Steric exclusion is the principal source of the preferential hydration of proteins in the presence of polyethylene glycols

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Abstract

The preferential interactions of bovine serum albumin, lysozyme, chymotrypsinogen, ribonuclease A, and β -lactoglobulin with polyethylene glycols (PEGs) of molecular weight 200–6,000 have been measured by dialysis equilibrium coupled with high precision densimetry. All the proteins were found to be preferentially hydrated in all the PEGs, and the magnitude of the preferential hydration increased with increasing PEG size for each protein. The change in the chemical potentials of the proteins with the addition of the PEGs had highly positive values, indicating a strong thermodynamic destabilization of the system by the PEGs. A viscosity study of the PEGs showed them to be randomly coiled polymers, as their radii of gyration were related to the molecular weight by $R_g = aM^{0.55}$. The thickness of the effective shell impenetrable to PEG around protein molecules, calculated from the preferential hydration, was found to vary with PEG molecular weight in similar fashion as the PEG radius of gyration, supporting the proposal (Arakawa, T. & Timasheff, S.N., 1985a, *Biochemistry* 24, 6756–6762) that the preferential exclusion of PEGs from proteins is due principally to the steric exclusion of PEG from the protein domain, although favorable interactions with protein surface residues, in particular nonpolar ones, may compete with the exclusion. These thermodynamically unfavorable preferential exclusion interactions lead to the action of PEGs as precipitants, although they may destabilize protein structure at higher temperatures.

Keywords: chemical potential; hydrodynamics; polyethylene glycol; preferential exclusion; preferential hydration; protein solubility; protein stability; radius of gyration; random coil polymers; steric exclusion

Polyethylene glycols (PEGs) are used extensively for the precipitation and crystallization of proteins (McPherson, 1976; Atha & Ingham, 1981). McPherson has suggested that PEGs may be the best reagent for crystallizing proteins and that the optimum PEG molecular weight for this purpose is between 2,000 and 6,000 (McPherson, 1985). Their precipitant, "salting-out" action is caused by the strong preferential hydration of proteins in their presence (Lee & Lee, 1979, 1981; Arakawa & Timasheff, 1985a; Arakawa et al., 1990a). Their preferential exclusion has also been reported from liposome surfaces (Arnold et al., 1990). On the other hand, PEGs have been found to destabilize proteins in thermal denaturation (Lee & Lee, 1987), although this is not universal as they have

little effect on the thermal unfolding of ribonuclease A (RNase A) (Atha & Ingham, 1981; Knoll & Hermans, 1981; Lee & Lee, 1987) and, in fact, act as stabilizers of penicillin acylase (Andersson & Hahn-Hägerdal, 1987) and Ca-ATPase (Welte et al., 1989). This behavior is contrary to that of a number of solvents, such as sugars, polyols, salting-out salts, and most amino acids, that induce a strong preferential hydration of native proteins either by raising the surface tension of water or by increasing solvophobicity and universally stabilize globular proteins against thermal denaturation (Back et al., 1979; Uedaira & Uedaira, 1980; Gekko & Morikawa, 1981; Gekko & Timasheff, 1981; Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a,b, 1985b). The preferential hydration induced by the PEGs must have a different origin, because the PEGs lower the surface tension of water (Arakawa & Timasheff, unpubl.) and display a strong nonpolar character (Hammes & Schimmel, 1967). Another effective agent in the promotion of the growth of usable

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crystals for structural studies, 2-methyl-2,4-pentanediol (MPD) shares with the PEGs the properties of lowering the surface tension of water (Arakawa & Timasheff, unpubl.) and of having a good affinity for nonpolar residues (Pittz & Bello, 1971; Arakawa et al., 1990b). The preferential exclusion of MPD is believed to be due to repulsion from charges on the surface of globular proteins (Pittz & Timasheff, 1978).

In early studies, Lee and Lee (1979, 1981) have proposed that the preferential exclusion of PEGs from protein surface has contributions from protein hydrophobicity and charge, as well as the bulkiness of the molecules. Using PEGs of low molecular weights (between 200 and 1,000), Arakawa and Timasheff (1985a) found that, for β -lactoglobulin (β -LG), the preferential hydration increased with the PEG size and proposed that the primary cause of the preferential exclusion of PEGs from proteins is their steric exclusion from the protein surface, a mechanism of preferential hydration that was first proposed by Kauzmann in 1949 (quoted in Schachmann & Lauffer, 1949). In order to test this hypothesis, studies have been carried out on the preferential interactions of several proteins, that vary in molecular weight and surface polarity, with PEGs both of low (200–1,000) and high molecular weights (2,000–6,000). These measured interactions have been related to the hydrodynamic and thermodynamic properties of these polymers and the results are reported in this paper.

Results

The preferential interactions of several proteins (bovine serum albumin [BSA], lysozyme, chymotrypsinogen, and RNase) with 10% (w/v) solutions of PEG 200–1,000 were determined by high precision densimetry. Similar measurements of the same proteins and β -LG were made in more dilute solutions of PEGs 2,000–6,000. The results are presented in Tables 1 and 2 together with the derived preferential interaction parameters.² The essential constancy of the values of the isomolal partial specific volume (ϕ_2^o) for each protein testifies to the absence of PEG-induced unfolding. The higher value for BSA at pH 3.0 than at pH 7.0 is consistent with the known expansion of this protein at low pH (Yang & Foster, 1954) and that of RNase A at pH 2.0 than 3.0 with its partial unfolding at the lower pH (Brandts & Hunt, 1967). Using the notation that components 1, 2, and 3 are water, protein, and PEG, respectively (Scatchard, 1946; Stockmayer, 1950), the preferential binding parameter, $(\partial g_3/\partial g_2)_{T, \mu_1, \mu_3}$, was calculated from

$$(\partial g_3/\partial g_2)_{T, \mu_1, \mu_3} = \rho_o(\phi_2^o - \phi_2'^o)/(1 - \rho_o \bar{v}_3), \quad (1)$$

² A simplified and more detailed explanation of the interpretation of these types of results can be found in, e.g., Timasheff and Arakawa (1988a).

where T is the thermodynamic (Kelvin) temperature, g_i is the concentration of component i in grams i per gram of water, μ_i is its chemical potential, and ρ_o is the density of the solvent. The measured values of $\bar{v}_3$, the partial specific volumes of the PEGs in water at 20 °C, are listed in Tables 3 and 4. All of the values of $(\partial g_3/\partial g_2)_{T, \mu_1, \mu_3}$ measured for the various proteins were found to be negative, meaning that both the high and low molecular weight PEGs are preferentially excluded from all the proteins studied. The increase in the negativity of this parameter with PEG molecular weight for all of the proteins points to the size of the cosolvent molecule as a determining factor of the extent of exclusion. Because the preferential binding parameter is the extent of "binding" of the ligand (PEG) to the protein measured in a dialysis equilibrium experiment, the exclusion of solvent components from the protein domain is equivalent to an excess of water in the domain of the protein, i.e., to preferential hydration of the protein. The two parameters are related by (Timasheff & Kronman, 1959; Timasheff, 1963)

$$(\partial g_1/\partial g_2)_{T, \mu_1, \mu_3} = -(g_1/g_3)(\partial g_3/\partial g_2)_{T, \mu_1, \mu_3}. \quad (2)$$

The values listed in column 6 of Tables 1 and 2 are seen to be increasingly positive with an increase in the PEG molecular weight. They attain magnitudes much higher than the usual protein hydration, which is 0.2–0.4 g/g (Bull & Breese, 1968; Kuntz, 1971).

Preferential binding (positive or negative) is a consequence of the mutual perturbations of the protein and PEG chemical potentials (Kirkwood & Goldberg, 1950; Casassa & Eisenberg, 1964)

$$\begin{aligned} (\partial \mu_3/\partial m_2)_{T, P, m_3} &= (\partial \mu_2/\partial m_3)_{T, P, m_2} \\ &= -(\partial m_3/\partial m_2)_{T, \mu_1, \mu_3}(\partial \mu_3/\partial m_3)_{T, P, m_2} \\ (\partial m_3/\partial m_2)_{T, \mu_1, \mu_3} &= (M_2/M_3)(\partial g_3/\partial g_2)_{T, \mu_1, \mu_3}, \end{aligned} \quad (3)$$

where m_i is the molal concentration of component i , M_i is its molecular weight, and P is pressure; $(\partial \mu_3/\partial m_3)_{T, P, m_2}$ is the self-interaction parameter of PEG, given by

$$(\partial \mu_3/\partial m_3)_{T, P, m_2} = [(RT/m_3) + RT(\partial \ln \gamma_3/\partial m_3)_{T, P, m_2}], \quad (4)$$

where γ_3 is the activity coefficient of PEG, and R is the universal gas constant. The values of $(\partial \ln \gamma_3/\partial m_3)$ used for PEGs 200–1,000 were those calculated by Arakawa and Timasheff (1985a) from activity coefficient and osmotic coefficient data (Elworthy & Florence, 1966; Rogers & Tam, 1977). For PEGs 2,000–6,000 the second term on the right-hand side of Equation 4 was evaluated from the osmotic coefficient data measured by Rogers and

Table 1. Preferential interaction parameters for proteins in 10% w/v low molecular weight polyethylene glycol solutions

Molecular weight PEG	m_3 (mol PEG/1,000 g H ₂ O)	ϕ_2^0 (mL/g)	$\phi_2^{0'}$ (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial \mu_2/\partial m_3)_{T,P,m_2}$ (kcal(mol of protein) ⁻¹ (mol of PEG) ⁻¹)
BSA (pH 7.0)							
200	0.54	0.734 ± 0.001	0.742 ± 0.001	-0.062 ± 0.014	0.574 ± 0.130	-21.08 ± 4.76	24.8 ± 5.6
400	0.27	0.735 ± 0.001	0.744 ± 0.001	-0.066 ± 0.014	0.611 ± 0.130	-11.22 ± 2.38	27.3 ± 5.8
600	0.181	0.734 ± 0.001	0.748 ± 0.002	-0.101 ± 0.020	0.935 ± 0.185	-11.45 ± 2.27	55.4 ± 11.0
1,000	0.108	0.734 ± 0.001	0.752 ± 0.002	-0.128 ± 0.020	1.185 ± 0.185	-8.70 ± 1.36	73.0 ± 11.4
BSA (pH 3.0)							
200	0.54	0.744 ± 0.002	0.750 ± 0.001	-0.046 ± 0.022	0.426 ± 0.204	-15.64 ± 7.48	18.4 ± 8.8
400	0.27	0.744 ± 0.001	0.752 ± 0.001	-0.058 ± 0.014	0.537 ± 0.130	-9.86 ± 2.38	24.0 ± 5.8
600	0.181	0.742 ± 0.001	0.753 ± 0.001	-0.079 ± 0.014	0.731 ± 0.130	-8.95 ± 1.59	43.1 ± 7.7
1,000	0.108	0.742 ± 0.001	0.757 ± 0.001	-0.106 ± 0.013	0.981 ± 0.120	-7.21 ± 0.88	60.5 ± 7.4
Lysozyme (pH 7.0)							
200	0.54	0.713 ± 0.001	0.715 ± 0.001	-0.015 ± 0.014	0.138 ± 0.128	-1.07 ± 1.00	1.25 ± 1.2
400	0.27	0.718 ± 0.002	0.722 ± 0.001	-0.028 ± 0.021	0.257 ± 0.193	-1.00 ± 0.75	2.4 ± 1.8
600	0.181	0.716 ± 0.001	0.724 ± 0.001	-0.055 ± 0.014	0.505 ± 0.128	-1.31 ± 0.33	6.3 ± 1.6
1,000	0.108	0.715 ± 0.001	0.729 ± 0.001	-0.095 ± 0.013	0.827 ± 0.119	-1.36 ± 0.19	11.3 ± 1.6
Chymotrypsinogen (pH 3.0)							
200	0.54	0.729 ± 0.001	0.736 ± 0.002	-0.052 ± 0.022	0.477 ± 0.202	-6.68 ± 2.83	7.8 ± 3.3
400	0.27	0.728 ± 0.001	0.743 ± 0.002	-0.107 ± 0.021	0.982 ± 0.193	-6.87 ± 1.35	16.6 ± 3.3
600	0.181	0.733 ± 0.001	0.747 ± 0.001	-0.098 ± 0.014	0.899 ± 0.128	-4.20 ± 0.60	20.2 ± 2.9
1,000	0.108	0.732 ± 0.001	0.753 ± 0.002	-0.145 ± 0.020	1.330 ± 0.183	-3.73 ± 0.51	31.1 ± 4.3
RNase A (pH 2.0)							
200	0.54	0.697 ± 0.002	0.706 ± 0.002	-0.067 ± 0.029	0.615 ± 0.266	-4.59 ± 1.99	5.4 ± 2.3
400	0.27	0.692 ± 0.001	0.711 ± 0.001	-0.136 ± 0.014	1.248 ± 0.128	-4.66 ± 0.48	11.3 ± 1.2
600	0.181	0.696 ± 0.002	0.720 ± 0.001	-0.168 ± 0.020	1.541 ± 0.183	-3.84 ± 0.46	18.5 ± 2.2
1,000	0.108	0.695 ± 0.001	0.723 ± 0.001	-0.194 ± 0.013	1.780 ± 0.119	-2.66 ± 0.18	22.2 ± 1.5

^a The g_3 value used to calculate $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (Equation 2) was 0.108 g of PEG/g of water.

Table 2. Preferential interaction parameters for some proteins in aqueous high molecular weight polyethylene glycol solutions at 20 °C

Molecular weight PEG	Concentration (% [w/v])	ϕ_2^0 (mL/g)	ϕ_2^{10} (mL/g)	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	$(\partial \mu_2/\partial m_3)_{T,P,m_2}$ (kcal/mol protein) ⁻¹ (mol PEG) ⁻¹	m_3 (mol PEG/1,000 g H ₂ O)
BSA (pH 7.0)								
2,000	4	0.740 ± 0.001	0.754 ± 0.001	-0.093 ± 0.012	2.214 ± 0.286	-3.16 ± 0.41	137.6 ± 17.9	0.021
3,000	4	0.742 ± 0.001	0.757 ± 0.001	-0.099 ± 0.012	2.357 ± 0.286	-2.42 ± 0.27	164.2 ± 19.8	0.014
	6	0.741 ± 0.002	0.759 ± 0.001	-0.121 ± 0.019	1.921 ± 0.302	-2.74 ± 0.43	162.9 ± 25	0.021
4,000	4	0.742 ± 0.001	0.760 ± 0.002	-0.119 ± 0.019	2.830 ± 0.452	-2.02 ± 0.32	237.9 ± 37.7	0.0105
6,000	4	0.741 ± 0.001	0.765 ± 0.001	-0.158 ± 0.012	3.762 ± 0.286	-1.79 ± 0.14	394.7 ± 30.9	0.007
Lysozyme (pH 7.0)								
2,000	4	0.716 ± 0.001	0.731 ± 0.002	-0.095 ± 0.019	2.262 ± 0.452	-0.68 ± 0.14	29.6 ± 6.1	0.021
3,000	4	0.717 ± 0.001	0.734 ± 0.001	-0.107 ± 0.012	2.548 ± 0.286	-0.51 ± 0.06	37.4 ± 4.4	0.014
4,000	2	0.715 ± 0.001	0.739 ± 0.002	-0.147 ± 0.018	7.350 ± 0.900	-0.53 ± 0.06	94.8 ± 10.7	0.005
6,000	2	0.716 ± 0.002	0.744 ± 0.002	-0.172 ± 0.024	8.600 ± 1.200	-0.41 ± 0.06	128.7 ± 18.8	0.0033
α -Chymotrypsinogen (pH 3.0)								
2,000	1	0.730 ± 0.002	0.743 ± 0.001	-0.082 ± 0.018	8.20 ± 1.8	-1.05 ± 0.23	138.9 ± 30.4	0.005
	2	0.732 ± 0.001	0.748 ± 0.002	-0.101 ± 0.018	5.05 ± 0.9	-1.30 ± 0.23	96.3 ± 17.0	0.010
3,000	1	0.732 ± 0.001	0.749 ± 0.002	-0.106 ± 0.018	10.60 ± 1.8	-0.91 ± 0.15	189.5 ± 31.2	0.0033
	2	0.731 ± 0.002	0.755 ± 0.001	-0.151 ± 0.018	7.55 ± 0.9	-1.29 ± 0.15	151.4 ± 17.6	0.0068
4,000	1	0.730 ± 0.002	0.753 ± 0.002	-0.143 ± 0.024	14.30 ± 2.4	-0.92 ± 0.15	271.7 ± 44.3	0.0025
6,000	1	0.730 ± 0.001	0.753 ± 0.002	-0.144 ± 0.018	14.40 ± 1.8	-0.62 ± 0.08	297.6 ± 38.4	0.0017
β -LG (pH 2.0)								
2,000	4	0.746 ± 0.001	0.781 ± 0.001	-0.219 ± 0.012	5.214 ± 0.286	-2.01 ± 0.11	87.5 ± 4.8	0.021
3,000	4	0.743 ± 0.001	0.789 ± 0.002	-0.286 ± 0.019	6.809 ± 0.452	-1.75 ± 0.12	128.3 ± 8.8	0.014
4,000	4	0.744 ± 0.001	0.799 ± 0.002	-0.342 ± 0.019	8.143 ± 0.452	-1.57 ± 0.09	184.9 ± 10.6	0.0105
6,000	4	0.744 ± 0.001	0.810 ± 0.002	-0.410 ± 0.019	9.762 ± 0.452	-1.26 ± 0.06	277.8 ± 13.2	0.007
RNase A (pH 2.0)								
2,000	2	0.693 ± 0.002	0.721 ± 0.002	-0.177 ± 0.024	8.85 ± 1.2	-1.21 ± 0.16	89.6 ± 11.8	0.01
3,000	2	0.693 ± 0.001	0.728 ± 0.002	-0.221 ± 0.018	11.05 ± 0.9	-1.01 ± 0.08	118.5 ± 9.4	0.0068
4,000	2	0.689 ± 0.001	0.733 ± 0.002	-0.274 ± 0.018	13.70 ± 0.9	-0.94 ± 0.06	168.1 ± 10.7	0.005
6,000	2	0.693 ± 0.002	0.740 ± 0.002	-0.295 ± 0.024	14.75 ± 1.2	-0.67 ± 0.05	210.3 ± 15.7	0.0033
RNase A (pH 3.0)								
2,000	2	0.686 ± 0.002	0.728 ± 0.001	-0.265 ± 0.018	13.25 ± 0.9	-1.81 ± 0.12	134.0 ± 8.9	0.01
3,000	2	0.684 ± 0.001	0.729 ± 0.002	-0.283 ± 0.018	14.15 ± 0.9	-1.29 ± 0.08	151.4 ± 9.4	0.0068
4,000	2	0.684 ± 0.001	0.730 ± 0.001	-0.286 ± 0.012	14.30 ± 0.6	-0.98 ± 0.04	175.2 ± 7.2	0.005
6,000	2	0.685 ± 0.002	0.744 ± 0.002	-0.369 ± 0.024	18.45 ± 1.2	-0.84 ± 0.05	263.6 ± 15.7	0.0033

^a The g_3 values used to calculate $(\partial g_1/\partial g_2)$ (Equation 2) were 0.010 g of PEG/g of water at 1%, 0.020 at 2%, 0.042 at 4%, and 0.063 at 6% PEG concentration.

Table 3. Characteristic parameters of low molecular weight PEGs in a 10% w/v solution

Molecular weight	$\bar{v}_3$ (mL/g)	RT/m_3 (cal/mol of PEG) ⁻²	$RT(\partial \ln \gamma_3/\partial m_3)$ (cal/mol of PEG) ⁻²	R_g (Å)
200	0.848	1,060	100	5.6
400	0.841	2,120	280	8.1
600	0.838	3,190	1,600	10.0
1,000	0.836	5,340	3,000	13.0

Tam (1977) for PEGs 1,500, 3,000, and 8,000, using the relation

$$\ln \gamma_3 = (\phi_3 - 1) + \int_0^{m_3} [(\phi_3 - 1)/m_3] dm_3, \quad (5)$$

where ϕ_3 is the osmotic coefficient of the PEG solution. The values are listed in Table 4. No PEG concentration dependence of $(\partial \ln \gamma_3/\partial m_3)_{T,P,m_2}$ could be established for PEGs 2,000–6,000 due to the paucity of the data, which introduces an uncertainty into the absolute values of $(\partial \mu_2/\partial m_3)_{T,P,m_2}$ for high molecular weight PEGs, calculated by Equation 3. Comparison of the magnitudes of the two terms on the right-hand side of Equation 4 shows that neglect of the activity coefficient term in the evaluation of $(\partial \mu_2/\partial m_3)_{T,P,m_2}$ would lead to serious errors, in particular with the larger PEG molecules, for which it can be greater than RT/m_3 .

The perturbation of the chemical potential of the proteins by the addition of PEG, given in column 8 of Tables 1 and 2, is highly positive and increasingly so with increase in PEG molecular weight. This means that the protein-PEG interactions are unfavorable in a PEG-size dependent manner. Conversely, the exclusion of PEG from proteins on a molar basis (column 7 of Tables 1 and 2) decreases with an increase in PEG molecular weight. This is due to the increase of the PEG nonideality with

increasing molecular weight, as is evident from the parameters listed in Tables 3 and 4.³

The increase of the preferential exclusion and of the chemical potential perturbation with PEG molecular weight suggested a possible correlation with molecular dimension parameters of the polymers. With this in view, the hydrodynamic properties of PEG 2,000, 3,000, 4,000, and 6,000 were examined by viscosity measurements. Figure 1 presents the concentration dependence of η_{sp}/C , where η_{sp} is the specific viscosity. Extrapolation to zero PEG concentration gave the intrinsic viscosity, $[\eta]$, values listed in Table 4. These were found to obey the relation

$$[\eta] = K'M^{0.645}, \quad (6)$$

which indicates a hydrodynamic behavior typical for a randomly coiled polymer in a relatively good solvent (Tanford, 1961). For a flexible polymer, knowledge of its intrinsic viscosity permits the calculation of its radius of gyration, R_g , by (Tanford, 1961)

$$R_g = \left[\frac{3[\eta]M}{10\pi N\xi^3} \right]^{1/3}, \quad (7)$$

where the intrinsic viscosity is expressed in cm³/g, M is the molecular weight of the PEG, N is the Avogadro number, and ξ is the Flory-Fox parameter, taken as 0.80 in the present calculation. The resulting values of R_g for PEGs 2,000–6,000 are listed in Table 4 and plotted as a function of PEG molecular weight in Figure 2. It is seen that this plot is curved. The logarithmic plot, shown in

³ The nonideality of the high molecular weight PEGs in aqueous solution was found to be proportional to molecular weight, as $(\partial \ln \gamma_3/\partial g_3)_{T,P,m_2} = (0.0066 \pm 0.0008)M_3$. This suggests a major contribution of the polymer excluded volume to its nonideality in aqueous solution. The equivalent sphere model predicts a dependence on R_g^3/M_3 , i.e., on $M_3^{1/2}$. This power dependence should increase in a complex manner as a consequence of interactions with water molecules (Tanford, 1961).

Table 4. Characteristic parameters of high molecular weight PEGs in aqueous solution at 20 °C

	PEG concentration (% [w/v])	PEG 2,000	PEG 3,000	PEG 4,000	PEG 6,000
$\bar{v}_3$ (mL/g)	1	0.836	0.835	0.835	0.836
	2	0.835	0.835	0.833	0.834
	4	0.835	0.834	0.834	0.834
	6		0.834		
$RT(\partial \ln \gamma_3/\partial m_3)$ (cal/mol PEG) ⁻²		15,800	31,700	62,300	137,300
$[\eta]$ (cm ³ /g)		8.7	11.3	13.9	17.1
R_g (Å)		18.0	22.6	26.6	32.7

Fig. 1. Dependence of the specific viscosity of high molecular weight PEGs on concentration. a, PEG 2,000; b, PEG 3,000; c, PEG 4,000; d, PEG 6,000.

the inset, on the other hand, gave a straight line that fitted the equation:

$$R_g = aM^{0.55}. \quad (8)$$

This is consistent with a random coil shape of the polymer (Tanford, 1961). The effective bond length, ℓ , was then calculated using the relation for free-draining coils

Fig. 2. Radius of exclusion of high molecular weight PEGs in contact with various proteins: $\circ$, BSA; Δ , lysozyme; $\square$, chymotrypsinogen; $\bullet$, β -lactoglobulin; $\blacktriangle$, RNase, pH 2.0; $\blacksquare$, RNase, pH 3.0; $\diamond$, radius of gyration (R_g) of PEG as calculated from viscosity measurements. The solid line is the best curve drawn through the R_g values; the dashed lines are the best straight lines drawn through the data points (no lines were drawn through the chymotrypsinogen and β -LG data because of crowding). Inset: Logarithmic dependence of R_g on the PEG molecular weight.

$$\ell = \frac{3^{1/2} R_g}{\sigma^{1/2}}, \quad (9)$$

where σ is the number of bonds, taken as three for each $-(CH_2-CH_2-O)-$ repeat unit of PEG. The resulting value was $\ell = 2.75 \pm 0.04 \text{ \AA}$. Its difference from the minimal value of $\ell = 1.54 \text{ \AA}$ (the C—C bond distance with unrestricted rotation) indicates that polyethylene glycol is not a fully flexible random coil in aqueous solution, but that there is some restriction in the rotation about the bonds along the chain.

Discussion

All the proteins examined in this study were preferentially hydrated in the presence of both low and high molecular weight PEGs, and the magnitude of the cosolvent exclusion increased with the size of PEG. The results are consistent with what had been found earlier with β -LG for PEGs 200–1,000 (Arakawa & Timasheff, 1985a) and with the only previous measurements on a high molecular weight PEG, namely the study of Lee and Lee (1981) on the interaction of PEG 4,000 with tubulin and lysozyme. It would appear, therefore, that the principal mechanism of the preferential exclusion of both low and high molecular weight PEGs from proteins is steric exclusion of the bulky PEG molecules, as proposed earlier (Arakawa & Timasheff, 1985a). Although for high molecular weight PEGs the selection of PEG concentration was limited by the low solubility of some proteins, in the case of BSA and chymotrypsinogen it was found that an increase in the PEG concentration decreased the preferential hydration, just as had been found earlier with β -LG in low molecular weight PEG (Arakawa & Timasheff, 1985a). This, within the uncertainty of the calculated values of $\log \gamma_3$, reflects the nonideality of the polymer solutions.

The positive perturbations of the chemical potentials of the proteins on addition of PEG indicate that the predominant interactions between PEG and proteins in aqueous medium are repulsive in nature and that this repulsion becomes stronger with an increase in PEG size. Such a thermodynamically unfavorable situation can be relieved either by a change in the protein structure, i.e., denaturation, or by the formation of interprotein contacts that reduce the surface area of protein exposed to the solvent and hence reduce the interactions. This can be accomplished either by protein self-association or precipitation, i.e., by a reduction of protein solubility (Arakawa et al., 1990a). The salting-out of a protein by an additive, PEG in the present case, is related to the chemical potential perturbation by⁴

⁴ For a detailed discussion of the relation between protein solubility and preferential interactions, see Timasheff and Arakawa (1988b) as well as the Appendix of Arakawa et al. (1990a).

$$\log(S_{2,m_3}/S_{2,w}) = \frac{1}{2.303RT} (\Delta\mu_{2,tr}^{pr} - \Delta\mu_{2,tr}^{sol}) \quad (10a)$$

$$\Delta\mu_{2,tr} = \int_0^{m_3} (\partial\mu_2/\partial m_3)_{T,P,m_2} dm_3, \quad (10b)$$

where S_{2,m_3} and $S_{2,w}$ are the protein solubilities in PEG solution and water, respectively, $\Delta\mu_2$ is the transfer free energy of the protein from water to the PEG solution, and the superscripts *pr* and *sol* refer to precipitate and dispersed solution, respectively. In the salting-out process, by definition, the structure of the protein does not change. Therefore, the nature of the interactions between the protein and solvent components can be regarded as not changing during precipitation. Therefore, if $\Delta\mu_{2,tr}$ is positive in both end states as deduced from the positive values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, it follows from the decrease in protein-solvent contact area that $\Delta\mu_{2,tr}^{pr} < \Delta\mu_{2,tr}^{sol}$, and $S_{2,m_3} < S_{2,w}$. It is clear, therefore, that the basis of the salting-out properties of PEGs 200–6,000 is the thermodynamically unfavorable state of the protein solutions in their presence relative to pure water as medium.

The conclusion that steric exclusion of the bulky PEG molecules is the decisive factor in their preferential exclusion from proteins was tested more quantitatively by calculating the volume of the effective shell surrounding the protein that is impenetrable to the cosolvent, i.e., the volume of exclusion (V_s) of the PEGs from proteins, and comparing the evolution of its thickness with PEG molecular weight with that of the polymer radius of gyration, R_g . This model, which was first proposed by Kauzmann (cited in Schachman & Lauffer, 1949), is shown in the inset of Figure 3. According to this model, the thickness of the shell, defined as the radius of exclusion, R_e , is related to the measured preferential hydration by, if the protein is assumed to have a spherical shape,

$$(4\pi N/3)[(R_p + R_e)^3 - R_p^3] \times 10^{-24} = (M_2/\rho_w)(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}^{(e)} \equiv V_s, \quad (11)$$

where N is Avogadro's number, R_p is the radius of the protein, M_2 is the molecular weight of the protein, ρ_w is the density of water, and $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}^{(e)}$ is the preferential hydration value extrapolated to infinite dilution of the cosolvent. This last quantity was calculated from the experimentally measured preferential hydrations at a finite concentration of PEG and the assumption that $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is concentration independent by using the relation:

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = g_3 \frac{M_2}{M_3} (\partial g_1/\partial g_2)_{T,\mu_1,\mu_3} (\partial\mu_3/\partial m_3)_{T,P,m_2}. \quad (12)$$

Fig. 3. Dependence on PEG molecular weight of the radius of exclusion of low molecular weight PEGs in contact with various proteins: $\circ$, BSA, pH 3.0; Δ , BSA, pH 7.0; $\square$, lysozyme; $\bullet$, chymotrypsinogen; $\blacktriangle$, RNase, pH 2.0; $\blacksquare$, β -LG; $\diamond$, radius of gyration of PEG calculated from the chain length as described in the text. (The β -lactoglobulin data are taken from Arakawa & Timasheff [1985a].) The solid line is the best curve through the R_g values; the dashed lines are the best straight lines drawn through the data points (no lines were drawn through the chymotrypsinogen and RNase data because of crowding). **Inset:** Schematic representation of the steric exclusion model.

This assumption was based on the previous observation (Arakawa & Timasheff, 1985a) for β -LG in the low molecular weight PEGs and the findings in a number of studies that $\log(\text{solubility})$ of proteins decreases linearly with PEG concentration (Lee & Lee, 1979; Atha & Ingham, 1981; Stevenson & Hageman, 1988). This, by Equation 10, requires that the preferential interaction parameter be independent of PEG concentration. The values of R_p were calculated from the molecular weights and partial specific volumes of the proteins in dilute buffer, assuming spherical shape. This gave $R_p = 27$ Å for BSA, 19.5 Å for chymotrypsinogen, 15.8 Å for lysozyme, 18.0 Å for β -LG, and 15.5 Å for RNase A.

The resulting values of R_e for the PEGs in contact with the different proteins are listed in Tables 5 and 6 and plotted as a function of PEG molecular weight in Figures 2 and 3.⁵ For both sets, all increase in similar fashion with increasing PEG molecular weight. The spread in slopes reflects the accumulation of uncertainties in the sequential calculations needed to derive these numbers, such as the assumption of spherical symmetry for the proteins, and errors in the calculation of PEG activity coefficients, especially for high molecular weight PEGs for which no concentration dependence of $(\partial \ln \gamma_3/\partial m_3)_{T,P,m_2}$ could

⁵ The values of R_e used in this analysis should not be regarded as exact molecular parameters, but only as relative quantities calculated in a consistent manner from the experimental results for the sole purpose of comparing the PEG molecular weight dependence of the preferential exclusion from the various proteins.

Table 5. Radius of exclusion of low molecular weight PEGs in contact with various proteins

Molecular weight PEG	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}^{(e)}$ (g/g)	R_e (Å)
BSA (pH 3.0)		
200	0.463	4.80
400	0.605	5.99
600	1.086	9.53
1,000	1.525	12.30
BSA (pH 7.0)		
200	0.625	6.17
400	0.688	6.67
600	1.396	11.47
1,000	1.840	14.00
Lysozyme (pH 7.0)		
200	0.150	1.06
400	0.288	1.92
600	0.756	4.34
1,000	1.356	6.78
Chymotrypsinogen (pH 3.0)		
200	0.520	3.84
400	1.107	7.00
600	1.347	8.06
1,000	2.074	11.0
RNase A (pH 2.0)		
200	0.675	3.98
400	1.413	7.00
600	2.313	9.80
1,000	2.775	11.10

Table 6. Thickness of the preferential hydration shell in the presence of high molecular weight PEGs

Molecular weight PEG	$(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}^e$ (g/g)	R_e (Å)
2,000		
BSA	3.47	21.4
Lysozyme	3.52	13.0
Chymotrypsinogen	7.9	24.7
β -LG	4.08	22.6
RNase, pH 2	11.2	24.6
RNase, pH 3	16.8	30.2
3,000		
BSA	4.12	23.8
Lysozyme	4.49	15.1
Chymotrypsinogen	11.4	30.2
β -LG	5.98	27.7
RNase, pH 2	14.8	28.3
RNase, pH 3	18.9	31.9
4,000		
BSA	6.0	29.6
Lysozyme	11.4	25.2
Chymotrypsinogen	18.1	38.2
β -LG	8.62	33.2
RNase, pH 2	21.0	33.5
RNase, pH 3	21.9	34.2
6,000		
BSA	9.95	39.1
Lysozyme	15.4	29.2
Chymotrypsinogen	19.8	39.9
β -LG	12.9	40.3
RNase, pH 2	26.3	34.7
RNase, pH 3	33.0	41.3

be established. From these results, it would appear that the increment in shell thickness (radius of exclusion) per identical increase in PEG molecular weight is independent of the nature of the protein. The mutual displacement of the parallel lines must reflect both experimental uncertainty and possible contributions of protein-specific factors to the preferential exclusion. The slopes of the R_e vs. PEG molecular weight plots were 0.0083 ± 0.0010 and 0.0042 ± 0.0008 for the low and high molecular weight PEGs, respectively. The corresponding slopes of the best straight lines drawn through the R_g vs. PEG molecular weight data were 0.0091 and 0.0037, respectively. The similarity of the slopes of the R_g and R_e linear plots as a function of molecular weight both for the high and the low molecular weight PEGs, but their decrease when passing from the low molecular weight to the high molecular weight polymers suggests that R_e should follow a dependence other than linear on PEG molecular weight. The variation of R_g with the square root of the PEG molecular weight suggests that the thickness of the excluded shell should vary in similar manner. Such plots for several proteins, shown in Figure 4, are consistent with a dependence of the radii of exclusion, R_e , on the square root of the PEG molecular weight. This similarity to the dependence of the polymer radius of gyration yields fur-

ther support to the proposed mechanism of the preferential exclusion.

The displacements of the radii of exclusion and, hence, preferential hydrations between proteins suggest that factors other than steric exclusion contribute to the interaction, as proposed by Lee and Lee (1981). Preferential interactions are in general determined by a fine balance between exclusion and binding interactions (Arakawa et al., 1990b). In the case of the PEGs, the volume swept out by these molecules makes a major contribution to the preferential exclusion. The difference between the values obtained for the various proteins must reflect then direct interactions of PEG with proteins. This can be analyzed best by comparing the chemical potential perturbations per unit mass of protein, $(\partial \mu_3/\partial g_2)_{T,P,m_3}$. Attempts to correlate this parameter with protein hydrophobicity, protein hydrophilicity, and charge density did not give any direct correlation. Lee and Lee (1981) reported a correlation with hydrophobicity, with a contribution from charge. The only trends possibly identified in the present study were with hydrophilicity and possibly negatively charged groups. RNase is by far the most hydrophilic (least hydrophobic) protein of those studied here. β -LG

Fig. 4. Dependence of the radius of exclusion of proteins and of the radius of gyration of PEGs on the square root of PEG molecular weight: $\circ$, BSA, pH 7.0; $\blacktriangle$, RNase, pH 2.0; $\triangle$, lysozyme; $\diamond$, radius of gyration (R_g) of PEG.

is extreme at the other end of the scale. The others are clustered in between. PEGs, being hydrophobic in nature (Hammes & Schimmel, 1967), tend to bind to nonpolar regions of proteins, whereas a high hydrophilicity should increase exclusion of PEG by the strong affinity for water. This is consistent with the ordering of the proteins. The high negative charge density on BSA should also increase its preferential hydration, consistent with the reported repulsion of PEGs from negative charges (Lee & Lee, 1981). Thus, although it may be possible to get an inkling of specific features on the protein surface that contribute to the preferential interactions, it would seem that several types of interactions can contribute and a universal correlation is difficult to attain.

That the binding of PEG to nonpolar regions of proteins, as expected from their hydrophobic character (Hammes & Schimmel, 1967), makes a contribution to the measured interactions is apparent from a comparison of the preferential exclusions of low molecular weight PEGs measured with BSA at pH 3.0 and 7.0 and of high molecular weight PEGs measured with RNase A at pH 3.0 and 2.0. The decrease in unfavorable interaction for both proteins when the pH is lowered is clearly brought out in Figure 5 in which the increments in the chemical potentials of the proteins induced by the addition of the PEGs are shown to be consistently less positive at the lower pH. At pH 2.0, RNase A exists in a partially unfolded state (Brandts & Hunt, 1967), whereas BSA is known to expand below pH 4.5 (Yang & Foster, 1954). Both processes must expose nonpolar groups to contact with solvent. The decrease in preferential exclusion can be ascribed, then, to some favorable interactions of PEG with these regions as a consequence of its nonpolar na-

ture (Hammes & Schimmel, 1967). This property of PEG should facilitate its penetration to the protein surface via local favorable interactions. This should have as consequences a reduction of the exclusion and a less positive perturbation of the PEG chemical potential by the protein, just as is observed. This effect of pH on the preferential interactions is strikingly opposite to the effect of glucose on the preferential hydrations of BSA at pH 3.0 and 6.0 (Arakawa & Timasheff, 1982a). Contrary to the current observations with the PEGs, for the sugar solution system the hydration is larger at the lower pH. For example, for BSA in 2 M glucose, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ has values of 0.212 g/g at pH 6.0 and 0.294 g/g at pH 3.0. In the case of 10% PEG 600, the corresponding values were 0.935 g/g at pH 7.0 and 0.731 g/g at pH 3.0. Sugars, including glucose, induce preferential hydration of proteins by increasing the surface tension of water. As a consequence, preferential interactions in the presence of sugars are determined strictly by the surface area of the protein (Lee & Timasheff, 1981). Because for BSA the protein-solvent interfacial area is larger at pH 3.0 than at pH 6.0, the interaction between glucose and the expanded BSA molecule is thermodynamically much more unfavorable at the lower pH (Timasheff et al., 1976). This is manifested by the increase in preferential hydration.

On the basis of observations such as that cited above, the PEGs have been assigned to the class of cosolvents in which the preferential interactions are determined by the balance between exclusion and binding (Arakawa et al., 1990b). The results reported in this paper fully support this assignment. In the light of this, the preferential exclusion of PEGs from native proteins does not necessarily mean that they should also be structure stabilizers, as are sugars and other compounds that act only by recog-

Fig. 5. Perturbation of the PEG chemical potentials by BSA and RNase in the compact and expanded states. $\circ$, BSA, pH 7.0; $\bullet$, BSA, pH 3.0; $\triangle$, RNase, pH 3.0; $\blacktriangle$, RNase, pH 2.0. The interaction parameter is plotted in terms of kcal per mol of PEG per 1,000 g of protein.

nizing a surface. The data on BSA and RNase in the compact and expanded forms suggest this, and PEGs are known to lower the transition temperature of some proteins (Arakawa & Timasheff, 1985a; Lee & Lee, 1987). Nevertheless, at room temperature (20 °C) all the proteins studied have been found to be strongly preferentially hydrated, and the CD spectra of those examined (Lee & Lee, 1979, 1981) have been found to be identical with those of native proteins in dilute buffer. This makes it possible to take advantage of the preferential exclusion of PEGs from proteins and use them effectively for protein precipitation and crystallization, although great care should be exercised at higher temperatures.

Materials and methods

BSA (lot 84F-9300), lysozyme (lot 65F-8170), chymotrypsinogen (lot 73F-8120), RNase A (lot 124F-0334), and β -LG (lot 106F-8120) were procured from Sigma. All proteins were dialyzed at 4 °C against distilled deionized water and then lyophilized after passing through a sintered glass funnel. The lyophilized proteins were dried over phosphorous pentoxide for 3 days at 40 °C and then stored in a desiccator in a refrigerator. Polyethylene glycols 200, 400, 600, and 1,000 were purchased from Sigma, and PEGs 2,000, 3,000, 4,000, and 6,000 from Fluka. All were used without further purification.

Solution densities were measured on an Anton-Paar digital densimeter (model DMA-02C) at 20 °C, as described previously, at isomolal (protein dissolved in the solvent with no dialysis) and isopotential (protein dissolved in the solvent followed by dialysis to equilibrium) conditions (Lee & Timasheff, 1974; Lee et al., 1979). The detailed experimental procedure is described in Lee et al. (1979). The apparent isomolal (ϕ_2^o) and isopotential (ϕ_2^{*o}) partial specific volumes of the proteins were calculated from the density values. For the isopotential measurements, protein solutions were dialyzed against the solvent for 22 h at 4 °C and for another 2–3 h at 20 °C. The solvents used were: BSA, in 0.2 M NaCl, 10^{-3} M HCl, pH 3.0; and in 0.2 M NaCl, 0.01 M sodium phosphate, pH 7.0; lysozyme, in 0.02 M KCl, 0.01 M sodium phosphate, pH 7.0; chymotrypsinogen, in 0.1 M KCl, 10^{-3} M HCl, pH 3.0; RNase A, in 0.1 M KCl, 10^{-2} M HCl, pH 3.0; and in 0.1 M KCl, 10^{-3} M HCl, pH 3.0; and β -LG in 10^{-2} M HCl, pH 2.0.

Protein concentrations were measured spectrophotometrically with a Perkin-Elmer Lambda 3B UV-visible instrument. The values of the absorptivities were: BSA, 0.658 mL/(mg·cm) at 278 nm and pH 7 (Noelken & Timasheff, 1967); lysozyme, 2.635 mL/(mg·cm) at 281.5 nm (Sophianopoulos et al., 1962); chymotrypsinogen, 1.97 mL/(mg·cm) at 282 nm (Jackson & Brandts, 1970); β -LG, 0.96 mL/(mg·cm) at 278 nm (Townend et al., 1960); and RNase A, 0.738 mL/(mg·cm) at 278 nm (Scott & Scheraga, 1963). The extinction coefficients of

BSA at pH 3.0 and RNase A at pH 2.0 were measured by diluting to the same final concentration aliquots of the stock protein solutions in pH 7.0 and pH 3.0 solvents, respectively, with equal volumes of the same buffer and of the pH 3.0 and 2.0 solvents, respectively, and comparing the absorptivities. This resulted in a value of 0.632 mL/(mg·cm) for BSA at pH 3.0, and of 0.726 mL/(mg·cm) for RNase A at pH 2.0. The extinction coefficients of the proteins in various PEG solutions were determined in a similar manner, as described previously (Gekko & Timasheff, 1981).

Viscosity measurements of PEG solutions were performed using an Ubbelohde viscometer that was calibrated with water at 20 °C. The viscometer was housed in a glass water bath whose temperature was controlled to within ± 0.01 °C by passing temperature-controlled water from a cooling unit through copper coils immersed in the bath.

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