Mechanism of Protein Stabilization by Glycerol: Preferential Hydration in Glycerol–Water Mixtures†

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ABSTRACT: A densimetric investigation of the interactions between solvent components in glycerol–water mixtures (between 10 and 40 vol % glycerol) and seven proteins have been carried out in the acid pH region. All the proteins were found to be preferentially hydrated at all conditions used, i.e., addition of the proteins to the mixed solvent results in an increase in the chemical potential of glycerol. It is considered that this thermodynamically unfavorable interaction should tend to minimize the surface of contact between proteins and glycerol and in this way stabilize the native structure of globular proteins.

Glycerol has been used for many years by biochemists to stabilize the activity of enzymes and the native structure of proteins (Jarabak et al., 1966; Ruwart & Suelter, 1971; Jarabak, 1972; Bradburg & Jakoby, 1972; Myers & Jakoby, 1973; Hoch, 1973) as well as that of assembled systems (Green et al., 1972; Behnke, 1975; Shifrin & Parrott, 1975). Recently, Lee & Timasheff (1975, 1977) have shown that high concentrations of glycerol enhance the self-assembly of purified tubulin to form microtubules. As a possible explanation of this effect they have proposed a mechanism for this enhancement which consists of totally nonspecific interactions between protein and solvent components in an aqueous glycerol medium. According to this model, glycerol exerts its boosting action on the self-association reaction by being preferentially excluded from the immediate domain of tubulin. Such exclusion has been shown to exist in a preliminary study on α-chymotrypsin in 4 M glycerol (Timasheff et al., 1976). Since this exclusion is thermodynamically unfavorable, the system will tend to reduce it by decreasing the area of solvent–protein contact through enhancement of the protein self-association. For a clarification of the mechanism of protein structure stabilization and the enhancement of self-association by glycerol, a systematic study has been undertaken of the preferential interaction of proteins with solvent components in aqueous glycerol medium.

The concept of "selective" or "preferential" solvation of proteins was discussed by Schachman & Lauffer already in 1949. The rigorous thermodynamic foundation of this concept (Kirkwood & Goldberg, 1950; Stockmayer, 1950) has made possible quantitative studies of protein solvation in three component systems, predominantly in salts (Kuntz & Kauzmann, 1974). Previous studies from our laboratory have shown that, in the case of denaturing cosolvents, protein unfolding is directly related to the "binding" of denaturant molecules to particular groups on proteins, namely to hydrophobic residues in the case of denaturing alcohols (Inoue & Timasheff, 1968, 1972; Timasheff & Inoue, 1968; Timasheff, 1970; E. P. Pittz and S. N. Timasheff, unpublished results) and to peptide groups in the case of guanidine hydrochlorides (Lee & Timasheff, 1974) and urea (Prakash et al., 1981). On the other hand, up to the present, there have been few studies of solvent–protein interactions in aqueous solutions of polyhydric compounds, such as sugars and glycerol, which are well-known as stabilizing agents of protein conformation and enzyme activity (Stauff & Mehrotra, 1961; Timasheff et al., 1976; Bull & Breese, 1978). Timasheff et al. (1976) have reported that protein structure stabilizing solvents are preferentially excluded from the domain of the protein. In the case of 2-methyl-2,4-pentanediol–water, Pittz & Timasheff (1978) found that preferential exclusion of the organic cosolvent is sufficiently strong to induce separation of the system into two phases, leading to crystallization in the case of ribonuclease A. The exclusion of 2-methyl-2,4-pentanediol seems to be related to its unfavorable interactions with charged groups on the surface of protein molecules (Pittz & Timasheff, 1978; K. Gekko and S. N. Timasheff, unpublished results). On the other hand, the preferential hydration of proteins in aqueous sucrose medium and their stabilization appear to be related to the increase in the surface tension of water induced by the addition of sucrose (Timasheff et al., 1976; Lee & Timasheff, 1981). This is similar to the proposal of Sinanoglu & Abdulnur (1965) that DNA structure is stabilized by the free energy of cavity formation.

Since glycerol decreases slightly the surface tension of water, factors other than surface tension must be operative in the glycerol stabilization of proteins.[1] Jarabak et al. (1966) and Ruwart & Suelter (1971) have proposed that the stabilization of enzymes by glycerol may be explained in terms of the formation of water–glycerol structure around the protein molecules. Gerlsma (1968, 1970) and Gerlsma & Sturr (1972, 1974), on the other hand, have proposed that the stabilizing effect of polyhydric alcohols is the result of a decrease in the hydrogen-bond-rupturing capacity of the medium. These reports suggest that a dominant factor in protein stabilization by glycerol may be enhancement of the structure of the medium or of the solvation layer of the protein. Such effects should manifest themselves in the free energy of interaction, or binding, of solvent components to proteins in these mixed solvent systems. Therefore, a systematic study has been undertaken of the preferential interactions of solvent components with proteins in water–polyhydric alcohols systems. This paper will be devoted to the description of such preferential interactions of several globular proteins in glycerol–water mixtures. The possible mechanism of the glycerol-induced stabilization

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[1] The surface tension arguments cannot be applied quantitatively to mixed solvents, since the local surface tension may depend on preferential solvation effects. They should be still valid for qualitative comparisons.

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of proteins will be discussed in the following paper (Gekko & Timasheff, 1981). A preliminary report of this work has been presented earlier (Timasheff et al., 1976).

Experimental Procedures

Chymotrypsinogen A (lot 114C-8330, type II from bovine pancreas), ribonuclease A (lot 46C-8080, type IIA from bovine pancreas), β-lactoglobulin (lot 75C-8126, from milk), and bovine serum albumin (lot 56B-1990) were purchased from Sigma. Egg-white lysozyme (LYSF 35J 881) and α-chymotrypsin (CDI 3AF) were purchased from Worthington. Insulin (bovine pancreatic, lot s3252) was obtained from Mann Research Laboratories.

The ribonuclease A was further fractionated on a Sephadex G-75 column in phosphate buffer, using the procedure of Crestfield et al. (1962); α-chymotrypsin was purified on CM-sephadex C-50 (Nakagawa & Bender, 1970); β-lactoglobulin was recrystallized from NaCl solution before use by the procedure of Townend et al. (1960). All the proteins were deionized either by exhaustive dialysis against distilled water (containing 10^{-3} M HCl in some cases) at 4 °C or by passage through a mixed-bed ion-exchange column (Amberlite MB-1) and lyphilized before use.

Spectroquality glycerol from Matheson, Coleman & Bell and reagent grade glycerol from Fisher Scientific were used without further purification. There was no difference in the results obtained with the two grades of glycerol. Fisher standardized 1 N HCl was used for pH adjustments of solutions. All other chemicals were reagent grade and were used without further purification. The dialysis tubing (no-Jax) was obtained from Union Carbide.

Density Measurements. The densities of the solvents and of the protein solutions were measured with a Precision DMF-02 density meter (Anton Paar, Gratz). The principles of the method have been described elsewhere (Stabinger et al., 1967; Kratky et al., 1973; Lee & Timasheff, 1974; Lee et al., 1979). In this technique, the density of an unknown liquid, d_1, is measured by reference to a standard of known density, d_2, through the determination of the time lapse of each solution, T_1 and T_2, for a preset number of periods:

$$d_1 - d_2 = (1/A)(T_1^2 - T_2^2) \quad (1)$$

The instrument constant A was determined by calibration with samples of known density NaCl solutions (International Critical Table, 1928; Lee et al., 1979). The constant A did not change during the entire duration of this study. All measurements were made at a temperature of 20 ± 0.02 °C, which was maintained with a refrigerated and heated Forma Scientific circulating bath.

The apparent partial specific volume, ϕ, was calculated from the densities of the solvent and protein solutions at a given concentration by using the standard equation (Kielley & Harrington, 1960; Cassasa & Eisenberg, 1961a,b)

$$\phi = (1/\rho_0)[1 - (\rho - \rho_0)/c] \quad (2)$$

where ρ is the density of the solution in grams per milliliter, ρ_0 is that of the solvent, and c is the protein concentration in grams per milliliter. The values of ϕ were plotted as a function of protein concentration, and the value extrapolated to infinite dilution of protein was taken as the partial specific volume of the protein, $\bar{v}_2$. In preferential interaction measurements, two types of apparent specific volumes are measured. The first, ϕ_2, is measured under conditions at which the molal concentration, m_3, of diffusible component 3 (here, glycerol) is kept identical in the solvent and the solutions. The second, ϕ_2', is measured under conditions at which it is the chemical potential of component 3, μ_3, which is kept constant between solution and reference solvent (operationally this condition can be attained to a close approximation by dialyzing the protein solution against the solvent). In this paper, components 1, 2, and 3 are water, protein, and added glycerol, respectively, following the notation of Scatchard (1946) and Stockmayer (1950).

Constant Molality Measurement. Five samples of protein (3–15 mg) were weighed into acid-washed tared tubes, placed into a beaker covered with a Kimwipe, and dried for 15–20 h in a vacuum oven at 40 °C in the presence of phosphorus pentoxide. After the oven had cooled down to room temperature, air was admitted into it by slowly bubbling it through concentrated sulfuric acid. As rapidly as possible after that, 1.0 mL of solvent was added to each tube and the tubes were sealed quickly with parafilm. The protein was entirely dissolved, taking care to avoid the formation of bubbles or foam in the solution, especially in glycerol solutions of high concentration. If bubbles were present, the solution was kept standing until they had disappeared before the density measurements were started. Samples were introduced into the density meter cell with disposable syringes fitted with clean Luer adaptors. After sample addition, 15–20 min were permitted to elapse to attain thermal equilibrium. During this time, the syringes were covered tightly with small plastic caps. This prevented evaporation of water from the solution, a fact which was checked by constancy of the measured density values. After the time lapse, T, was read, the sample in the cell was transferred with the same syringe to a new tube covered with Parafilm. This solution was used to determine the concentration. Density measurements were done on the solvent first, then the protein solutions, and finally solvent again to control the stability of the machine. In most cases the time lapse for solvent before and after measurements with the samples did not change within experimental error. In cases where a significant change of time lapse was observed, the data were discarded.

Constant Chemical Potential Measurement. Of the protein solution remaining from the constant molality experiment 1.3–1.5 mL or a freshly prepared sample in the concentration range of 3–15 mg/mL was placed into a dialysis bag and dialyzed for at least 15–20 h against a large excess of clean solvent in a cold room at 4 °C. The dialyzing system was shifted to 20 °C for 3–4 h prior to the density measurements. Density measurements were done first on the dialysate, then the samples, and finally the dialysate again. Just before measurement, each dialysis bag was taken out individually from the dialysis system with stainless-steel forceps and the protein solution contained in it was removed with a sterile disposable 1-mL syringe. The needle was then replaced by a female Luer adaptor, and the solution was injected into the density meter cell. The rest of the procedure was identical with that of the constant molality experiment.

Protein Concentration Determination. The protein concentration was determined following the density measurements by measuring the absorbance of the solution on a Cary Model 118 spectrophotometer. For the absorbance measurements, the protein solutions were diluted gravimetrically on a Mettler balance sensitive to 0.000 05 g as soon as possible after the density measurements in order to minimize concentration changes caused by the evaporative loss of water. The gravimetrically obtained dilution factor was converted to a volumetric one from density data on the solvent and the solution, with the assumption of the additivity of their volumes. The absorption spectrum was recorded between 240 to 400 nm to

Table I: Extinction Coefficients of Proteins in Glycerol-Water Mixtures

protein	λ (nm)	pH [a]	absorptivity [dL/(cm g)] [b] 0	10	20	30	40
ribonuclease A	278	2.0	7.08			7.56	
		2.8	7.38 [c]	7.48	7.61	7.71	7.79
		4.3	7.38 [c]			7.65	
		5.8	7.38 [c]			7.65	
chymotrypsinogen A	282	2.0	19.7 [d]	19.85	19.98	20.17	20.32
		5.8	19.7 [d]			20.05	
β-lactoglobulin	278	2.0	9.6 [e]	9.68	9.75	9.83	9.87
		5.8	9.6 [e]			9.89	
α-chymotrypsin	280	3.0	20.3 [f]	20.45	20.59	20.78	20.94
lysozyme	281	2.0	27.4 [g]			28.2	
		5.8	27.4 [g]			28.2	
insulin	277	2.0	10.4 [h]			10.8	
bovine serum albumin	278	2.0	6.58 [i]			6.87	
		4.0	6.58 [i]			6.72	
		5.8	6.85 [i]			6.68	

[a] The following solvent compositions were used: 0.01 M HCl for pH 2.0; 10^{-3} M HCl and 0.1 M NaCl for pH 3.0; 0.01 M sodium acetate and 0.02 M NaCl for pH 4.0, 4.3, and 5.8; 0.04 M glycine for pH 2.0. [b] The numbers under $A^{1\%}_{1cm}$, 0, 10, 20, 30 and 40, show glycerol concentration by volume. [c] Scott & Scheraga (1963). [d] Jackson & Brandts (1970). [e] Townend et al. (1960). [f] Aune & Timasheff (1971). [g] Roxby & Tanford (1971). [h] Herskovitz (1965). [i] Noelken & Timasheff (1967).

ascertain the absence of light-scattering contribution and of absorbance shifts resulting from protein denaturation.

Since the extinction coefficient of a protein generally can be expected to be affected by the glycerol medium, this quantity was corrected for each glycerol concentration. To do this, the absorbance of identical concentrations of protein was measured in the glycerol solutions and in dilute buffer. From a ratio of these values, the absorptivity in each glycerol concentration was calculated. The obtained values are summarized in Table I, those of the native form of each protein being taken from the literature. The value for ribonuclease at pH 2.0 was measured by comparing the absorbance with that at pH 5.8, because this protein may be partly unfolded at pH 2.0 even at room temperature, although the extent of unfolding may be small (Brandts & Hunt, 1967). The values for chymotrypsin were estimated by assuming the same glycerol concentration dependence of the extinction coefficient as for chymotrypsinogen. As shown in Table I, the extinction coefficients of proteins increase almost in direct proportion to the glycerol concentration, the absorptivity in 30% glycerol being 1.5 to 4.5% higher than that in water, with the value for ribonuclease at pH 2.0 being exceptionally high. While the various assumptions made in the determination of the extinction coefficients in the presence of glycerol may lead to some uncertainty in the absolute values of partial specific volumes, no significant errors should result in the difference between ϕ_2^0, and $\phi_2'^0$ which is the quantity required for calculating the preferential interaction parameters.

Calculation of Preferential Interactions. The preferential interaction parameter $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \equiv \xi_3$ of solvent component 3 with protein can be determined from a combination of ϕ_2^0 and $\phi_2'^0$, where the superscript 0 means extrapolation to zero protein concentration, by using the equation (Cohen & Eisenberg, 1968)

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = [(\partial \rho/\partial g_2)_{T,P,\mu_3} - (\partial \rho/\partial g_2)_{T,P,m_3}]/(\partial \rho/\partial g_3)_{T,P,m_2} = [(1 - \rho_0\phi_2'^0) - (1 - \rho_0\phi_2^0)]/(1 - \rho_0\bar{v}_3) \quad (3)$$

where g_i is the concentration of component i in grams per gram of principal solvent, i.e., water, m_i is molal concentration of component i, T is thermodynamic (kelvin) temperature, P is pressure, and μ_i is the chemical potential of component i, defined as

$$\mu_i = RT \ln a_i + \mu_i^0(T,P) = RT \ln m_i + RT \ln \gamma_i + \mu_i^0(T,P) = RT \ln m_i + \mu_i^{(e)} + \mu_i^0(T,P) \quad (4)$$

where a_i is the activity of component i and γ_i is its activity coefficient. The preferential interaction, or binding, on a molal basis, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ i.e., the number of moles of component 3 preferentially bound to 1 mol of component 2, is given by

$$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3} = (M_2/M_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (5)$$

where M_i is the molecular weight of component i. Positive values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ signify preferential binding of component 3 (glycerol) to the protein; negative values indicate preferential exclusion of component 3, or preferential hydration, which, in turn, is obtained from (Timasheff & Kronman, 1969; Reisler et al., 1977) eq 6.

$$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = -(g_1/g_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (6)$$

Preferential interaction is strictly an activity coefficient effect, since

$$(\partial m_3/\partial m_2)_{T,P,\mu_3} = -(\partial \mu_3/\partial m_2)_{T,P,m_3}/(\partial \mu_3/\partial m_3)_{T,P,m_2} = (\partial \mu_2/\partial m_3)_{T,P,m_2} \quad (7)$$

Within the approximation that $(\partial \mu_3/\partial m_3)_{T,P,m_2} = RT/m_3$,[2] the intercomponent thermodynamic interaction is equal to

$$(\partial \mu_3/\partial m_2)_{T,P,m_3} = (\partial \mu_2/\partial m_3)_{T,P,m_2} = -(RTM_2/1000g_3)(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} \quad (8)$$

Equations 7 and 8 clearly show us the true meaning of the term "binding", as measured by dialysis equilibrium, or similar methods. It is essentially a measure of the perturbation of the activity coefficient of one solution component by another, and it should be regarded as an expression of the summation of the entire spectrum of solvent–solute (or ligand–protein) interactions, attractive or repulsive, from strong complexing at specific sites to momentary perturbations of the freedom of motion of a molecule of one component by another. While a true thermodynamic quantity, the preferential interaction, or binding, parameter ξ_3 is also a measure of the difference in concentrations of component 3 in the immediate domain of the protein and in the bulk solvent. Since at equilibrium the activity of component 3, $a_3 = m_3\gamma_3$, must be identical in the two domains, a perturbation of its activity coefficient by component 2 must necessarily result in a change in concentration of component 3 in the vicinity of component 2. Normally, we interpret this perturbation in terms of binding (complexing), although no information on protein–ligand contact is given by the usual "binding" experiments followed by Scatchard (or similar) plotting of the data.

Results

The preferential interaction of solvent components with proteins as a function of glycerol concentration was determined for α-chymotrypsin, chymotrypsinogen A, ribonuclease A, and β-lactoglobulin at conditions at which these proteins are susceptible to a thermal conformational transition. Typical plots of apparent partial specific volume against protein concentration are presented in Figure 1 for chymotrypsinogen A and β-lactoglobulin. In all cases, there was little or no protein

[2] Rigorously, $\partial\mu_3/\partial m_3 + RT(\partial \ln \gamma_3/\partial m_3)$. Frequently there is no information on the variation of the activity coefficient of a component with respect to its own concentration, and the last term on the right-hand side of this equation is neglected. This results generally in errors not greater than 10%.

Table II: Partial Specific Volumes and Preferential Interaction Parameters of Proteins in Glycerol-Water Mixtures at 20 °C

glycerol (vol %)	g_3 [a]	ϕ_2^0 (mL/g)	$\phi_2'^0$ (mL/g)	$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$ [b]
			Chymotrypsinogen A [c]				
0	0	0.7327 ± 0.001	0.7323 ± 0.001				
10	0.1398	0.7301 ± 0.001	0.7384 ± 0.002	-0.040 ± 0.012	0.285	-11.3 ± 3.4	4300
20	0.3134	0.7294 ± 0.001	0.7438 ± 0.001	-0.081 ± 0.008	0.258	-22.6 ± 2.4	3900
30	0.5348	0.7269 ± 0.001	0.7454 ± 0.001	-0.123 ± 0.010	0.229	-34.2 ± 2.8	3400
40	0.8273	0.7271 ± 0.002	0.7474 ± 0.002	-0.161 ± 0.032	0.195	-45.0 ± 8.9	2900
			α-Chymotrypsin [d]				
0	0	0.736 ± 0.001	0.736 ± 0.001				
10	0.1402	0.735 ± 0.001	0.740 ± 0.001	-0.025 ± 0.010	0.176	-6.7 ± 2.7	2610
20	0.3143	0.734 ± 0.001	0.744 ± 0.001	-0.057 ± 0.011	0.182	-15.5 ± 2.0	2600
30	0.5363	0.733 ± 0.001	0.748 ± 0.001	-0.101 ± 0.012	0.189	-27.5 ± 3.4	2800
40	0.8303	0.732 ± 0.001	0.750 ± 0.003	-0.146 ± 0.029	0.176	-39.6 ± 7.9	2600
			Ribonuclease A [e]				
0	0	0.699 ± 0.001	0.699 ± 0.001				
10	0.1401	0.698 ± 0.001	0.702 ± 0.001	-0.020 ± 0.010	0.140	-2.9 ± 1.4	1100
20	0.3141	0.697 ± 0.001	0.705 ± 0.001	-0.045 ± 0.011	0.144	-6.7 ± 1.7	1200
30	0.5361	0.696 ± 0.002	0.708 ± 0.001	-0.080 ± 0.020	0.149	-11.9 ± 3.0	1200
40	0.8297	0.695 ± 0.002	0.711 ± 0.001	-0.128 ± 0.024	0.154	-19.1 ± 3.6	1200
			β-Lactoglobulin [f]				
0	0	0.750 ± 0.002	0.750 ± 0.001				
10	0.1398	0.750 ± 0.001	0.754 ± 0.001	-0.019 ± 0.010	0.193	-3.9 ± 1.9	1500
20	0.3134	0.749 ± 0.001	0.757 ± 0.001	-0.045 ± 0.011	0.144	-9.0 ± 2.2	1500
30	0.5348	0.749 ± 0.001	0.760 ± 0.002	-0.073 ± 0.020	0.138	-14.7 ± 4.0	1500
40	0.8273	0.748 ± 0.001	0.762 ± 0.001	-0.111 ± 0.016	0.135	-22.2 ± 3.2	1400

[a] Grams of glycerol per gram of water. [b] Calories per mole of glycerol per mole of protein in 1000 g of H_2O. [c] In 0.01 M HCl, pH 2.0. [d] In 10^{-3} M HCl and 0.01 M NaCl, pH 3.0. [e] In 0.04 M glycine, pH 2.9. [f] In 0.01 M HCl, pH 2.0.

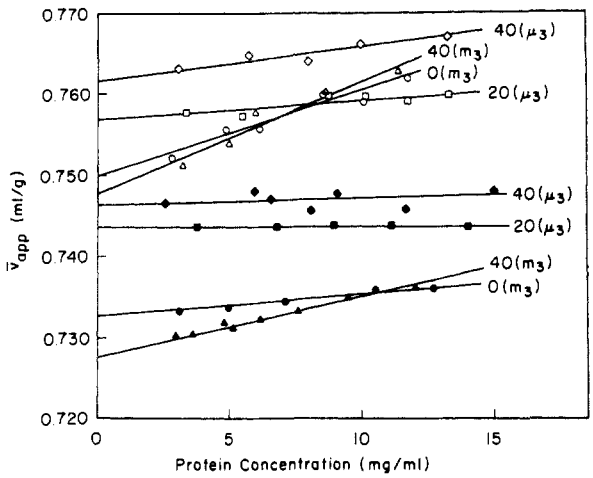

FIGURE 1: Protein concentration dependence of the partial specific volume of chymotrypsinogen A (♦, ■, ●, ▲) and β-lactoglobulin (◇, □, ○, △). The numbers in the figure show the glycerol concentration in volume percent; μ_3 and m_3 in parentheses indicate the constant chemical potential and constant molality experiments, respectively.

concentration dependence of the apparent partial specific volume in the systems with or without glycerol at conditions of constant chemical potential. For some proteins, however, a definite concentration dependence of the partial specific volume was observed at conditions of constant molality, the extent of which was a function of the glycerol concentration. In all cases, however, there was a good linear relationship between the apparent partial specific volume and protein concentration, which allowed extrapolation of $\bar{v}_{app}$ to infinite dilution of protein by a least-squares procedure.

Results of the partial specific volume measurements over the glycerol concentration range between 0 and 40% by volume at 20 °C are shown in Table II for the four proteins. The partial specific volumes of chymotrypsinogen, β-lactoglobulin, and α-chymotrypsin in dilute buffer without glycerol are absolutely identical with literature values. In the case of ribonuclease the partial specific volume seems to lie between literature values, 0.696 (Lee & Timasheff, 1974), 0.695 (Ulrich et al., 1964), and 0.703 (Crouch & Kupke, 1977). These values, however, seem to depend both on the experimental conditions and on the protein lot used; e.g., values of 0.694 and 0.693 were observed for lot 63C-1650 from Sigma and for lot 65165 from Calbiochem, respectively, while the value reported in the table (0.699) was obtained with lot 46C-8080 from Sigma. It is obvious that, in the absence of glycerol, ϕ_2^0 coincides with $\phi_2'^0$ in all systems, satisfying the basic requirement for the calculation of the preferential binding parameter. With all four proteins, the value of $\phi_2'^0$ was found to increase with increasing glycerol concentration, and conversely, the value of ϕ_2^0 had a trend to decrease slightly.

The preferential binding parameters $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ and $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, calculated with eq 3 and 5, are listed in Table II. In these calculations, the values of the partial specific volume of glycerol, $\bar{v}_3$, at concentrations of 10, 20, 30, and 40% by volume used were 0.7674, 0.7680, 0.7699, and 0.7716. These had been measured in the densimeter at 20 °C. The molecular weights of proteins used for the calculation of $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ are listed in Table III. For all four proteins and at all glycerol concentrations, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was found to be negative, indicating a deficiency of glycerol in the immediate domain of the protein, i.e., preferential hydration. The extent of this negative interaction increases in monotone fashion with an increase of glycerol. The corresponding values of preferential hydration, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ calculated with eq 6 are shown in column 6 of Table II and in Figure 2. The values of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ were found to be almost independent of glycerol concentration for α-chymotrypsin and β-lactoglobulin and possibly slightly increasing with glycerol concentration for ribonuclease A. A striking difference was found,

Table III: Molecular Parameters of the Proteins Investigated

protein	M_r	$H\phi_{av}$ [a]	P [a]
ribonuclease A	13 700	870	1.73
bovine serum albumin	68 000	1120	1.22
lysozyme	14 300	970	1.18
β-lactoglobulin	18 400	1230	0.96
insulin	12 000	1180	0.86
α-chymotrypsin	25 000	1020	0.85
chymotrypsinogen A	25 700	1040	0.83

[a] $H\phi_{av}$ is the total hydrophobicity divided by the number of residues (calories/residue) and P is the ratio of polar volume to nonpolar volume (Bigelow, 1967).

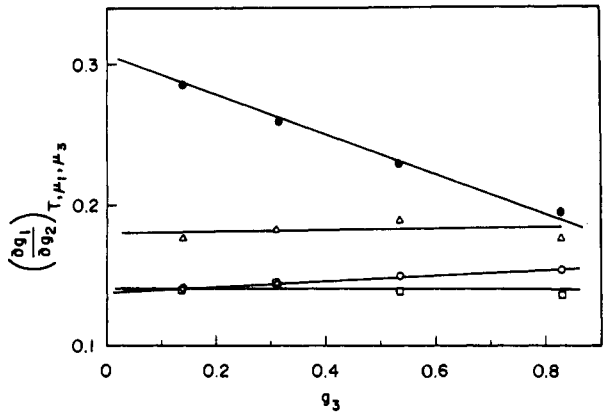

FIGURE 2: Glycerol concentration dependence of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ of the various proteins in glycerol–water mixtures at 20 °C: (●) chymotrypsinogen A, (Δ) α-chymotrypsin, (○) ribonuclease A, and (□) β-lactoglobulin.

however, for chymotrypsinogen A for which this parameter decreases linearly with glycerol concentration.

The extent of preferential interaction, or binding, observed is the resultant of the total interactions of the protein with the solvent components, given by (Inoue & Timasheff, 1972; Kupke, 1973; Reisler et al., 1977).

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = g_1A_3 - g_3A_1 \qquad (9)$$

where A_3 is total solvation, i.e., the actual amount of glycerol in the immediate domain of the protein (grams of glycerol/gram of protein), and A_1 is total hydration (grams of water/gram of protein). If, as a limit, it is assumed that glycerol is totally excluded from the domain of the protein, i.e., if one sets $A_3 = 0$ in eq 9, the minimum hydration is given by the values of column 6 of Table II, since now $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} = A_1$. In fact, a plot of the data according to eq 9, $(\partial g_3/\partial g_2)$ vs. g_3, or according to a combination of eq 6 and 9, $(\partial g_1/\partial g_2)$ vs. g_3^{-1}, (Kupke 1973; Reisler et al., 1977), results in curves from which it may be estimated that, for chymotrypsinogen, A_3 is negative (~–0.02 g/g), indicating actual exclusion of glycerol from the protein, while this parameter is close to zero for the other three proteins. These plots, furthermore, suggest that A_3 may be a function of glycerol concentration. It appears, therefore, that there is an effective layer of water around the protein molecules which is impenetrable to glycerol. The values of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ are considerably smaller than hydration measured by vapor adsorption, NMR, or hydrodynamic techniques. For example, Bull & Breese (1968) reported protein hydration values measured by vapor pressure of 0.35, 0.29, 0.33, and 0.32 g of H_2O/g of protein for ribonuclease, chymotrypsinogen, α-chymotrypsin, and β-lactoglobulin, respectively. Nevertheless, close to the protein molecule there is a considerable region in which the solvent composition is perturbed from that in the bulk. The result that $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, is almost independent of g_3 for chymotrypsin and β-lactoglobulin suggests that, for these proteins, there is no binding of glycerol at specific sites. This may also be the case for ribonuclease. In the case of chymotrypsinogen, there actually appears to be repulsion of glycerol from the protein surface.

Table IV: Protein–Solvent Interactions in Glycerol–Water Mixtures at 20 °C

protein	glycerol [a] (vol %)	Bull and Breese [b] A_1	Bull and Breese [b] A_3	Kuntz [c] A_1	Kuntz [c] A_3
chymotrypsinogen A	10	0.290	0.001	0.273	0.000
	20	0.290	0.010	0.273	0.005
	30	0.290	0.032	0.273	0.023
	40	0.290	0.079	0.273	0.065
α-chymotrypsin	10			0.308	0.018
	20			0.308	0.040
	30			0.308	0.064
	40			0.308	0.110
ribonuclease A	10	0.355	0.030	0.332	0.027
	20	0.355	0.067	0.332	0.059
	30	0.355	0.110	0.332	0.098
	40	0.355	0.167	0.332	0.147
β-lactoglobulin	10	0.320	0.026	0.296	0.022
	20	0.320	0.055	0.296	0.048
	30	0.320	0.097	0.296	0.085
	40	0.320	0.154	0.296	0.134

[a] The buffers used are same as those in Table I. [b] Taken from hydration data at water activity of 0.92 (Bull & Breese, 1968). [c] Taken from hydration calculated by assuming that all amino acid residues are fully hydrated and all carboxyl groups are uncharged at pH 3.0 (Kuntz, 1971).

The maximal amount of glycerol present in the immediate domain of the protein may be estimated in the following way. At the glycerol concentrations used, protein hydration should not differ greatly from that in pure water, since glycerol does not change significantly the activity of water in the concentration range used (Scatchard, 1946; Kozak et al., 1968) nor is the dielectric constant of these mixed solvents (43–45 for pure glycerol at 20 °C) sufficiently low to favor ion-pair formation and neutralization of ionized groups (Singer, 1961) between 10 and 40% glycerol. Assuming, therefore, that A_1 remains constant at all solvent compositions used and is equal to the hydration in pure water, A_3 was calculated as a function of glycerol concentration, using two sets of hydration values, namely those of Bull & Breese (1968), determined by water vapor binding measurement at isopiestic equilibrium, and those calculated from the amino acid composition of the proteins, according to the method of Kuntz (1971). As seen in Table IV, these values do not differ greatly. The resulting values of A_3 are presented in Figure 3 as a function of glycerol concentration. It is evident that in all the systems the amount of glycerol present within the protein hydration layer increases with increasing glycerol concentration.

The values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ are presented in the last column of Table II. This parameter is found to be essentially invariant with glycerol contents for all proteins studied, with the exception of chymotrypsinogen for which it decreases linearly with an increase in glycerol concentration, according to

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = -165m_3 + 4370 \qquad (10)$$

Integration of this equation with respect to m_3 yields

$$\mu_{2_G} - \mu_{2_W} = -82.5m_3^2 + 4370m_3 \qquad (11)$$

where μ_{2_G} and μ_{2_W} are the chemical potentials of protein in the glycerol–water mixture and water, respectively. The re-

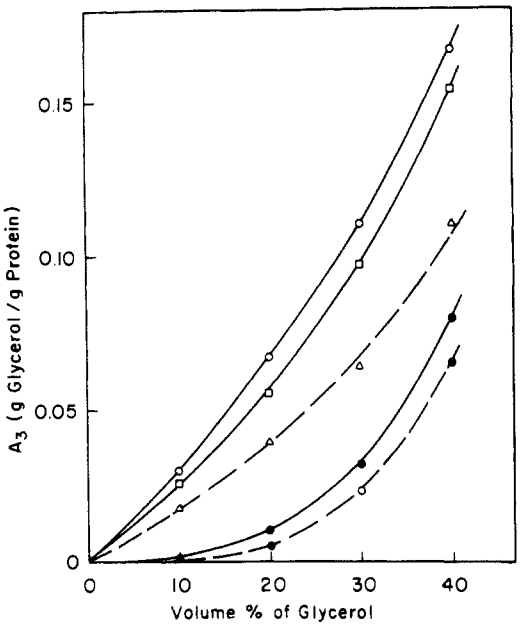

FIGURE 3: Glycerol concentration dependence of glycerol bound to proteins at 20 °C: (●) chymotrypsinogen A, (△) α-chymotrypsin, (○) ribonuclease A, and (□) β-lactoglobulin. The solid and dotted lines are based on hydration values measured at a water activity of 0.92 (Bull & Breese, 1968) and those determined by the NMR method (Kuntz, 1971), respectively.

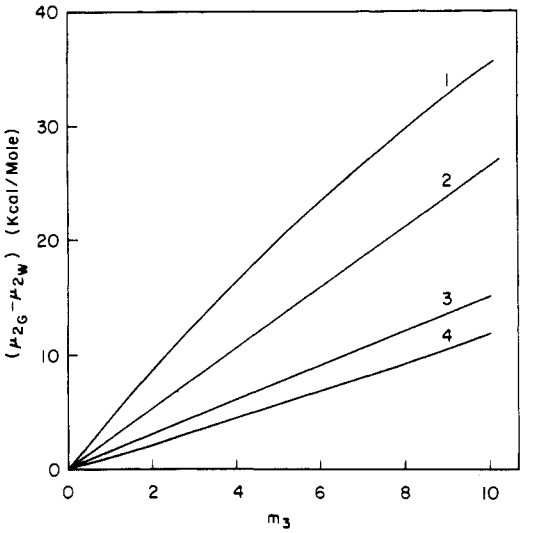

FIGURE 4: Glycerol concentration dependence of the chemical potential change of various proteins on transferring them from water to glycerol–water mixtures at 20 °C. Curves 1–4 in the figure represent chymotrypsinogen A, α-chymotrypsin, β-lactoglobulin, and ribonuclease A, respectively.

sulting change in the chemical potential of the protein, $(\mu_{2G} - \mu_{2w})$, on transfer from water to the water–glycerol solvent is shown for all four proteins as a function of m_3 in Figure 4. The positive values of $\mu_{2G} - \mu_{2w}$ indicate that the free energies of the proteins are increased by the addition of glycerol. In the cases of ribonuclease, α-chymotrypsin, and β-lactoglobulin, this increase is proportional to the molality of glycerol. An extension of the concentration dependence for chymotrypsinogen predicts that $(\mu_{2G} - \mu_{2w})$ will increase with glycerol concentration to a maximal value at $m_3 \sim 26$ (mole ratio of glycerol to water of about 1:2), then decreases to zero at $m_3 \sim 53$ (mole ratio of glycerol to water of about 1:1), and finally assume a negative value at higher m_3. The preferential interaction parameter should vary in a similar fashion, becoming positive at high concentrations of glycerol. This extrapolation could not be tested, however, since at glycerol concentrations higher than 40% the solution is so viscous that the persistence of the bubbles precludes the use of the densimetric technique. It is interesting to note, however, that a similar variation in preferential interaction with glycerol has been reported for bovine serum albumin by Stauff & Mehrotra (1961), who did the measurements by the techniques of light-scattering and refractive index increment. These authors found that this protein is preferentially hydrated in a complicated manner, preferential binding of glycerol occuring above 70 to 80% glycerol.

The change in volume when protein is transferred from water to aqueous glycerol solution was calculated from

$$\Delta V = M_2[\phi_2^0 \text{ (in glycerol solution)} - \phi_2^0 \text{ (in water)}] \quad (12)$$

where ΔV is the molar volume change of the protein. As seen in Table II, the values of ϕ_2^0 for all proteins become smaller with increasing glycerol content, indicating a negative volume change, $\Delta V < 0$, for the transfer of the protein from water into aqueous glycerol. For example, the volume changes of chymotrypsinogen and ribonuclease on transfer from water to 40% glycerol amount to −144 and −55 mL per mol of protein, respectively. It should be noted that ΔV is the summation of all the volume changes occurring in the system. The partial molar volume of a protein consists of two contributions: the intrinsic volume of the protein itself, V_{int} (positive value), expressed as the sum of the constitutive atomic volumes and the void volume due to imperfect atomic packing, and the volume change arising from solvation around the protein, ΔV_{sol} (generally a negative value) (Kauzmann, 1959)

$$M_2\phi_2^0 = V_{int} + \Delta V_{sol} \quad (13)$$

The intrinsic volume of the protein should not differ significantly between water and aqueous glycerol solutions, since no conformational change seems to occur on transfer between the two media, at least as probed by circular dichroism. Thus, the observed volume change may be attributed mainly to the solvation of the protein. The negative value of ΔV means that ΔV_{sol} is more negative in the glycerol-containing solvents than in water. This would be true if glycerol could penetrate into the solvation layer of the protein and form a larger solvation sheath containing now both water and glycerol molecules or if the presence of glycerol induced a greater order in the hydration layer than exists in dilute aqueous buffer.

In order to get further insight into the properties of proteins which govern the preferential hydration in glycerol–water solutions, the preferential interaction experiments were extended to several other globular proteins at 30% glycerol by volume at pH 5.8 and 2.0. Furthermore, additional experiments were done at a pH of 4.0 with bovine serum albumin and at pH 2.8 and 4.3 with ribonuclease. The results are summarized in Table V. In all the systems studied, the interaction was that of preferential hydration, the extent being a function both of protein and of pH. In attempts to relate the results to some general properties of the proteins, hydrophobicity and polarity were selected as possible common parameters. The molecular parameters of the proteins used are listed in Table III, where $H\phi_{av}$ is the total hydrophobicity divided by the number of residues and P is the ratio of polar volume to nonpolar volume (Bigelow, 1967). Since, as a rule, most hydrophobic groups of globular proteins are buried in the interior of the protein molecule while polar residues are located preferentially on the surface, $H\phi_{av}$ may be regarded as a parameter characteristic mostly of the interior of a protein and P as one that reflects the degree of polarity of the protein surface. In fact, Bull & Breese (1968) have found a linear correlation between the amount of hydration and the polarity

Table V: Partial Specific Volumes and Preferential Interaction Parameters or Proteins in 30% Glycerol Solution at 20 °C

pH [a]	ϕ_2^0 (mL/g)	ϕ'^0_2 (mL/g)	$\left(\frac{\partial g_3}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial g_1}{\partial g_2}\right)_{T,\mu_1,\mu_3}$ (g/g)	$\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}$ (mol/mol)	$\left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$ [b]
			Ribonuclease A			
2.0	0.698 ± 0.002	0.700 ± 0.002	−0.013 ± 0.027	0.025	−1.97 ± 3.94	200
2.8	0.696 ± 0.002	0.708 ± 0.001	−0.080 ± 0.020	0.149	−11.9 ± 3.0	1200
4.3	0.694 ± 0.001	0.709 ± 0.003	−0.099 ± 0.027	0.186	−14.8 ± 3.9	1500
5.8	0.695 ± 0.003	0.708 ± 0.001	−0.087 ± 0.027	0.163	−12.9 ± 4.0	1300
			Bovine Serum Albumin			
2.0	0.726 ± 0.001	0.729 ± 0.001	−0.020 ± 0.013	0.037	−14.7 ± 0.7	1500
4.0	0.731 ± 0.002	0.749 ± 0.001	−0.119 ± 0.020	0.223	−88.2 ± 14.7	8900
5.8	0.732 ± 0.002	0.749 ± 0.001	−0.113 ± 0.020	0.212	−83.4 ± 14.7	8400
			Lysozyme			
2.0	0.709 ± 0.001	0.715 ± 0.002	−0.040 ± 0.018	0.074	−6.17 ± 2.83	600
5.8	0.709 ± 0.001	0.720 ± 0.002	−0.073 ± 0.018	0.137	−11.4 ± 2.85	1100
			β-Lactoglobulin			
2.0	0.749 ± 0.001	0.760 ± 0.002	−0.073 ± 0.020	0.138	−14.7 ± 4.0	1500
5.8	0.750 ± 0.001	0.760 ± 0.002	−0.067 ± 0.020	0.125	−13.3 ± 4.0	1300
			Chymotrypsinogen A			
2.0	0.727 ± 0.001	0.745 ± 0.001	−0.123 ± 0.010	0.229	−34.2 ± 2.8	3400
5.8	0.727 ± 0.002	0.746 ± 0.001	−0.127 ± 0.020	0.237	−35.4 ± 5.6	3600
			Insulin			
2.0	0.715 ± 0.002	0.734 ± 0.001	−0.126 ± 0.020	0.236	−16.4 ± 2.6	1700
			α-Chymotrypsin			
3.0	0.733 ± 0.001	0.748 ± 0.001	−0.101 ± 0.012	0.189	−27.5 ± 3.4	2800

[a] The solvent compositions are the same as in Table I. [b] Calories per mole of glycerol per mole of protein in 1000 g of H_2O.

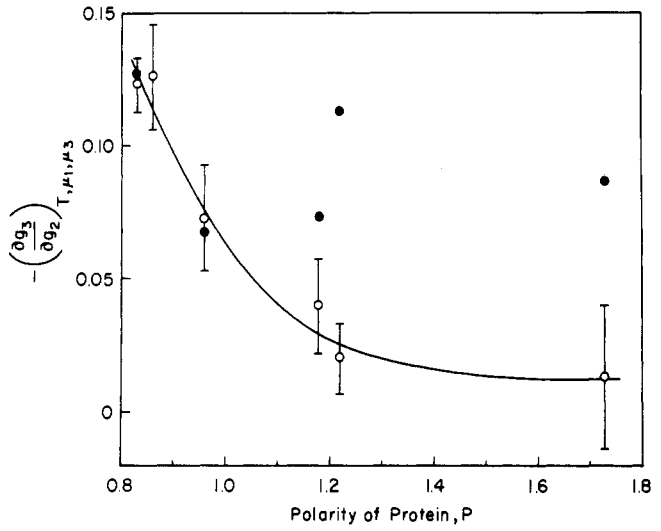

FIGURE 5: Dependence of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ on protein polarity, P, in 30% glycerol solution at pH 2.0 (○) and pH 5.8 (●) and 20 °C.

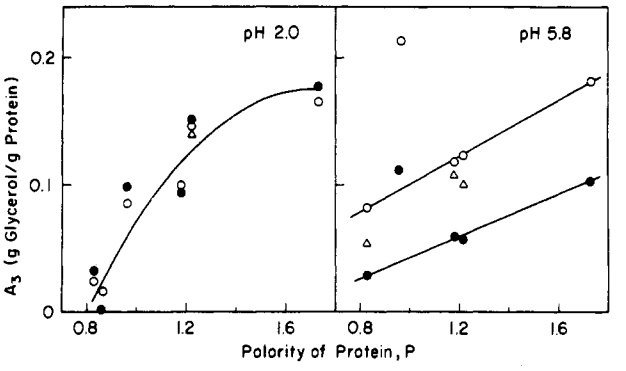

FIGURE 6: Dependence of glycerol binding on protein polarity, P, in 30% glycerol solutions, at pH 2.0 and 5.8 and 20 °C: (●) calculated from hydration values measured at a water activity of 0.92 (Bull & Breese, 1968); (○) calculated from hydration values obtained by assuming that all amino acid residues are fully hydrated at pH 5.8 (Kuntz, 1971); (△) calculated from hydration values measured by the NMR method (Kuntz, 1971).

estimated as the sum of the hydroxyl, carboxyl, and basic residues minus the amide residues per gram of protein. A priori, it may be expected that, in the aqueous glycerol system, the preferential interactions with solvent components will be related to P rather than to $H\phi_{av}$ since glycerol is a structure-stabilizing cosolvent while correlation with $H\phi_{av}$ has been found only with strong denaturants such as 2-chloroethanol which interacts directly with hydrophobic residues of proteins (E. P. Pittz and S. N. Timasheff, unpublished results). Keeping these considerations in mind, attempts were made to correlate $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ with P. As shown in Figure 5, a clear correlation seems to exist between $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ and P at pH 2.0: the preferential hydration decreases with an increase in the polarity of the protein. Data at pH 5.8, however, do not show a similar correlation. No correlation at all was found with hydrophobicity at either pH.

Preferential interaction is an expression of the difference between the interactions of the two solvent components with the protein. Since these may vary independently for different proteins, a better way to compare the different proteins would be through the actual amount of glycerol present in the domain of the protein. Assuming that total hydration, A_1, is independent of glycerol concentration, the values of A_3 calculated with eq 9 from the data of Table V are presented as a function of protein polarity in Figure 6. A clear correlation exists between A_3 and P at both pH's, with the exception of β-lactoglobulin at pH 5.8. The amount of glycerol within the protein domain increases with increasing protein polarity. The scatter in the data at pH 2.0 may be related to the assumption that the amount of hydration at pH 2.0 is identical with that

at neutral pH. At pH 5.8, glycerol binding calculated using hydration values obtained according to the method of Kuntz (1971) is considerably greater than that calculated from the hydration data of Bull & Breese (1968). Since glycerol binding calculated according to the Kuntz and Bull and Breese hydration data can be considered as maximal and minimal, respectively, the actual amount of glycerol binding may be expected to be between these two sets of values. In any case, for the purpose of the present analysis, the important consideration is that the interactions be calculated in a consistent manner for all proteins used.

The variations of the preferential interaction parameters shown in Figure 5 and Table V are quite striking. Chymotrypsinogen and β-lactoglobulin, which are less polar, showed no pH dependence of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$. On the other hand, there is a large increase in the negative value of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ for lysozyme, bovine serum albumin, and ribonuclease when pH is raised from 2.0 to 5.8. It should be noted, however, that ribonuclease may be slightly denatured at pH 2.0 (Brandts & Hunt, 1967) and bovine serum albumin is considerably swollen at pH <4.0 (Aoki & Foster, 1957). No substantial variation was observed for ribonuclease between pH 2.8 and 5.8 and for bovine serum albumin at pH >4.0, conditions at which both proteins have their native conformation.

Discussion

The present measurements of the preferential interactions of proteins with solvent components in the water–glycerol solvent system have shown that of six proteins examined, all are preferentially hydrated in this solvent system. Recently the same situation has been shown to be true for calf brain tubulin (Na & Timasheff, 1978) and for egg albumin (Bull & Breese, 1978). It would appear reasonable, therefore, to generalize this situation for other proteins. Furthermore, it has been known empirically for a long time that the conformation of proteins is stabilized by the presence of glycerol. One may wish, therefore, to ask the questions: why is the protein preferentially hydrated in glycerol–water mixtures and how is the protein stabilized in the preferentially hydrated state? Let us show that these two questions are closely related.

The preferential binding data indicate that the chemical potential of a protein (or its activity coefficient) increases with increasing glycerol concentration. An increase in the activity coefficient of a solute corresponds to a decrease in its concentration at constant activity or a decrease in its solubility. Glycerol is known to repel nonpolar substances quite effectively (Sinanoglu & Abdulnur, 1965), and its antagonism to butane is comparable to that of water (Tanford, 1973). Since glycerol interacts favorably with water (Scatchard et al., 1938), by entering into the water lattice and strengthening solvent structure (McDuffie et al., 1962), its presence in the aqueous medium could increase the hydrophobicity of the protein. The nonpolar groups on the protein surface should be expected, then, to react unfavorably to contact with the mixed solvent. Surface hydrophobic groups would prefer to migrate into the interior of the protein out of contact with solvent in order to relieve this situation. Such migration is, of course, mechanically hindered by the tight packing of the three-dimensional structure of proteins and the fact that the hydrophobic groups are covalently linked to the polypeptide chain of the protein. As a result, only the converse can take place, i.e., the water and glyceol molecules redistribute themselves in the vicinity of the protein molecule, keeping the chemical potential, and hence the activity, of solvent components constant. In other words, the phenomenon of preferential binding of solvent components manifests its essence as an expression of the general change in activity coefficients.

Being a truly thermodynamic quantity, the preferential binding parameter by itself can give no information about the solvation structure around the protein molecules. However, the total solvation, i.e., the actual amount of each solvent component "bound" to a protein, may be useful at this point. The obtained results were that the extent of glycerol "bound" to protein increases with glycerol concentration and that A_1 calculated by assuming $A_3 = 0$ is considerably smaller than the actual amount of hydration. This suggests that the glycerol molecules may actually penetrate into the solvation sheath of proteins as a result of the delicate balance between repulsion from nonpolar regions, attraction from polar regions of the protein surface, and attraction between glycerol and water molecules. Any interaction between glycerol and protein in these mixed solvents must, however, be nonspecific, since these effects take place only at high glycerol concentrations (of the order of 1–4 M). Talalay (196) has suggested that it is the ability of glycerol to stabilize networks of "structured" water molecules which is essential to the maintenance of the proper spatial configuration of the protein in the native state, and the anomalous dielectric relaxation behavior of glycerol–water mixtures has been interpreted in terms of "structural groups" or clusters containing glycerol and water in which dielectric orientation occurs through a cooperative effect (McDuffie et al., 1962). Thus, the formation of water–glycerol structures around protein molecules appears to be as possible as the formation of water structures alone. This concept is fully consistent with the observed small decrease of the partial molar volumes of proteins upon transfer from dilute buffer to aqueous glycerol mixtures. At present, it is difficult to evaluate quantitatively whether such solvent ordering is the dominant factor in the increase in protein chemical potential in aqueous glycerol solutions or not. In the absence of specific interaction between glycerol and protein, one can expect a deficiency of the bulky solvent component in the immediate vicinity of the protein molecule as explained by the steric exclusion principle ascribed to Kauzmann (Schachman & Lauffer, 1949). This effect, however, does not seem sufficient to account for the specificity suggested by the present results, which indicate a relation between the extent of exclusion and the chemical nature of the protein surface. Thus, the dependence of the exclusion of glycerol on the surface polarity of proteins argues against the operation solely of a simple statistical exclusion but suggests the contribution also of a chemically more defined type of interaction. Other factors that may affect the chemical potential of proteins include a change in the dielectric constant of the solvent and the lowering of water activity. In the glycerol system, however, both effects are small and can make only a minor contribution to the increased activity coefficients of proteins (Lakshmi & Nandi, 1976).

A considerable fraction of the surface of a typical native globular protein is hydrophobic in the sense that the surface is occupied by atoms which have no ability to form hydrogen bonds (Bull & Breese, 1968; Lee & Richards, 1971; Shrake & Rupley, 1973; Chothia, 1974, 1975). The polar or ionic groups on the protein bind strongly the water molecules around them through dipole–dipole or ion–dipole interactions. Water molecules also make cagelike structures around hydrophobic groups (Tanford, 1978), with a compressibility between those of normal water and ice (Conway & Verrall, 1966; Gekko & Noguchi, 1974). This compressibility is considerably greater than that of electrostricted hydration around ionizable groups (Desnoyers et al., 1965). These differences in the hydration behavior between polar and nonpolar groups suggest that the

preferential interactions of the hydrophobic areas of a protein should be different from those of polar regions. Since the surface of a protein may be regarded as a mosaic of regions of different degrees of polarity and hydrophobicity, it seems useful for the sake of analysis to divide the preferential interaction into two distinct contributions, one due to polar regions, $(\partial g_3/\partial g_2)_p$, the other to nonpolar areas, $(\partial g_3/\partial g_2)_{np}$. Then

$$(\partial g_3/\partial g_2)_{obsd} = (\partial g_3/\partial g_2)_p + (\partial g_3/\partial g_2)_{np} = (A_3^p + A_3^{np}) - g_3(A_1^p + A_1^{np})$$

where the subscripts in $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ have been omitted. It seems reasonable that the polar proteins, such as ribonuclease which is one of the most polar globular proteins, "bind" glycerol essentially in direct proportion to glycerol concentration through the interaction of glycerol with the water of hydration around polar groups (see Figure 3). On the other hand, in the case of chymotrypsinogen which is a very hydrophobic protein, the binding of glycerol may involve significant contributions from interactions both on the polar and the nonpolar surfaces. This could give rise to the strong curvature in Figure 3 because the glycerol concentration dependences of $(\partial g_3/\partial g_2)_p$ and $(\partial g_3/\partial g_2)_{np}$ need not be the same. A change in pH should have a large effect on the $(\partial g_3/\partial g_2)_p$ term but cause no significant variation in the $(\partial g_3/\partial g_2)_{np}$ term. In general, A_1^p should be greater at pH 5.8 than at pH 2.0 because most carboxyl groups are ionized at pH 5.8 and are neutral at pH 2.0. This factor would make $(\partial g_3/\partial g_2)$ more negative at high pH, resulting in a decrease in $(\partial g_3/\partial g_2)_{obsd}$ at pH 5.8. On the other hand, the effect of protein polarity on $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ cannot be predicted in straightforward fashion. It is likely that A_1^p increases and A_1^{np} decreases with increasing polarity of proteins. This should contribute to a decrease in $(\partial g_3/\partial g_2)_p$ and an increase in $(\partial g_3/\partial g_2)_{np}$. A correlation between $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ and P is more likely, therefore, at pH 2.0 than at pH 5.8, since at the low pH all the proteins show good solubility and A_1 should make a smaller contribution. The results that A_3 increases with increasing protein polarity (see Figure 6) is consistent with the concept that glycerol is essentially a hydrophilic compound capable of occupying a part of the solvation sheath around a protein with a concomitant stabilization of solvent structure around the protein.

A final question needs to be asked: In what manner do the present observations on preferential interactions bear on the problem of the structural stabilization of proteins by addition of glycerol to the aqueous medium? A general answer seems to be clear. In aqueous medium, glycerol is preferentially excluded from the domain of the protein. This means that addition of glycerol raises the chemical potential of the protein. Such a situation is thermodynamically unfavorable. Since this effect is general and nonspecific and can, as a first approximation, be regarded as statistically distributed over the entire protein–solvent interface, it would seem that any reduction of this interface will render the system less unfavorable thermodynamically. Denaturation, or unfolding, involves an increase in the surface of contact between protein and solvent, and in particular exposes additional hydrophobic residues to contact with solvent. In the presence of glycerol, this would increase the thermodynamically unfavorable situation and require the use of more free energy for unfolding than in water. As a result, the presence of glycerol should tend to favor the more folded, or native, state.

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