Hayashi, R. (1977b) *Methods Enzymol. 47*, 339-351.
Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., & Walsh, K. A. (1972) *Biochemistry 11*, 4493-4502.
Hill, R. L., & Delaney, R. (1967) *Methods Enzymol. 11*, 339-351.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 59-62.
IUPAC-IUB Commission on Biochemical Nomenclature: A One-Letter Notation for Amino Acid Sequences (1968) *J. Biol. Chem. 243*, 3557-3559.
Kägi, J. H. R., & Vallee, B. L. (1960) *J. Biol. Chem. 235*, 3460-3465.
Kägi, J. H. R., & Nordberg, M., Eds. (1979) *Metallothionein*, Birkhäuser, Basel.
Kägi, J. H. R., Vašák, M., Lerch, K., Gilg, D. E. O., Hunziker, P., Bernhard, W. R., & Good, M. (1984) *EHP, Environ. Health Perspect. 54*, 93-103.
Karin, M., Najarian, R., Haslinger, A., Valenzuela, P., Welch, J., & Fogel, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 337-341.
Kissling, M. M., & Kägi, J. H. R. (1977) *FEBS Lett. 82*, 247-250.
Kojima, Y., Berger, C., & Kägi, J. H. R. (1979) in *Metallothionein* (Kägi, J. H. R., & Nordberg, M., Eds.) Birkhäuser, Basel.
Kuiper, H. A., Finazzi-Agro, A., Antonini, E., & Brunori, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2387-2389.
Lastowsky-Perry, D., Otto, E., & Maroni, G. (1985) *J. Biol. Chem. 260*, 1527-1530.
Lerch, K. (1980) *Nature (London) 284*, 368-370.
Lerch, K. (1981) *Met. Ions Biol. Syst. 13*, 299-316.
Lerch, K., & Fischer, E. (1975) *Biochemistry 14*, 2009-2014.
Lerch, K., & Beltramini, M. (1983) *Chem. Scr. 21*, 109-115.
Lerch, K., Ammer, D., & Olafson, R. W. (1982) *J. Biol. Chem. 257*, 2420-2426.
Margoshes, M., & Vallee, B. L. (1957) *J. Am. Chem. Soc. 79*, 4813-4814.
Meisch, H.-U., Schmitt, J. A., & Reinle, W. (1977) *Z. Naturforsch., C: Biosci. 32C*, 172-181.
Mendez, E., & Lai, C. Y. (1975) *Anal. Biochem. 68*, 47-53.
Minkel, D. T., Poulson, K., Wielgus, S., Shaw, C. F., III, & Petering, D. H. (1980) *Biochem. J. 191*, 475-485.
Münger, K., Lerch, K., & Tschierpe, H. J. (1982) *Experientia 38*, 1039-1041.
Münger, K., Germann, U. A., Beltramini, M., Niedermann, D., Baitella-Eberle, G., Kägi, J. H. R., & Lerch, K. (1985) *J. Biol. Chem. 260*, 10032-10038.
Otvos, J. D., & Armitage, I. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7094-7098.
Overnell, J., Berger, C., & Wilson, K. J. (1981) *Biochem. Soc. Trans. 9*, 217-218.
Parker, C. A., & Rees, W. T. (1960) *Analyst (London) 85*, 587-600.
Seger, R. (1978) *Z. Lebensm.-Unters. -Forsch. 166*, 23-34.
Stijve, T., & Besson, R. (1976) *Chemosphere 2*, 151-158.
Tyler, G. (1980) *Trans. Br. Mycol. Soc. 74*, 41-49.
Vašák, M., & Kägi, J. H. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6709-6713.
Weser, U., & Rupp, H. (1979) in *Metallothionein* (Kägi, J. H. R., & Nordberg, M., Eds.) pp 221-230, Birkhäuser, Basel.
Weser, U., Hartmann, H.-J., Fretzdorff, A., & Strobel, G.-J. (1977) *Biochim. Biophys. Acta 493*, 465-477.

Mechanism of Poly(ethylene glycol) Interaction with Proteins†

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ABSTRACT: Poly(ethylene glycol) (PEG) is one of the most useful protein salting-out agents. In this study, it has been shown that the salting-out effectiveness of PEG can be explained by the large unfavorable free energy of its interaction with proteins. Preferential interaction measurements of β-lactoglobulin with poly(ethylene glycols) with molecular weights between 200 and 1000 showed preferential hydration of the protein for those with $M_r \geq 400$, the degree of hydration increasing with the increase in poly(ethylene glycol) molecular weight. The preferential interaction parameter had a strong cosolvent concentration dependence, with poly(ethylene glycol) 1000 having the sharpest decrease with an increase in concentration. The preferential hydration extrapolated to zero cosolvent concentration increased almost linearly with increasing size of the additive, suggesting steric exclusion as the major factor responsible for the preferential hydration. The poly(ethylene glycol) concentration dependence of the preferential interactions could be explained in terms of the nonideality of poly(ethylene glycol) solutions. All the poly(ethylene glycols) studied, when used at levels of 10-30%, decreased the thermal stability of β-lactoglobulin, suggesting that caution must be exercised in the use of this additive at extreme conditions such as high temperature.

A large number of compounds with a variety of chemical structures are known to be preferentially excluded from the immediate domain of proteins in aqueous solution; i.e., when they are present at high concentration proteins are preferentially hydrated. These include amino acids (Arakawa & Timasheff, 1983), sugars (Lee & Timasheff, 1981; Lee et al., 1975; Arakawa & Timasheff, 1982a), salts (Arakawa & Timasheff, 1982b; Aune & Timasheff, 1970; Timasheff et al., 1976), glycerol (Gekko & Timasheff, 1981a; Na & Timasheff, 1981), 2-methyl-2,4-pentanediol (MPD)[1] (Pittz & Timasheff,

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[1] Abbreviations: PEG, poly(ethylene glycol); MPD, 2-methyl-2,4-pentanediol; β-LG, β-lactoglobulin; Gdn·HCl, guanidine hydrochloride; RNase A, ribonuclease A.

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1978), and poly(ethylene glycol) (PEG) (Lee & Lee, 1979, 1981). The diversity in the physical and chemical properties of these compounds suggests that there must exist a variety of mechanisms leading to preferential hydration in their presence. For some, such as sugars, amino acids, and some salts, the main factor was found to be the cohesive force of water (Timasheff et al., 1976; Lee & Timasheff, 1981; Arakawa & Timasheff, 1982a,b, 1983). For MPD, the exclusion appears to be due to unfavorable interactions between this compound and charges on the protein surface (Pittz & Timasheff, 1978). For glycerol, the same effect seems to stem from the solvophobicity of protein surface nonpolar residues (Gekko & Timasheff, 1981a). For PEG two possible mechanisms have been suggested (Lee & Lee, 1979, 1981), steric exclusion and protein charge. Preferential hydration due to steric exclusion, as first proposed by Kauzmann, and Schachman & Lauffer (1949), arises simply from the difference in the size between the water and the additive molecules. This mechanism generally should be in effect to a more or less significant degree since most of these substances have molecular sizes larger than water. Being very large, it seems reasonable that PEG should be excluded from the protein surface by the steric exclusion mechanism. This hypothesis was tested by examining densimetrically the preferential interactions of a well-characterized protein, β-lactoglobulin, with PEG as a function of the PEG molecular weight.

A second result of the solvent interaction studies has been the formulation of a general rule: substances that lead to protein preferential hydration can also stabilize the native structure of globular proteins in aqueous solutions. This is true of sugars (Lee & Timasheff, 1981), some salts (Arakawa & Timasheff, 1982b), amino acids (Arakawa & Timasheff, 1983), and glycerol (Gekko & Timasheff, 1981b). An exception to this rule, however, was found in MPD, which was analyzed to be a destabilizer of RNase A (Pittz & Bello, 1971), although its effectiveness is very low. In the case of PEG, the situation is uncertain. Ingham (1977) reported that PEG may have a stabilizing effect on proteins. Knoll & Hermans (1981) and Atha & Ingham (1981), on the other hand, have found that PEG does not alter significantly the transition temperature of RNase A. To probe this question further, we have examined the effect of PEGs of various molecular weights on the thermal denaturation of β-lactoglobulin, and the results are reported in this paper along with those of the preferential interaction measurements.

Materials and Methods

Poly(ethylene glycol) 200, 400, 600, and 1000 and β-LG (lot 106C-8070) were obtained from Sigma. The PEG was used without further purification. The protein was dialyzed against distilled deionized water and lyophilized after passage through a sintered glass filter.

Partial specific volume measurements were carried out as described previously (Lee & Timasheff, 1974; Lee et al., 1979; Gekko & Timasheff, 1981a; Arakawa & Timasheff, 1982a). Protein concentration was determined spectrophotometrically on a Cary Model 118 spectrophotometer using the absorptivity value of 9.6 dL/(g·cm) at 278 nm (Townend et al., 1960) in the absence of PEG. Absorptivities in the presence of PEG were measured by the previously described method (Gekko & Timasheff, 1981a,b), being 9.69 and 9.83 in 20 and 40% PEG 200, 9.73 and 9.77 in 20 and 40% PEG 400, 9.67 and 9.79 in 15 and 30% PEG 600, and 9.67 and 9.70 in 15 and 30% PEG 1000. Where not determined directly, the values were estimated, assuming a linear increase with PEG concentration. Calculations of partial specific volumes and preferential interaction parameters were performed as described previously (Lee et al., 1979).

Thermal denaturation was followed on a Gilford 2600 spectrophotometer with a thermoprogrammer. Sample protein solutions were prepared by diluting 1 mL of a stock protein solution in HCl, pH 2.0, in 25-mL volumetric flasks with the desired solvent, pH 2.0, and then bubbling helium gas through the solution for about 1 min to avoid the formation of air bubbles at high temperature, in particular in the presence of concentrated PEG. The protein concentration was adjusted to about 1 mg/mL. For each run, the three samples to be compared were scanned simultaneously. The temperature scan rate was set at 0.25 °C/min, and absorbance readings were taken at 287 nm at 1-min intervals.

Results

The partial specific volume of β-LG was measured in HCl solution, pH 2.0, as a function of PEG concentration, and the values are listed in Table I.[2] The partial specific volume of the protein at constant solvent molality, ϕ_2^0, was not different from that at constant chemical potential, ϕ'^0_2, for PEG 200, whereas ϕ'^0_2 was significantly higher than ϕ_2^0 for higher molecular weight PEGs. Setting component 1 = water, component 2 = protein, and component 3 = PEG (Scatchard, 1946; Stockmeyer, 1950), the preferential interaction parameter

$$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3} = \rho_0(\phi_2^0 - \phi'^0_2)/(1 - \rho_0\bar{v}_3) \qquad (1)$$

was calculated by using values of the partial specific volumes, $\bar{v}_3$, of PEG, given in Table II. In eq 1, g_i is the concentration of component i in grams per gram of water, μ_i is its chemical potential, T is the thermodynamic temperature, and ρ_0 is the density of the solvent. The values are given in Table I. The same parameter on the molal basis, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, is also given, where m_i is the molality of component i. For all cases, except PEG 200, the above parameters are negative, indicating that PEG 400, PEG 600, and PEG 1000 are preferentially excluded from the protein surface. The preferential hydration parameter, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ is, of course, positive for them, as shown in column 6 of Table I; i.e., the protein is preferentially hydrated. If it is assumed that PEG does not bind to β-LG, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ becomes the total binding of water to the protein (Inoue & Timasheff, 1972). It is evident that in some cases, notably 10% PEG 1000, the total hydration is much higher than the usual value of this parameter, i.e., 0.2–0.4 g/g (Kuntz, 1971; Kuntz & Kauzmann, 1974; Bull & Breese, 1968). This means, in fact, extensive exclusion of PEG from the protein, leaving a large amount of water free from PEG in the protein domain. In Figure 1, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ is plotted vs. PEG concentration, g_3. For PEGs with a molecular weight, M_3, larger than 200, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ showed a strong, almost linear dependence on g_3, the negative slope increasing with an increase in the PEG molecular weight.

The perturbation of the chemical potential of the protein by addition of PEG was calculated from (Kirkwood & Goldberg, 1950; Casassa & Eisenberg, 1964)

$$(\partial\mu_2/\partial m_3)_{T,P,m_2} = -(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}(\partial\mu_3/\partial m_3)_{T,P,m_2} = -(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}[(RT/m_3) + RT(\partial \ln \gamma_3/\partial m_3)_{T,P,m_2}] \qquad (2)$$

where γ_3 is the activity coefficient of component 3, R is the universal gas constant, and P is the pressure. For PEG 200,

[2] The measurements were done at pH 2.0, since, at that pH, β-lactoglobulin exists essentially in the state of monomeric subunits (Timasheff & Townend, 1961) and no complications from self-association need to be taken into account. Furthermore, at that pH, β-lactoglobulin is known to be in its native conformation (Timasheff et al., 1966a,b).

Table I: Preferential Interaction Parameters of β-Lactoglobulin in PEG/Water Mixture at pH 2.0

PEG concn [% (w/v)]	g_3 (g/g)	ϕ_2^0 (mL/g)	ϕ'^0_2 (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)	m_3 (mol of PEG/1000 g of H_2O)	$(\partial\mu_2/\partial m_3)_{T,P,m_2}$ [cal (mol of protein)$^{-1}$ (mol of PEG)$^{-1}$]	RT/m_3 [cal (mol of protein)$^{-1}$ (mol of PEG)$^{-1}$]	$RT(\partial \ln \gamma_3/\partial m_3)^b$ [cal (mol of protein)$^{-1}$ (mol of PEG)$^{-1}$]
					PEG 200					
10	0.110	0.754 ± 0.001	0.753 ± 0.001	0.0072 ± 0.0143	−0.065 ± 0.131	0.66 ± 1.32	0.55	−800 ± 1500	1060	100
20	0.241	0.754 ± 0.002	0.757 ± 0.001	−0.0237 ± 0.0237	0.098 ± 0.0098	−2.18 ± 2.18	1.20	1400 ± 1400	480	150
30	0.403	0.757 ± 0.001	0.760 ± 0.001	−0.0269 ± 0.0179	0.067 ± 0.044	−2.47 ± 1.65	2.02	1200 ± 800	290	200
40	0.606	0.755 ± 0.001	0.757 ± 0.001	−0.0214 ± 0.0321	0.035 ± 0.053	−1.97 ± 2.95	3.03	900 ± 1300	190	250
					PEG 400					
30	0.402	0.756 ± 0.001	0.771 ± 0.002	−0.103 ± 0.026	0.256 ± 0.064	−4.74 ± 1.20	1.00	4900 ± 1200	580	450
40	0.603	0.755 ± 0.002	0.765 ± 0.002	−0.100 ± 0.040	0.166 ± 0.066	−4.60 ± 1.84	1.51	4800 ± 1900	390	650
					PEG 600					
10	0.109	0.755 ± 0.002	0.765	−0.0686 ± 0.0137	0.627 ± 0.125	−2.10 ± 0.42	0.182	10100 ± 2000	3190	1600
20	0.241	0.757 ± 0.001	0.772 ± 0.001	−0.112 ± 0.015	0.464 ± 0.062	−3.43 ± 0.46	0.401	11100 ± 1500	1450	1800
30	0.402	0.758 ± 0.002	0.769 ± 0.001	−0.0934 ± 0.0255	0.232 ± 0.063	−2.86 ± 0.78	0.670	9400 ± 2600	870	2400
					PEG 1000					
10	0.109	0.754 ± 0.002	0.774 ± 0.001	−0.133 ± 0.020	1.22 ± 0.18	−2.45 ± 0.37	0.109	20400 ± 3100	5340	3000
20	0.241	0.754 ± 0.002	0.775 ± 0.001	−0.154 ± 0.022	0.640 ± 0.091	−2.84 ± 0.40	0.241	15400 ± 2200	2420	3000
30	0.401	0.754 ± 0.002	0.774 ± 0.001	−0.165 ± 0.025	0.411 ± 0.0062	−3.04 ± 0.46	0.401	13500 ± 2000	1450	3000

[a] M_2 = 18 400. [b] $\partial \ln \gamma_3/\partial m_3$ was calculated from the activity coefficient data for PEG 200, 400, and 600 and from the osmotic coefficient data for PEG 1000.

Table II: Partial Specific Volumes of PEG

PEG [% (w/v)]	$\bar{v}_3$ (mL/g)			
	PEG 200	PEG 4000	PEG 600	PEG 1000
10	0.847		0.840	0.835
20	0.845		0.836	0.833
30	0.845	0.838	0.837	0.832
40	0.849	0.840		

400, and 600 the self-interaction term, $(\partial \ln \gamma_3/\partial m_3)_{T,P,m_2}$, was calculated from activity coefficient data obtained with hexaoxyethylene (M_r 282), nonaoxyethylene (M_r 414), and dodecaoxyethylene (M_r 546) glycols (Elworthy & Florence, 1966). The logarithm of γ_3 was plotted vs. m_3, and the tangent was taken at the PEG concentrations listed in Table I. For PEG 1000, the activity coefficient was calculated from osmotic coefficient data obtained at 35 °C (Rogers & Tam, 1977). Due to the paucity of these data, only a very rough estimate of γ_3 could be made for PEG 1000. The calculated values of $RT(\partial \ln \gamma_3/\partial m_3)_{T,P,m_2}$ at 20 °C, given in Table I, indicate that the nonideality of PEG solutions is very large, in particular at higher PEG concentrations and for larger PEG. Comparison of the RT/m_3 and the nonideal terms shows that their magnitudes are of the same order. Therefore, neglect of the nonideality term would lead perforce to totally incorrect conclusions.

The results listed in Table I show that, except for 10% PEG 200, $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ had high positive values increasing for higher molecular weight PEG. Thus, addition of PEG to an aqueous protein solution leads to an increase in the chemical potential of the protein; i.e., the system is destabilized thermodynamically. A plot of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ as a function of g_3,

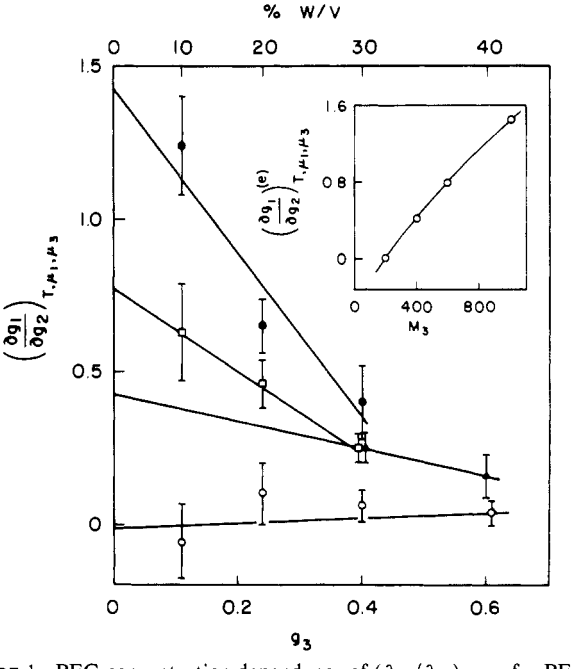

FIGURE 1: PEG concentration dependence of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ for PEG 200 (○), 400 (▲), 600 (□), and 1000 (●). The lines were obtained by a least squares method. (Inset) Dependence of $(\partial g_1/\partial g_2)^{(e)}_{T,\mu_1,\mu_3}$ on PEG molecular weight. $(\partial g_1/\partial g_2)^{(e)}_{T,\mu_1,\mu_3}$ was obtained as described in the text.

shown in Figure 2, indicates little dependence on PEG concentration except possibly for PEG 1000 for which the uncertainty in the estimation of the nonideality term precludes any definite conclusions on this question. The essential lack

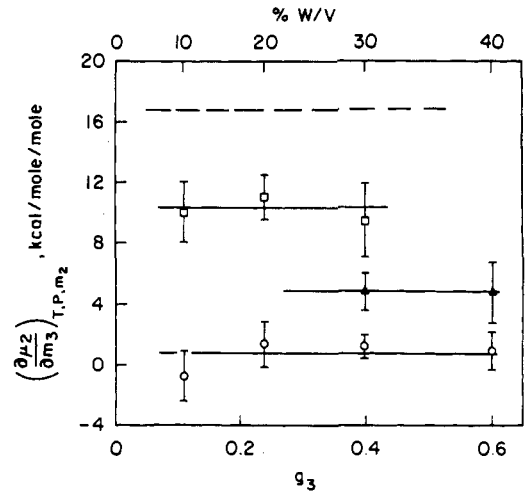

FIGURE 2: Concentration dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ for PEG 200 (○), 400 (▲), and 600 (□). The dashed line is the average of the values calculated for PEG 1000; it reflects the large uncertainty in the estimation of PEG 1000 nonideality.

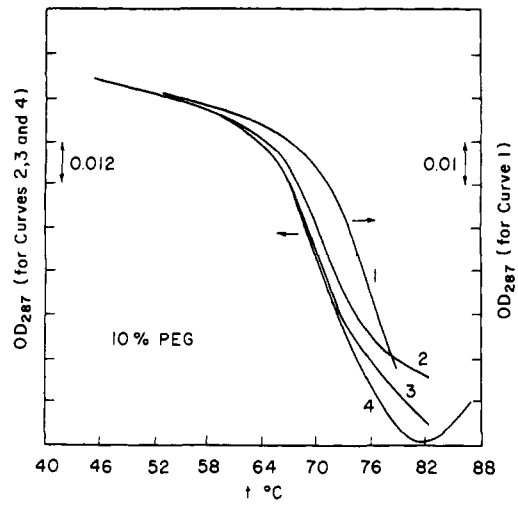

FIGURE 3: Thermal denaturation of β-LG at pH 2.0 in 0.01 M HCl. Curve 1, no PEG; curve 2, 10% PEG 200; curve 3, 10% PEG 400; curve 4, 10% PEG 1000.

Table III: Transition Temperature of β-Lactoglobulin at pH 2.0[a]

	temp (°C) for PEG [% (w/v)]				
	0	10	20	30	40
PEG 200	65–	60–78		46–66	44–57
PEG 400	58–[b]	60–77		46–[b]	44–[b]
PEG 1000	65–85	60–79	57–		
PEG 600[c]	55–		48–	47–	
PEG 1000[c]	53–	50–			

[a] Only initial transition temperatures are given for those samples that did not give a clear end temperature. [b] 1.5 M Gdn·HCl added. [c] 1.8 M Gdn·HCl added.

of concentration dependence of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ is in sharp contrast to the strong concentration dependence of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$, suggesting that the decrease of the "binding" parameter with PEG concentration is strictly a reflection of the nonideal behavior of PEG in aqueous solution.

The thermal denaturation of β-LG in the presence of PEG at different concentrations was examined, and the results are summarized in Table III. The thermal denaturation curve of the protein in a pH 2.0 solution without PEG indicates that the protein is very stable, the denaturation being incomplete even at 80 °C. The result is consistent with the fact that β-LG is highly stable at low pH (Timasheff et al., 1966a,b). Addition of PEG 200 or 1000 decreases significantly the transition temperature. The effects of 10% PEG 200, 400, and 1000 are compared in Figure 3 and Table III, indicating that these additives decrease the transition temperature to essentially the same extent. This suggests that the destabilizing effectiveness of PEG is not related to its molar concentration but rather to the concentration of the repeating $-CH_2-CH_2-O-$ unit. Since the protein is highly stable, thermal denaturation experiments were also carried out in the presence of small amounts of Gdn·HCl (1.5–1.8 M) which does not cause denaturation near room temperature but lowers the transition temperature of the protein by ca. 10 °C. The results obtained with PEG 400, 600, and 1000 are summarized in Table III. The decrease in the transition temperature indicates that these additives destabilize the protein in a manner consistent with the results obtained without Gdn·HCl.

Discussion

The results described in this paper for the preferential interaction of β-LG with solvent components in aqueous PEG systems are similar to those reported by Lee and Lee for tubulin (1979) and other proteins (1981): large preferential hydrations of the proteins in the presence of PEGs of higher molecular weight than those used in this study and sharp decrease of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ with an increase in PEG concentration. In their second paper (Lee & Lee, 1981), these authors have suggested as a possible contributing mechanism the exclusion of PEG from the protein surface due to unfavorable interaction with charges on proteins, as had been proposed for the preferential exclusion of MPD from RNase A at pH 5.6 (Pittz & Timasheff, 1978). The two systems, however, differ in the dependence of preferential hydration on the concentration of the additive. For the MPD system, $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ increases strongly with MPD concentration, with values of 0.196 and 1.031 g/g at 20 and 50% MPD, respectively, indicating an increase in MPD exclusion from the protein with an increase in its concentration. For PEG, on the other hand, this parameter decreases with concentration as seen in Figure 1, with values of, e.g., 1.22 and 0.411 g/g at 10 and 30% PEG 1000, showing that an increase in PEG concentration decreases its exclusion. It would seem, therefore, that the mechanism responsible for the negative interactions of PEG and MPD with proteins need not be identical. On the other hand, the same authors (Lee & Lee, 1979) had also proposed the large excluded volume of PEG as a source of the preferential hydration in its presence. The effects of PEG on protein solubility have also been analyzed in terms of its large excluded volume (Juckes, 1971; Atha & Ingham, 1981).

The distinction between the two mechanisms can be tested by examining this system as a function of PEG molecular weight, since higher molecular weight PEGs should have a larger excluded volume, leading to a larger protein preferential hydration. This in fact is what has been observed (Table I and Figure 1). This difference, however, may be obscured at high additive concentrations by the strong concentration dependence of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ for high molecular weight PEGs. Extrapolation of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ to zero PEG concentration should give the preferential exclusion of PEG in the ideal state, i.e., the excluded volume of PEG in dilute solutions, $(\partial g_1/\partial g_2)^{(e)}_{T,\mu_1,\mu_3}$. The value of this parameter for the various PEGs was obtained by extrapolation of the data of Figure 1 to $g_3 = 0$ using a least-squares method. The results are listed in Table IV together with the slopes, $\Delta(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}/\Delta g_3$. A plot of $(\partial g_1/\partial g_2)^{(e)}_{T,\mu_1,\mu_3}$ as a function of PEG molecular weight, given in the inset of Figure 1, reveals a monotone increase in this parameter, i.e., the preferential exclusion increases with

Table IV: Interaction Parameters for PEG[a]

PEG	$(\partial g_1/\partial g_2)^{(e)}_{T,\mu_1,\mu_3}$ (g/g)	slope	R_e (Å)	R_g (calcd) (Å)
200	−0.01	0.08		3.0
400	0.43	−0.44	2.8	4.5
600	0.78	−1.37	4.6	6.0
1000	1.44	−2.57	7.4	7.5

[a] These parameters were calculated as described in the text.

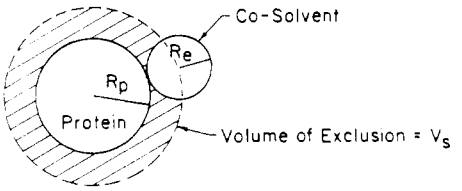

FIGURE 4: Schematic representation of the steric exclusion mechanism of preferential hydration.

an increase in the size of PEG. This result argues in favor of steric exclusion being a major factor in determining the preferential interaction of PEG with proteins, although the detailed analysis was performed only for β-LG and over a limited range of PEG sizes.

As proposed by Kauzmann, the excess water due to the steric exclusion mechanism is located in the shell between the protein surface and the effective radius, R_e, of the additive molecule as shown schematically in Figure 4. The volume of this shell, V_s, expressed in units of milliliters per mole, is related to the preferential hydration generated by steric exclusion by

$$V_s = (M_2/\rho_w)(\partial g_1/\partial g_2)^{(e)}_{T,\mu_1,\mu_3} \quad (3)$$

where ρ_w is the averaged density of water in the hydration layer. Assuming a spherical shape for the protein molecule, with a radius, R_p, the effective radius of the additive molecule, R_e, can be calculated from V_s by

$$V_s = (4\pi N/3)[(R_p + R_e)^3 - R_p^3] \times 10^{-24} \quad (4)$$

where N is Avogadro's number. Setting for β-LG, R_p = 18 Å (Witz et al., 1964) and ρ_w = 1, R_e was calculated from the values of $(\partial g_1/\partial g_2)^{(e)}_{T,\mu_1,\mu_3}$ listed in Table IV, except for PEG 200 which gave a negative value of $(\partial g_1/\partial g_2)^{(e)}_{T,\mu_1,\mu_3}$. The results, given in the fourth column of Table IV, indicate that the effective radius of PEG for the purpose of steric exclusion increases greatly with molecular weight. This is in good agreement with the fact that an increase in the degree of polymerization of flexible polymers leads to an increase in their radius of gyration and, hence, in their effective hydrodynamic radius (Tanford, 1961).

An approximate calculation of the expected radii of gyration of the various PEGs used in this study was carried out by using equations derived by Tanford (1961) for polymethylene chains. For these polymers Tanford (1961) showed that the radius of gyration, R_g, is related to the bond length, l, and the number of bonds, σ, by

$$R_g = (2\sigma l^2/6)^{1/2} \quad (5)$$

Simulating the PEGs by polymethylene chains with an identical number of bonds along the chain, values of R_g were obtained which are listed in the last column of Table IV. It is seen that their magnitudes are similar to the experimentally determined values of R_e. This agreement, however, must be regarded strictly as qualitative. This is due to the assumptions made in the calculation (i) that the structural parameters of PEG can be represented by those of polymethylene and (ii) that the chain statistics on which eq 5 is based are valid for such small polymers as the PEGs used in this study.

The PEG concentration dependence of $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$ (Figure 1), expressed by the negative slopes in Table IV, can be understood in terms of the strongly nonideal behavior of PEG solutions. Since the free energy perturbation of the protein solution is essentially of the same magnitude at high PEG concentrations as at low ones, the decrease in PEG exclusion observed at higher PEG concentrations must be the result of the increase of the activity coefficient of PEG with PEG concentration. The essential constancy of values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$ at different PEG concentrations for all the PEGs examined means that, thermodynamically, the excluded volume effect of PEG is not concentration dependent. Therefore, the sharper concentration dependence of preferential hydration for higher molecular weight PEGs is due to the greater increase in the activity coefficients of PEG with an increase in its concentration.

The decrease of the PEG exclusion from proteins at high PEG concentrations can be understood in terms of the solution properties of PEG, namely, of the changes in solute–solvent interactions related to the nonideal behavior of PEG solutions. It has been proposed that PEG, which is a flexible, essentially nonpolar polymer (Hammes & Schimmel, 1967), can assume compact structures stabilized by intramolecular hydrophobic interactions. These structures must have lower solute–solvent interactions than the fully extended ones (Rogers & Tam, 1977). According to this mechanism, the effective exclusion size of PEG should be reduced at higher PEG concentrations where the nonideality becomes extremely large, and this change in the PEG exclusion size should be greater for larger PEGs, which, in fact, has been observed.

A necessary consequence of the increase in the chemical potential of the protein upon addition of PEG (except for PEG 200) should be a decrease in its solubility. The transfer free energy of the protein from water to PEG solution, $\Delta\mu_2$, was calculated by assuming constant values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, although the values for PEG 1000 show a slight decrease with m_3, which may simply reflect the uncertainty in the estimation of $(\partial\mu_3/\partial m_3)_{T,P,m_2}$. If the free energy change contains no significant contributions of other interactions such as changes in the binding of protons or other ionic species to the proteins due to the addition of PEG, these values of $\Delta\mu_2$ express the change in the activity of the protein upon transfer from water to a PEG solution. If the usual assumption is made that the chemical potential of the protein in the solid phase is independent of solvent composition, $\Delta\mu_2$ is related directly to the change in protein solubility, since

$$\Delta\mu_2 = -\log (S_{2,p}/S_{2,w}) \quad (6)$$

where $S_{2,p}$ and $S_{2,w}$ are the protein solubilities in PEG solution and water, respectively. As is shown in Figure 5, the solubility of β-LG is expected to be about 10 times lower in 30% PEG 200 than that in water, whereas it should decrease by several orders of magnitude in 30% PEG 600. In fact, the protein solubility should decrease 10-fold at less than 10% PEG 600. This is in agreement with the general salting-out effectiveness of PEG on proteins and suggests that the observed decrease in β-LG solubility by addition of PEG 20 000 (Middaugh et al., 1979) is due mainly to its preferential interaction with the protein.

Substances that show a large preferential hydration have been found in general to be protein-structure stabilizers. The interactions between these substances and proteins are thermodynamically unfavorable, as manifested by large positive values of $(\partial\mu_2/\partial m_3)_{T,P,m_2}$, and become even more unfavorable for denatured proteins whose surface areas are larger than

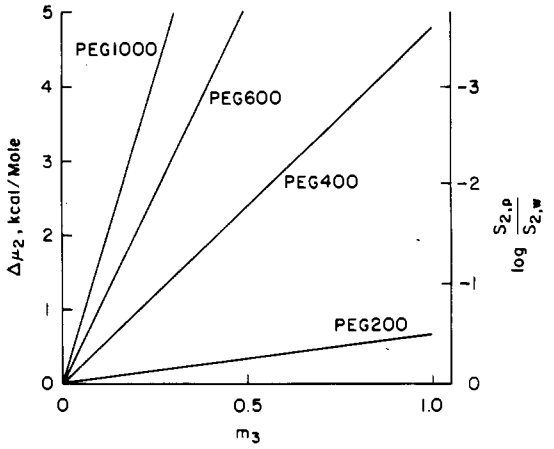

FIGURE 5: Concentration dependence of $\Delta\mu_2$ and relative protein solubility for the indicated systems.

those of native ones. As a result, their addition should stabilize the native, compact state of the proteins. From the observed large preferential hydrations of β-LG (this study) and other proteins (Lee & Lee, 1979, 1981) in the presence of a variety of PEGs, PEG could be expected to be a strong protein-structure stabilizer. Knoll & Hermans (1981), however, have shown that addition of PEG 6000 at 6% does not change the thermal transition of RNase A. Atha & Ingham (1981) found an insignificant effect of PEG 400 and 4000 at 30% on the thermal denaturation of RNase A. Ingham (1977) suggested a protein-stabilizing role for PEG from the observation that PEG decreased the cloud point of poly(vinyloxazolidinone). This macromolecule assumes a random coil shape at a low temperature and becomes insoluble in aqueous solution above a certain temperature, known as the cloud point (Klotz, 1965). Klotz (1965) has observed that those substances which stabilize proteins decrease the cloud point, which indicates a stabilizing effect on the high-temperature form of this macromolecule. This process, however, consists of two coupled reactions, i.e., a transition from an expanded to a compact structure, followed by aggregation. Since most protein stabilizers are also protein precipitants, they should affect both phases of the above reaction. The situation need not be identical for all substances. The lowering of the cloud point by PEG may be the result just of its strong salting-out action, but not of any stabilizing effect of the compact form. In this study, we found that all the PEGs studied lower the melting point of β-LG, although the effect was not strong enough to cause denaturation at room temperature. There must be, therefore, a factor that overcomes the expected stabilizing effectiveness of PEG.

The decrease in preferential exclusion of PEGs at their higher concentrations should permit these molecules to penetrate the hydration layer of the protein. We propose that, at high concentrations, PEG can bind to hydrophobic sites on proteins based on the fact that PEG is essentially nonpolar (Hammes & Schimmel, 1967; Ingham, 1977). In such a case the binding of PEG should be stronger to denatured proteins than to native ones, with, as a consequence, stabilization of the denatured form. It seems, then, that the observed effects of PEG on protein stability can be interpreted in terms of a fine balance between two opposing factors, namely, the stabilizing effect due to PEG exclusion and the destabilizing effect due to binding through hydrophobic interactions. The difference in the effect of PEG on RNase A and β-LG may be due to the difference in the chemical natures of the two proteins. It is known that RNase A is extremely hydrophilic, while β-LG is relatively hydrophobic (Bigelow, 1967). PEG should then bind more to β-LG than to RNase A in the denatured state and, as a consequence, have a stronger destabilizing effect on β-LG. This is in agreement with the finding of Pace & Marshall (1980) that nonpolar compounds, in general, destabilize β-LG more than RNase A. These possible destabilizing effects of PEG suggest that care should be exercised when PEG is used for protein salting-out or crystallization, in particular for hydrophobic proteins, although it can be expected not to denature the proteins except under extreme conditions such as at elevated temperatures.

Registry No. PEG, 25322-68-3.

REFERENCES

Arakawa, T., & Timasheff, S. N. (1982a) *Biochemistry 21*, 6536–6544.

Arakawa, T., & Timasheff, S. N. (1982b) *Biochemistry 21*, 6545–6552.

Arakawa, T., & Timasheff, S. N. (1983) *Arch. Biochem. Biophys. 224*, 169–177.

Atha, D. H., & Ingham, K. C. (1981) *J. Biol. Chem. 256*, 12108–12117.

Aune, K. C., & Timasheff, S. N. (1970) *Biochemistry 9*, 1481–1484.

Bigelow, C. C. (1967) *J. Theor. Biol. 16*, 187–211.

Bull, H. B., & Breese, K. (1968) *Arch. Biochem. Biophys. 128*, 488–496.

Casassa, E. F., & Eisenberg, H. (1964) *Adv. Protein Chem. 19*, 187–211.

Elworthy, P. H., & Florence, A. T. (1966) *Kolloid Z. Z. Polym. 208*, 157–162.

Gekko, K., & Timasheff, S. N. (1981a) *Biochemistry 20*, 4667–4676.

Gekko, K., & Timasheff, S. N. (1981b) *Biochemistry 20*, 4677–4686.

Hammes, G. G., & Schimmel, P. R. (1967) *J. Am. Chem. Soc. 89*, 442–446.

Ingham, K. C. (1977) *Arch. Biochem. Biophys. 184*, 59–68.

Inoue, H., & Timasheff, S. N. (1972) *Biopolymers 11*, 737–743.

Juckes, I. R. M. (1971) *Biochim. Biophys. Acta 229*, 335–546.

Kirkwood, J. G., & Goldberg, R. J. (1950) *J. Chem. Phys. 18*, 54–57.

Klotz, I. M. (1965) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24*, 524–533.

Knoll, D. A., & Hermans, J. (1981) *Biopolymers 20*, 1747–1750.

Kuntz, I. D. (1971) *J. Am. Chem. Soc. 93*, 514, 516–518.

Kuntz, I. D., & Kauzmann, W. (1974) *Adv. Protein Chem. 28*, 239–345.

Lee, J. C., & Timasheff, S. N. (1974) *Biochemistry 13*, 257–265.

Lee, J. C., & Lee, L. L. Y. (1979) *Biochemistry 18*, 5518–5526.

Lee, J. C., & Lee, L. L. Y. (1981) *J. Biol. Chem. 256*, 625–631.

Lee, J. C., & Timasheff, S. N. (1981) *J. Biol. Chem. 256*, 7193–7201.

Lee, J. C., Frigon, R. P., & Timasheff, S. N. (1975) *Ann. N.Y. Acad. Sci. 253*, 284–291.

Lee, J. C., Gekko, K., & Timasheff, S. N. (1979) *Methods Enzymol. 61*, 26–49.

Middaugh, C. R., Tisel, W. A., Haire, R. N., & Rosenberg, A. (1979) *J. Biol. Chem. 254*, 367–370.

Na, G. C., & Timasheff, S. N. (1981) *J. Mol. Biol. 151*, 165–178.

Pace, C. N., & Marshall, H. F., Jr. (1980) *Arch. Biochem. Biophys. 199*, 270–276.

Pittz, E. P., & Bello, J. (1971) *Arch. Biochem. Biophys. 146*, 513-524.
Pittz, E. P., & Timasheff, S. N. (1978) *Biochemistry 17*, 615-623.
Rogers, J. A., & Tam, T. (1977) *Can. J. Pharm. Sci. 12*, 65-70.
Scatchard, G. (1946) *J. Am. Chem. Soc. 68*, 2315-2319.
Schachman, H. K., & Lauffer, M. A. (1949) *J. Am. Chem. Soc. 71*, 536-541.
Stockmayer, W. H. (1950) *J. Chem. Phys. 18*, 58-61.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Wiley, New York, NY.
Timasheff, S. N., & Townend, R. (1961) *J. Am. Chem. Soc. 83*, 470-473.
Timasheff, S. N., Mescanti, L., Basch, J. J., & Townend, R. (1966a) *J. Biol. Chem. 241*, 2496-2501.
Timasheff, S. N., Townend, R., & Mescanti, L. (1966b) *J. Biol. Chem. 241*, 1863-1870.
Timasheff, S. N., Lee, J. C., Pittz, E. P., & Tweedy, N. (1976) *J. Colloid Interface Sci. 55*, 658-663.
Townend, R., Winterbottom, R. J., & Timasheff, S. N. (1960) *J. Am. Chem. Soc. 82*, 3161-3168.
Witz, J., Timasheff, S. N., & Luzzati, V. (1964) *J. Am. Chem. Soc. 86*, 168-173.

Amino Acid Sequence of the Calcium-Dependent Photoprotein Aequorin†

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ABSTRACT: The Ca(II)-dependent photoprotein aequorin produces the luminescence of the marine coelenterate *Aequorea victoria*. The complete amino acid sequence of aequorin has been determined. A complete set of nonoverlapping peptides was produced by cyanogen bromide cleavage. These peptides were aligned by using the amino-terminal sequence of the intact protein and the sequences of selected arginyl and lysyl cleavage products. Although the aequorin preparations employed in these studies were homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the presence of a minimum of 3 isotypes was demonstrated by the location of 17 sites of sequence microheterogeneity. Two amino acid variants were observed at each of 16 positions while 1 position had 3 different replacements. The protein as isolated has 189 amino acids with an unblocked amino terminus. According to the sequence reported here, the molecular weight of the apoprotein is 21 459 while that of the holoprotein is 21 914. The molecule possesses three internally homologous domains which were judged to be EF-hand Ca(II) binding domains by several different criteria. Aequorin is homologous to troponin C and to calmodulin. These findings demonstrate that aequorin is a member of the Ca(II) binding protein superfamily.

Aequorin is the protein responsible for the bioluminescence of the marine coelenterate *Aequorea victoria* (Arai & Brinckman-Voss, 1980). Aequorin was first isolated from these jellyfish by Shimomura et al. (1962) and was shown to produce a blue luminescence in response to added Ca(II). The luminescent reaction of aequorin and other photoproteins is initiated solely by the addition of Ca(II) and requires neither additional molecular oxygen nor any other cofactor for production of the energy (about 60 kcal/mol) released as light and heat.

Aequorin is comprised of a single polypeptide chain and contains 1 mol of a tightly bound chromophore termed coelenterate-type luciferin [cf. reviews by Cormier (1978), Blinks et al. (1976), and Cormier et al. (1974)]. This chromophore is a substituted dihydropyrazinimidazolone ring system (M_r 423) which has been found to be a common substrate in the bioluminescent reaction of the photoproteins as well as a large number of diverse marine organisms (Shimomura & Johnson, 1978; Ward & Cormier, 1975; Hori et al., 1977). Upon the addition of Ca(II), the luciferin of aequorin is converted to oxyluciferin plus CO_2. Coelenterate-type oxyluciferin is a 2,3,6-substituted pyrazine compound which is generated by the oxidative decarboxylation of luciferin. The light emission (λ_{max} = 469 nm) occurs from the first singlet excited state of oxyluciferin (Shimomura & Johnson, 1973, 1975a; Hori et al., 1973). Studies of other related bioluminescent systems have clearly established the dependency of light emission on the concentration of molecular oxygen (Cormier et al., 1975). Consequently, the lack of an O_2 dependency for the aequorin reaction has led to the postulate that aequorin has some form of tightly bound oxygen at a site that is independent of the luciferin binding site (Cormier, 1978). In this model, the binding of Ca(II) to aequorin triggers luminescence by inducing a conformational change in the protein that allows the oxygen species to react with luciferin to produce luminescence. An alternative hypothesis, based largely on chromophore extraction experiments and chemical modification of luciferin derivatives, speculates that the oxygen occurs as a linear peroxide that covalently links the luciferin to an amino acid

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