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Comparison between the thermodynamic features of α1-antitrypsin and human albumin partitioning in aqueous two-phase systems of polyethyleneglycol-dextran

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Abstract

The partitioning features of human serum albumin and α1-antitrypsin in aqueous two-phase systems of dextran and polyethyleneglycol were studied. The effect of factors that affect the electrostatic term of Albertsson equation such as pH, ionic strength, presence of neutral salts as well as those which affect the non-electrostatic term such as polyethyleneglycol mol. wt. and temperature were assayed. At room temperature, the positive entropy and enthalpy changes associated to the partition may be due to a release of part of the structured water in the domain of proteins caused by H-bonds rupture when the proteins are transferred to the upper phase. This behaviour may be explained on the basis of a preferential hydration of the proteins in presence of dextran (bottom phase) and a preferential interaction of polyethyleneglycols with the protein domain (top phase). The electrostatic interactions were similar for both proteins due to the proximity of their isoelectric point and similar dissociation profiles of their prototropic groups.

Keywords: Albumin; Polyethyleneglycol; Two-phase aqueous system; α1-Antitrypsin

Abbreviations: α1-Antitrypsin (AAT); Human serum albumin (HSA); Dextran of average mol. wt. 500 000 Dx; 1-anilino-8-naphthalene sulfonate (ANS); Polyethyleneglycols of average mol. wt. 1000, 3350, 6000, 8000 and 10 000 as PEG 1000, PEG 3350, PEG 6000, PEG 8000 and PEG 10 000, respectively

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1. Introduction

α1-Antitrypsin (AAT) is a protein of the family of the serine proteinase inhibitors (serpins). It is a glycoprotein of 394 residues with a mol. wt. of 52 000 Da. It has three asparagine-linked carbohydrate side chains at positions 46, 83 and 247, the total content of carbohydrate being approximately 12% [1]. The most important physiological role of AAT is to act as an inhibitor of elastase, a scavenger protein enzyme, which destroys elastin on connective tissues. AAT is present in plasma at approximately 2 g/l, thus representing approximately 3% of total proteins, while albumin, the most abundant plasma protein, is present in approximately 40 g/l. In this way, albumin may interfere either, in the quantitative detection of AAT or in the isolation and purification of this protein from plasma [2]. The standard purification method of AAT from plasma involves salt precipitations to remove globulins and affinity chromatography with cibacron blue to eliminate albumin, based on the well-known ability of this protein to bind dyes. However, albumin is not completely eliminated, due to diverse causes: (i) the isoelectric point of AAT varies from 4.9 to 5.1, which is very near the isoelectric point of the serum albumin (5.1); (ii) both proteins are hydrophilic, therefore, they are very soluble and stable because of their low content of hydrophobic residues exposed to the solvent.

On the other hand, aqueous polymer–polymer two-phase systems provide a powerful method for separating and purifying proteins by extraction. This is due to several advantages: (i) high biocompatibility and low interfacial tension, thereby, minimizing product degradation often observed in other methods; (ii) good resolution and yields which can be enhanced dramatically varying experimental medium conditions such as pH, ionic strength, polymer mol. wt. [3–5]. In this work we studied the partitioning properties of the AAT in two-phase aqueous systems of polyethyleneglycol-dextran and compared their thermodynamic features with those associated to human serum albumin partitioning in identical conditions. In this way, our findings might be useful to use partitioning as a putative method to isolate AAT from other plasma proteins.

2. Materials and methods

2.1. Chemicals

Polyethleneglycols of mol. wt. 1000, 3350, 6000, 8000 and 10 000 (PEG 1000, 3350, 6000, 8000 and 10 000, respectively). Dextran T500 (Dx) of average mol. wt. 500 000, α1-antityrpsin (AAT), human serum albumin (HSA), 1-anilino-8-naftha-lene sulfonate (ANS), α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma Chem. Co. USA and used without further purification.

2.2. Preparation of the biphasic aqueous systems

To prepare phase systems, stock solutions of the phase components, PEG 40% w/w, Dx 20% w/w and the adequate buffer were mixed in order to obtain a total system composition of PEG 8.4% w/w and Dx 5.8% w/w, at 22°C according to the binodal partition diagram reported by Albertsson for PEG 8000 and Dx 500 [3]. When PEGs of different mol. wt. were employed, we selected tie lines from the binodal diagrams in order to make both polymers concentrations similar at the top and bottom phases. Low-speed centrifugation to speed up phase separation was used after thorough gentle mixing of the system components, then 1 ml of each phase was mixed to reconstitute several two-phase systems in which the protein partition was assayed.

2.3. Determination of the partition coefficient (K)

Partition of the proteins in both phases was analysed by dissolving increasing amounts of protein (5–15 μM of total concentration) in the two-phase pre-formed systems containing 1 ml of each equilibrated phase. The protein aliquots added to the systems varied from 1 to 10 μl, 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 the separated phases and after dilution, the protein content in each phase was determined by measuring the native fluorescence emission of the proteins at 340 nm (while exciting at 280 nm). For AAT, the enzymatic activity measurement was also employed. Equally diluted samples from identical phase systems without protein were used as blanks, which had been prepared in parallel. The partition coefficient (K) was defined as:

$$K = \frac{[P]_{top}}{[P]_{bottom}} \quad (1)$$

where $[P]_{top}$ and $[P]_{bottom}$ are the equilibrium concentrations of partitioned protein in the PEG and Dx enriched phases, respectively. In the protein concentration range assayed, a plot of $[P]_{top}$ vs. $[P]_{bottom}$ showed a linear behaviour, K value being its slope.

2.4. Thermodynamic parameters associated to the protein partition

The partitioning coefficient was determined at different temperatures and by applying the van't Hoff equation:

$$\frac{\partial \ln K}{\partial T} = \frac{\Delta H^{\circ}}{R T^2} \quad (2)$$

the enthalpic change (ΔH°) associated to the protein partitioning was calculated from the slope of the van't Hoff plot after fitting ln K vs. the reciprocal of the temperature by a non-linear regression analysis of data. The free energy change (ΔG°) was determined from the $\Delta G^{\circ} = -RT \ln K$ and the entropic change (ΔS°) from:

$$\Delta S^{\circ} = \frac{(\Delta H^{\circ} - \Delta G^{\circ})}{T} \quad (3)$$

In the case of the previous two thermodynamic relationships it is generally assumed, that temperature may affect the equilibrium composition of the phases and therefore, the partition coefficient in a manner quite independent of the effect of temperature on the partition coefficient per se. From inspection of binodal curves for PEG/Dx systems [4] we note a slight shift in the position of the binodal curve at different temperatures, whereas, the tie lines remain nearly parallel. This means that a system of a given total composition separates into phases with rather similar contents at the two temperatures. In this way we can affirm that any modification in the partition coefficient with increasing temperature is due to changes in the forces which govern protein partitioning and not to changes in the phase composition.

2.5. Determination of AAT activity

The AAT inhibits the hydrolysis of α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) by trypsin in Tris buffer 20 mM, pH 8.2 at 37°C. The reaction is stopped by adding acetic acid and the liberated reaction product, *p*-nitroaniline, is determined by absorbance at 400 nm with an absorptivity of 10 500. At first, aliquots from the top and bottom phases (with AAT) were mixed with trypsin and then with BAPNA solutions. After incubation for 10 min at 37°C, the reaction was stopped and the absorbances were determined. The control assay was carried out without AAT in the top and bottom phases. The inhibitory capacity of AAT in each phase is proportional to the difference between the control and sample absorbances [6].

2.6. Measurement of the protein surface hydrophobicity (So)

Stock solutions of 8 mM ANS, 460 μM AAT and 1000 μM HSA were prepared in 20 mM Tris–HCl buffer, pH 8.2. Aliquots of AAT and HSA stock solutions, respectively, were added to a sample containing 1 ml of the corresponding equilibrated phase (top or bottom) and 2.5 μl of ANS stock solution. The final concentration of ANS was 20 μM while the protein concentration varied from 0 to 3 μM. Relative fluorescence intensity was measured at a Jasco FP 770 spec-

trofluorometer, the excitation and emission wavelengths being 390 and 470 nm, respectively. Under the above mentioned conditions with excess probe (ANS), the initial slope (S_o) of the fluorescence intensity vs. protein concentration plot has been shown to be correlated to the effective hydrophobicity of the proteins [7].

2.7. Spectroscopic analysis

Fluorescence emission spectra were obtained on a Jasco FP 770 spectrofluorometer by exciting the proteins in 1.0-cm rectangular quartz cuvette at 280 nm and recording the emission from 310 to 500 nm. Absorbance measurements were carried out in Spekol 2000 spectrophotometer

3. Results

3.1. Comparison between the AAT partition coefficient determined by fluorescence and activity measurements

The quantitative measurement of the partition constant (K) of AAT was perfomed using fluorescence emission and enzymatic activity of the protein. The following results were observed: (i) neither the native fluorescence emission at 345 nm (while exciting at 280 nm) nor the enzymatic activity of the protein were affected by the presence of Dx and PEGs of different mol. wt. at different concentrations; (ii) a linear relationship was observed between the native fluorescence intensity of the AAT and its concentration in the range 4 to 15 μM. A similar behaviour was observed for enzymatic activity; and (iii) a straight line was obtained when either the activity or the fluorescence emission of the protein in the top phase was plotted vs. the bottom activity or fluorescence emission (Fig. 1). No significant difference between their slopes (coefficient partition) was observed. Therefore, we decided to carry out the determination of the partition coefficient of AAT in the following experiences through fluorescence spectroscopy since it is both a simple and reliable method.

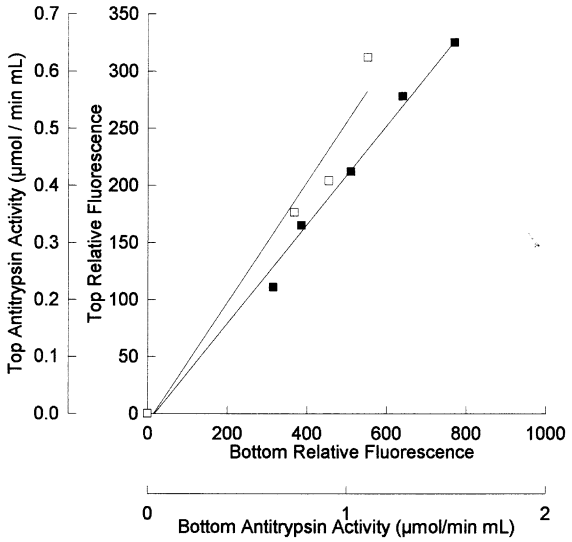


Fig. 1. Dependence of the fluorescence intensity (■) and the enzymatic activity (□) in the top vs. the bottom phases at increasing AAT concentrations. Medium buffer Tris–HCl, 50 mM, pH 8.2. Temperature 25°C.

3.2. pH effect on the AAT and HSA partition in aqueous two-phase systems

Fig. 2 shows the dependence of the ln K on the pH variation in the range 4.0–8.2 at constant ionic strength. When the pH increased from pH 4.0 to 6.0, an important decrease in the transfer of both proteins to the PEG phase was observed. Ln K reached a minimun value at approximately pH 6.0, while the partition was favoured to the PEG-rich phase when the pH increased in the range 6.0–8.2 for the two proteins. However, HSA proved to be more influenced by the pH change than AAT did. At pH 8.2 the major difference between the ln K for both proteins was observed.

3.3. Effect of ionic strength

The effect of the ionic strength on the partition of both proteins was tested as shown in Fig. 3. Both proteins decreased their K linearly when the ionic strength of the medium was increased from 50 to 400 mM at 25°C, this effect being more pronounced for HSA. A similar behaviour was observed when the experience was carried out at 10°C.

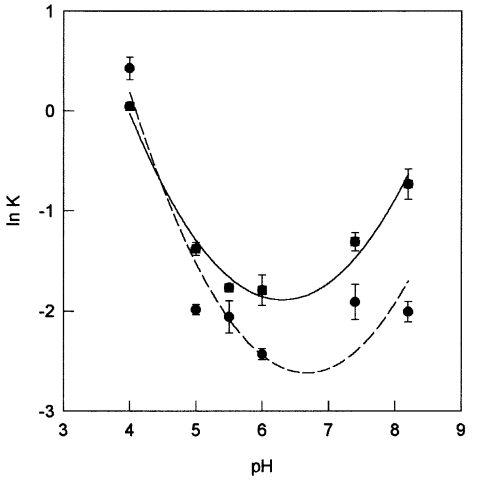


Fig. 2. pH effect on the partition coefficient for AAT (■) and HSA (●) in two-phase systems of PEG 8000 (5.8% w/w) and Dx 500 (8.4% w/w). Ionic strength 150 mM, temperature 25°C.

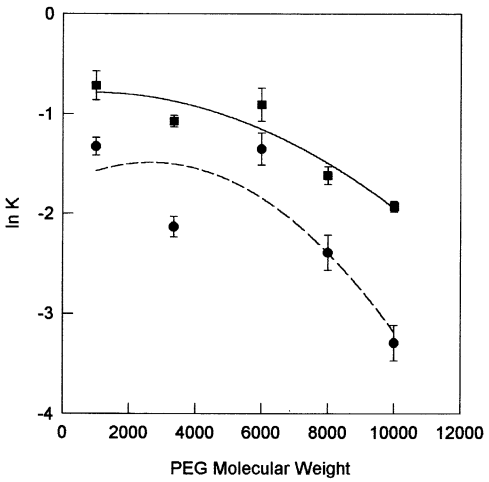


Fig. 4. Effect of PEG mol. wt. on the partition coefficient (K) of AAT (■) and HSA (●). Buffer Tris–HCl 150 mM, pH 8.2. Temperature 25°C.

3.4. Effect of mol. wt. of PEG

The effect of the mol. wt. of PEGs on the partition of AAT and HSA is shown in Fig. 4. Both proteins are partitioned more to the bottom phase in the phase systems with high-mol. wt. PEG, which has been demonstrated for many proteins in PEG/Dx systems, this effect being a general rule for other proteins [8]. The magnitude of the change observed was minor for AAT. Fig. 4 also shows that PEG 8000 and 10 000 induce the major difference between their ln K in both proteins.

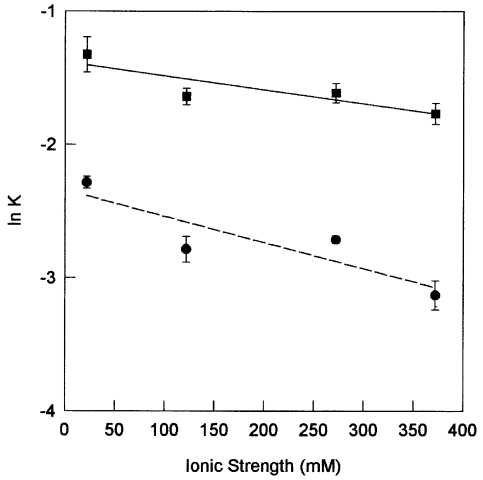


Fig. 3. Ionic strength effect on the K value for AAT (■) and HSA (●) in two-phase systems of PEG 8000 (5.8% w/w) and Dx 500 (8.4% w/w). Medium buffer Tris–HCl 50 mM, pH 8.2. The ionic strength was given by addition of adequate quantities of sodium chloride. Temperature 25°C.

3.5. Determination of the surface hydrophobicity of the proteins

Fig. 5 shows the titration curves of the fluorescent probe ANS, at constant concentration, with increasing concentration of both proteins. The S_o values obtained from the slope of these curves at the origin are shown in Table 1 and indicate that HSA has more surface hydrophobicity than AAT.

Table 1
Surface hydrophobicity (S_o) of HSA and AAT obtained from titration curves of fluorescent probe ANS, at constant concentration, with increasing concentration of both proteins

Protein	S_o (M^{-1})
HSA	56.8 ± 0.3
AAT	36.0 ± 0.5

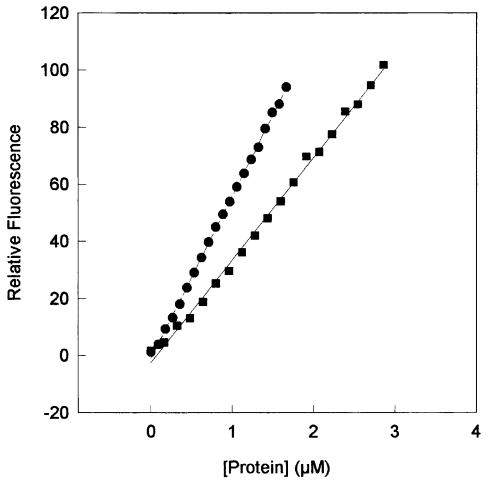


Fig. 5. Titration curves of ANS (20 μM) with AAT (■) and HSA (●). Medium buffer Tris–HCl 150 mM, pH 8.2. λ_{exc} = 360 nm, λ_{em} = 460 nm. Temperature 25°C.

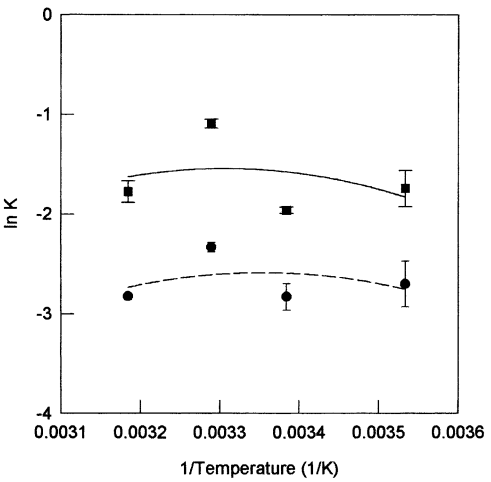


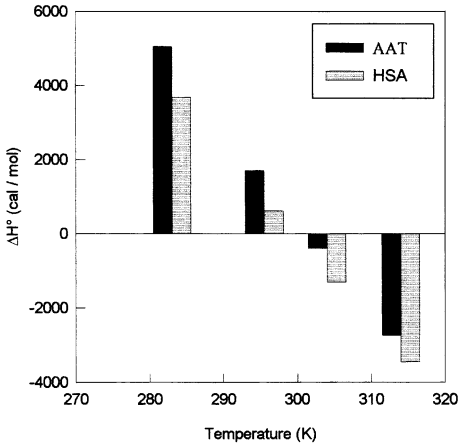
Fig. 6. Van't Hoff plot for the partition coefficient of AAT (■) and HSA (●). Two-phase systems of PEG 8000 (5.8% w/w) and Dx 500 (8.4% w/w). Ionic strength 150 mM.

3.6. Temperature effect on the partition of AAT and HSA

Fig. 6 shows a non-linear relation between ln K and the reciprocal of the temperature, which suggests that the enthalpic change associated to the protein partition is temperature-dependent. The $\Delta H°$ and $\Delta S°$ values calculated at different temperatures are shown in Fig. 7. The transfer of both proteins to the top phase was endothermic in the temperature range 8–20°C, while at temperatures between 30 and 40°C, an inversion of the sign in $\Delta H°$ was observed. A similar inversion in the $\Delta S°$ sign was observed.

3.7. Presence of neutral salts

The effect of chlorides of monovalent cations which belong to the Hofmeister series: Li^+; Na^+;



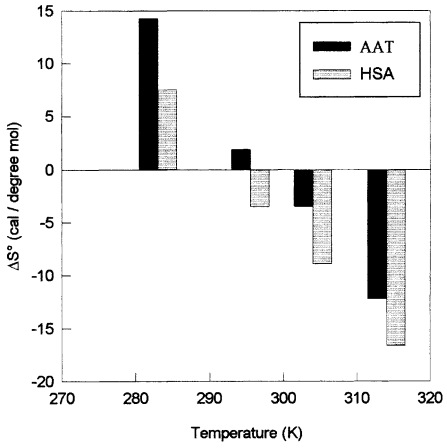


Fig. 7. Thermodynamic parameters associated to the transfer of AAT and HSA to the PEG-rich phase. Two-phase systems of PEG 8000 (5.8% w/w) and Dx 500 (8.4% w/w), buffer Tris–HCl 150 mM, pH 8.2.

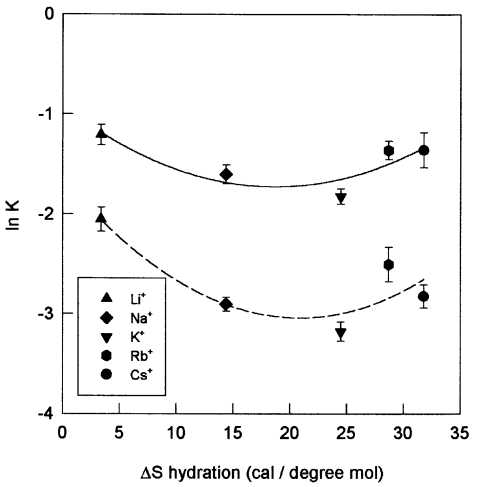


Fig. 8. Effect of presence of chlorides of different monovalent cations: Li^+, Na^+, K^+, Rb^+ and Cs^+ (150 mM) on partition coefficient of AAT (—) and HSA (---) vs. hydration entropy change associated to the correspondent cation. Two-phase systems of PEG 8000 (5.8% w/w) and Dx 500 (8.4% w/w). Medium buffer Tris–HCl, pH 8.2, ionic strength 150 mM, temperature 25°C.

K^+; Rb^+; and Cs^+ on the partition behaviour of the proteins, at constant ionic strength was assayed. Fig. 8 shows that the observed K values for HSA and AAT decrease (from Li^+ to K^+) and slightly increase (from Rb^+ to Cs^+) when the hydration entropy change of the cation, ΔS_h, increase. The ΔS_h values were obtained from [9].

4. Discussion

At present no model has yet been developed which allows the a priori calculation of protein partitioning. In this way, a more complete understanding of protein partitioning is desirable for the prediction and interpretation of extraction behaviour in laboratory and commercial applications. The quantitative modelling of protein partitioning in two-phase aqueous polymer systems is an extremely complex problem, since such behaviour depends on a broad array of factors including: protein size, conformation and surface structure; the interactions of different salts with each other, the proteins and the phase polymers; and the interactions of the polymer chains with the proteins. Albertsson [10], derived an equation which describes the relation between the partition coefficient (K) of a protein and some medium factors such as mol. wt. and charge of the partitioned particle, ionic strength of the medium, ionic composition, mol. wt. of the flexible polymer and temperature, where:

$$\ln K = -\ln\left(\frac{f_T}{f_B}\right) - \frac{\Delta\mu^o}{RT} - \frac{Z_p\mathcal{F}\Delta\psi}{RT} \qquad (4)$$

f is the activity coefficient for the protein at the top (T) and bottom (B) phases, respectively, Z_p: the net electrical charge of the protein; $\mathcal{F}$: the Faraday constant; R: the universal gas constant; T: the absolute temperature; and $\Delta\mu^o$ is the standard chemical potential difference between the two phases. From Eq. (4) it is clear that K is expected to be sensitive to:

1. the protein–protein interaction which is expressed by the f coefficients;
2. the electrical potential difference ($\Delta\psi$) between the two coexisting phases which act on the charges of the proteins (Z_p); and
3. the free energy change when an isolated protein molecule without electrical charge is transferred from the bottom phase to the top phase, expressed by the term ($\Delta\mu^o$). The first factor may be diminished or become zero when the protein partitioning is assayed at low protein concentration, the third factor is the contribution of all electrostatic factors which act on the partitioning, while the second term is the contribution to the K of all non-electrostatic factors. This term includes some factors which drive the partition of the protein, such as:
   - the affinity of the protein for the polymer of flexible chain. Polyethyleneglycols (PEG) bind to proteins in an unspecific way [11], because of their hydrophobic nature therefore, the extension of the protein–PEG affinity is related to the hydrophobic superficial area of the protein exposed to the solvent and the mol. wt. of the flexible polymers; and

- the temperature, which indirectly affects the entropy of the water molecules in their interaction with the polymers of flexible chain [12].

Our results clearly indicate that the partition coefficient (K), of both proteins is independent of the protein concentration under the experimental conditions assayed therefore, the ratio of the activity coefficients of the protein in both phases is near the unit and the first term of the Eq. (4) becomes zero which indicates that the protein–protein interactions can be neglected.

On the other hand, the net charge of the protein (Z_p) is assumed to be the same in both phases, that is $Z_{p_{\text{top}}} = Z_{p_{\text{bottom}}} = Z_p$. This assumption is supported by the small pH difference between the two coexisting phases caused by the difference in the distribution of the buffer ions [13]. In presence of a neutral salt in excess the Eq. (4) can be written as:

$$\ln K = \ln K_0 - \gamma Z_p \tag{5}$$

where $K_0 = \exp\left(-\frac{\Delta\mu^\circ}{RT} - \ln\frac{f_T}{f_B}\right)$ is the partition coefficient of the protein in the system at zero interfacial potential or if the net charge of the protein, Z_p becomes zero, e.g. at the isoelectric point and γ is a factor that depends on the neutral salt used and is directly related to the interfacial potential [14,15]. From inspection of Fig. 2, the complex curves obtained for the two proteins, with a minimum close to their isoelectric points, indicate that the linear character of the ln K vs. Z_p predicted by the Eq. (5) is not reached. This behaviour implies that the partition coefficient of the protein with zero charge is dependent of pH and salt composition due to solute–solvent interactions. Moreover, the similar shape of the plots obtained for the two proteins suggests a similar pattern of their prototropic groups dissociation with the pH variation.

Fig. 3 shows that ln K for the two proteins decreases when the ionic strength of the medium increases (at constant pH 8.2). This behaviour may be explained on the basis of Eq. (5). At pH 8.2 both proteins are negatively charged, the magnitude of Z_{HSA} being greater than the Z_{AAT} [16]. For a given ionic strength γ is a positive factor which adopts similar values for both proteins because both two-phase systems have the same composition with respect to dextran, PEG and neutral salt. As it was pointed out, γ is directly related to the interfacial potential, which becomes smaller when the sum of the number of charges on the ions of the salt is larger, i.e. the ionic strength of the medium increases. The more pronounced decay of ln K vs. ionic strength for HSA is in agreement with the different magnitude of Z_p.

Fig. 4 shows the effect of the mol. wt. of PEGs on the partition of AAT and HSA. Both proteins are partitioned more to the bottom phase in the phase systems with high-mol. wt. PEG, which has been shown for many proteins in PEG/Dx systems [8]. Moreover, from inspection of Fig. 4, it can be observed that the change was greater for HSA than for AAT. This behaviour is in agreement with the experimental observation that the rate of the variation of ln K with the mol. wt. of PEG depends on the mol. wt. of the protein, being a small rate for the low mol. wt. proteins (i.e. 10 000 Da) and a more significative one for proteins of mol. wt. between 40 000 and 200 000 Da. [10]. Albertsson et al. (1987) based their discussion on the Flory Huggins theory for polymers in solution. They expressed the partition coefficient of the protein as a function of the mol. wt. of the flexible polymer and the Flory coefficient of interaction between the protein and the polymer. Our approach has been different and simpler, we explain the above mentioned behaviour based on Timasheff theory [17,18]. He has defined the preferential binding parameter as: $\partial m_3/\partial m_2$; m_2 and m_3 being the molar concentrations of the protein and a cosolute (in this case, PEG), respectively. It is the expression of the amount of cosolute that would have to be added to (or removed from) the system to restore thermodynamic equilibrium when protein is added. This parameter is similar to υ_3, the binding parameter of Scatchard notation (υ_3 = moles ligand/mole protein). Positive values of the preferential binding parameter suggest a good interaction of the cosolute with the protein domain,

such as denaturant agents, PEGs of low mol. wt. (1000 and 3300), while other solutes such as Dx and PEG of large mol. wt. (6000–10 000), which have negative preferential binding parameter are excluded from the protein domain. The Wyman linkage relation applied to the partition process shows:

$$\frac{\partial \ln K}{\partial \ln a_3} = \left(\frac{\partial m_3}{\partial m_2}\right)_{\text{top}} - \left(\frac{\partial m_3}{\partial m_2}\right)_{\text{bottom}} = \nu_3^{\text{top}} - \nu_3^{\text{bottom}} \quad (6)$$

where K is the partition coefficient; a_3: the activity of the cosolute (PEG); and $(\partial m_3/\partial m_2)_{\text{top}} - (\partial m_3/\partial m_2)_{\text{bottom}}$, the change in preferential binding parameter of the protein and PEG, in the top and bottom phases, respectively. As the bottom phase is poor in PEG component, the $\upsilon_3^{\text{bottom}}$ will be negligible. In this way, at any given cosolute concentration, the change in the ln K may be related with the υ_3^{top}. As it was pointed out above, this parameter decreases when the mol. wt. of PEG increases because of its exclusion from the protein domain. This finding is in agreement with previous reports, which demonstrated that PEG has little affinity for some proteins such as albumin which binds this polymer in an unspecific way [11]. The smaller effect observed for ln K vs. mol. wt. of PEGs for AAT might be due to the low surface hydrophobicity of this protein (Fig. 5), as a result of its chemical nature and its smaller mol. wt., which conduce to a lower preferential binding parameter.

The obtainment of non-linear van't Hoff plot for both proteins (Fig. 6) suggests the presence of at least two temperature-dependent processes involved in the partition molecular mechanism.

Fig. 7 shows that the transfer of both proteins to the PEG-rich upper phase is endothermic in the range of 8–20°C. This is a frequent behaviour for several proteins with an enthalpic change associated to the partition between 1 and 4 kcal/mol, which suggests a common molecular mechanism of protein transfer [14]. Proteins in aqueous solutions are generally stabilized by a combination of hydrogen bonding, electrostatic and hydrophobic interactions. It is known that sugars and polyols such as Dx stabilize proteins principally due to their effects on hydrophobic interactions. The hydrophobic interactions between pairs of hydrophobic groups are stronger in solvents in which the three-dimensional hydrogen bonded structure of water is most developed. The addition of these polyhydric alcohols or sugars to an aqueous solution of the protein results in a preferential hydration of the protein, sugars interact in water lattice and their structure, allows them to form the proper hydrogen bonds that reinforce water interaction, having a 'structure making effect' [19,20]. For the AAT, a glycoprotein, this stabilizing effect is enhanced due to its hydrophilic nature. On the other side, as it was pointed out above, PEG has little affinity for some proteins [11], having a positive preferential binding parameter. At room temperatures PEG 8000 adopts a compact structure [21], because of intramolecular hydrophobic interactions between its flexible chains, which allow it to penetrate into the hydration layer of the protein. Therefore, when the two proteins are transferred from the Dx to the PEG-rich phase a release of part of the structured water in the domain of proteins occurs and so H-bonds rupture, which is in agreement with the positive values of the enthalpic and entropic changes associated to the partition, the magnitude of such change being greater for AAT.

When temperature increases, PEG structure becomes more extended, decreases its preferential interaction with the protein, the hydrophobic side chains in the protein are more exposed to the solvent and the water molecules form clusters around these hydrophobic residues thus, leading to an inversion in the sign of the enthalpic and entropic changes associated to the partition.

The effect of chlorides of different monovalent cations on the partition coefficient of the two proteins are shown in Fig. 8. We plotted: ln K vs. the entropy of hydration changes of the different cations because this property is related with their faculty of desordering the water structure. By ordering the cations according their entropy of hydration change, we obtain the sequence Li^+, Na^+, K^+, Rb^+ and Cs^+, which is known as the Hofmeister series.

On the other side, the effect of salts on protein

partitioning has been correlated with the anion and cation partition coefficients which determine the electrical potential difference between the two phases ($\Delta\psi$) thus modifying the γ value of Eq. (5). After a theoretical framework, Albertsson [3] calculated the partition coefficient value for different cations and anions and predicted the sign and magnitude of the electrical potential difference between the two phases when these ions were present. According to this assumption, the partition coeffcient of negatively charged proteins in the presence of chlorides of the cations above mentioned would decrease in the same order of the Hofmeister series, because of their influence in the electrical term of Albertsson equation. Our results are in agreement with this prediction for the first three cations, but not for Rb^+ and Cs^+. Since Rb^+ and Cs^+ are the cations with the more important water structure 'breaking effect' we believe that they affect the electrical as well as the non-electrical term of Albertsson equation, but in opposite sense. Taking into account that we assumed a hydrophobic interaction between PEG and proteins domain, the presence of these cations would entropically favour the proteins transfer to the top phase, therefore, would increase the 'ln K_o, term of Eq. (5), being in this effect more important than their 'lowering effect' on electrical term.

Finally, we conclude that the partitioning behaviour of AAT and HSA is governed by the combination of several factors. The electrostatic interactions are similar for both proteins due to the proximity of their isoelectric point and the similar dissociation profiles of their prototropic groups. The preferential interaction of the phase polymers with the protein domain and the structure of water, which determines the strength of hydrophobic interactions play an important role in the mechanism of partitioning.

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