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Polymer–protein interactions in aqueous two phase systems: fluorescent studies of the partition behaviour of human serum albumin

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Abstract

This study describes the partitioning of fluorescent macromolecules in aqueous two-phase systems (ATPS) comprising phosphate salt and poly(ethylene glycol) of three different molecular masses (i.e. 1000, 1450 and 2000 Da). The impact of system assembly was studied with fluorescent macromolecules introduced in contact with either (i) first salt, then polymer or (ii) first polymer, then salt, or (iii) with both salt and polymer simultaneously. Native human serum albumin (HSA) and derivatives labelled with *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid (1,5-IAEDANS) were partitioned using selected ATPS. Partitioning behaviour was characterised by molecular rotational studies of recovered proteins based upon changes of depolarisation. Measurements were undertaken by steady-state fluorescence or time-decay fluorescence using a single-photon counting system. In addition, circular dichroism was used as a tool for the study of macromolecular secondary structure. Two discrete categories of stable molecular structure have been identified that exist irrespective of the phase environment. The findings form the basis for a discussion of polymer–protein interactions and the molecular micro-environment of proteins in ATPS.

Keywords: Aqueous two-phase systems; Polymer–protein interactions; Human serum albumin

1. Introduction

Aqueous two-phase systems (ATPS) have been widely investigated as an alternative to conventional unit operations for the fractionation of biological extracts [1]. ATPS result from the mixing of two water-soluble solutes (e.g. polymer and salt), and provide a highly biocompatible environment for biomaterials that is amenable to process scale-up [2]. Although the partitioning behaviour of biomolecules has been studied extensively, many questions remain unanswered concerning a true molecular understanding of the mechanisms of the partition process, in particular, the interactions between phase components and target molecules [3]. Previous work has underlined their role in terms of the design of partitioning processes, for example, in respect of the nature of the polymer [4] and the way in which ATPS are assembled [5]. Moreover, it has been suggested that changes of protein conformation may occur during, and be strongly influential upon, the separation process in terms of yield and quality of the products [5].

Proteins are known to exist in a large number of discrete structural states, such as intermediates between native folded and denatured forms [6,7]. Such

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deviation from the native protein structure may have strong implications for the biological function of proteins [8] and may promote large changes in protein conformational dynamics [9]. For instance, fluorescence techniques have been reported to monitor unfolding transitions [10], protein–ligand interactions [11], polyelectrolyte–protein interactions [12] and polymer free volume [13]. They have also been applied to the study of native human serum albumin (HSA) conformation in reverse micelles, reporting changes of local and overall conformation of the protein relative, as an example, to the water-pool size [14,15]. Fluorescence spectroscopy has been investigated herein in the context of the study of both the mechanism of aqueous two phase partitioning and the impact of ATPS in the context of the quality of the partitioned product.

This work describes a molecular rotational study of fluorescent macromolecules based upon changes of polarisation. HSA and derivatives labelled with the fluorophore *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid (1,5-IAEDANS) were partitioned using selected ATPS having various different tie-line lengths (TLL) [16], assembled from polyethyleneglycol and potassium phosphate at pH 7.5. Both steady-state and time-decay anisotropy measurements were performed on samples taken from top and bottom phases separated by such ATPS. Circular dichroism analyses were also conducted on native HSA molecules recovered from appropriate top and bottom phase environments.

2. Experimental

2.1. Labelling reaction

A 9-mg quantity of 1,5-IAEDANS (purchased from Molecular Probes Europe, Leiden, Netherlands) was added to 20 ml of HSA (90 μ*M*) in 0.1 *M* phosphate buffer, pH 7.5. The reaction was continued for 1 h at 37°C under stirring conditions. The resulting solution was passed through prepacked gel-permeation columns of Sephadex G25 (PD10 column from Pharmacia), equilibrated with deionised water in order to desalt the solution and to significantly reduce the excess of fluorescent reagent. Three successive extractions were then conducted to achieve further purification using ATPS comprising poly(ethylene glycol) (PEG 1450) and potassium phosphate at apparent pH 7.5 (see Table 1 for ATPS composition). In the first extraction (ATPS of TLL 41.3), the labelled proteins remained in the bottom phase whilst any residual free reagent was extracted on the basis of top phase preference. The second extraction (ATPS of TLL 47.8), achieved the separation between labelled and unlabelled proteins, the former being preferentially extracted into the top phase, and the latter remaining in the bottom phase. The third step (ATPS of TLL 35.6) enabled back extraction of the labelled material into the salt phase. A final desalting operation was undertaken using gel-permeation on a PD10 column, followed by freeze-drying of the labelled protein.

2.2. Aqueous two-phase partition

ATPS comprising potassium phosphate and PEG, of three different molecular masses (i.e. 1000, 1450 and 2000 Da), were constructed by the simultaneous addition of the phase forming chemicals to the protein solution unless stated otherwise (see Table 1 for the composition of ATPS). Each system was characterised by a TLL, as described by Huddleston et al. [16]. Potassium phosphate was used as a combination of K_2HPO_4 and KH_2PO_4 (ratio, 18:7) to buffer the pH of ATPS to an apparent value of

Table 1
Composition and tie-line length of aqueous two-phase systems comprising potassium phosphate and PEG of three molecular masses (i.e. 1000, 1450 and 2000 Da) at apparent pH 7.5

PEG 1000–phosphate salt			PEG 1450–phosphate salt			PEG 2000–phosphate salt		
TLL	% PEG	% salt	TLL	% PEG	% salt	TLL	% PEG	% salt
28.8	15.0	13.0	29.0	13.6	12.4	43.4	19.0	15.0
36.7	6.5	4.0	5.6	5.4	3.2	46.9	0.0	16.0
41.6	7.7	5.0	1.3	7.3	4.5	0.8	1.0	7.0
47.0	0.0	6.5	7.8	9.7	6.1	3.2	2.0	8.0

All compositions are indicated in % w/w.

7.5. The resulting solutions were mixed until total dissolution of the chemicals was achieved, whereupon, phases were separated by centrifugation at 1700 *g* for 10 min. Samples were carefully extracted from each separated phase, and the protein contents were evaluated by the Bradford method [17] in order to determine the partition coefficient, K_p, representing the ratio of protein concentrations in top and bottom phases. The viscosity, η, of each sample was determined using a Rheomat 30 (Contraves, Zürich, Switzerland).

2.3. Steady-state and time-decay fluorescence measurements

Steady-state analyses were performed using an LS-50B spectrofluorimeter (Perkin Elmer, Beaconsfield, UK) at an excitation wavelength of 340 nm. The intensity maximum was recorded and the fluorescent partition coefficient, K_f, defined as the ratio of fluorescent intensities in top and bottom phases, was determined. In the field of fluorescence spectroscopy, anisotropy is commonly applied to fluorescent molecules in order to evaluate changes in the polarisation of fluorescence, which occurs with molecular rotation. Anisotropy measurements strictly indicate the average angular displacement of the fluorophore that occur between the absorption and subsequent emission of a photon [18]. For the steady-state fluorescence analysis, anisotropy values were calculated from the fluorescent intensities resulting from changes of polarisation. The excitation and emission polarisers were alternately in vertical or horizontal positions, as described elsewhere [19].

In order to maximise the relevant information retrievable from this experimental system, the steady-state data were best analysed in conjunction with time-decay fluorescence analysis, since the latter is a more powerful analytical technique in terms of system resolution. Such measurements were conducted using a correlated time single-photon counting system, with a hydrogen-filled coaxial nanosecond flashlamp as the excitation source [20]. Fluorescence emission was selected by a Schott cut-off filter and a Philips XP2020Q photomultiplier tube was used for the detection. Anisotropy measurements were conducted using Glan-Thompson polarisers with emission at each polarisation detected at 30 s intervals by automated rotation of the emission polariser. An IBH software library performed all data analyses. The closeness of fit is represented by normalised χ^2 values and errors correspond to three standard deviations, as determined by the analytical software.

2.4. Aqueous two-phase system assembly

A series of four ATPS comprising PEG 1450 and potassium salt were assembled by three different methods. In Method 1, the salt was first dissolved in the labelled protein solution before the addition of the polymer. In Method 2, the polymer was first dissolved in the labelled protein solution before the salt was added, whereas in Method 3, both phase component chemicals were added simultaneously to the protein solution. The resulting solutions were mixed for 30 min until total dissolution of all of the chemicals was achieved. The phases were separated by centrifugation at 1700 *g* for 10 min. The protein content of each phase was determined by the Bradford method [17] and steady-state anisotropy was measured.

2.5. Circular dichroism

Circular dichroism was studied using Jasco 600 and 720 spectropolarimeters flushed with evaporated nitrogen. The absorption of protein solution was monitored by UV spectroscopy using an AVIV spectrophotometer model 17DS UV–vis–IR. Submilligram quantities of protein were weighed using a Mettler Toledo MT5 balance and were transferred to a 3-ml vial. Solvent was added to reach a required concentration of 130 μg/ml, where the solvent represented a blank ATPS phase. Protein solutions were centrifuged at 2400 *g* for 15 min to obtain a clear solution in order to reduce light scattering. In all cases, the acquired spectra were corrected by subtraction of the corresponding solvent baseline before data analysis.

3. Results and discussion

3.1. Partition studies

In fundamental studies of ATPS, proteins have commonly been chemically modified to change their

surface properties [21,22] and to investigate the partitioning of the derivatised material as a function of the modification. However, in the present study, the protein was labelled to conduct extrinsic fluorescence measurements to aid the interpretation of protein conformation when partitioned in ATPS. The labelling reaction exploited a thiol reaction between the fluorescent reagent and the free cysteine residue, Cys34, of HSA [23]. As judged from similar partition coefficients estimated by protein content (K_p) and fluorescence measurements (K_f), purification was necessary to recover a homogeneous preparation of the derivatised HSA molecules, referred to as labelled HSA. Three successive extractions were then conducted using selected ATPS, as described in Section 2.1.

Fig. 1 depicts the partitioning behaviour of labelled HSA in comparison to native HSA in ATPS comprising potassium phosphate and PEG of three different molecular masses, 1000, 1450 and 2000 Da, at pH 7.5. Increasing the molecular mass of the PEG promoted an increasing bottom phase preference for native HSA molecules. Extraction to the top phase occurred at a higher TLL, that is, at a higher phase component concentration. Labelled HSA partitioned in a similar manner to native HSA, but its extraction to PEG-rich top phases occurred best at lower TLL, regardless of the molecular mass of PEG. The thiol reagent, 1,5-IAEDANS, exploited in labelling, possesses a naphthalene ring and is therefore a relatively strongly hydrophobic molecule. Its reaction with HSA slightly modified the protein in terms of molecular mass, but more strongly in terms of partitioning in ATPS. It has been proposed that reaction with Cys34 could be accompanied by a ''flip-out'' of that residue, resulting in a greater exposure of the derivatised moiety to free solvent [24]. The covalent binding of the fluorophore incrementally modified the surface hydrophobicity of the protein so as to promote an increase in affinity for the PEG-rich top phase compared to the partitioning of native HSA molecules.

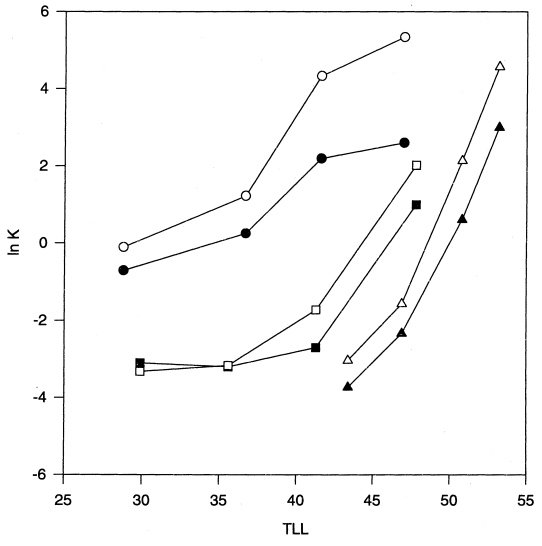

Fig. 1. Partitioning behaviour of HSA and labelled HSA in aqueous two-phase systems comprising phosphate salt and PEG of different molecular masses. The partitioning behaviour of native HSA (●, ■ and ▲) and that of labelled HSA (○, □ and △) are represented for ATPS comprising phosphate salt and PEG 1000 (●, ○), PEG 1450 (■, □) and PEG 2000 (▲, △).

3.2. Steady-state fluorescent analysis

For a globular protein, i.e. a spherical particle at a given conformation, assuming that the rotation is mainly due to Brownian motion, the inverse of steady-state anisotropy, A, is a linear function of the ratio of absolute temperature over viscosity of the sample, T/η [18]:

$$\frac{1}{A} = \frac{1}{A_0} \cdot \left(1 + \frac{\tau \cdot kT}{V_m \eta}\right) \quad (1)$$

where A_0 is the anisotropy in the absence of fluorophore rotation, which is equivalent to that of a protein in a vitrified solution, τ is the lifetime of the fluorophore, k is the Boltzmann constant, and V_m is the molecular volume of the rotating unit. The lifetime of the fluorophore, which was measured by time-decay fluorescence, was found to vary slightly, being close to 19 ns irrespective of the sample, water, bottom or top phase and of the phase concentration in salt or PEG. The fluctuations of anisotropy should thus be due mainly to variation of the molecular volume of the protein, the viscosity of the samples and the temperature at which the measurement was conducted. The inverse of anisotropy was thus plotted as a function of the ratio of the temperature divided by the viscosity.

The resulting plot for the partitioned samples is

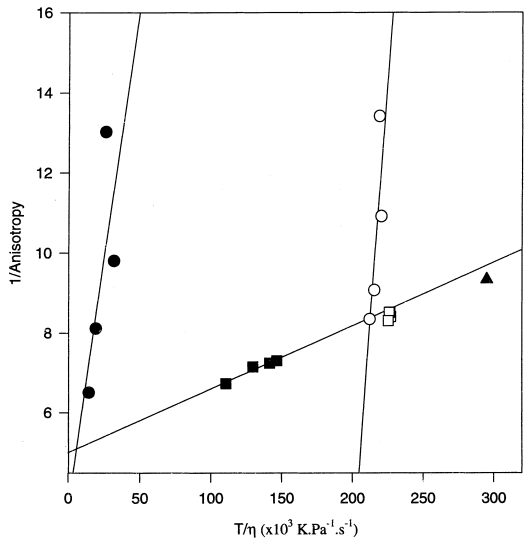

Fig. 2. $1/A$ versus T/η for partitioned samples of labelled HSA in PEG1450–phosphate aqueous two-phase systems. Extracted samples from top (●, ○) and bottom (■, □) phases were diluted both in the corresponding blank phase (●, ■) and in water (○, □). Labelled HSA dissolved in water was also analysed (▲).

depicted in Fig. 2. It demonstrates two different linear relationships, representing states where the protein was either in an aqueous solution, with or without salt, or in the polymer-rich phase, which could be associated with estimated molecular volume. Since the viscosity in the polymer-rich top phase was higher than in the salt-rich bottom phase, higher anisotropy values should have been expected together with lower inverse values of anisotropy. However, the opposite was observed, suggesting a different local environment and molecular volume for the fluorophore in the presence of the PEG polymer compared to other aqueous solutions. It should be noted that the calculation of molecular volume did not yield a value that was comparable to that reported by He and Carter [23], which may be attributed to the assumptions made. Those workers compared HSA molecules to equilateral triangles with sides of 80 Å and a depth of 30 Å and preferred to analyse only qualitatively the steady-state anisotropy data in terms of the local environment of the fluorophore.

When the top phase samples were diluted in water, the anisotropy profile was very similar to that of samples diluted in the corresponding blank phase. The bulk viscosity then corresponded to that of an aqueous solution. Such results possibly indicate an equivalent micro-viscosity of the protein when diluted in a blank top phase or in water. In such a micro-environment, a polymer film driven by polymer–protein interactions may be proposed to surround protein molecules. In respect of PEG–protein interactions, PEG has been shown to display an affinity for certain hydrophobic residues or domains of proteins [25]. Moreover, the concept of a ''polymer atmosphere'' has already been addressed in the case of affinity partitioning, where a ligand was covalently attached to a PEG chain and anchored to the binding site of albumin [26]. Affinity binding maintained the PEG chain in contact with specific areas of the protein surface. In the present study, although polymer and protein molecules were not directly associated by either covalent or affinity binding, a similar conclusion of polymer film and contact around the protein molecule can be proposed.

When the labelled protein molecules were partitioned in ATPS comprising salt and different molecular masses of PEG (i.e. 1000, 1450 and 2000 Da), samples extracted from both phases were also analysed using steady-state fluorescence methods. Fig. 3 shows the inverse of steady-state anisotropy plotted as a function of the temperature divided by viscosity. A clear distinction can be made between top polymer-rich phases, and bottom salt-rich phases, which can be expressed in terms of linear regression. The top phase samples are characterised by a greater slope than that of the bottom phase. Although the viscosity was higher in the top phase for the three molecular masses of PEG, the anisotropy had lower values in the polymer-rich phase than in the salt phase, which was the opposite of the relationship expected (refer to Eq. (1)). Moreover, three different linear relations were to be found for the top phase samples, irrespective of the molecular mass of PEG in the systems. The slope of the linear regression was higher for PEG 2000 when compared to PEG 1000, suggesting a different interaction strength between the protein and polymer as a function of the average number of ethylene oxide units (EO units) in the polymer chain. The longer the polymer, the weaker seemed to be the interactions between the polymer and protein. Such findings can be correlated with protein adsorption to polyethyleneoxide (PEO) sur-

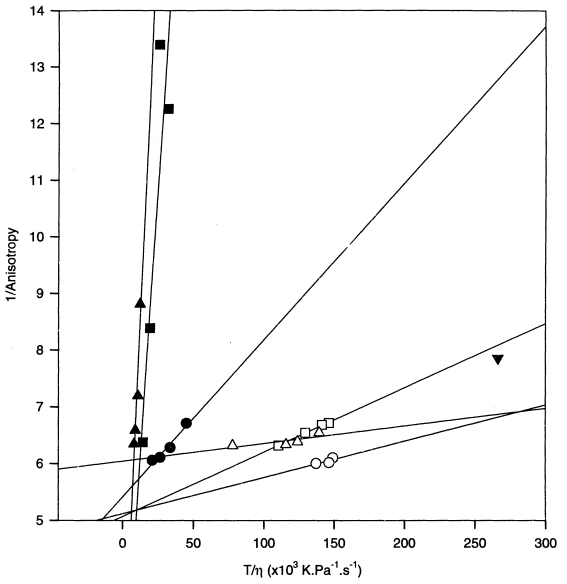

Fig. 3. Impact of the different molecular masses of PEG upon the conformation of labelled HSA partitioned in ATPS. ATPS comprising phosphate salt and PEG of three molecular masses, i.e. 1000 (●, ○), 1450 (■, □) and 2000 Da (▲, △), were assembled. The steady-state anisotropy of labelled HSA in water (▼) and of top phase (●, ■, ▲) and bottom phase samples (○, □, △) was measured as described in Section 2.3.

faces [27]. The adsorption of bovine serum albumin decreased with increasing PEO molecular mass, up to 3500 Da. Moreover, minimal adsorption of proteins has been reported for PEO surfaces characterised by molecular masses of between 2000 and 3500 Da [27].

3.3. Time-decay fluorescence study

The time-decay fluorescence study was conducted to determine anisotropy decays of labelled HSA under various solvent conditions using the single-photon counting system. The anisotropy of the fluorophore was analysed using a single rotational correlation time, T_r [20]. For spherical molecules and according to the Stokes–Einstein relationship, the rotational correlation time, T_r, is a function of the size of the molecule expressed as a molecular volume of the rotating unit, V_m, where:

$$T_r = \frac{V_m \cdot \eta}{k \cdot T} \quad (2)$$

with η, as the viscosity in Pa·s, k as the Boltzmann constant and T as the temperature in degrees Kelvin [24]. The molecular volume resulting from time-decay analysis is equal to the anhydrous volume plus an increment reflecting the degree of hydration, assuming that the rotational correlation time measured is comparable to that of the protein. Any conformational changes of resident biomolecules should then be described by a non-linear relationship between T_r and η/T. The time decay anisotropy was studied for phase samples of a series of ATPS comprising PEG 1450 and phosphate salt (refer to Table 1 for the ATPS composition). The rotational correlation times of partitioned derivatives are shown in Table 2. The samples S1B to S4B and S1T to S4T corresponded to bottom (salt-rich) and top (PEG-rich) phases, respectively, with increasing TLL from 29.0 to 47.8 [16]. The angle, θ, has been evaluated using the wobbling-in-cone theory of Kinosita and Ikegami [28], which is based on the existence of residual anisotropy, $r\infty$.

Firstly, the rotational correlation times of the 1,5-IAEDANS group, covalently attached to HSA molecules in water, was found to be equivalent to 32.7 ns. Referring to Eq. (2), such a correlation time could be associated with the molecular volume of the protein with the assumption of an immobile fluorophore within the protein matrix [18]. The molecular volume, V_m, reflects the sum of the specific volume of the protein plus the degree of hydration. It has been previously reported that the correlation time of anisotropy decay of native HSA was 31 ns at a temperature of 8°C. Such a value and the present result were found to be comparable to the calculated value of 40 ns for a spherical particle of molecular mass 64 kDa with a hydration degree of 0.2 g of water per gram of protein. Such results indicated that the rotational correlation time estimated by time-resolved analysis could be related to the dynamics of the protein and not only to that of the fluorophore.

Secondly, the static study showed a residual anisotropy characteristic of a restricted motion for the fluorophore. The cone angle demonstrated that this restricted motion increased in both phases when the phase component concentration was raised. Such an increasing restriction could be attributed to variations of the geometrical confinement imposed by the neighbouring amino acid residues as the viscosity of

Table 2
Time-decay anisotropy measurements of labelled HSA partitioned in a series of four aqueous two-phase systems comprising PEG 1450 and potassium phosphate at pH 7.5 (see composition of the systems in Table 1)

Sample	TLL	T_r (ns)	r_0	r_∞	θ (°)	χ^2
Water	0	32.7±0.3	0.19±0.01	0.01±0.01	(nd)	1.09
S1B	29.0	51.3±9.1	0.19±0.01	0.04±0.01	54	1.31
S2B	35.6	44.8±9.4	0.19±0.01	0.05±0.01	51	1.20
S3B	41.3	52.4±11.7	0.20±0.01	0.05±0.01	52	1.17
S4B	47.8	66.6±7.5	0.22±0.01	0.08±0.01	45	1.23
S1T	29.0	1.3±0.3	0.20±0.01	0.04±0.01	55	1.19
S2T	35.6	1.0±0.2	0.20±0.01	0.04±0.01	39	1.36
S3T	41.3	22.9±0.3	0.19±0.01	0.10±0.01	25	1.28
S4T	47.8	59.4±1.3	0.22±0.01	0.16±0.01	25	1.21

TLL stands for tie-line length. T_r corresponds to the rotational correlation time, r_0 and r_∞ are the anisotropy in the absence of fluorophore rotation and the residual anisotropy, respectively. Those three latter parameters directly resulted from the analysis of anisotropy decays [19]. The semi-angle, θ, was calculated from both limiting and residual anisotropies, based on the wobbling-in-cone theory [28].

the sample increased with increasing TLL. This could also be explained in terms of changes of local dielectric properties surrounding the fluorophore. Stokes shifts of fluorescence spectra were observed as the environment of the fluorophore changed from a salt-rich solution to a polymer-rich solution, and as the phase component concentration increased (data not shown). Kimura and Ikegami [29] have actually used such Stokes shifts to estimate local dielectric constants for the polar regions of lipid bilayer membranes. It is thus suggested that both salt and polymer had an effect upon the fluorophore environment, which induced a restricted motion of two different types due to the nature of each solvent.

Thirdly, the rotational correlation time was less in the top phase than in the bottom phase, indicating a faster rotational diffusion of the fluorophore in the upper phase despite a greater viscosity. T_r was then plotted as a function of η/T (Fig. 4). This figure exhibits two distinct linear domains; one for bottom phase samples and the labelled HSA in water, and one for the top phase samples. Such dynamic data are concordant with the steady-state analysis, demonstrating different local environments for the fluorophore. We conclude that the protein has two different conformational substates, i.e. two discrete local conformations, depending on the phase environment and the interactions between residue and solvent. The presence of salt in the bottom phase does not apparently modify the conformation of the labelled protein in comparison with the water environment. However, when the HSA molecules are located in a polymer environment, the fluorophore has a faster rotational diffusion than expected.

3.4. Aqueous two-phase systems assembly

ATPS can be assembled in various different ways depending upon the circumstances of experimental

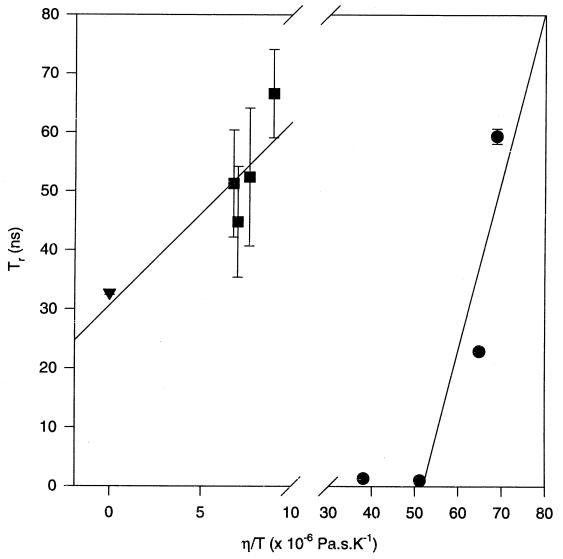

Fig. 4. Rotational correlation time, T_r, of partitioned derivatised protein as a function of η/T. Samples from the top (●) and bottom (■) phase were analysed as described in Section 2.2. The time-decay anisotropy labelled HSA in water was also measured (▼).

process operation. As a result, the partitioning of proteins at apparent equilibrium may be expected to vary as a function of that assembly. Three methods were investigated (refer to Section 2.4) and each was applied to four selected ATPS comprising PEG 1450 and potassium phosphate at pH 7.5 (see Table 1 for ATPS composition). Fig. 5a depicts the partitioning behaviour of labelled HSA in those ATPS. When the protein was first in contact with the salt followed by the polymer, i.e. ATPS assembled by Method 1, the partition coefficient was slightly lower than for other methods of assembly. On the other hand, the partition coefficient of labelled HSA was higher when the systems were constructed following Method 2 (i.e. polymer followed by salt). It is interesting to note that the protein had an apparent preference for the first phase component chemical with which contact was made. This may be due in part to different rates of equilibration in the three methods and deserves further study. Greater variation of protein partitioning with the method of ATPS assembly has also been noted by Rito-Palomares [5] for ATPS comprising PEG 1000 and phosphate salt. The data of steady-state anisotropy measurements were plotted as the inverse of the anisotropy versus temperature over the viscosity, as shown in Fig. 5b. The distinction between top and bottom phase samples was again demonstrated in terms of anisotropy profile. However, no significant differences were observed when the ATPS were assembled by the three different methods, although the partition of the protein was slightly modified.

3.5. Circular dichroism

Circular dichroism is a common tool for the analysis of protein secondary structure based upon the difference in the absorption of left and right polarised light [30]. Responses in the far UV (260–190 nm) relate to the backbone structure in respect of α-helix and β-sheet content, while near UV measurements (400–240 nm) allow the study of the aromatic region. Both far and near UV analyses were conducted on native HSA. The far UV measurements clearly exhibited similar spectra for HSA molecules either in an aqueous solution or in a salt-rich bottom phase sample, regardless of the concentration of phosphate salt. When the protein was dissolved in a

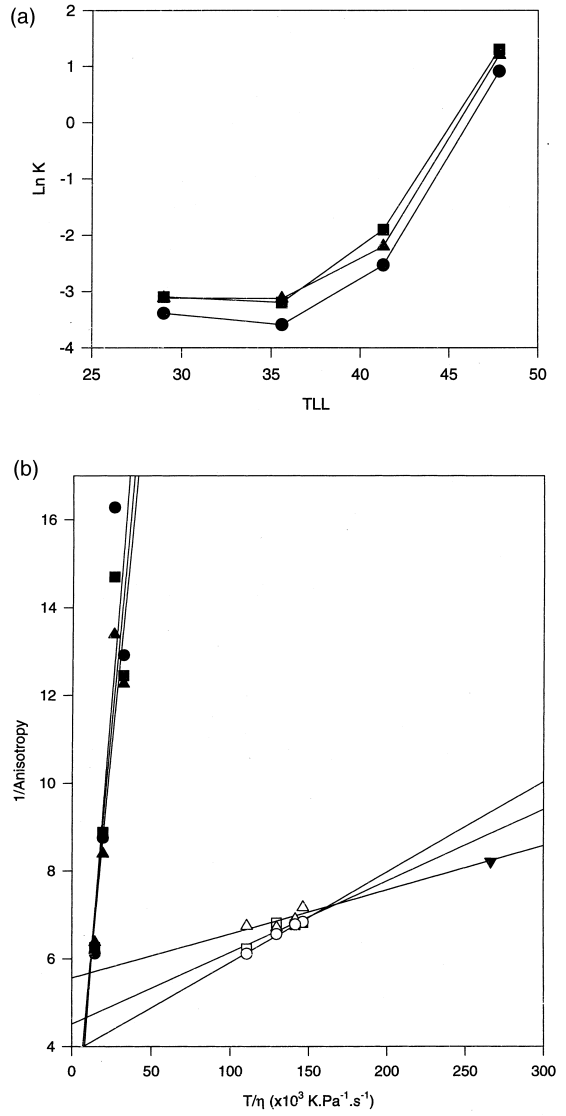

Fig. 5. (a) Partitioning behaviour of labelled HSA in ATPS assembled by three different methods. In Method 1 (●), the salt was added first to the protein solution, then the polymer was added; in Method 2 (■), the polymer was added first, then the salt and in Method 3 (▲), both the salt and polymer were added simultaneously. (b) Impact of the aqueous two-phase assembly upon the steady-state anisotropy profile of labelled HSA dissolved in water (▼) and partitioned in PEG–salt systems (top phase samples, ●, ■, ▲; bottom phase samples, ○, □, △). ATPS were assembled by three different methods: in Method 1 (●, ○), salt was added first, then the polymer; in Method 2 (■, □), polymer was added first, then the salt and in Method 3 (▲, △), both salt and polymer were added simultaneously.

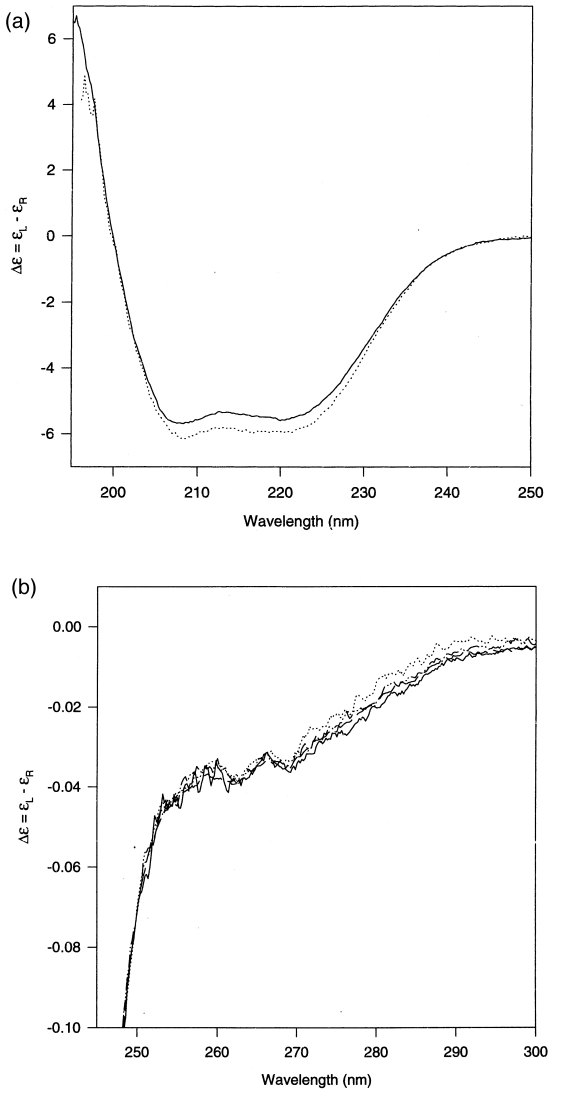

Fig. 6. (a) Far UV circular dichroism spectra of native HSA dissolved in blank ATPS phases as described in Section 2.5. Top phase from ATPS comprising PEG 1450 and phosphate salt (———; TLL of 29.0) and bottom phase from ATPS comprising PEG 2000 and phosphate salt (.....; TLL of 43.4) were used for the calculation of α-helix and β-sheet content, as described in Section 3.5. (b) Near UV circular dichroism spectra of native HSA dissolved in blank aqueous two-phase systems. The ATPS comprised phosphate salt and polyethyleneglycol of different molecular masses: (——— PEG 1000, S4T; - - PEG 2000, S1T; .... PEG 2000, S1B; — — PEG 1450, S3T; Refer to Table 1 for the ATPS composition).

polymer-rich top phase, the far-UV spectra had a different profile to the bottom phase samples, as shown in Fig. 6a, which can be interpreted in terms of α-helix and β-sheet content using principle component regression ([31]; see Table 3 for the results). However, no difference was observed in the aromatic region of the protein for either polymer or salt sample, as shown in Fig. 6b. When different average molecular masses of PEG polymer were studied, the far-UV analysis did not exhibit any modification of the spectra, while differences were observed for similar samples by steady-state spectroscopy. It should be noted that circular dichroism did not detect any PEG–protein interactions with respect to variations in the molecular mass of the polymer.

4. Conclusion

The dynamic study of proteins partitioned in aqueous two-phase systems was based herein on extrinsic fluorescence. Two distinct trends were observed regarding the phase environment of the proteins. A first substate was related to the molecules present in water or in a salt-rich bottom phase. A second substate concerned the polymer-rich top phase, where the fluorophore rotated in a faster manner than expected, despite an elevated bulk viscosity, and where a polymer film surrounding the protein has been proposed. Such conformational changes may particularly affect hydrophobic domains of partitioned proteins, since the fluorophore is a hydrophobic moiety. Polymer–protein interactions may therefore have been promoted based upon mild hydrophobic interactions [32,33]. However, no changes in the aromatic region of native HSA were observed by circular dichroism. Small changes of the overall protein conformation were also observed by far UV circular dichroism for native HSA in top and bottom phase samples. Such results could be attributed to hydrogen bonding between protein and solvent, or to various degrees of hydration of the protein. Polymer–protein interactions seemed to decrease as the molecular mass of PEG was increased from 1000 to 2000 Da, but in a rather weak manner. The nature and strength of the PEG–protein interactions are not clearly defined and require

Table 3
α-Helix and β-sheet contents estimated by protein component regression [31] of HSA molecules dissolved in blank top and bottom phases of ATPS (refer to Section 2.5)

Solvent	Salt-rich bottom phase (%)	Polymer-rich top phase (%)
α-Helix content	56.5	62.3
β-Sheet content	15.2	10.2
Other	28.3	27.5

further investigation based on fluorescence spectroscopy.

No significant changes of conformation were observed when the ATPS were assembled by three different methods, although the partitioning of the protein was slightly modified. We conclude that the manner in which ATPS are constructed should not affect the molecular quality (in terms of protein conformation) of recovered products. The PEG–protein interactions indicated herein for HSA seem to be relatively weak and are unlikely to impact upon the partitioning of biomolecules in aqueous two phase systems. However, this may not be true for all protein species. This, together with the conformational influences noted above, should be considered when designing and operating realistic aqueous two phase systems loaded with complex feedstock.

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