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Journal of Chromatography B, 798 (2003) 25–33
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Study of the serum albumin-polyethyleneglycol interaction to predict the protein partitioning in aqueous two-phase systems

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Received 12 May 2003; received in revised form 20 August 2003; accepted 25 August 2003

Abstract

The theoretical framework based only on the excluded volume forces is not enough to explain the bovine serum albumin partitioning behaviour in aqueous biphasic systems. The goal of this work is to look at the phase separation via the polymer effect on the water structure. Our findings suggest that polyethyleneglycol 600-protein interaction is conducted by van der Waals forces between the hydrophobic surfaces from PEG and protein molecules, which implies the rupture of hydrogen bonds from the structured water in their neighbours. Therefore, the protein will concentrate in the most water-structured phase (polyethyleneglycol) in order to reach the minimal free energy condition. When polyethyleneglycol molecular weight increases, its exclusion from protein surface prevails, thus pushing the bovine serum albumin to the bottom phase.

Keywords: Protein partitioning; Aqueous two-phase systems; Albumin; Polyethyleneglycol

1. Introduction

Aqueous biphasic systems are widely used in the biotechnology of isolation and purification of macromolecules. The method consists in partitioning a macromolecule between the two aqueous phases of a system formed by mixtures of either two polymers of flexible chains or one polymer and a high concentration salt, the polyethyleneglycol and potassium phosphate being the most popular [1]. The partition coefficient of a protein (K_r) depends, among other factors, on the molecular weight and charge of the partitioned particle, the ionic strength of the medium, the type and molecular weight of the flexible polymer and the temperature [2]. Several theoretical models have been proposed to explain the thermodynamics of protein partitioning in aqueous two-phase systems. Diamond and Hsu [3] presented a simple linear semilogarithmic relationship for correlating biomolecule partitioning with respect to the polyethyleneglycol concentration difference between the phases. The relationship proved to be adequate for dipeptide and low-molecular-weight protein partitioning. Edmon and Ogston and more recently King and co-workers [1,4] have derived an equation based on the lattice model of Flory Huggins that predicts that the partition coefficient of a protein, K_r, in a biphasic system formed by two uncharged flexible chain polymers not only depends on the differences in polymer concentration but on the second virial coefficients between each polymer and the protein also. From another point of view, the phase separation in aqueous-polymer systems may be considered as a consequence of the polymer effect on the water structure of coexisting phases. By studying PEG-protein interaction through some physico-chemical techniques, the aim of this paper is to explain the effect of PEG molecular weight on BSA (as model protein) partitioning behaviour via its influence on the water structure.

Abbreviations: PEG600, polyethyleneglycol of average molecular weights 600; PEG1000, polyethyleneglycol of average molecular weights 1000; PEG1450, polyethyleneglycol of average molecular weights 1450; PEG3350, polyethyleneglycol of average molecular weights 3350; PEG6000, polyethyleneglycol of average molecular weights 6000; PEG8000, polyethyleneglycol of average molecular weights 8000; BSA, bovine serum albumin; ANS, 1 anilino-8-naphthalene sulfonate; S_0, relative protein surface hydrophobicity; D_f, diffusion hydrodynamic coefficient; r, anisotropy; CD, circular dichroism

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doi:10.1016/j.jchromb.2003.08.044

2. Experimental

2.1. Material

2.1.1. Chemicals

Bovine serum albumin (BSA); polyethyleneglycols of the following average molecular weights: 600 (PEG600); 1000 (PEG1000); 1450 (PEG1450); 3350 (PEG3350); 6000 (PEG6000); 8000 (PEG8000) and 1 anilino-8 naphthalene sulfonate (ANS) were purchased from Sigma and used without further purification. All the other reagents were of analytical quality.

2.2. Methods

2.2.1. Fluorescence and absorbance spectra of BSA in the presence of PEGs

Absorption spectra were performed on a Jasco VS550 spectrophotometer with a slit of 0.1 nm and a scan rate of 20 nm min^{-1}; fluorescence measurements were performed on an Aminco Bowman S2 spectrofluorometer using a thermostatized cuvette of 1 cm of optical pathway. Emission fluorescence spectra were corrected using the software supplied by the manufacturer. For globular proteins, the fluorescence anisotropy (r) reciprocal is a function of the absolute temperature and the medium viscosity according to:

$$\frac{1}{r} = \frac{1}{r^{\circ}}\left(1 + \frac{\tau kT}{V_{\mathrm{m}}\eta}\right) \quad (1)$$

where r° is the anisotropy in the absence of fluorophore rotation, τ the life time of the fluorophore, k the Boltzman constant and V_{m} is the molecular volume of the rotating unit. In media of increasing PEG concentrations, τ and T are constant, then r will vary due to the V_{m} and η changes induced by the PEG presence. Polarized light fluorescence measurements were corrected by the G factor according to Lakowicz [5].

2.2.2. Light scattering measurements

Dynamic and static light scattering measurements were made at 25 °C, onto Malvern 7032 spectrometer of 72 channels instrument equipped with a He/Ne laser, operating at 633 nm with vertically polarized light (Spectra Physics 124B). Dynamic light scattering measurements were carried out at scattering angles from 30° to 120° using digital auto correlator. The homodyne intensity–intensity correlation function $G(q, t)$ with q (the amplitude of the scattering wavelength vector) is given by $q = (4\pi n/\lambda)\sin(\theta/2)$ where n is the medium refractive index, λ is the vacuum wavelength of the incident light in the vacuum and θ is the scattering angle. $G(q, t)$ is related to the time correlation function of concentration fluctuations $g(q, t)$ by $G(q, t) = A[1 + bg(q, t)^2]$, where A is the experimental base line and b is the fraction of the scattered intensity arising from concentration fluctuations. The mean decay constant, Γ, was obtained by the method of the cumulants and used to calculate the apparent diffusion coefficient $D = \Gamma/q^2$ [6,7]. Neither precipitation nor turbidity appeared in any of the samples examined. Protein solutions do not absorb at 633 nm, so the laser beam does not perturb the protein. All solutions were filtered through a 0.2 μm filter. Benzene was used as a reference with a Rayleigh ratio value of 1.18×10^{-5} m^{-1}.

2.2.3. Effect of PEG presence on circular dichroism spectrum

Circular dichroism spectra (CD) were performed in a Jasco J810 spectropolarimeter, using a thermostatized cuvette of 10 or 1 mm of pathlength, the slit was varied in an automatic manner from 0.2 to 0.7 nm. Repetitive scanning of 5 cycles was used [8].

2.2.4. Measurements of the protein surface hydrophobicity (S_0)

Stock solutions of 8 mM ANS, 1 mM of BSA were prepared in 40 mM potassium phosphate buffer pH 7.4. Aliquots of the protein were added to a sample containing 2 ml of the corresponding equilibrated phase (top or bottom) and 2.5 μl of ANS solution. The final ANS concentration was 20 μM while the protein concentration varied from 0 to 3 μM. Relative fluorescence intensity was measured on a Jasco FP 770 spectrofluorometer, the excitation and emission wavelength being 360 and 470 nm, respectively. Under the above mentioned conditions with excess probe (ANS), the initial slope (S_0) of the fluorescence intensity versus protein concentrations plot has been shown to be correlated to the effective hydrophobicity of the protein [9].

2.2.5. Chemical denaturation of the protein by PEG

Chemical stability of the protein was assayed measuring the native fluorescence emission of the protein at 340 nm (while exciting at 280 nm) in media of increasing urea and constant PEG concentrations. We assumed that the unfolding reaction is accompanied by the formation of a complex between the unfolded protein (U) and the denaturant agent (D) according to the following equilibrium equation, as it was previously demonstrated [10]:

$$U + nD \rightleftarrows UDn$$

pH measurements of the protein-denaturant media were carried out in all the experiments. Analysis of the data was performed assuming a two-state denaturation model [11]. The unfolded fraction of protein, α, was calculated as:

$$\alpha = \frac{F_{\mathrm{N}} - F_i}{F_{\mathrm{N}} - F_U} \quad (2)$$

where F_{N} and F_U are the fluorescence of the native (in absence of denaturant) and the unfolded states (at high denaturant concentration) of albumin, respectively; and F_i is the fluorescence of the protein at i denaturant concentration, α versus the denaturant agent concentration [urea] was fitted by using a least squares non-linear method.

The free energy change of unfolding, ΔG_U was obtained by applying the equation:

$$\Delta G_U = -RT \ln K \quad (3)$$

where K is the unfolding equilibrium constant, calculated as $K = \alpha/(1-\alpha)$.

2.2.6. Isothermal titration calorimetry of BSA with PEG

Heats of mixing of BSA as a function of PEG concentration were determined at pH 7.40 and 25 ± 0.1 °C using an isothermal titration calorimeter (ITC) (MicroCal, Inc.). Stock solutions of BSA 1.5 mM were prepared and then dialized with a 50 mM potassium phosphate buffer pH 7.40. The ITC experiments were performed by 10 injections (using 10 μl of a 10 mM stock solution of PEG per injection) into the calorimeter cell (ca. 1.5 ml) containing the 1.5 mM BSA solution. Control experiments involving injections of buffer into BSA solutions and injections of PEG stock solutions in buffer were also conducted. Heats of mixing experiments for BSA solution with PEG ($\Delta H_{\text{mix (BSA–PEG)}}$), BSA solution with buffer ($\Delta H_{\text{mix (BSA)}}$) and buffer with PEG ($\Delta H_{\text{mix (PEG)}}$) were separately measured. By plotting ΔH_{mix} versus PEG concentration the ΔH_{mix} at infinite dilution was obtained. The net calorimetric heat of interaction of BSA with PEG (ΔH_{intter}) was obtained using:

$$\Delta H_{\text{inter.}} = \Delta H_{\text{mix (BSA–PEG)}} - \Delta H_{\text{mix (BSA)}} - \Delta H_{\text{mix (PEG)}} \quad (4)$$

2.2.7. Preparation of the aqueous biphasic system

To prepare the aqueous biphasic systems, stock solutions of the phase component PEG 40% (w/w) and potassium phosphate were mixed in order to obtain a total system composition of Table 1 according to the binodal partition diagram reported by Zaslavsky [12]. PEG concentrations in both top and bottom equilibrium phases are lower than the polymer crossover concentration—the concentration where the extensive overlap of polymer coils begins to occur—for most of PEGs assayed. Under these conditions the polymer coils are longer separated by regions of solvent and a dilute polymer solution regime can be assumed [13]. Low-speed centrifugation to speed up phase separation was used after a gentle mixing of the system components, then 2 ml of each phase were mixed to reconstitute several two-phase systems in which the protein partition was assayed.

2.2.8. Determination of the partition coefficient (K_r)

Partition coefficient of the protein in both phases was analysed by dissolving increasing amounts of protein in the two phase pre-formed system containing 2 ml of each equilibrated phase. The protein aliquots added to the system varied from 1 to 10 μl (total protein concentration varied from 5 to 15 μM), the change of the total volume of each phase being negligible. After mixing by inversion for 1 min and leaving it to settle for at least 30 min, the system was centrifuged at low speed for the two phase separation. Samples were withdrawn from separated phases, and after dilution, the protein content in each phase was determined by measuring the absorption at 280 nm. The partition coefficient (K_r) was defined as:

$$K_r = \frac{[P]_{\text{top}}}{[P]_{\text{bottom}}} \quad (5)$$

where $[P]_{\text{top}}$ and $[P]_{\text{botton}}$ are equilibrium concentrations of the partitioned protein in the PEG and phosphate enriched phases, respectively. In the protein concentration range assayed, a plot of $[P]_{\text{top}}$ versus $[P]_{\text{bottom}}$ showed a linear behaviour, K_r value being its slope. Absorbances were measured with a Spekol 1200 spectrophotometer.

3. Results

3.1. Absorption and fluorescence spectra of BSA in the presence of PEG

The presence of PEGs of different MW (600–8000) at 7% (w/w) did not produce any modification in the protein absorption spectrum while it induced a perturbation of the native protein fluorescence at 340 nm when it was excited at 280 nm. Table 2 shows that both PEG1000 and PEG600 induce significant shifts to the left of the emission peak position, which suggests the presence of a specific interaction between these flexible chain polymers and the protein. The other PEGs did not induce any appreciable modification either on the position or in the intensity of the fluorescence emission band.

Table 1
Phase diagram and phase composition of biphasic systems

PEG molecular weight (Da)	Total system		Top phase		Bottom phase	
	PEG (%, w/w)	Salt (%, w/w)	PEG (%, w/w)	Salt (%, w/w)	PEG (%, w/w)	Salt (%, w/w)
600	14.00	15.50	23.01	9.04	5.41	22.06
1000	13.00	15.00	25.02	6.64	3.08	21.56
1450	12.40	12.83	22.22	6.64	3.79	18.33
3350	10.10	10.90	18.55	6.60	2.76	14.80
6000	8.26	10.65	18.45	6.13	0.90	13.84
8000	12.20	9.70	22.19	4.68	2.00	14.77

Systems of polyethyleneglycol (of different molecular weight)–potassium phosphate pH 7.00 were used at temperature = 25 °C.

Table 2
Effect of PEG molecular weight on BSA fluorescence spectrum

PEG molecular weight	Fluorescence emission	
	Peak position	Fluorescence intensity
Buffer alone	338.8	6.193
600	336.8	6.208
1000	335.2	6.443
1450	339.2	6.499
3350	339.2	5.875
6000	338.8	6.373
8000	339.2	6.302

Position and intensity of the fluorescence emission peaks for BSA (9 μM) in different media of 8% (w/w) PEG and buffer potassium phosphate 50 mM pH 7.40 (λ_{exc} = 280 nm).

3.2. PEG effect on the BSA native anisotropy (r)

The anisotropy for the native fluorescence of albumin tryptophan (measured at 340 nm, while exciting at 280 nm) was plotted as a function of PEG concentration at constant temperature. Fig. 1 shows the anisotropy of native albumin fluorescence in the presence of increasing PEG concentration. According to Eq. (1) the anisotropy variation can be due mainly to the variation of the protein molecule volume, the medium viscosity and the temperature. Taking into account the fact that the measurements were performed at constant temperature (25 °C) and that solutions of low MW PEGs have low viscosity, the *r* increase with PEG concentration may be due to the protein volume changes. In presence of PEG 600 and PEG1000 (at low concentration) a great value of $\partial r/\partial$[PEG] is observed, which suggests a loss of freedom in the BSA molecule due to PEG-protein interaction. At higher PEG concentration, a saturation anisotropy value is reached which is also in agreement with the presence of a protein–PEG interaction.

Table 3
Effect of PEG presence on BSA diffusion coefficients

Medium	D_f (10^{-7} cm^2/s)	η (mPa s)	$D_f\eta/T$ (10^{-16} J/K m)
Buffer	6.32	0.83	1.76
PEG600	0.79	1.04	0.27
PEG1000	1.20	1.10	0.44
PEG3350	0.82	1.80	0.49
PEG8000	1.15	2.82	1.08

Media viscosities and diffusion coefficients of BSA in absence and presence of PEGs of different molecular weight. BSA concentration 10 μM, PEG concentration 7% (w/w), temperature = 25 °C.

3.3. Dynamic light scattering measurements

The diffusion coefficient of BSA (D_f) was measured at 25 °C in a medium containing PEG 7% (w/w) of different molecular weights. This was determined by measuring the scattered light of a PEG-BSA solution at 90° because the slope of the ln *G* versus time plot was found to show a linear behaviour with the scattering angle, which implies that scattered light intensity is angle independent (data not shown). It was assumed that the system under study is a multicomponent system of PEG and protein. Taking into account that the measured light scattering signal for PEG solution was of similar magnitude to that obtained for the buffer alone, we assume that the contribution of free PEG to the total scattered light in PEG-protein solution is negligible. Table 3 shows the diffusion coefficients obtained for BSA in the absence and presence of different PEGs, the media viscosities (η) of each medium without protein and the product $\eta D_f/T$.

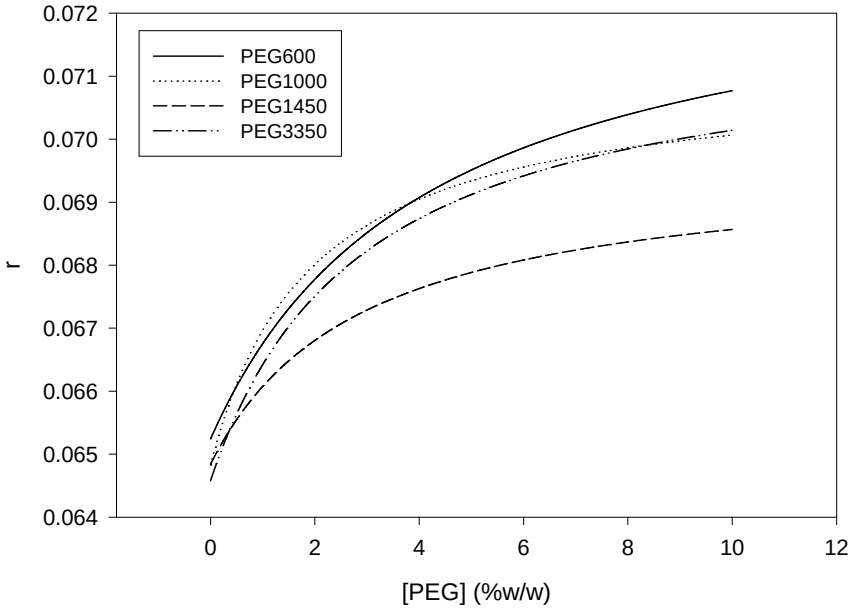

Fig. 1. Effect of PEG concentration on the BSA native anisotropy (*r*) for PEGs of different molecular weight (λ_{exc} = 280 nm, λ_{em} = 340 nm). Buffer potassium phosphate 50 mM, pH 7.40, [BSA] = 30 μM. Each point is the mean of four independent measurements.

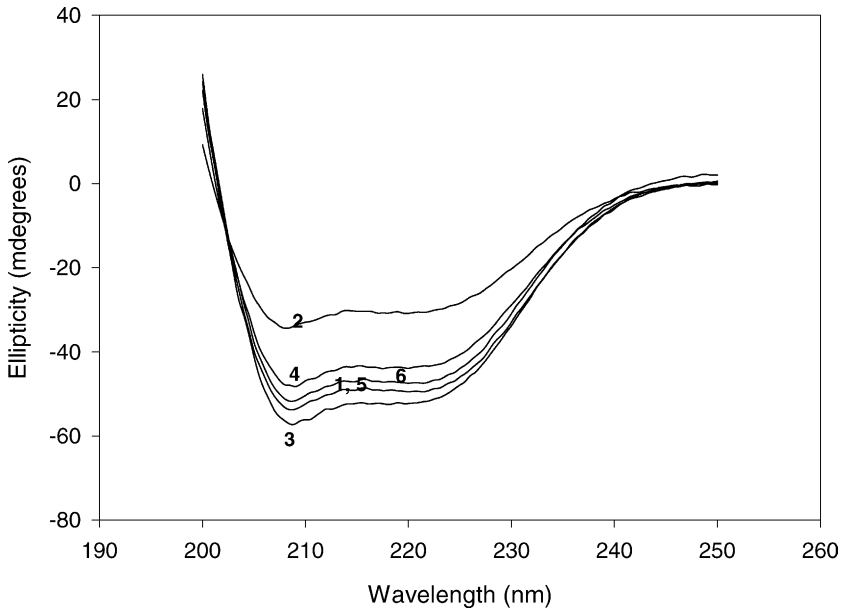

Fig. 2. Circular dichroism spectra of BSA 5 μM in absence and presence of PEGs of different molecular weight. (1) Without PEG, (2) PEG600, (3) PEG1000, (4) PEG1450, (5) PEG3350 and (6) PEG8000, temperature = 25 °C. Buffer potassium phosphate 50 mM, pH 7.40. Each point is the mean of four independent measurements.

As expected, the measured D_f scale with viscosity, therefore the PEG presence induces a dramatic decrease in the D_f coefficient of BSA for the totality of the PEGs assayed. However, the sharp decrease in the calculated product $\eta D_f/T$, only observed for PEGs of lower molecular weight, suggests a large change in the average dimension of the BSA which is in agreement either with the presence of an atmosphere of PEGs molecules in the protein neighbours or with a PEG–protein complex formation.

3.4. PEG effect on the CD spectrum of BSA

Fig. 2 shows the effect of PEG at a concentration of 7% w/w on the CD protein spectrum. It can be seen that PEG600 induced a significant change in the far UV CD signal in agreement with a decrease of the protein alpha helix content (see Table 4). No significant effects were observed for the other PEGs assayed. The presence of a perturbation in the CD spectrum of a protein by the specific binding of a ligand has been shown to induce a significant perturbation in the CD spectrum in the far UV region (200–250 nm) due to a conformational change in the environment of the tryptophan residues. No effect was observed in the near UV region of the CD protein spectrum (270–300 nm) suggesting that PEG does not induce microarragements at the BSA tryptophan residues accessible to solvent.

Table 4
Effect of PEG presence on BSA alpha helice content

	Alpha helice content (%)
Without PEG	58.3
PEG600	36.4
PEG1000	60.7
PEG1450	50.9
PEG3350	58.3
PEG8000	58.0

BSA alpha helice content in absence and presence of PEGs of different molecular weight at 7% (w/w), [BSA] = 5 μM, temperature = 25 °C.

3.5. Effect of PEG on the protein relative surface hydrophobicity

Fig. 3 shows that the presence of the totality of PEGs assayed induced a significant decrease in protein relative surface hydrophobicity (S_0), this effect being more important for PEG1000 and PEG8000. These results suggest that PEG presence modifies the protein hydrophobic area exposure to the solvent, and therefore the ANS binding to the protein.

3.6. Chemical denaturalization of BSA in the presence of PEG

The native fluorescence of albumin at 345 nm was measured versus the urea concentration (while exciting the protein at 280 nm). By plotting ΔG_U values (calculated from Eqs. (2) and (3)) versus urea concentration, a linear relationship was obtained according to:

$$\Delta G_u = \Delta G_{H_2O} - m\,[\text{urea}] \quad (6)$$

where ΔG_{H_2O} is the protein free energy of unfolding in the absence of a denaturant agent, and m is the slope of this equation (the dependence of free energy on the denaturant concentration) which is proportional to the difference in solvent-exposed surface area between the denatured and

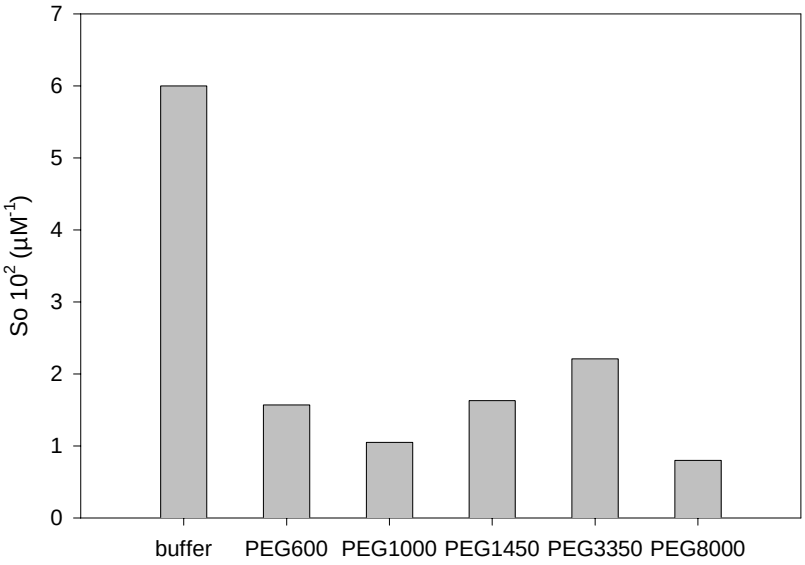

Fig. 3. Relative surface hydrophobicity (S_0) of BSA in medium of PEGs (7% (w/w)) of different molecular weight. Medium potassium phosphate buffer 50 mM, pH 7.4, temperature = 25 °C. Each point is the mean of four independent measurements.

native states [11]. Thus, *m* can be taken as an indicator of the compactness of the denatured state. Table 5 summarizes the PEG presence effect on the ΔG_{H_2O} and m the values obtained for BSA chemical unfolding. PEG600 induced a significant increase in both *m* and ΔG_{H_2O} values. This finding suggests an increase in BSA thermodynamic stability. For the other PEGs assayed, a low decrease in *m* values is observed when PEG molecular weight increases.

3.7. Isothermal calorimetry titration of albumin by PEG

Table 6 shows the integral heat of solution of PEGs with buffer and BSA respectively (ΔH_{mix}) at infinite dilution. The PEG–buffer mix can be seen to be an exothermic process, the ΔH_{mix} magnitude increasing at higher PEG MW. These PEGs have a greater number of oxhidriles in their molecule and, consequently, release more energy in the hydration process. On the other hand, the opposite effect is observed when the PEGs–BSA mix occurs. Positive ΔH_{mix} values are observed which may indicate the breaking of hydrogen bonds of the structured water in both the protein and the flexible polymer neighbour. This effect is more significant for PEG600.

Table 5
Parameters characterizing the urea unfolding of serum bovine albumin

	ΔG_{H_2O} (kcal/mol)	*m* (cal/mol M)
Without PEG	3.9	980
PEG600	6.4	1705
PEG1450	3.5	929
PEG3350	3.4	811
PEG6000	3.9	741

ΔG_{H_2O} and *m* values obtained for BSA in potassium phosphate buffer 50 mM, temperature = 25 °C.

3.8. Effect of MW of PEG on partition coefficient BSA

The effect of protein concentration on partition coefficient (K_r) was tested. A plot of the top versus bottom absorbance at 280 nm yielded a linear behaviour (data not shown), demonstrating that the partition coefficient was independent of the protein concentration in each phase in the range 0–20 μM. Fig. 4 shows the partition coefficient obtained for BSA in the different PEG-phosphate biphasic systems. The increase of PEG MW can be seen to induce a significant transfer of BSA to the phosphate rich phase. These results are in agreement with the findings of other authors [1,2,4] and it is a generalized rule for the partition constant dependence with the PEG molecular weight. In systems of phosphate with PEG6000 and PEG8000, the K_r values were not reported because the BSA partition equilibria were totally displaced to the bottom phase (phosphate rich phase); therefore, the protein concentration at the top phase was practically impossible of measuring.

Table 6
Calorimetric measurements for PEG–BSA interaction

	$\Delta H_{mix(PEG)}$ (kcal/mol)	$\Delta H_{mix(BSA–PEG)}$ (kcal/mol)[a]	$\Delta H_{inter.}$ (kcal/mol)	$\Delta H_{inter.}$ (cal/g)
PEG600	−0.170	1.323	1.493	2.49
PEG1000	−0.157	0.062	0.219	0.219
PEG3350	−0.346	0.078	0.424	0.126
PEG8000	−0.28	0.628	0.908	0.113

Integral heats of solution at infinite dilution for the different PEGs in buffer potassium phosphate 50 mM, pH 7.40 ($\Delta H_{mix(PEG)}$) and in a 1.5 mM BSA solution in identical buffer ($\Delta H_{mix(BSA–PEG)}$). The $\Delta H_{inter.}$ associated to the BSA–PEG interaction was calculated according Eq. (4) at temperature = 25 °C.

[a] The heat of solution of BSA was subtracted.

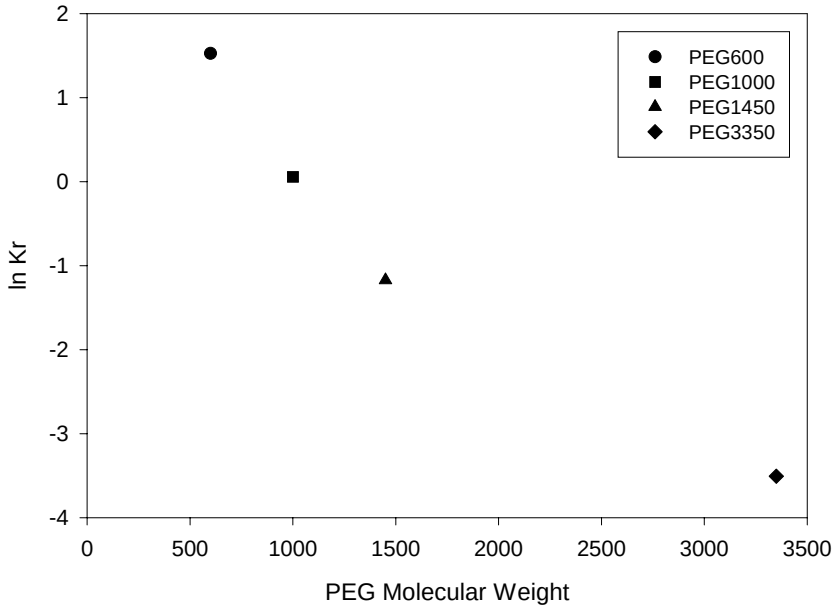

Fig. 4. Variation of logarithm of BSA partition coefficient (K_r) with PEG molecular weight. Two-phase PEG-phosphate systems composition are those from Table 1 (temperature = 25 °C). Each point is the mean of four independent measurements.

4. Discussion

PEG molecule has hydrophilic and hydrophobic properties, as it is soluble both in water and many organic solvents is frequently described as amphiphilic molecule. Due to its low capacity to interact with proteins, it is very difficult to determine the protein–PEG affinity constant. Farruggia et al. [14] found a value of about $10^2\ M^{-1}$ for serum albumin-low molecular weight PEG complex formation by using an indirect method, which suggests a very weak interaction. This finding agrees with the modification of the fluorescence emission spectrum of the protein at 340 nm when it is excited at 280 nm for the low molecular weight PEGs. The small blue shift in the fluorescence emission peak of the protein is in accordance with an increase in the environment non polar character of the BSA tryptophan residues accessible to solvent. BSA has two tryptophan residues, the 214 residue is buried in the protein but only the 114 one is accessible to the solvent and therefore the fluorescence emission will be very sensitive to changes in the polarity of its environment. The theory of the chromophore–solvent interaction predicts that the decrease in the polarity of the medium induces a blue shift of the emission fluorescence peak. The 2 nm shift observed in presence of PEG for the BSA fluorescence emission peak might suggest the presence of an atmosphere of this polymer around the protein molecule. On the other hand, the drastic diminution of BSA relative surface hydrophobicity (S_0) in presence of PEGs indicates a decrease of the ANS accessibility to the protein domain and is in agreement with the presence of a PEG atmosphere in the protein hydrophobic surface neighbours. A minor effect was observed for PEGs of higher MW in agreement with an increasing exclusion effect due to their size.

In general, the dissolution of PEG in water is an exothermic process, in accordance with the high PEG capacity to form hydrogen bonds between the oxygen ether linkages and water molecules, which leads to the formation of structured water around the flexible chain polymer. The magnitude of the enthalpic change increases as the PEG molecular weight does. However, the interaction between different PEGs and albumin is associated to a positive enthalpic change, which can be attributed both to the unfavourable interaction of PEG molecules with charges present on the surface of the protein [15] and to the rupture of hydrogen bonds from structured water on both PEG molecule and hydrophobic protein surface [16]. Taking into account the fact that we are comparing systems of similar compositions expressed in the percentage weight on weight, it is convenient to recalculate $\Delta H_{\text{inter.}}$ per gram of PEG. In this way, the maximal enthalpic change observed for albumin-PEG600, is probably due to its low size and the possibility of forming a complex. This finding is also in agreement with the far UV CD spectrum of BSA which shows a perturbation in presence of PEG600. This suggests an important decrease in the protein alpha helice content. On the other hand, the $\eta D_f/T$ ratio calculated for the totality of PEGs assayed, shows a significant decrease for PEG600 that may be explained on the basis of an increase of protein size due to the formation of a complex between the flexible chain polymer and the protein [6].

Anysotropy polarization results are not conclusive about the effect of different PEGs. However, r versus [PEG] plots which reached a plateau at high PEG concentration suggest the presence of an interaction with the protein.

The theory of chemical globular proteins denaturation [17] predicts that the free energy variation of protein

unfolding (ΔG_U) depends on the denaturant agent concentration (c) according to:

$$\frac{\partial \Delta G_{\mathrm{U}}}{\partial c} = kT\,\Delta A \frac{\partial \chi}{\partial c} \tag{7}$$

where k is the Boltzman constant and T the absolute temperature. χ is the free energy of transfer of an average hydrophobic residue from aqueous medium to solution of denaturant. ΔA is the difference between the exposed area in the unfolded and folded protein states. χ has been demonstrated to depend on both the temperature and the chemical composition of the solution but slightly on the protein structure. On the other hand, ΔA depends largely on the protein structure because it is related not only to the number of aminoacids per protein molecule but to the change of the hydrophobic residues fraction on the protein surface associated to the unfolding process. Therefore, the size and composition of the protein affect $\partial \Delta G_U/\partial c$ because they affect the amount of hydrophobic surface area that can be exposed upon unfolding (ΔA).

In our work, ΔG_U versus (denaturant agent [urea]) plot followed a linear relationship, therefore the $\partial \Delta G_U/\partial$[urea] values can be obtained from its slope, m. By comparing Eqs. (6) and (7) it is clear that m will be directly related to ΔA. Previous works have demonstrated that BSA unfolding follows a two-state mechanism [18]. In this case, the m value depends on the amount of polypeptide chain that is freshly exposed to solvent on unfolding. By comparing the presence of different PEGs on BSA unfolding, the larger m value observed for PEG600 presence must reflect a larger increase in the extent of BSA unfolding because the folded conformation is less accessible [19]. The protein chemical denaturation may be explained on the basis of an unfolding equilibrium displacement to the unfolded state due to the binding of the denaturant agent to peptide bonds, which are more exposed as a protein unfolds. The highest $\Delta G_{\mathrm{H_2O}}$ value observed for PEG600 presence suggests an increase of the BSA stability in agreement with a previous PEG600-albumin interaction, which prevents the urea from binding and therefore the protein from unfolding. On the other hand, a protein preferential hydration [20] occurs in presence of PEGs of higher molecular weight which leads to an exclusion of PEG from the protein and to a destabilization of the protein in the presence of chemical denaturants.

The partition coefficient of BSA decreases with increasing PEG molecular weight, but the magnitude of this decrease is larger for the smaller PEG molecular weights and tends to level off at high PEG molecular weight. In order to explain this fact, the theoretical framework based only in the excluded volume forces is not sufficient. Zaslavsky [12] attempted to look at phase separation in aqueous polymer systems from the point of view of the polymer effects on the water structure. In this way, the effects of all factors on phase partition are realized via their influence on the water structure. It is reasonable to assume that there is a condition of the minimal free energy of a two-phase system. To comply with this condition, the difference between the water structures in the coexisting phases should be minimal. This may be achieved when a water structure making solute concentrates in the less water-structured (phosphate-rich) phase and the water-structure-breaking solute concentrates in the other (PEG) with more structured water. The presence of a significant interaction between PEGs of low molecular weight—specially PEG600—and the protein surface has been experimentally shown in this work. This interaction would be conducted by van der Waals forces (as a result of the fluctuations in the charges of the proteins and dipoles or multipoles in the polymer) between hydrophobic surfaces from PEG and BSA molecules, which would imply the rupture of hydrogen bonds from the structured water in their neighbours [16,21] and thus an increase in the system disorder. As consequence both enthalpic and entropic changes are positive, the $T\,\Delta S$ magnitude being higher than the ΔH. In this way a negative free energy change will be associated to the protein partition equilibrium thus favouring the BSA transfer from the bottom to the top (PEG enriched) phase (higher partition coefficient). In other words, BSA behaviour in PEG600 is that of a water-structure-breaking solute and, according to Zaslavsky, it will concentrate in the PEG rich phase. When the PEG molecular weight increases, the exclusion effect prevails [22], thus pushing the protein to the bottom phase.

In summary, via simple arguments, a qualitative explanation to the observed trends could be given assuming that the partitioning of a solute in an aqueous two-phase system is governed by the difference between the solute–solvent interactions in the coexisting phases.

Acknowledgements

This work was supported by a grant from FONCyT No. 06-03806 and CONICET, PIP 0771/98. We thank Maria Robson, Susana Spirandelli and Marcela Culasso for the language correction of the manuscript. We also thank Luciano Abriata for the alpha helice content calculation. This work was partially carried out during our visits to the Alcala and Granada Universities in Spain. We are grateful to Prof. Maria Melia Rodrigo López and Prof. Bartolomé Quintero Osso for helping us with the light scattering and calorimetric measurements.

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