$ value in their regression line yields $\beta(0) = 15.7 \times 10^{-6}$ atm^{-1}, which is in good agreement with our $\beta(0)$ value ($(16.3 \pm 3.2) \times 10^{-6}$ atm^{-1}). However, the glycerol-induced decreases in V observed by us is much larger than that reported by Gekko and Timasheff (1981). It is possible that the discrepancy results from the different methods of preparation; Gekko and Timasheff dehydrated the proteins before solvation and then dissolved in water prior to mixing with the cosolvent. A better understanding of this discrepancy requires the measurements to be done with and without dialysis in a number of protein concentrations, as done by these researchers. This requires a separate work. The hysteresis associated with gradual protein hydration (Gregory, 1995; Lumry et al., 1962) could be of some relevance, too.

DISCUSSION

The results depicted in Figure 3 demonstrate that the addition of 30% glycerol to water *decreases* the apparent specific volume (V) and the apparent compressibility (K) of proteins but *increases* both V and K of amino acids.

Effect of Glycerol on the Protein−Solvent Interface. Charged groups at the solute−solvent interface are known to give negative contributions to V and K in proteins and amino acids (Gucker et al., 1950; Miyahara, 1952; Millero et al., 1976, 1978; Gekko & Noguchi, 1979; Gavish et al., 1983; Kharakoz, 1989; Chalikian et al., 1994). This effect is explained by the large electric field generated by the electric charge, which compresses neighboring water molecules (Noyes, 1964). In addition, the packing of water around proteins and amino acids leaves voids, which can be described as an empty interfacial layer having a thickness of about 0.5 Å (Kharakoz, 1992). However, since glycerol molecules are preferentially excluded from the vicinity of proteins, in general, and charged groups, in particular (Gekko & Timasheff, 1981), the glycerol is unlikely to affect directly electrostricted water and such interfacial voids. Since glycerol enhances hydrogen bonding at the bulk solvent, this makes the values of the apparent volume and compressibility of the bulk water closer to that of electrostricted water. Thus, the differences between the V and K values of electrostricted and bulk water, which give the dominant contribution to ΔV_s and ΔK_s in eqs 1 and 3 (negative values), become smaller. This means positive values for $\delta\Delta V_s$ and $\delta\Delta K_s$ (see eqs 2 and 4). Since this contribution dominates V and K in their small-size limit, glycerol is expected to increase V and K in the case of amino acids, as indeed observed here.

What region at the protein interior is affected by the glycerol? The net effect of the glycerol on the V and K of the protein interior can be evaluated by taking the large-size limit of these quantities. This extrapolation (Figure 3) shows that glycerol makes the protein core denser (by 8%) and less compressible (by 32%). Our basic considerations, as expressed by eqs 2 and 4, suggest that the *glycerol may reduce the total voids volume at the protein core* (region 3 in Figure 1). However, more information about this region can be obtained from our results by calculating its compressibility: the adiabatic compressibility β of a composite system consisting of species $i = 1, 2, ...$ that occupy *relative* volumes v_i can be shown to be $\beta = \sum v_i\beta_i$, where $\sum v_i = 1$. If for the sake of argument species 1 gives the dominant contribution to β, and it undergoes a variation δv_1, then $\delta\beta \approx \delta v_1\beta_1$. Taking for the glycerol effect $\delta\beta = -3.9 \times 10^{-6}$ atm^{-1} (calculated by using the relation from calculus $\delta\beta = \delta(K/V) = (\delta KV - \delta VK)/V^2$ with our experimental values at the large-size limit) and $\delta v_1 = -0.078$ (see Results), we obtain $\beta_1 = (50 \pm 10) \times 10^{-6}$ atm^{-1}, which is comparable, within the experimental error, to the compressibility of free water. These calculations suggest that *if the internal compressibility of the protein is mainly contributed by a structural component which is eliminated by glycerol, this component has the compressibility of water*. Thus, it is plausible to conclude that the glycerol-induced reduction in the protein volume and compressibility is due to *release of water* from the protein interior. This water species is a major contribution to the protein compressibility, and it interacts with the polypeptide chain in a way that its elimination leads to a decrease in the total volume of the voids (or cavities). A water species that fits this description is the so-called "lubricant" water, which keeps apart neighboring segments of the polypeptide chain (thick lines) that would otherwise stick (Chirgadze & Ovsepyan, 1972). This water plays an important functional role in proteins by keeping its conformational flexibility, which is essential for its function (Zak & Klibanov, 1988). A similar type of water has been found in dextran using ultrasonic spectroscopy (Kato et al., 1980). The elimination of such water molecules, which are located

in a cavity, causes the "collapse" of the cavity. This leads to enhanced intramolecular bonding, which locally rigidifies the protein and decreases the amplitude of the thermal motion (Gavish, 1983). As a result, the volume and compressibility of the protein interior are decreased, as observed in the present study. This relation between the decrease of the volume and structural mobility of the protein fits the mobile-defect picture of Lumry and Rosenberg (1976) and the "cavity-traps" description of protein dynamics (Gavish, 1981).

Glycerol Effect on Thermal Motions of the Protein Structure. The isothermal compressibility β_T, defined by $-(1/V)\partial V/\partial P|_T$, is related to mean-square volume fluctuation by eq 7 (Landau & Lifshitz, 1958)

$$\langle \delta \mathbf{V}^2 \rangle = kT\mathbf{V}\beta_T \tag{7}$$

where the brackets mark thermal averaging. k, T, and $\mathbf{V}$ are, respectively, the Boltzmann factor, the absolute temperature, and the molar volume of the tested solute. Similarly, the mean-square atomic displacement, x, in a molecular structure can be expressed by eq 8 (Priev et al., 1990; Kharakoz & Sarvazyan, 1993)

$$\langle \delta x^2 \rangle = kT\beta_T/(36\pi d^2 \upsilon^{1/3}) \tag{8}$$

where d is the packing density, i.e. the volume fraction occupied by the constitutive atoms and groups, and υ is the average volume occupied by an atom. The isothermal and adiabatic compressibilities are related thermodynamically (Landau & Lifshits, 1958). The isothermal compressibility has been shown to be larger than the adiabatic one by an amount independent of the specific protein. β_T was calculated from our measurements of β using the difference $\beta_T - \beta$ obtained from the literature (Gekko & Hasegawa, 1986). Using the relation $\mathbf{V} = MV$ and estimating d by 0.7 (Klapper, 1971) and using eqs 7 and 8, we have calculated $\delta\mathbf{V}_{rms}/\mathbf{V}$ (where $\delta\mathbf{V}_{rms} = \langle \delta\mathbf{V}^2 \rangle^{1/2}$) and $\delta x_{rms} = \langle \delta x^2 \rangle^{1/2}$. The results for hemoglobin, BSA, β-lactoglobulin, myoglobin, and lysozyme are, respectively, $\delta\mathbf{V}_{rms}/\mathbf{V} = 0.28$, 0.28, 0.34, 0.53, and 0.48% and $\delta x_{rms} = 0.17$, 0.164, 0.082, 0.040, and 0.027 Å in water. The decrease in δx_{rms} after the addition of glycerol was, respectively, 0.035, 0.03, 0.018, 0.007, and 0.012 Å. Using the extrapolation to the large-size limit, we find that for the protein interior in water $\delta\mathbf{V}_{rms}/\mathbf{V} = 0.8\%$ and $\delta x_{rms} = 0.39 \pm 0.08$ Å. In 30% glycerol δx_{rms} is decreased by 0.06 ± 0.01 Å, which is 17%, in comparison with its value in water. We may conclude that *glycerol decreases the amplitude of thermal motion in proteins*. This conclusion is compatible with the effect of glycerol on protein dynamics, studied by the Mossbauer effect, reported by Goldanskii and Krupyanskii (1995).

Comparisons with Other Works. Dielectric measurements have shown that the amount of water associated with the protein flexibility is about 13−20% of the protein volume (Bone & Pethig, 1985). Thus, the glycerol-induced 8% decrease in the protein volume is in the range of the flexibility-associated water.

A 6% decrease in protein volume upon dehydration was calculated by Kachalova et al. (1991) from the X-ray diffraction of cross-linked lysozyme crystals. These authors suggested that the tendency of water to leave the protein interior in the presence of glycerol could be the result of it being squeezed out by the compression of the protein following the process of reducing its surface of contact with the solvent.

The possibility that the compressibility of a protein decreases in any water−cosolvent system in which preferential hydration exists follows from the Gibbs−Duem equation (Gregory, 1988). It has been shown by the same author that such a decrease in volume and compressibility can explain the glycerol effect on hydrogen exchange rates.

Our conclusions on the basis of direct measurements of the apparent volume and compressibility of proteins are compatible with the suggestions that glycerol dehydrates the protein and decreases the free internal volume and compressibility and thermal motion of the polypeptide chain, as well as increases the protein stability (Gregory, 1988; Oliveira et al., 1994; Cioni & Strambini, 1994; Goldanskii & Krupyanskii, 1995). The present study, concluding that the elimination of water induces the collapse of voids in the protein interior, provides a mechanism for these phenomena. We have previously predicted (Gavish, 1980) and further found (Almagor et al., 1992) that viscous cosolvents increase the internal friction of protein, as measured by its ultrasonic absorption. This has been suggested to be associated with an effect of the cosolvent of the "lubricating" water. The collapse of the voids proposed here may provide a mechanism for this phenomenon as well.

Gekko and Hasegawa (1986) have identified the voids in the protein interior as the most compressible elements in the protein and suggested that they make an important contribution to the protein function (Gekko & Yamagami, 1991). The present study suggests that this contribution originates from the water in the voids ("lubricant" water).

Conclusion. The present study provides for the first time direct evidence that the addition of glycerol to aqueous solutions of proteins decreases the specific volume and the adiabatic compressibility of the protein interior. We suggest that the mechanism involves the collapse of voids in the protein core following a glycerol-induced elimination of lubricant water.

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