Matthews, C. R., & Crisanti, M. M. (1981) *Biochemistry 20*, 784–792.
Merkler, D. J. (1985) Ph.D. Thesis, The Pennsylvania State University.
Merkler, D. J., Wedler, F. C., Pendleton, T. P., & Sturtevant, J. M. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, Abstr. 2046.
Mozhaev, V. V., & Martinek, K. (1982) *Enzyme Microb. Technol. 4*, 299–309.
Nall, B. T., Garel, J.-R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317–330.
Norris, R. D., & Fowden, L. (1972) *Phytochemistry 12*, 2109–2121.
Pohl, F. M. (1968) *Eur. J. Biochem. 7*, 146–152.
Rudolph, R., Zettlmeissel, G., & Jaenicke, R. (1979) *Biochemistry 18*, 5572–5575.
Shapiro, B. M., & Stadtman, E. R. (1967) *J. Biol. Chem. 242*, 5069–5079.
Shapiro, B. M., & Ginsburg, A. (1968) *Biochemistry 7*, 2153–2167.
Shapiro, B. M., & Stadtman, E. R. (1971) *Methods Enzymol. 17*, 910–922.
Shibuya, H., Abe, M., Sekiguchi, T., & Nosoh, Y. (1982) *Biochim. Biophys. Acta 708*, 300–304.
Singleton, R., Jr., & Amelunxen, R. E. (1973) *Bacteriol. Rev. 37*, 320–342.
Stadtman, E. R., & Ginsburg, A. (1974) *Enzymes (3rd Ed.) 10*, 755–807.
Stearn, A. E., & Eyring, H. (1937) *J. Chem. Phys. 5*, 113–124.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Voordouw, G., & Roche, R. S. (1975) *Biochemistry 14*, 4659–4666.
Voordouw, G., Milo, C., & Roche, R. S. (1976) *Biochemistry 15*, 3716–3724.
Voordouw, G., DeHaard, H., Timmermans, J. A. M., Veeger, C., & Zabel, P. (1982) *Eur. J. Biochem. 127*, 267–274.
Wedler, F. C. (1978) in *Biochemistry of Thermophily* (Friedman, S. M., Ed.) pp 325–343, Academic, New York.
Wedler, F. C., & Hoffmann, F. M. (1974a) *Biochemistry 13*, 3207–3214.
Wedler, F. C., & Hoffmann, F. M. (1974b) *Biochemistry 13*, 3215–3221.
Wedler, F. C., & Eismann, K. (1976) *Biochem. Biophys. Res. Commun. 69*, 1003–1010.
Wedler, F. C., & Merkler, D. J. (1985) *Curr. Top. Cell. Regul. 26*, 263–280.
Wedler, F. C., Kenney, R. M., Ashour, A. E., & Carfi, J. (1978) *Biochem. Biophys. Res. Commun. 81*, 122–126.
Wedler, F. C., Shreve, D. S., Kenney, R. M., Ashour, A. E., Carfi, J., & Rhee, S. G. (1980) *J. Biol. Chem. 255*, 9507–9516.
Wedler, F. C., Shreve, D. S., Fisher, K. E., & Merkler, D. J. (1981) *Arch. Biochem. Biophys. 211*, 276–287.
Wedler, F. C., Srikumar, K., Moore, K. S., & Premalatha, R. (1987) *Biochim. Biophys. Acta 912*, 144–146.
Yutani, K., Ogasahara, K., Suzuki, M., Sugino, Y., & Matsushiro, A. (1978) in *Biochemistry of Thermophily* (Friedman, S. M., Ed.) pp 233–249, Academic, New York.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1982) *Biochemistry 21*, 3946–3950.

Thermal Stability of Proteins in the Presence of Poly(ethylene glycols)†

Lucy L.-Y. Lee and James C. Lee*
Edward A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104
Received April 17, 1987; Revised Manuscript Received July 10, 1987

ABSTRACT: Thermal unfolding of ribonuclease, lysozyme, chymotrypsinogen, and β-lactoglobulin was studied in the absence or presence of poly(ethylene glycols). The unfolding curves were fitted to a two-state model by a nonlinear least-squares program to obtain values of ΔH, ΔS, and the melting temperature T_m. A decrease in thermal transition temperature was observed in the presence of poly(ethylene glycol) for all of the protein systems studied. The magnitude of such a decrease depends on the particular protein and the molecular size of poly(ethylene glycol) employed. A linear relation can be established between the magnitude of the decrease in transition temperature and the average hydrophobicity of these proteins; namely, the largest observable decrease is associated with the protein of the highest hydrophobicity. Further analysis of the thermal unfolding data reveals that poly(ethylene glycols) significantly effect the relation between $\Delta H°$ of unfolding and temperature for all the proteins studied. For β-lactoglobulin, a plot of ΔH versus T_m indicates a change in slope from a negative to a positive value, thus implying a change in ΔC_p in thermal unfolding caused by the presence of poly(ethylene glycols). Results from solvent–protein interaction studies indicate that at high temperature poly(ethylene glycol) 1000 preferentially interacts with the denatured state of protein but is excluded from the native state at low temperature. These observations are consistent with the fact that poly(ethylene glycols) are hydrophobic in nature and will interact favorably with the hydrophobic side chains exposed upon unfolding; thus, it leads to a lowering of thermal transition temperature.

Organic solvents have been utilized extensively in various aspects of studies on macromolecules, especially proteins. Empirically, polyhydric alcohols and sugars are introduced into the solvent medium in order to stabilize biological macromolecules in solution (Ball et al., 1943; Boyer, 1945; Bradbury & Jakoby, 1972; Frigon & Lee, 1972; Gerlsma, 1968; Neucere & St. Angelo, 1972). However, as a result of the detailed and

† Supported by National Institutes of Health Grants NS-14269 and DK-21489.

0006-2960/87/0426-7813$01.50/0

thorough studies of Timasheff and co-workers (Pittz & Timasheff, 1978; Timasheff et al., 1976; Lee & Timasheff, 1981; Gekko & Timasheff, 1981a,b; Na & Timasheff, 1981; Arakawa & Timasheff, 1982a,b), it is becoming evident that the ability of organic solutes to stabilize the structure of proteins is related to the preferential hydration of the macromolecule in an aqueous solution containing these organic compounds. Preferential hydration is a thermodynamic phenomenon that reflects the inability of organic additives to interact with proteins; thus, it leads to an exclusion of these solvent components from the protein surface. Depending on the magnitude of this interaction, a phase separation between proteins and the solvent may occur. A small negative interaction may lead to only a microscopic phase separation, which can be manifested in the maintenance of biological activity or native structure under conditions that are known to denature the proteins, e.g., an increase in the thermal transition temperature (Gerlsma, 1968; Lee & Timasheff, 1981; Gekko & Timasheff, 1981a). A more negative interaction may lead to a macroscopic phase separation such as precipitation or crystallization of proteins. Results of these past studies have led to the following questions: Is preferential hydration of a macromolecule a common thermodynamic phenomenon observed in all aqueous solutions containing solvent components that stabilize macromolecules against thermal unfolding? And also, what are the common physicochemical characteristics of these components that enable them to stabilize these macromolecules?

For organic additives such as sucrose, glycerol, poly(ethylene glycols), and some amino acids, preferential hydration of proteins has been reported in all of these studies (Lee & Timasheff, 1981; Gekko & Timasheff, 1981a; Lee & Lee, 1979, 1981; Arakawa & Timasheff, 1983, 1985a,b; Na & Timasheff, 1981). These polyhydric alcohols and sugars all stabilize the native properties of proteins such as the enzymic activities (Bradbury & Jakoby, 1972) or the secondary/tertiary structure of proteins (Lee & Lee, 1979, 1981). Furthermore, PEG[1] has been shown to be a powerful precipitant and crystallizing solvent for macromolecules (McPherson, 1976; Ingham, 1977, 1978; Juckes, 1971). Apparently within the few cited systems, preferential hydration of macromolecules appears to be associated with stabilization of structures of these macromolecules. The molecular mechanism that leads to the stabilization effect is the subject of reports on sucrose (Lee & Timasheff, 1981), glycerol (Gekko & Timasheff, 1981a,b), and some amino acids (Arakawa & Timasheff, 1983, 1985a,b). Although it has been reported that a correlation can be established between preferential hydration and the surface tension of water, an increase in surface tension is not the only factor responsible for these observation. For example, glycerol and betaine do not increase the surface tension of water. Hence, the common thermodynamic phenomenon of preferential hydration reported for all these organic additives may be induced by various factors with an increase in surface tension of water being only one of them. Nevertheless, the interesting and prevalent observation is the parallel association between preferential hydration and stabilization of macromolecules.

In the case of sucrose, glycerol, and some amino acids, these additives have all induced an elevation in the thermal transition temperature (Lee & Timasheff, 1981; Gekko & Timasheff, 1981b; Arakawa & Timasheff, 1983; Gerlsma, 1968). However, PEG have been reported to not have any effect on the thermal transition temperature of proteins (Atha & Ingham, 1981; Knoll & Hermans, 1981), although they are known to induce phase separations (Ingham, 1977, 1978; Knoll & Hermans, 1983) and preferential hydration of macromolecules (Lee & Lee, 1979, 1981; Arakawa & Timasheff, 1985b). In fact, glycols are reported to be denaturants of proteins (Herskovits et al., 1970) and also to lower the thermal transition temperatures of proteins (Gerlsma & Stuur, 1972; Arakawa & Timasheff, 1985b). In view of the apparent conflicting results on the effects of PEG on the thermal stability of protein, this study was initiated to monitor the effect of PEG on the thermal stability of a series of proteins.

Materials and Methods

EG, PEG 1000, annd PEG 4000 were purchased from Fisher Scientific Co. while PEG 200 and PEG 400 were obtained from Sigma Chemical Co. These were used without further purification. The following proteins were obtained from Sigma Chemical Co.: egg white lysozyme (lot 57C-8025) and bovine β-lactoglobulin (lot 106C-8072). Bovine pancreas ribonuclease A (lot 1438331) and chymotrypsinogen (lot 37E 837) were purchased from Boringer Mannheim Biochemicals and Worthington Biochemical Corp., respectively.

The protein concentrations were determined by measuring the absorbance, and their respective values are as follows: RNase A, 0.738 mL/(mg·cm) at 278 nm (Scott & Scheraga, 1963); lysozyme, 2.635 mL/(mg·cm) at 281.5 nm (Sophianopoulos et al., 1962); CTG, 1.97 mL/(mg·cm) at 282 nm (Jackson & Brandts, 1970), and β-lactoglobulin, 0.96 mL/(mg·cm) at 278 nm (Townend et al., 1960).

The equilibrium thermal denaturation study was conducted by monitoring the difference spectra of protein solutions at various temperatures in 1-cm fused silica cuvettes on a Cary 118 spectrophotometer. The protein solutions in both the sample and reference compartments were aliquots of the same stock solutions. Base lines were determined before and after each experiment by comparison of two identical protein solutions. The difference in absorbance at the wavelength of maximum change was then plotted against temperature at 1 °C increments to obtain the thermal denaturation profiles of the proteins. Having established the experimental conditions by this procedure using difference spectra, most of the denaturation studies were then conducted with a Gilford 250 spectrophotometer equipped with a Thermoset temperature regulator. In this case the change in absorbance was monitored at one wavelength.

The solvents and the proteins used in the thermal denaturation measurements were as follows: 10^{-3} M HCl or 4×10^{-2} M glycine at pH 3.0 for CTG; 10^{-2} M HCl or 4×10^{-2} M glycine for lysozyme, β-lactoglobulin, and RNase at pH 2.9; 4×10^{-2} M glycine for RNase at pH 4–12.

Interactions between solvent components and proteins were monitored by density measurements according to previously published procedures (Lee et al., 1979; Lee & Lee, 1979, 1981). The densities of the solvents and protein solutions were measured with a Precision density meter DMA-02D (Mettler/Paar). All measurements were made at 20 °C, except when stated specifically.

Results

Thermal denaturation of CTG was monitored at pH 3.0 as a function of PEG 1000 concentration. Figure 1 shows typical denaturation profiles, and it is evident that the presence of PEG 1000 affects the thermal unfolding temperature, T_m, of CTG

[1] Abbreviations: EG, ethylene glycol; PEG, poly(ethylene glycols); PEG 200, 400, 1000, and 4000, PEG with an average M_r of 200, 400, 1000, and 3350, respectively; RNase, ribonuclease A; CTG, chymotrypsinogen; β-Lg, β-lactoglobulin.

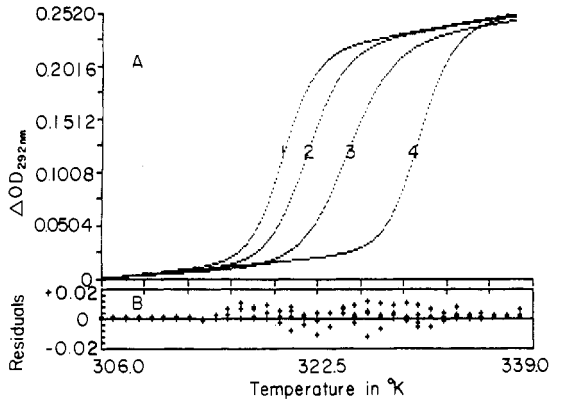

FIGURE 1: Thermal denaturation of CTG in 10^{-3} M HCl, as a function of PEG 1000 concentration. (A) The specific curves and PEG 1000 concentrations in percent (w/v) are as follows: 1, 30; 2, 20; 3, 10; 4, 0. Specific data points are omitted from the graph for clarity, and data were collected at 1-deg increments throughout the temperature interval shown. (B) Residuals of the least-squares fits of all the data sets.

under the experimental conditions. With increasing concentrations of PEG 1000, the observed value of T_m decreases. This unfolding process is thermodynamically reversible as indicated by the return of the difference spectrum to the base line when the temperature was lowered to the initial value. Hence, the data are amenable to quantitative analysis. However, an observable decrease in T_m is not consistent with literature reports (Atha & Ingham, 1981; Knoll & Hermans, 1981). The source of this contradictory observation could be the presence or absence of contaminants in PEG (Ray & Puvathingal, 1985) or the nature of the protein being studied. In order to eliminate or decrease the effect of contaminants on the T_m of proteins, different lots of PEG from two commercial sources were employed. Furthermore, PEG solutions were purified by chromatography using a gel permeation or an ion-exchange column [procedure 1 of Ray and Puvathingal (1985)]. All of these control experiments yielded the same results; hence, the lowering of T_m by PEG cannot be due to contaminants in PEG. In addition, the nature of the buffer component does not seem to significantly affect the results, since the replacement of 4×10^{-2} M glycine with 10^{-3} M HCl yielded the same results. To test whether lowering of T_m by PEG is particular to specific proteins, additional proteins were subjected to thermal denaturation in the presence of different concentrations of PEG 1000. The proteins of choice included RNase, lysozyme, and β-lactoglobulin. In each case, there is a detectable decrease in T_m as a function of PEG 1000 concentration. Results of these studies are shown in Figure 2. The most significant change in T_m is associated with the β-lactoglobulin system, followed by the lysozyme and CTG systems. PEG 1000 has the smallest effect on the T_m of RNase under these experimental conditions. Essentially no change in T_m at 15% (w/v) PEG 1000 was observed for RNase as compared to the 12 °C drop in T_m for β-lactoglobulin at the same concentration of PEG 1000. The effect of PEG on T_m of RNase is apparently pH dependent since a decrease of up to 3 °C was observed for RNase at pH 12.0. In this case, the denaturation process was not completely reversible, although at higher concentration of PEG 1000 (e.g., 20%) the absorbance did return to the base line upon lowering of temperature. In the absence of PEG, only 50–60% of the absorbance change can be reversed.

An effort was made to correlate this change in T_m with $H\phi_{av}$, average hydrophobicity, of these proteins. $H\phi_{av}$ is a term defined by Bigelow (1967) as the average free energy of

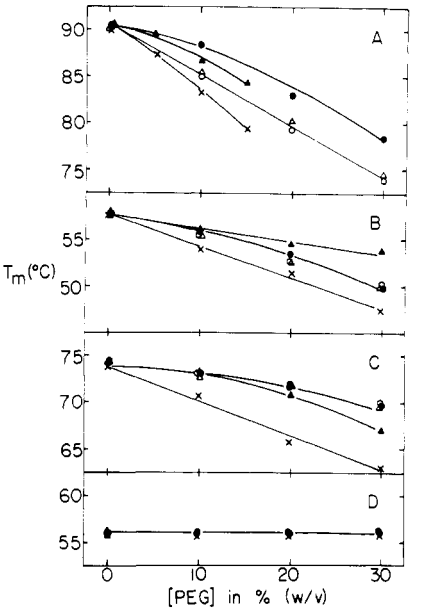

FIGURE 2: Dependence of transition temperatures, T_m, on PEG concentration. The proteins are (A) β-Lg, (β) CTG, (C) lysozyme, and (D) RNase. The symbols and identities of PEG are (●) EG, (△) PEG 200, (○) PEG 400, (×) PEG 1000, and (▲) PEG 4000.

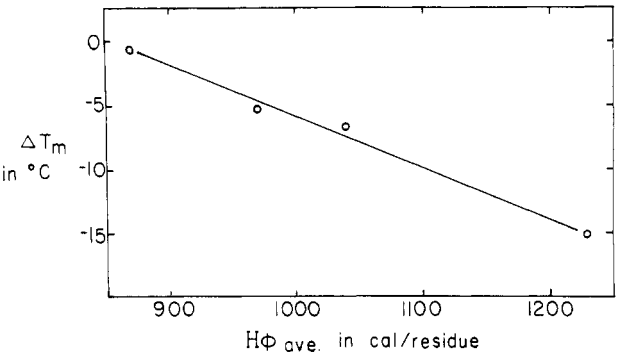

FIGURE 3: Dependence of ΔT_m on the average hydrophobicity of proteins in pH 3.0 15% PEG 1000. The proteins and their average hydrophobicity are as follows: RNase, 870; lysozyme, 970; CTG, 1040; β-Lg, 1230.

transfer of the amino acid side chains of a protein from an aqueous environment to a nonpolar environment, based on the values given by Tanford for ethyl alcohol (Tanford, 1962). Within the few proteins studied, there is a linear relationship between the magnitude of the decrease in T_m and $H\phi_{av}$. A greater decrease in T_m is observed with proteins of higher $H\phi_{av}$, as shown in Figure 3. With RNase being the least hydrophobic, there is, therefore, only a minimal observable effect of PEG on its T_m.

The effect of PEG molecular weight was tested by conducting thermal denaturation studies for all four proteins in the presence of EG, PEG 200, PEG 400, and PEG 4000. It is interesting to note that there is a dependence on PEG molecular weight. At any concentration of PEG, a larger decrease in T_m is associated with the larger synthetic polymer; e.g., for β-Lg at 10% PEG, decreases of 2, 5, and 7 deg in T_m were observed in the presence of EG, PEG 200, and PEG 1000, respectively, as shown in Figure 2A. However, in all cases tested, the effect of PEG 4000 does not follow that trend. The presence of PEG 4000 does not induce as significant a decrease in T_m as PEG 1000, as shown in Figure 2.

Since the thermal unfolding of proteins reported in this study is reversible at low pH, the data can be further analyzed to obtain the thermodynamic parameters governing the unfolding

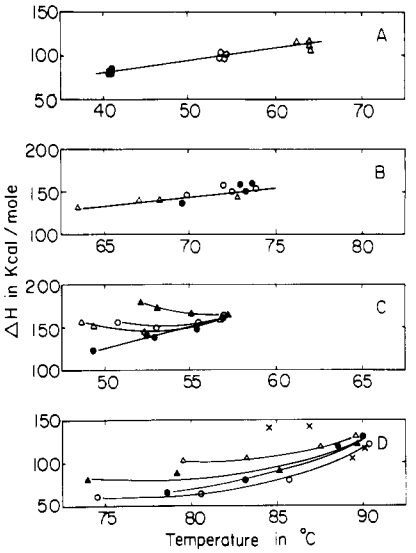

FIGURE 4: Relation between ΔH and transition temperature. The proteins are (A) RNase, (B) lysozyme, (C) CTG, and (D) β-Lg. The symbols and identities of PEG are as follows: (A) (●) PEG 1000 at pH 2.0, (○) PEG 1000 at pH 2.9, and (△) PEG 1000 at pH 6.0; (B–D) (●) EG, (○) PEG 200, (▲) PEG 400, (△) PEG 1000, and (×) PEG 4000. The lines were drawn to indicate the trend of the data only.

process. A two-state model was used to derive thermodynamic parameters from the melting curves. The equilibrium constant for thermal unfolding K is related to α, the degree of conversion from the initial to the final state in the two-state process, by $K = \alpha/(1 - \alpha)$. α can be calculated from the melting curves since

$$\alpha = \frac{A^T - A^T_0}{A^T_\infty - A^T_0} \quad (1)$$

where A^T, A^T_0, and A^T_∞ are the observed absorbance and the absorbances of the native and unfolded states at temperature T, respectively. Values for A^T_0 and A^T_∞ were obtained by linear least-squares analysis of the base lines and extrapolation into the transition zone. A similar procedure had been employed by Biltonen and Lumry (1969) and Lee and Timasheff (1981). Having determined values for A^T_0 and A^T_∞, a nonlinear least-squares program was developed to determine simultaneously ΔH, ΔS, and T_m, since

$$K = \frac{\alpha}{1 - \alpha} = \exp\left(-\frac{\Delta H}{RT} + \frac{\Delta S}{R}\right) \quad (2)$$

and T_m is the temperature where $K = 1$.

A typical set of results is shown in Figure 1. The fitted and experimental results are in good agreement, and within experimental error the residuals are randomly distributed. The relations between ΔH and T_m or ΔS and log T_m were explored. For RNase, PEG do not shift the T_m values significantly; hence, values of ΔH for each additive are grouped together. However, an increase in pH causes significant change in values of T_m, as shown in Figure 4A. A slope of 1.4 ± 0.3 kcal/(mol·deg) was obtained, indicating a positive change in heat capacity, ΔC_p, upon thermal unfolding of RNase. This change in heat capacity is consistent with the values reported by Tsong et al. (1970) and Privalov and Khechinashvili (1974). In the case of lysozyme, the presence of EG or PEG does cause changes in T_m and ΔH values, as shown in Figure 4B. Since there is no specific trend in the data for each additive, the data were analyzed as a whole, and an apparent linear dependence of ΔH on T_m can be observed. A value of 2.4 ± 0.5 kcal/(mol·deg) was obtained for ΔC_p. In the case of CTG, there is no longer a linear relation between ΔH and T_m or PEG concentration. With the exception of EG, a concave relationship is observed for all the additives, as shown in Figure 4C. The presence of PEG decreases T_m; thus, the data points at lower T_m represent those in the presence of increasing PEG concentration. Since the slope of ΔH vs T_m changes from a positive to a negative value with increasing PEG concentration, it implies that ΔC_p of unfolding changes by the addition of PEG in the solvent. A similar trend is also observed for β-Lg, as shown in Figure 4D. A series of concave relationships are observed for all solvent additives, and the degree of curvature is apparently related to the size of solvent additive, with the larger PEG inducing a more severe curvature, indicating that the magnitude of ΔC_p for unfolding decreases with larger PEG and at high concentrations of PEG ΔC_p actually assumes a negative value.

Table I: Preferential Solvent Interaction and Thermal Unfolding of RNase in 10% (w/v) PEG 1000–Water Systems

pH	$\partial g_3/\partial g_2$ (g/g)	ΔT_m	net charge[a]
2.9	−0.17 ± 0.02	+0.3	16
4.0	−0.22 ± 0.02	+0.5	12
6.0	−0.25 ± 0.02	−0.0	6
8.0	−0.12 ± 0.02	−0.5	3
10.0	−0.19 ± 0.02	−1.0	−2
12.0	−0.17 ± 0.02	−1.0	−10

[a] Data from Tanford and Hauenstein (1956).

Having established the effect of PEG on the thermal denaturation of proteins, it is of interest to monitor the interaction between solvent and proteins at the protein surface. Density measurements were carried out with RNase at various pH values ranging from 4 to 12 so as to correlate T_m with protein–solvent interactions. From density measurements done at both constant chemical potential and constant composition of solvent components, the preferential interaction between PEG (component 3) and protein (component 2) in water (component 1) can be calculated since

$$\left(\frac{\partial \rho}{\partial C_2}\right)_{T,\mu_1,\mu_3} = (1 - \phi'_2\rho_0)^\circ = (1 - \phi_2\rho_0)^\circ + \xi_3(1 - \bar{v}_3\rho_0) \quad (3)$$

The superscript indicates infinite dilution of protein; $\bar{v}_3$ is the partial specific volume of component 3; ρ_0 is the density of the solvent; ϕ'_2 and ϕ_2 is the apparent partial specific volume of the protein measured at constant chemical potential and constant molality of PEG, respectively; $\xi_3 \equiv (\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ is the preferential interaction parameter.

Solvent–protein interaction is also a reflection on the perturbation of the chemical potential of PEG by the presence of protein since (Timasheff, 1973; Lee et al., 1979)

$$(\partial \mu_3/\partial m_2)_{T,\mu_1,\mu_3} = -(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}\frac{M_2}{M_3}RT\left(\frac{1}{m_3} + \frac{\partial \ln \gamma_3}{\partial m_3}\right) \quad (4)$$

where M_2 and M_3 are the molecular weights of protein and PEG, respectively; m_3 is the molal concentration of PEG, and γ_3 is the activity coefficient of PEG, values of which were taken from Arakawa and Timasheff (1985b). Results from density measurements were analyzed according to eq 3 and 4. The calculated values of $(\partial g_3/\partial g_2)$ as a function of pH are summarized in Table I. It is evident that under the experimental conditions studied RNase is preferentially hydrated; i.e., PEG is excluded from the protein surface. The chemical potential of PEG is increased in the presence of protein, indicating a

Table II: Preferential Solvent Interactions in 20% PEG 1000–Water Systems

sample	ϕ'° (mL/g)	ϕ^0 (mL/g)	$\partial g_3/\partial g_2$ (g/g)	$\partial\mu_3/\partial m_2$ [cal (mol of protein)$^{-1}$ (mol of PEG in 1000 g of H_2O)$^{-1}$]	$\partial\mu_3/\partial m_3$ [cal (mol of PEG)$^{-1}$ (mol of PEG in 1000 g of H_2O)$^{-1}$]
RNase					
predenaturation	0.715 ± 0.002	0.678 ± 0.002	−0.29 ± 0.04	−21 000 ± 3000	5328
postdenaturation	0.651 ± 0.008	0.671 ± 0.001	0.14 ± 0.06	11 000 ± 5000	5790
CTG					
predenaturation	0.754 ± 0.003	0.744 ± 0.003	−0.08 ± 0.03	−11 000 ± 4000	5328
postdenaturation	0.754 ± 0.005	0.766 ± 0.005	0.09 ± 0.1	13 000 ± 15 000	5790

destabilization of the solution. The degree of destabilization, as indirectly reflected by the value of $(\partial g_3/\partial g_2)$, does not seem to be significantly altered by the number of charges on RNase. It is interesting to observe that the apparently constant values of $(\partial g_3/\partial g_2)$ are associated with an absence of a significant PEG effect on the T_m of RNase as a function of pH, as summarized in Table I.

The interaction between solvent and native/denatured proteins was also monitored. Density measurements were conducted with RNase and CTG at predenaturation and postdenaturation conditions, which are represented by pH 1.5, 15 °C, and pH 1.5, 40 °C, respectively. these experimental conditions were chosen since the T_m values of both proteins are between 30 and 35 °C in the presence or absence of 20% PEG 1000. Under native conditions, both proteins show preferential hydration in the presence of 20% PEG 1000, as shown in Table II. Results in postdenaturation conditions are just the opposite. Both proteins show preferential interaction with PEG. These results are consistent with the thermal denaturation studies, which indicate that PEG lower the T_m of proteins studied by preferentially interacting with the unfolded state of proteins.

Discussion

PEG is a solvent that is being used as an agent to precipitate or crystallize proteins (McPherson, 1976; Juckes, 1971; Laurent, 1963; Ingham, 1977, 1978). One of the factors responsible for its rising popularity is that PEG is not known to denature proteins. Actually, Atha and Ingham (1981) and Knoll and Hermans (1981) have reported negligible effects of PEG on the thermal denaturation of RNase. However, results from this study indicate that PEG actually induces a decrease in the thermal transition temperatures for all the proteins studied. The magnitude of this decrease is related to the hydrophobicity of these proteins. Let us examine the factors that lead to these observations.

The thermal stability of protein is governed by the fine balancing between various forces involving the interactions among solvent components and the folded or unfolded state of the protein. The unfolded state will consist of conformations in which the hydrophobic side chains are exposed to the solvent. Hence, if the added solvent components would favorably interact with these exposed groups, then the unfolded state will be stabilized in comparison with an aqueous environment, thus leading to a lower thermal transition temperature. Apparently, PEG is partially hydrophobic in nature. Hammes and Schimmel (1967) investigated the interactions between water and poly(ethylene glycol) by ultrasonic attenuation measurements. A relaxation process was identified and was attributed to a cooperative change in the local water and the hydrophobic structure associated with the polymer. Furthermore, Ingham (1977) has shown that tryptophan, the amino acid with the most hydrophobic side chain (Bigelow, 1967), is more soluble in PEG solutions. These results indicate that PEG does assume hydrophobic characteristics. Possessing partly hydrophobic properties, PEG would interact favorably with hydrophobic side chains made available when the protein is unfolded, leading to a preferential stabilization of the unfolded state. This is manifested as a lowering of the thermal transition temperature, such as the result shown in this study. A consequence of such an interpretation is that for proteins which are more hydrophobic PEG would induce a greater effect, i.e., a more substantial lowering of the thermal transition temperature. The linear relation between ΔT_m and average hydrophobicity (Figure 3) provides a strong indication that the expected result is indeed obtained. β-Lactoglobulin is the most hydrophobic protein of the four studied; thus, it is logical to observe the greatest decrease in the thermal transition temperature in this system. On the contrary, RNase is the least hydrophobic. It is not surprising that PEG has a negligible effect on the thermal transition temperature up to 20% (w/v) PEG 1000 as reported in this study. However, a significant change in the thermal transition temperature is observed at 50% (w/v) PEG 1000. This observation indicates that the general effect of PEG on the thermal transition can be detected in RNase, provided an appropriate experimental condition can be chosen to manifest the phenomenon.

The thermodynamic parameters associated with the thermal denaturation of proteins in the presence of PEG are consistent with the interpretation that PEG exerts its effect partially because of its hydrophobic nature. ΔH of unfolding for RNase and lysozyme increases with temperature (Figure 4), thus yielding a positive value for ΔC_p. This observation is consistent with the literature (Brandts, 1964; Brandts & Hunt, 1967) and is a consequence of the unfolding of the protein molecule. In the folded state hydrophobic side chains are shielded from water as they are "buried" inside the proteins. Upon unfolding they are exposed to the solvent, and the water molecules in the solvent will form clusters around these hydrophobic residues thus leading to an increase in ΔC_p for the system. However, in the presence of PEG, which is hydrophobic, these exposed side chains would interact favorably with PEG. Not only would there be a lesser chance for water clusters formed around these newly exposed side chains, the interaction between these side chains with PEG will help break up the water structure associated with PEG, thus leading to a decrease in ΔC_p such as that observed in this study for CTG and β–Lg. The hydrophobic nature of PEG is apparently size dependent with the larger one assuming a greater degree of hydrophobicity. This speculation is based on the apparent increasing curvature in ΔH vs T plots with the larger PEG. It may be concluded, therefore, that the decrease in thermal transition temperature induced by PEG can be accounted for by the hydrophobic characteristics of PEG, which leads to a stabilization of the unfolded state of proteins. The presence of more hydrophobic side chains in the protein yields a more stable unfolded state in the presence of PEG, thus a more significant decrease in transition temperature. Results on PEG–protein interactions at pre- and postdenaturation conditions are totally consistent with the thermal denaturation study. A preferential

interaction between the unfolded state and PEG was observed, a result that reflects the stabilization of the unfolded state by PEG. The explanation presented contains an assumption that the ΔH of interaction between PEG and denatured proteins is insignificant.

On the other hand, a PEG solution is more destabilized in the presence of charged species (Lee & Lee, 1981). At high enough concentrations of $MgSO_4$, an actual phase separation can be detected. Knowing that PEG exhibits hydrophobic characteristics and that it does not like to be in the presence of charged species, one may further search for a rationale that can explain why PEG is such a useful solvent for precipitating and crystallizing proteins. Ogston and co-workers (Ogston, 1958; Ogston & Phelps, 1961) have proposed that the partition of proteins between buffer solutions and solutions containing other polymers is that proteins are excluded from a part of the volume of a solution occupied by these synthetic polymers. Recently, Hermans (1982) extended Ogston's theory to treat the polymers as randomly coiled chains with Gaussian statistics and the protein as impenetrable spheres. Experimental results show that the excluded volume can account for the solution behavior of proteins in PEG solutions (Knoll & Hermans, 1983). Being excluded from the protein surface, as in the case for glycerol (Gekko & Timasheff, 1981a), PEG most likely might behave in a similar manner to decrease the magnitude of dynamic motion of proteins (Calhoun & Englander, 1985). Such an interpretation is consistent with and analogous to the results of studies on solvent–protein interactions (Gekko & Timasheff, 1981a,b) and the change in the rates of hydrogen exchange in myoglobin and lysozyme in the presence of glycerol (Calhoun & Englander, 1985; Knox & Rosenberg, 1980). Globular proteins are preferentially hydrated in glycerol, and the dynamic motions of myoglobin and lysozyme are apparently decreased by glycerol.

Evidently, the solution behavior of PEG depends on the effectiveness of utilizing its excluded volume. Why can the excluded volume of PEG be so effectively utilized? Remembering that the protein surface can be considered as a mosaic of charges and that PEG does not like to be in the presence of charged species, it is logical to conclude that at such an interface the interaction of PEG and these charges is thermodynamically unfavorable, leading to an exclusion of PEG from the protein domain. Such a proposed mechanism is consistent with the experimental results presented in this study and an earlier report from this laboratory (Lee & Lee, 1981). It is also consistent with the observation reported by Pittz and Timasheff (1978) on the interaction between proteins and 2-methyl-2,4-pentanediol, a protein crystallizing solvent. If PEG does not have such an unfavorable interaction with charges, it may not be able to utilize their excluded volume to such an advantage in precipitating and crystallizing proteins.

In conclusion, preferential hydration of the macromolecule is an observation associated with solvent additives that have been surveyed and shown to stabilize or induce crystallization of macromolecules. Being a measurement of thermodynamic interactions, preferential hydration does not infer any specific mechanism. It is interesting to note that Winzor and Wills (1987) showed that an equivalence of interpretation can be derived from the sucrose–protein interaction study both by excluded volume and by preferential solvation. Hence, in the presence of sucrose, glycerol, amino acids, and PEG, proteins are always preferentially hydrated even though the mechanisms that lead to the same thermodynamic observation are most likely different. In the case of sucrose, the exclusion of sucrose from the protein domain seems to be related to the higher cohesive force of the sucrose–water solvent system. This same mechanism does not apply to glycerol and PEG.

ACKNOWLEDGMENTS

We greatly appreciate the expert assistance of Thomas G. Consler in establishing the computer program for data analysis and the aid of Cai Jung. We are particularly grateful for the constructive criticism by the reviewers and Dr. George Na on this and an earlier version of the manuscript.

Registry No. PEG, 25322-68-3; CTG, 9035-75-0; RNase, 9001-99-4; lysozyme, 9001-63-2.

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.

Arakawa, T., & Timasheff, S. N. (1985a) *Methods Enzymol. 114*, 49–77.

Arakawa, T., & Timasheff, S. N. (1985b) *Biochemistry 24*, 6756–6762.

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

Ball, C. D., Hardt, C. R., & Duddles, W. J. (1943) *J. Biol. Chem. 151*, 163–169.

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

Biltonen, R., & Lumry, R. (1969) *J. Am. Chem. Soc. 91*, 4256–4264.

Boyer, P. D. (1945) *J. Biol. Chem. 158*, 715–716.

Bradbury, S. L., & Jakoby, W. B. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2373–2376.

Brandts, J. (1964) *J. Am. Chem. Soc. 86*, 4291–4301.

Brandts, J., & Hunt, L. (1967) *J. Am. Chem. Soc. 91*, 4826–4838.

Calhoun, D. B., & Englander, S. W. (1985) *Biochemistry 24*, 2095–2100.

Frigon, R. P., & Lee, J. C. (1972) *Arch. Biochem. Biophys. 153*, 587–589.

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

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

Gerlsma, S. Y. (1968) *J. Biol. Chem. 243*, 957–961.

Gerlsma, S., & Stuur, E. (1972) *Int. J. Pept. Protein Res. 4*, 377–383.

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

Hermans, J. (1982) *J. Chem. Phys. 77*, 2193–2203.

Herskovits, T. T., Gadegbeku, B., & Jaellet, H. (1970) *J. Biol. Chem. 245*, 2588–2598.

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

Ingham, K. C. (1978) *Arch. Biochem. Biophys. 186*, 106–113.

Jackson, W. M., & Brandts, J. F. (1970) *Biochemistry 9*, 2294–2301.

Juckes, I. R. M. (1971) *Biochim. Biophys. Acta 229*, 535–546.

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

Knoll, D., & Hermans, J. (1983) *J. Biol. Chem. 258*, 5710–5715.

Knox, D., & Rosenberg, A. (1980) *Biopolymers 19*, 1049–1068.

Laurent, T. C. (1963) *Biochem. J. 89*, 253–257.

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., Gekko, K., & Timasheff, S. N. (1979) *Methods Enzymol. 61*, 26–49.
McPherson, A., Jr. (1976) *J. Biol. Chem. 251*, 6300–6303.
Na, G. C., & Timasheff, S. N. (1981) *J. Mol. Biol. 151*, 165–178.
Neucere, N. J., & St. Angelo, A. J. (1972) *Anal. Biochem. 47*, 80–89.
Ogston, A. G. (1958) *Trans. Faraday Soc. 54*, 1754–1757.
Ogston, A. G., & Phelps, C. F. (1961) *Biochem. J. 78*, 827–833.
Pittz, E. P., & Timasheff, S. N. (1978) *Biochemistry 17*, 615–621.
Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665–684.
Ray, W. J., Jr., & Puvathingal, J. M. (1985) *Anal. Biochem. 146*, 307–312.
Scott, R. A., & Scheraga, H. A. (1963) *J. Am. Chem. Soc. 85*, 3866–3873.
Sophianopoulos, A. J., Rhodes, C. K., Holcomb, D. N., & Van Holde, K. E. (1962) *J. Biol. Chem. 237*, 1107–1112.
Tanford, C. (1962) *J. Am. Chem. Soc. 84*, 4240–4247.
Tanford, C., & Havenstein, J. D. (1956) *J. Am. Chem. Soc. 78*, 5287–5291.
Timasheff, S. N. (1973) *Protides Biol. Fluids 29*, 511–519.
Timasheff, S. N., Lee, J. C., Pittz, E. P., & Tweedy, N. (1976) *J. Colloid Interface Sci. 55*, 658–663.
Townend, R., Winterbotton, R. J., & Timasheff, S. N. (1960) *J. Am. Chem. Soc. 82*, 3161–3168.
Tsong, Y. T., Hearn, R. P., Wrathall, D. P., & Sturtevant, J. M. (1970) *Biochemistry 9*, 2666–2677.
Winzor, D. J., & Wills, P. R. (1987) *Biophys. Chem. 25*, 243–252.

Presclerotized Eggshell Protein from the Liver Fluke *Fasciola hepatica*†

J. Herbert Waite*

College of Marine Studies, University of Delaware, Lewes, Delaware 19958

Allison C. Rice-Ficht

Department of Medical Biochemistry and Genetics, Texas A&M University, College Station, Texas 77843

Received May 13, 1987; Revised Manuscript Received June 26, 1987

ABSTRACT: Trematode parasites protect their eggs with a tough tanned eggshell. Eggshell precursor proteins are synthesized and stockpiled within the extensive vitellaria of the animal. A major eggshell precursor protein with an apparent molecular weight of 31 000 and p*I* of 7.4 was isolated from the vitellaria of *Fasciola hepatica*. This protein, which represents 6–7% of the total protein in mature *Fasciola*, is unique in containing rather high levels of the amino acid 3,4-dihydroxyphenylalanine (DOPA), i.e., 110 residues per 1000. Other prominent amino acids are glycine, aspartic acid, and lysine. A prominent DOPA-containing tryptic peptide derived from eggshell precursor protein has the sequence Gly-Gly-Gly-DOPA-Gly-Gly-DOPA-Gly-Lys. DOPA residues disappear during the maturation of the eggshell and by treatment in vitro with mushroom polyphenol oxidase. This disappearance may be related to the formation of cross-links in the eggshell protein.

The liver fluke *Fasciola hepatica* is a parasitic trematode that occurs throughout the world and causes "liver rot" in infected sheep and milder cirrhotic complications in cattle, goats, and humans. Immature *Fasciola* migrates through the liver parenchyma, consuming copious amounts of tissue. Upon maturation, *Fasciola* moves from the parenchyma to the bile ducts of the liver where it feasts on blood, leaving a trail of eggs and fecal material. A mature liver fluke produces an average of 2500 eggs daily (Björkman & Thorsell, 1963). Some of these eggs find their way into the gut, through which they are passed to continue their life cycles. The entrapped eggs, however, eventually decompose, setting up serious inflammatory and fibrotic reactions in surrounding tissues (Pantelouris, 1965; Malek, 1980).

To protect the eggs from the host as well as the external environment, the liver fluke encapsulates the eggs with a mechanically tough and chemically resistant scleroprotein eggshell. The stability of this scleroprotein has been variously ascribed to quinone tanning (Stephenson, 1949; Nollen, 1971), dityrosine cross-links (Ramalingam, 1972), and keratin (Smyth & Halton, 1982) but has never been adequately investigated. Eggshell precursor proteins are synthesized and stockpiled by vitelline cells in the extensive vitellaria of *Fasciola* (Smyth, 1954; Björkman & Thorsell, 1963; Irwin & Threadgold, 1972). Mature vitelline cells sequester eggshell precursors in mottled globules that contain two immiscible substances. The discontinuous "polka dot" substance strongly binds heavy metals (Björkman & Thorsell, 1963), basophilic dyes such as malachite green (Johri & Smyth, 1958), and di- and tetrazotized salts and other reagents with affinities for polyphenols (Stephenson, 1949; Smyth, 1954; Bogitsch, 1984). These tests suggest the presence of a cationic protein rich in orthodiphenolic groups (Smyth & Clegg, 1959). The continuous

† Funding for this research was obtained from the Tropical Medicine and Parasitology Program of NIAID Grants 1 R22 AI24450 (J.H.W.) and 1 R01 AI GM 22713-01A1 (A.C.R.-F.). Additional support came from Office of Naval Research Contract N00014-86-K-0717 (J.H.W.).

0006-2960/87/0426-7819$01.50/0