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Journal of Chromatography B, 711 (1998) 19–29

JOURNAL OF CHROMATOGRAPHY B

Influence of temperature upon protein partitioning in poly(ethylene glycol)–salt aqueous two-phase systems close to the critical point with some observations relevant to the partitioning of particles[1]

Sylvie de Belval, Benedicte le Breton, Jonathan Huddleston*, Andrew Lyddiatt

Biochemical Recovery Group, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Abstract

The partition of proteins in a poly(ethylene glycol) (PEG)–potassium phosphate aqueous two-phase system has been studied in systems which were produced by manipulation of the equilibrium temperature of a monophasic mixture composition close to the critical point. Precise control of the composition enabled the formation of biphasic systems lying very close to the critical point. A high-performance liquid chromatographic method for analysis of the phase compositions is presented. The generally applicability of the method to the partition of proteins and other particles is discussed.

Keywords: Aqueous two-phase systems; Partitioning; Proteins

1. Introduction

In previous papers on the partitioning of proteins in poly(ethylene glycol) (PEG)–potassium phosphate aqueous two-phase systems we have shown that only a relatively small region of the phase diagram close to the critical point conforms to classical partitioning laws [1]. At extended tie line lengths the partition coefficient of proteins is concentration and volume ratio dependent, being dominated by strong salting out forces arising from the high concentrations of salt in the lower phase [2,3] counterposed by a phase in which excluded volume forces, arising from the high concentrations of PEG present, have been considered important [3–5]. An empirically extended form of the Brönsted relationship, as originally proposed by Diamond and Hsu [6,7], was found to be useful in the description of the partition of some simple monomeric proteins in PEG–potassium phosphate aqueous two-phase systems [3].

The well known Brönsted relationship [8] is often expressed as:

$$\ln k = \frac{\lambda M(C - C_0)}{KT} \quad (1)$$

where T is the absolute temperature, K is the Boltzmann constant and M is the molecular mass of the solute. Lamda (λ) is a potential energy term relating to the interaction of the solute with its environment. $C - C_0$ is a term which relates the change in the composition of the system to the composition at the critical point.

Diamond and Hsu's empirically extended relationship was expressed in the form [6,7]:

$$\ln k = A(w_1'' - w_1') + b(w_1'' - w_1')^2 \quad (2)$$

*Corresponding author. Address for correspondence: Department of Chemistry, University of Alabama, Tuscaloosa, Alabama, AL 35487, USA.

[1]Paper dedicated to the memory of the late Ernest Boucher.

0378-4347/98/$19.00
PII: S0378-4347(97)00561-6

where *w* represents the weight/weight (w/w) percent concentration of the polymer, the subscript refers to PEG and the double and single primes refer to the top and bottom phase, respectively.

It is obvious that these descriptions become equivalent as the physical difference between the two phases decreases. However, our previous data conspicuously lacked information on the partition coefficients of proteins close to the critical point. Tie line length increases rapidly with increase in overall system composition away from the critical point and thus construction of consistent systems of different tie line length is more difficult and error prone the closer the critical point is approached. Under these circumstances extrapolation to the critical point was previously made by assuming a theoretical distribution coefficient of 1 [3]. The late Ernest Boucher, in what appears to have been his final contributions [9–11], recommended the elegance of Brönsted and Warming's original method [12], in which precise control of the compositions of the coexisting phases of biphasic systems close to the critical point was achieved by manipulation of their equilibrium temperature. In this way biphasic systems with increasingly divergent compositions of the two phases could be produced which, nevertheless, remained very close to the critical point. Boucher also discusses the theoretical basis of Brönsted's approach to the study of partitioning [9,10]. More recently, the method has been applied to the study of the partition of colloidal particles in aqueous–organic biphasic systems in the context of surface wetting phenomena [13–17]. We have attempted to apply the method to the study of protein partitioning in PEG–phosphate aqueous two-phase systems. Our results, whilst less than perfect, indicate the essential utility of the approach and the applicability of Brönsted type relationships to the partitioning of proteins in these systems. Some aspects of the underlying physicochemical forces which may determine protein partitioning behavior is examined by reference to their molecular structures. The universal applicability of this approach recommends its application to partitioning studies in general and especially as to the study of colloidal and biological particles. Finally we attempt to place our work on protein partitioning in PEG–salt aqueous two-phase systems in the more general context of the distribution of particles in biphasic systems.

2. Experimental

2.1. Overview

Our aim was to produce systems of progressively divergent compositions of the two phases comprising a PEG–potassium phosphate aqueous two-phase system and to measure the partition coefficient of selected protein solutes. This was to be achieved by controlled manipulation of the equilibrium temperature of a monophasic system close to the critical point. To this end considerable effort was expended on developing methods for the determination of the equilibrium compositions of the two coexisting phases. This is because systems produced at different temperatures do not lie on a single binodal curve (see Fig. 1). The remainder of this section gives a detailed account of the methods employed.

2.2. Characterization of biphasic systems

Binodal curves were determined by the cloud point method [18] from stock solutions of 50% (w/w) PEG of molecular mass 1450 Da as stated by the manufacturer (Sigma P5402, Lot 115H0074,

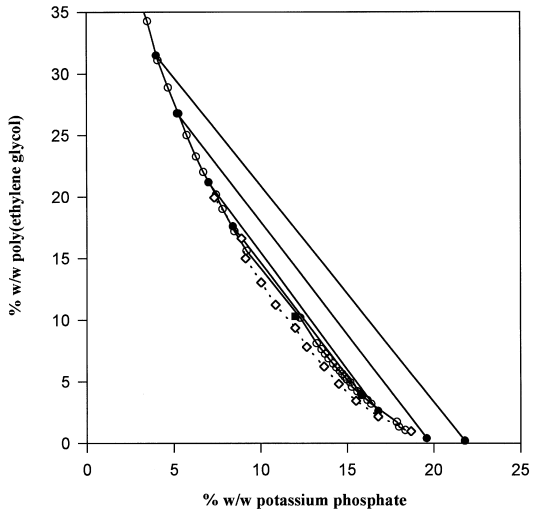

Fig. 1. The influence of temperature on the binodal curve of a PEG1450–potassium phosphate aqueous two-phase system. Binodal curves determined at 20°C (○–○) and 31°C (◊–◊) showing the near critical point system selected (■) [10.3% (w/w) PEG–12.0% (w/w) potassium phosphate] and a selection of tie lines determined at 20°C (●–●).

Poole, Dorset, UK) and 30% (w/w) potassium phosphate. Potassium phosphate solutions were composed of a mixture of dipotassium hydrogenorthophosphate–potassium dihydrogenorthophosphate (18:7, w/w) (Merck, Poole, Dorset, UK). The pH at this salt composition is approximately 7.4. Binodal curves determined at other than room temperature (22°C) were determined in the same way using solutions maintained at the appropriate temperature in a water bath.

2.3. Determination of the coordinates of the critical point

The densities of the separated phases and the volume ratio of six systems lying within the binodal curve were used to estimate the location of the tie lines by the known relationship between the mass ratio of the phases and the geometry of the phase diagram [4,17]. The point on each tie line giving a mass ratio of 1 was determined and the line joining successive points was extrapolated through the binodal curve. The point at which the mass ratio = 1 line crossed the binodal was taken to be the critical point. A similar method given by Treybal [20] using triangular coordinates to plot the phase diagram was also used to estimate the location of the critical point. In this method lines are constructed from the nodes of each tie line parallel to the axes of the triangular diagram. The critical point is interpolated from the point at which a line drawn through the intersections of these lines crosses the binodal curve [20].

2.4. Sample preparation

Direct preparation of each critical point phase system from weighed components or from dilution of more concentrated systems proved insufficiently reproducible resulting in the preparation of systems having different critical temperatures of phase separation. Thus all systems were made from a large stock solution having the composition 10.3% (w/w) PEG–12.01% (w/w) phosphate from which well mixed aliquots of 10 ml were withdrawn for blanks and samples and into which the proteins were directly dissolved at the rate of 1 mg/ml for the sample aliquots. All solutions were monophasic at room temperature. The solutions were heated in a water bath (Grant LTD 6G, manufacturers stated accuracy ±0.01°C) to the critical temperature for phase separation T_c (°C). Samples were withdrawn from the phases and assays made on separate tubes at each temperature T_c, T_c+1, T_c+2, T_c+3, T_c+4°C. For each temperature to be examined two tubes, a blank and a sample, were placed in the bath and agitated every 5 min for 15 min and finally allowed to stand for 1 h until phase separation was complete. Samples of each phase were then withdrawn and analyzed for protein content and phase composition.

2.5. Proteins used in the partitioning study

The following protein preparations obtained from Sigma were used in the partitioning study without further purification; α-amylase (A-6380), bovine serum albumin (A-3675), α-chymotrypsin (C-4129), cytochrome *c* (C-3006), lysozyme (L-6876), myoglobin (M-0630), ribonuclease-A (R-5000).

2.6. Determination of protein concentrations

A 400-μl sample of each phase was mixed with 1 ml of distilled water and its absorbance read at 280 nm using a Kontron Uvikon 922 spectrophotometry system. Absorbances of protein containing phases were corrected by using the absorbance of an appropriate blank phase and the partition coefficient calculated from the rectified absorbance.

2.7. Protein structure analysis

Structural analysis of the model proteins was carried out using IDITIS [21] and NACCESS [22] in conjunction with protein structure files of the Brookhaven Protein Data Bank mounted on the Silicon Graphics Crimson system of SEQNET (Daresbury, Warrington, UK). The following protein structure files were used; ''1BPL'' for α- amylase, ''4CHA'' for α-chymotrypsin, ''1HRC'' for cytochrome *c*, ''1LYZ'' for lysozyme, ''1YMB'' for myoglobin and ''1RAT'' for ribonuclease-A. The following deficiencies in this analysis must be noted. No structural information appears to be available for any of the serum albumins. Although the structure of human serum albumin has been published [23] it is

not lodged with the Protein Data Bank and thus could not be included in the analysis. The structure of α-amylase has been determined with a 30 amino acid proteolytic excision without which it cannot, apparently, be induced to form crystals [24]. Horse heart cytochrome *c* was analyzed in place of the beef heart cytochrome *c* used in the partitioning study. However, their partitioning behavior may be expected to be substantially the same. Indeed yeast cytochrome *c* could have been used for the structural analysis and the partitioning study without major effect on the results [5]. The proteins were analyzed in terms of the total surface accessibility ($Å^2$) of each amino acid residue of the protein sequence according to the method of Lee and Richards [25] using an 0.05 Å *Z*-slice and a probe of radius 1.4 Å. From this data the hydrophobic surface area was calculated by summing of areas for residues having side chains of negative hydrophobicity values (kcal/mol) according to the data of Roseman [26]. Hydrophilic surface area was calculated analogously but using zero or positive values of hydrophobicity, the two together giving the total solvent accessible surface area. The area of exposed side chains of tryptophan residues was also calculated and additionally expressed as a percentage of the total solvent accessible surface area. In the absence of reliable methods for calculating dipole moments of proteins from protein structure data [27,28] the relative charge at the protein surface was expressed, albeit crudely, as the molar percentage, over the whole sequence, of the strongly charged amino acid residues (D, E, K, R) using the Wisconsin Package [29].

2.8. Chromatographic analysis of phase compositions

Analysis of the compositions of the coexisting phases, sampled at each temperature, was by size-exclusion chromatography (SEC) on a high-performance liquid chromatography (HPLC) system comprising LKB 2152 controller, 2150 pump, 2158 Uvicord SD and an ERC 7515A refractive index (RI) detector with output to a chart recorder. UV absorbance detection was used to monitor the elution of protein from samples to ensure that it could not interfere with the detection of PEG and phosphate which was determined by RI changes. The SEC column used was a Superdex Peptide HR 10/30 (Pharmacia). The mobile phase was composed of 50 m*M* potassium phosphate pH 7.2 containing 0.25 *M* NaCl. Flow-rate was 1.0 ml/min and the injection volume 20 μl. The operating temperature of the RI detector was set to 30°C. All chromatographic peaks were well separated and consisted of a single peak except phosphate which consisted of two closely adjacent peaks separated by a negative inflection. These peaks are presumed to be related to the presence of the two different phosphate ions present under these conditions. Both peaks were present at constant ratio and the later eluting, larger peak was used to quantify phosphate. Concentration of PEG was linear with peak height in the range 0–15% (w/w) PEG. A second order polynomial fit was used to describe phosphate concentration in the range 0–25% (w/w). The ability to perform the phase analyses at concentrations close to those of the actual phase systems employed was viewed as a major advantage of this approach.

2.9. Phase compositions by fluorescence spectroscopy

The fluorescent dye 8 anilino-1-naphthelenesulphonic acid (ANS) was investigated as a means of determining the composition of the phases. A 100-μl volume of 2.5 m*M* ANS was added to 6 ml of sample. This dye was selected in preference to Nile Red because of its higher emission intensity and greater stability. A Perkin-Elmer LS50B spectrofluorimeter was used to measure the peak maximum of an emission scan from 380 to 560 nm using an excitation wavelength of 280 nm with excitation and emission slit widths of 5 nm.

3. Results

Fig. 1 shows binodal curves for the PEG–phosphate system determined at 20°C and 31°C. The binodal curve is shifted to lower concentrations of PEG and phosphate with increase in temperature. Away from the critical point the binodal curve is hardly affected by moderate increase in temperature. The effect on the phase diagram is small and affects

only the region close to the critical point despite a temperature difference of 11°C. Fig. 1 also shows a selection of tie lines determined at 20°C which were used to determine the coordinates of the critical point as outlined in the methods section. Using this method indicated a critical point of 11.5% (w/w) PEG–11.5% (w/w) phosphate. Using a triangular plot (not shown) and the method of Treybal [20] the coordinates of the critical point were found to be 10.3% (w/w) PEG–12% (w/w) phosphate. These points are in reasonable agreement, but it is the latter point, shown in Fig. 1, which was arbitrarily selected to give the composition of the near critical point system.

As described in Section 2.4 two 10-ml aliquots of the critical point system were placed in the water bath in the form of a blank system containing no protein or as a system containing each protein to be partitioned. The phase compositions of the blank and protein containing systems were determined on a different sample for each equilibrium temperature selected. The critical temperature for phase separation for all proteins and blank systems was 28°C except for the systems containing α-amylase and α-chymotrypsin where the critical temperature for phase separation was found to be 28.5°C. Fig. 2 shows the phase compositions of the average of all the blank systems as determined by SEC at each increment in the temperature of phase separation above T_c (°C). Consideration of the geometry of the phase diagram and the requirement for a material balance suggests that some error in the determination of the compositions of the phases may be responsible for the fact that the compositions of the systems and the overall composition of the critical point system do not lie on a common straight line. This may be accounted for by our use of peak height to evaluate the SEC results. More recently we have obtained improved results by the use of area measures of eluting peaks. It should also be pointed out that knowledge of the mass, as opposed to the volume, of HPLC sample injections is absolutely essential. The necessity to closely consider our binodals revealed an interesting aspect of the temperature method of increasing the divergence of the compositions of the coexisting phases. Whilst phases of equivalent divergence, in terms of tie line length (see below) are produced by change in temperature or change in composition, as illustrated schematically in Fig. 3, these phases are not of identical composition. Systems having the same tie line length at different temperatures do not have the same absolute com-

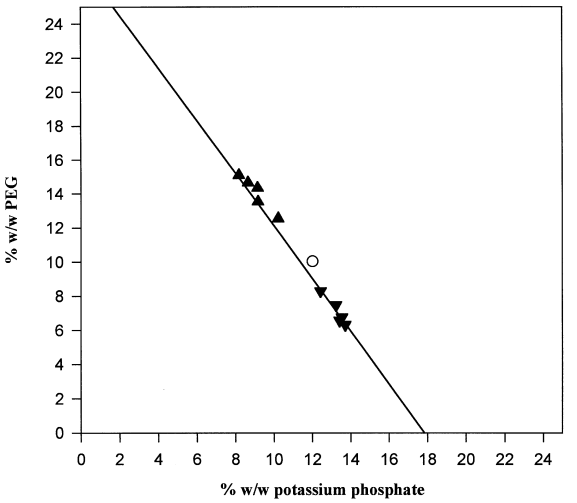

Fig. 2. Variation of phase compositions with temperature. The initial near critical point system – 10.3% (w/w) PEG–12.0% (w/w) phosphate – is shown (○) along with the compositions of the top (▲) and bottom (▼) phases resulting from an increase in temperature to T_c, T_c+1, T_c+2, T_c+3, T_c+4°C. Each point is an average of eight determinations performed on the blank systems of each protein to be examined.

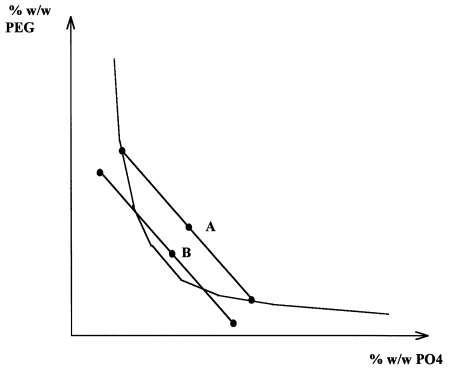

Fig. 3. Comparison between the phase composition of two biphasic systems having the same tie line but built by two different methods. The schematic diagram shows the way in which a system built by addition of components at one temperature (A) may have the same tie line length but differing absolute composition of the phases from another system built by increase in temperature close to the critical point (B).

positions of the phases and may not have the same absolute value of the partition coefficient.

In partitioning studies it is conventional to express the difference in the composition of the two phases by the tie line length which is determined, by simple Pythagorean procedure, from the difference in concentration of the phase forming components between the phases [18]. The variation in tie line length for all the systems examined is shown in Fig. 4. There is considerable variation in the data and the tie line length of the blank systems appears, for the most part, to be lower than that of the samples containing protein. This is not unexpected since the added protein takes part in the overall equilibrium in these near critical systems in which it is completely soluble and thereby affects the distribution of the phase forming components. The critical temperature for phase separation [T_c (°C)], in many cases, may be lower in the presence of protein. The tendency for increased temperature to increase the tie line length above T_c (°C) appears to decrease as the temperature is further increased which seems also to be implicit in Fig. 1 (see also Ref. [19], p. 71). The intercept of the regression line shown in Fig. 4 indicated a critical temperature for phase separation of about 25.9°C for a system having the present initial mixture composition. The experimentally realized temperature at which biphasic systems were observed to form of 28°C may simply be a consequence of our equilibrium procedure. The tie line lengths produced by manipulation of temperature are remarkably short varying between 5% (w/w) at a temperature about 2°C above the suggested true T_c (°C) and approaching 12% (w/w) at a temperature 6°C above T_c (°C). Such short tie line lengths would be difficult to achieve by mixing the components by weight at this scale.

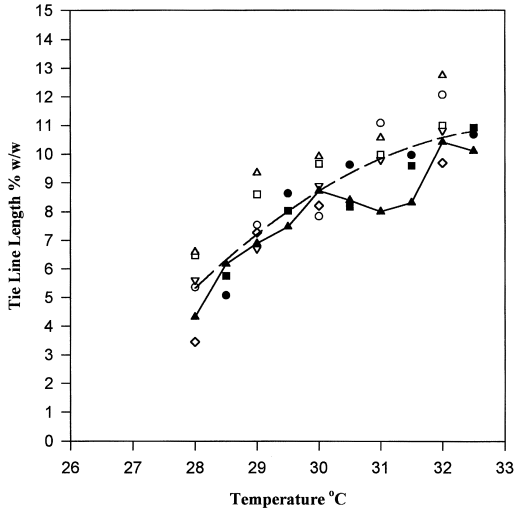

Fig. 4. Variation in tie line length with system equilibrium temperature. The line connects the tie line length of all the blank systems. The remaining symbols denote systems containing proteins; (○) BSA, (□) cytochrome *c*, (△) myoglobin, (▽) lysozyme, (●) ribonuclease, (◊) α-chymotrypsin, (■) α-amylase, (▲) system blanks. The dashed line shows a second order regression for the total data in the plot.

The protein containing systems were sampled and the partition coefficient determined as outlined in Section 2.6. According to Brönsted the effect of temperature on the partition coefficient is negligible and the change in partition coefficient with system composition resulting from change in temperature is constant given by [8–12]

$$\ln k = a(T - T_c)^{\frac{1}{2}} \quad (3)$$

where a is a constant. The partitioning results for the model proteins are shown in Fig. 5 where the assumption has been made that T_c is 25.9°C as indicated in Fig. 4. The intersection of the plots for

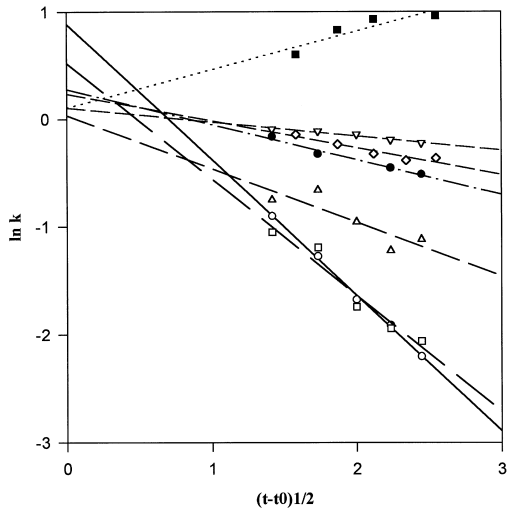

Fig. 5. Partition coefficients of the model proteins according to the Brönsted relationship. (○) BSA, (□) cytochrome *c*, (△) myoglobin, (▽) lysozyme, (●) ribonuclease, (◊) α-chymotrypsin, (■) α-amylase.

Table 1
Partitioning data for the model proteins derived from Fig. 5

Protein	Slope	Intercept	Regression coefficient
BSA	−1.259	0.885	0.998
Cytochrome *c*	−1.077	0.532	0.947
Myoglobin	−0.496	0.037	0.734
Ribonuclease A	−0.325	0.277	0.976
α-Chymotrypsin	−0.249	0.236	0.909
Lysozyme	−0.130	0.106	0.957
α-Amylase	0.336	0.107	0.803

each protein are reasonably close to a theoretical value of zero for both partition coefficient and temperature difference. It appeared to make very little difference to the quality of the regressions in Fig. 5 whether the exponent of the temperature difference was assumed to be 1/2 or 1/3. Although in the latter case, which is sometimes applicable very close to the critical point [30], the ordinate intercepts tended to be scattered further away from $\ln k = 0$. Only α-amylase has a positive slope and preference for the top phase. Slopes, intercepts and regression coefficients for all the proteins are shown in Table 1. For ''isochemical species'' the slope of the partition coefficient would depend on their molecular mass or area [31]. This is evidently oversimplified when applied to biological particles as may be judged from consideration of Table 2 which shows the results of our surface structural analysis of these proteins. It is a matter of some regret that the structure of human serum albumin is not lodged with the Protein Data Bank. Had the bovine serum albumin been omitted from the partitioning study interpretation of Table 2 would have appeared, superficially, straightforward. The influence of the surface exposure of tryptophan side chains combined with the hydrophobic surface area and the influence of the density of positively charged residues at the surface of these proteins might recommend themselves for reasonable consideration as additive components of the observed partition coefficient. In previous publications considering areas of the phase diagram relatively removed from the critical point salting out relationships of proteins have been considered important [1–5] and under these circumstances interpretation of the results in terms of protein hydrophobic surface area and dipole moment may be attempted [1–5]. Consideration of the likely surface properties of serum albumin in relation to its partitioning behavior makes that interpretation difficult to sustain in the present experiment where the same proteins are partitioned close to the critical point. Its hydrophobic surface area may be expected to be large since there are two hydrophobic binding pockets [23] and otherwise this parameter is largely size dependent. There are two tryptophan residues one of which lies in one of its

Table 2
Physical data relating to the partitioned proteins

Protein	Hphob[a]	Hphil[b]	Trp sch[c]	% Trp[d]	Charged[e] (mol %)	p*I*[f]	Mw[g]	Slope[h]
ALB					31.044	5.6	66.4	−1.3
CYT	1417	4364	14.4	0.25	30.769	10.3	11.6	−1.1
MYO	2153	5458	18.7	0.25	26.797	9.0	17.0	−0.5
RIBO	1757	5116	0	0	22.581	8.2	13.7	−0.3
CHY	3540	6892	136.4	1.31	13.978	8.1	25.7	−0.2
LYS	2063	4508	237.4	3.61	20.155	9.2	14.3	−0.1
AMY	5608	12 206	470.2	2.64	22.656	6.8	58.5	0.3

Abbreviations for the proteins are ALB, bovine serum albumin; CYT, cytochrome *c*; MYO; myoglobin; RIBO; ribonuclease A; CHY; α-chymotrypsin; LYS, lysozyme, AMY, α-amylase.
[a] Hydrophobic surface area (Å^2).
[b] Hydrophilic surface area (Å^2).
[c] Area of exposed tryptophan side chains (Å^2).
[d] Exposed tryptophan as % total solvent accessible surface area.
[e] Mol % D,E,K,R residues in the sequence.
[f] Isoelectric point.
[g] Molecular mass (Da).
[h] Slope of ln *K* from Fig. 5.

two hydrophobic pockets [23] and could reasonably be expected to be partially exposed. The degree of exposure of the other tryptophan residue is currently unknown, however the total degree of tryptophan exposure seems likely to lie in the mid range of the values for all the proteins shown in Table 2.

In order to consider the determinants of protein partitioning behavior close to the critical point it is necessary to examine in more detail their overall partitioning behavior throughout the phase diagram. Fig. 6 is a composite figure based on previous results and the current near critical point data. At extended tie line length the partition coefficient may be observed to rise from a minimum for most proteins except perhaps cytochrome *c*. In the case of α-amylase this occurs from the critical point. This behavior has been interpreted in terms of the onset of a salting out response [1–5]. Thus close to the critical point all these proteins, except α-amylase are partitioned in a salt regime closer to the salting-in region. Consideration of the solubility relationships of proteins in the salting in region leads to the conclusion that solubility should be a function of charge and size [32,33]. This seems to be approximately confirmed by the surface concentration of charged groups (mol %) for these proteins shown in Table 2. Simplistic interpretations of this sort cannot be expected to be entirely accurate since it is not at all clear whether each protein, under these conditions, is partitioned close to the maximum salting in effect of phosphate. α-Amylase is clearly not, on the other hand cytochrome *c* may well be nor is it clear how close each of these proteins is to its maximum intrinsic solubility which is likely to vary widely between them and will affect their apparent solubility in presence of salt. This in turn will affect their relative solubility between the phases. At the critical point proteins are equally soluble in either phase. Increasing the tie line length results in rapid loss of PEG from the lower phase and in the absence of any salting out effect, such as is shown in the case of α-amylase, protein solubility increases rapidly in the salt-rich phase, giving rise to a rapid decrease in the partition coefficient. Conventionally this would be ascribed to the reduction of the excluded volume effect of PEG leading to increasing protein solubility in the bottom phase as the PEG distributes increasingly to the top phase with increase in tie line length [34]. Protein solubility and partition would then, as previously stated be dependent on charge and size. However a mechanism in addition to, or in place of, molecular exclusion seems also possible which could lead to the same result.

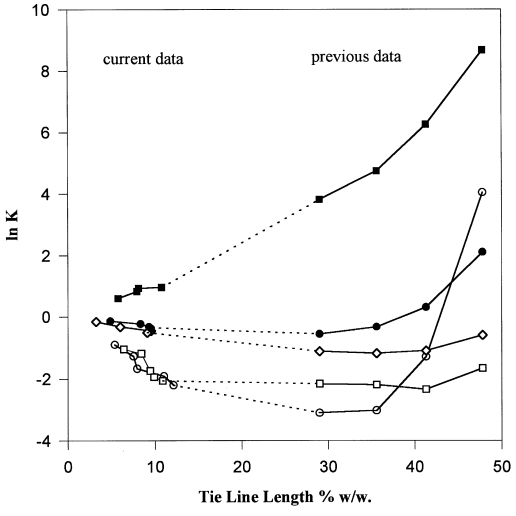

Fig. 6. Partition coefficients of the model proteins near the critical point obtained in the present study and at extended tie line length reported previously [2]. (○) BSA, (□) cytochrome *c*, (◊) ribonuclease A, (●) α-chymotrypsin, (■) α-amylase. (The data for myoglobin and lysozyme have been omitted for clarity).

Fig. 7 shows the results of a fluorometric assay using ANS on the separated phases resulting from increasing the temperature of a monophasic system at room temperature. We initially thought that this assay could provide a means of determining the compositions of the two phases. However, as has been discussed earlier the evolution of the phase compositions with temperature does not follow the perimeter of a single binodal curve These results are therefore far from easy to use quantitatively to give phase compositions and tie line length. They are of some interest however, since it is well known that solvatochromic dyes such as ANS or Nile Red respond to the dielectric permittivity of their environment showing increased emission intensity and Stoke's shift as the dielectric constant of the medium decreases [35]. This is also the case when these dyes are used as probes of hydrophobic surfaces [35]. Thus the results seem to imply that the dielectric constant of the two phases changes rapidly with increasing tie line length as the composition of the

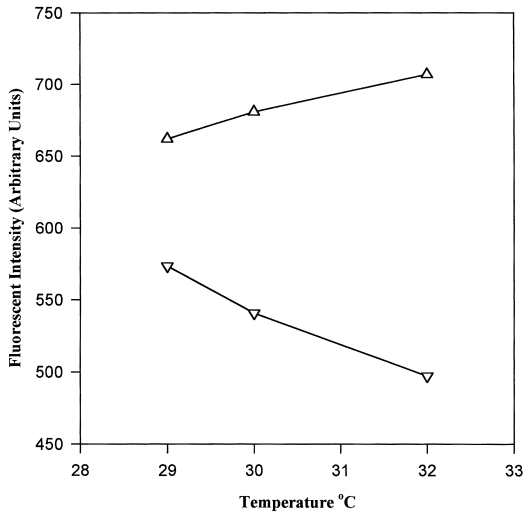

Fig. 7. Fluourescent intensity of 100 μl of 2.5 m*M* ANS added to 6 ml of the separated phases of a 10.3% (w/w) PEG-1450–12.01% (w/w) potassium phosphate system. (△) Top phase, (▽) bottom phase.

phases diverges. The dielectric constant is very important in determining protein solubility but it is not normally necessary to specifically account for it in excluded volume or precipitation studies. The effect of changes in dielectric constant should also be dependent on protein size and charge [32].

4. Discussion

The technique of controlling partition by manipulation of temperature is an interesting one which can illuminate the partitioning behavior of introduced particles. The method is also universally applicable whatever the nature of the phases comprising the two phase system. Favorable results recommend extending the method to the study of particles of colloidal size (e.g., viruses, cell organelles) and into polymer–polymer systems of lower interfacial tension. Note, however, that in the latter systems it is necessary to reduce rather than raise the temperature to promote phase separation of near critical point systems. In PEG–salt aqueous two phase systems significant changes in partitioning behavior occur with increase in tie line length. The results of applying the temperature method in combination with conventional methods reveals that there are essentially three regions of importance to protein partitioning in these phase systems. The first very close to the critical point where ideal solute behavior and conformation to the assumptions of Brönsted may be observed. A region further from the critical point where this behavior may be significantly modified and finally a region in which increasing precipitation and aggregation at the interface will occur.

The use of temperature to control the equilibrium distribution of biphasic systems has been rather infrequently applied. Most recently, Gallagher and coworkers [16,17] amongst others have applied the technique to study wetting phenomena in water lutidene biphasic systems. Gallagher and Maher [16], working within 0.5°C of the temperature for critical mixing studied the partitioning of latex polymer spheres in both lutidene–water and isobutyric acid–water systems. Phase preference close to the critical point was dependent on the charge density of the particles. The particles showed high preference for only one of the bulk phases in these systems within 0.1°C of T_c (°C). However, due to the onset of the population of the liquid–liquid interface by the particles as the temperature was increased they were not able to determine the exponent of the Brönsted equation. (The amazing precision attained by Brönsted and Warming in applying the temperature method [12] and an inconsistency in the evaluation of their results have been alluded to by Boucher [10]). In the study of Gallagher and Maher [16] further increase in temperature lead to increasing aggregation of the latex particles at the interface.

It is apparent that for larger particles and for increasing differences between the compositions of the phases there may exist a window in which material of interest will be distributed between the bulk phases, which later becomes a more and more one sided distribution and finally results in partition to the interface, the so-called Winkelblech effect [36]. For aqueous organic systems this window may be very small. Brönsted worked within 0.25°C of the critical point in order to partition a chromium hydroxide sol in a system in which the interfacial tension was reduced to a minimum [12]. The work of Albertsson [19,37] especially demonstrates that this window is likely to be much larger for polymer–polymer systems than for polymer–salt or aqueous

organic systems because of the decreased interfacial tension in these systems compared to the others.

In the present study where proteins have been partitioned in relatively low molecular mass PEG–salt systems a relatively wide range of temperature has been used without any tendency to partition to the interface but with increasing one sidedness of the partition. This may be compared to the results obtained in solvent water systems with much larger colloidal particles such as in the experiments of Brönsted and Warming [12] or of Lashmiller and McPherson [38] where partitioning is conducted very close to the critical point, which in temperature terms amounts to just few hundred mK. Lashmiller and McPherson (using the temperature method) surveyed a number of systems to discover ones in which one sided distribution rather than collection at the interface ensued. In colloidal partitioning the increasing tendency to collect at the interface, the Winkelblech effect, seems to have been first proposed as a ''reaction'' that could be used as a quantitative analytical tool for use with colloidal material [36]. It is interesting to note the redevelopment of this approach in PEG–salt systems as an assay method for PEG employing cibacron blue dye [39]. In this case the utility arises from the one sidedness of the partition as a result of the strong preference of the dye for the least polar phase. The dye is highly soluble in the PEG phase and almost completely insoluble in the phosphate phase [40].

5. Conclusions

The broad similarities in behavior of particles, from molecules to colloids, partitioned in aqueous two-phase and in conventional aqueous–organic systems is striking. By suitable choice of salts, polymers and operating conditions an almost infinite variety of aqueous two-phase systems may be produced. Important physical parameters such as the relative polarity of the phases and their interfacial tension may also be selectable over a wide range. That the ability to fine tune aqueous biphasic systems should find wide applicability in a biological context is perhaps not so surprising, because of the requirement to maintain molecular conformation and activity, but the comparative paucity of applications in other areas of analytical and separation science seems rather curious.

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