Partitioning of Proteins Using Two-Phase Aqueous Surfactant Systems

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Recently, there has been considerable interest in the utilization of two-phase aqueous surfactant systems to partition proteins and other biomaterials (Bordier, 1981; Pryde, 1986; Pryde and Phillips, 1986; Holm et al., 1986; Ramelmeier, 1991; Saitoh and Hinze, 1991; Nikas et al., 1992). The underlying reason is that under the appropriate solution conditions, many surfactant systems can separate into two water-based, yet immiscible, liquid phases, between which water-soluble proteins can partition unevenly while maintaining their native conformations and biological activities. This feature makes these systems particularly suitable for protein purification using liquid-liquid extraction techniques.

When compared to the more traditional *two-phase aqueous polymer systems* (Albertsson, 1986; Walter et al., 1985, 1991; Abbott et al., 1990, 1991, 1992), *two-phase aqueous surfactant systems* offer a number of unique, desirable features:

(1) In the simplest realization, only a *binary* surfactant-water system is required instead of the more complex *ternary* polymer 1-polymer 2-water or polymer-salt-water systems.

(2) The self-assembling, labile nature of the surfactant micelles present in the solution, as opposed to the unchanging identity of the polymers which is fixed upon synthesis, enables one to control and optimize the partitioning behavior by tuning micellar characteristics, including micellar shape and size.

(3) The dual character of micelles, which can simultaneously offer hydrophilic and hydrophobic environments to solute species, gives rise to a partitioning selectivity based on the hydrophobicity of biomaterials.

(4) The partitioning selectivity can be enhanced by utilizing mixed micelles containing surfactant-type ligands which can target a desired biomaterial.

(5) The separation of the desired biomaterial from the surfactant molecules after partitioning is completed can be facilitated by exploiting the self-assembling character of micelles, for example, micelles may be disassembled into their constituent surfactant monomers followed by filtration of the resulting protein-surfactant monomer solution.

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In view of the above features, two-phase aqueous surfactant systems may provide a useful alternative to two-phase aqueous polymer systems for the liquid-liquid extraction of biomaterials. Accordingly, it appears very important to identify as well as characterize the main driving forces and the underlying physical principles responsible for the observed partitioning behavior of biomaterials in two-phase aqueous surfactant systems.

In a previous publication (Nikas et al., 1992), we presented a theoretical formulation to describe and predict the partitioning of water-soluble (hydrophilic) proteins in two-phase aqueous surfactant systems containing cylindrical, noncharged micelles. The theory was based on the assumption that *excluded-volume interactions between the globular hydrophilic proteins and the noncharged cylindrical micelles* play the dominant role in determining the experimentally observed partitioning behavior. The excluded-volume formulation incorporated (i) the self-assembling character of micelles, which enables them to grow into long, cylindrical microstructures with changing temperature and/or surfactant concentration, and (ii) a broad polydispersity in micellar size. We also presented a detailed comparison of the similarities and differences in the partitioning of proteins in two-phase aqueous surfactant and polymer systems. The theoretically predicted protein partitioning behavior was compared with experimental measurements of the partitioning of the hydrophilic protein *ovalbumin* in a two-phase aqueous system of the *nonionic* surfactant *n*-decyl tetra(ethylene oxide), $C_{10}E_4$, and was found to be in good agreement.

In this article, we present new extensive experimental data on the partitioning behavior of several water-soluble (hydrophilic) proteins of various sizes, including *cytochrome c*, *soybean trypsin inhibitor*, *ovalbumin*, *bovine serum albumin*, and *calatase* (in the order of increasing size), in the *nonionic surfactant* $C_{10}E_4$*-water* two-phase system, as well as new partitioning data for three hydrophilic proteins, including *cytochrome c*, *ovalbumin*, and *catalase*, in the *zwitterionic surfactant dioctanoyl phosphatidylcholine* (C_8*-lecithin*)*-water* two-phase system. In addition, we compare this broad set of new experimental protein partitioning data with predictions based

on the recently proposed excluded-volume protein partitioning theory (Nikas et al., 1992). This comprehensive comparison will permit for the first time a detailed evaluation of the range of validity and applicability of the recently proposed excluded-volume theory, as well as shed light on the dominant driving forces responsible for the observed protein partitioning behavior.

Materials and Experimental Methods

The nonionic surfactant $C_{10}E_4$ and the zwitterionic surfactant C_8-lecithin were selected for this study because (i) they are nondenaturing and gentle to proteins, and (ii) they form two-phase aqueous systems over a temperature range below typical protein denaturation temperatures.

Homogeneous $C_{10}E_4$ (lot no. 1006) was obtained from Nikko Chemicals (Tokyo). C_8-lecithin powder (lot no. 80PC-34) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cytochrome *c* (from horse heart), soybean trypsin inhibitor (type I-S), ovalbumin, bovine serum albumin, and catalase (from bovine liver) were obtained from Sigma Chemicals (St. Louis, MO). All these materials were used as received. All other chemicals used were of reagent grade. All solutions were prepared using deionized water which had been fed through a Milli-Q ion-exchange system and were buffered at pH 7 by 10 mM citric acid and 20 mM disodium phosphate (McIlvaine buffer). Solutions also contained 0.02% sodium azide to prevent bacterial growth.

Coexistence curves for liquid-liquid phase separation of the buffered aqueous $C_{10}E_4$ and C_8-lecithin solutions without and with added proteins were determined by the cloud-point method (Nikas et al., 1992). This method consists of visually identifying the temperature T_{cloud} at which solutions of known surfactant concentration become cloudy as the temperature is raised (for $C_{10}E_4$) or lowered (for C_8-lecithin). The measured cloud-point temperatures were reproducible to within 0.03°C.

The protein partition coefficient K_p provides a useful quantitative measure of the protein partitioning behavior, and is defined as the ratio of the protein concentration in the top phase $C_{p,t}$ to that in the bottom phase $C_{p,b}$, that is, $K_p = C_{p,t}/C_{p,b}$. For the measurement of K_p, buffered solutions containing known concentrations of either $C_{10}E_4$ or C_8-lecithin and protein were prepared and subsequently allowed to equilibrate at a constant temperature for at least 6 h to form a two-phase system. The protein concentration in each coexisting phase was then determined by measuring the UV absorbance of that phase using a Shimadzu UV 160U spectrophotometer. Absorbance measurements were made at wavelengths of 545 nm for cytochrome *c* after it has been reduced by sodium ascorbate, 410 nm for catalase, and 280 nm for soybean trypsin inhibitor, ovalbumin, and bovine serum albumin, as well as referenced to the absorbance of the identical $C_{10}E_4$ or C_8-lecithin solution phase (but without protein).

For more details on the experimental procedures for measuring coexistence curves and protein partition coefficients, see Nikas et al. (1992).

Experimental Results

Figure 1 shows the experimental coexistence curves of aqueous $C_{10}E_4$ surfactant solutions without protein (○), and with 0.25 g/L cytochrome *c* (△), 0.5 g/L ovalbumin (*), and 0.5 g/L catalase (□) in McIlvaine buffer at pH 7. This figure indicates that over the range of surfactant concentrations examined, the added proteins have a negligible effect on the phase separation of the $C_{10}E_4$ solutions. Similar results were obtained for aqueous C_8-lecithin solutions containing proteins, but are not reported here due to space limitations. This important finding was utilized in the theoretical formulation (Nikas et al., 1992) to decouple the description of the protein partitioning from that of the surfactant solution phase separation.

Figure 2 shows the experimentally determined partition coefficients K_p of cytochrome *c* (▲), ovalbumin (●), and catalase (■) as a function of temperature over the range 18.8°C–21.1°C in two-phase aqueous $C_{10}E_4$ systems containing 0.25 g/L cytochrome *c*, 0.5 g/L ovalbumin, and 0.5 g/L catalase, respectively, in McIlvaine buffer at pH 7. The fact that $K_p < 1$ indicates that these three hydrophilic proteins partition preferentially into the *bottom micelle-poor phase*. It is also clear that as the temperature increases away from the critical temperature $T_c \approx 18.8$°C (corresponding to the minimum of the coexistence curve in Figure 1), K_p decreases and deviates further from unity for all three proteins. These observations suggest that (i) proteins are pushed into that phase which has a larger available free volume (which in this case is the bottom micelle-poor phase) due to excluded-volume interactions between $C_{10}E_4$ micelles and proteins, and (ii) this tendency becomes stronger as $(T - T_c)$ increases, that is, with increasing difference in the surfactant concentrations (or the volume fractions occupied by micelles) of the two coexisting

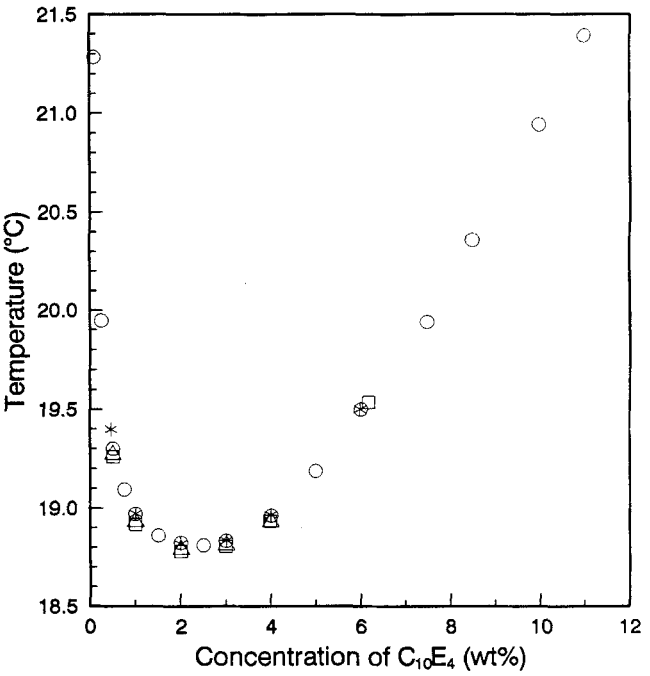

Figure 1. Experimentally measured coexistence curves of aqueous solutions of $C_{10}E_4$.

Without protein (○), and with 0.25 g/L cytochrome *c* (△), 0.5 g/L ovalbumin (*), and 0.5 g/L catalase (□).

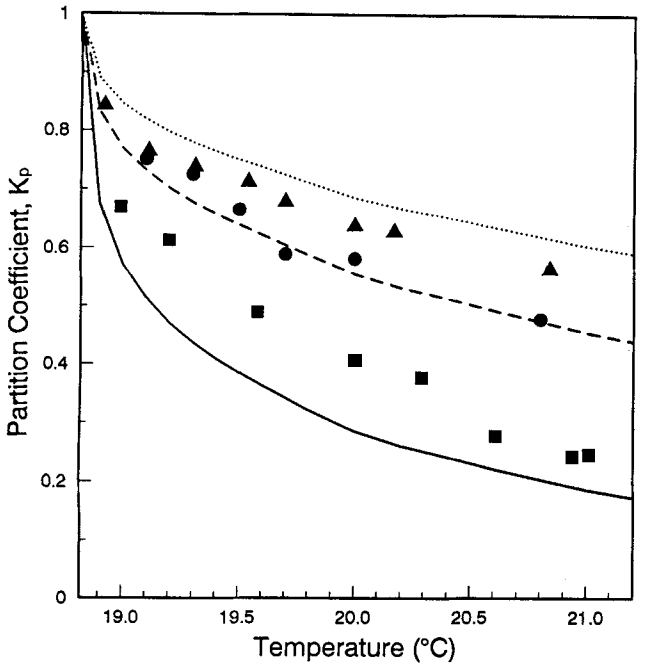

Figure 2. Experimentally measured partition coefficients K_p.

Cytochrome c (▲), ovalbumin (●), and catalase (■) at different temperatures in the two-phase aqueous $C_{10}E_4$ surfactant system. Predicted partition coefficients K_p of cytochrome c(· · ·), ovalbumin (– – –), an catalase (–) are also shown as a function of temperature (see Eq. 1).

phases (see Figure 1). One can also observe from Figure 2 that, at a fixed temperature, the extent of the protein partitioning into the bottom micelle-poor phase increases in the order cytochrome $c <$ ovalbumin $<$ catalase. This observed trend is consistent with the notion that excluded-volume interactions between proteins and $C_{10}E_4$ micelles play the dominant role in determining the observed partitioning behavior, since catalase has the largest size (M.W. 232,000 Da), followed by ovalbumin (M.W. 44,000 Da), and cytochrome c (M.W. 12,400 Da).

Theoretical Results and Comparison with Experimental Data

Results of various experimental and theoretical studies have shown (Blankschtein et al., 1986; Puvvada and Blankschtein, 1990; Lindman and Wennerström, 1991) that under appropriate solution conditions $C_{10}E_4$ and C_8-lecithin can form long, flexible, and polydisperse cylindrical micelles. The average length of a $C_{10}E_4$ or C_8-lecithin micelle greatly exceeds the hydrodynamic radius of a typical hydrophilic protein (20Å–50Å), and the micelle is rigid on the scale of a typical protein molecule (Nikas et al., 1992). Experimental results also indicate (Helenius and Simons, 1972; Makino et al., 1973; Makino, 1979) that hydrophilic proteins, including cytochrome c, ovalbumin, and bovine serum albumin, do not bind nonionic and zwitterionic surfactants of the type used in this study to any significant extent. In addition, our coexistence curve measurements indicate that the effect of these proteins on micellar characteristics is negligible. In view of this, it is reasonable to assume that, to a first approximation, hydrophilic proteins and $C_{10}E_4$ or C_8-lecithin micelles behave as mutually nonassociating entities interacting primarily through short-ranged, repulsive, excluded-volume interactions.

Specifically, we have assumed (Nikas et al., 1992) that an aqueous surfactant-protein solution containing cylindrical micelles and globular hydrophilic proteins can be modeled as a mixture of mutually penetrable, polydisperse, hard spherocylinders (micelles) and hard spheres (proteins). Due to space limitations, we will only present the final theoretical expression for K_p corresponding to this system. Nikas et al. (1992) provide complete details of the theoretical derivations.

We have found that, under conditions of low protein concentration, noncharged surfactants, and low salt concentration, in a two-phase aqueous surfactant system containing *cylindrical* micelles and *globular* hydrophilic proteins, K_p, is given by:

$$K_p = \exp\left[-(\phi_t - \phi_b)(1 + R_p/R_0)^2\right] \tag{1}$$

where ϕ_t and ϕ_b are the surfactant volume fractions in the top and bottom phases respectively, R_p is the protein hydrodynamic radius, and R_0 is the cross-sectional radius of the cylindrical micelles. It is interesting to note that if the shape of the micelles present in the two-phase system is *spherical* instead of cylindrical, K_p is given by:

$$K_p = \exp\left[-(\phi_t - \phi_b)(1 + R_p/R_0)^3\right] \tag{2}$$

where R_0 is the radius of the spherical micelles.

Equations 1 and 2 indicate that the uneven partitioning of a hydrophilic protein in the two-phase aqueous surfactant system is a direct consequence of the difference in the surfactant concentrations in the two coexisting micellar solution phases, that is, the $(\phi_t - \phi_b)$ term. In addition, the value of the partition coefficient depends on the shape of the micelle, as well as on the relative sizes of micelles and proteins, as reflected in the values of R_0 and R_p. In Eqs. 1 and 2, the major difference is in the power of the $(1 + R_p/R_0)$ term, which is 2 (3) for cylindrical (spherical) micelles. Clearly, this implies stronger excluded-volume interactions induced by spherical micelles as compared to cylindrical ones, and vividly illustrates the effect of micellar shape and size on the resulting protein partitioning behavior.

In order to predict the variation of K_p with temperature, values of R_0 and R_p and of $(\phi_t - \phi_b)$ as a function of temperature are needed. In general, R_0 is approximately equal to the length of the surfactant molecule. Calculations based on a recently developed molecular model of micellization (Puvvada and Blankschtein, 1990; Naor et al., 1992) yield $R_0 \approx 21$Å for both $C_{10}E_4$ and C_8-lecithin. The hydrodynamic radii of cytochrome c, ovalbumin, and catalase are $R_p = 19$, 29, and 52 Å, respectively (Abbott et al., 1992). Values of $(\phi_t - \phi_b)$ at various temperatures can be obtained from the experimentally measured coexistence curves of $C_{10}E_4$ or C_8-lecithin. As an illustration, we consider the $C_{10}E_4$ coexistence curve in Figure 1. At a given temperature, ϕ_t and ϕ_b are given by the intersections of the horizontal tie line corre-

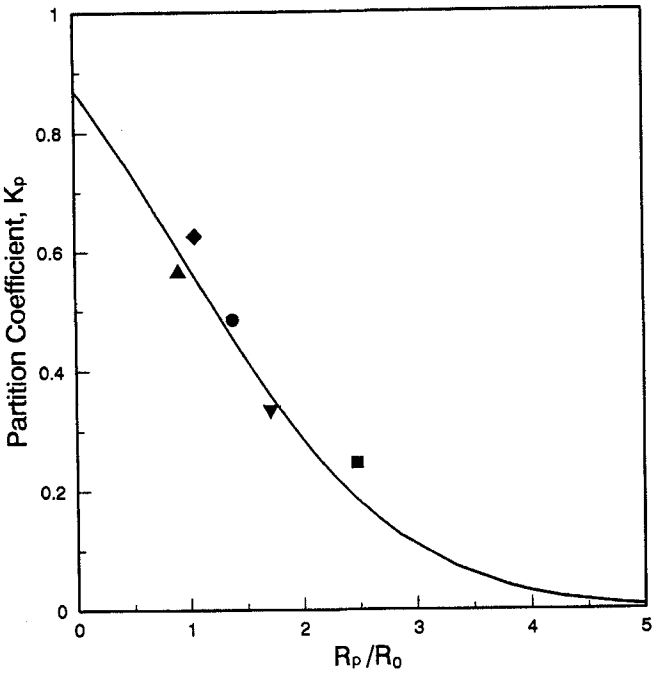

Figure 3. Predicted protein partition coefficient K_p as a function of the ratio R_p/R_0 in aqueous $C_{10}E_4$ surfactant solutions.

R_p is the protein hydrodynamic radius, $R_0 = 21$Å is the cross-sectional radius of a $C_{10}E_4$ cylindrical micelle, and $\phi_t - \phi_b = 10\%$, see Eq. 1. The various symbols correspond to the experimentally measured K_p values of the following proteins: (▲, $R_p = 19$ Å) cytochrome c; (◆, $R_p = 22$ Å) soybean trypsin inhibitor; (●, $R_p = 29$ Å) ovalbumin; (▼, $R_p = 36$ Å) bovine serum albumin; and (■, $R_p = 52$ Å) catalase.

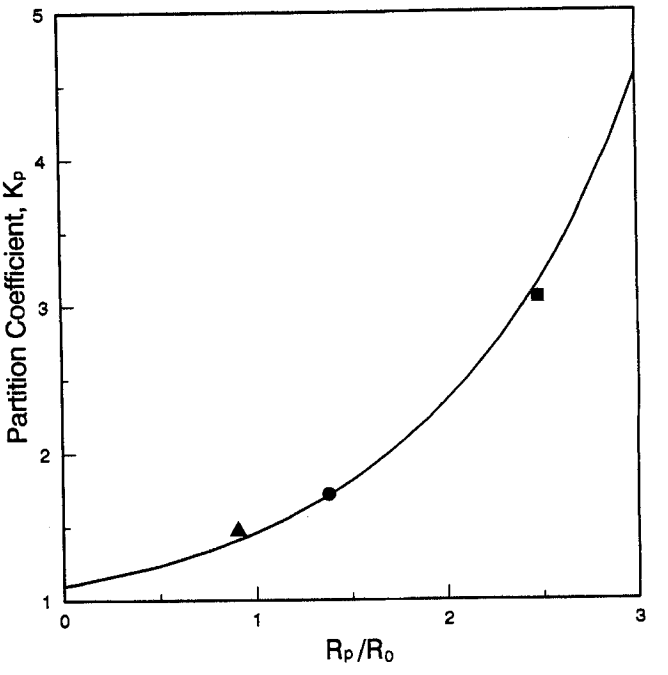

Figure 4. Predicted protein partition coefficient K_p as a function of the ratio R_p/R_0 in aqueous C_8-lecithin surfactant solutions.

R_p is the protein hydrodynamic radius, $R_0 = 21$ Å is the cross-sectional radius of a C_8-lecithin cylindrical micelle, and $\phi_b - \phi_t = 10\%$, see Eq. 1. The various symbols correspond to the experimentally measured K_p values of the following proteins: (▲, $R_p = 19$ Å) cytochrome c; (●, $R_p = 29$ Å) ovalbumin; (■, $R_p = 52$ Å) catalase.

sponding to that temperature with the concentrated and dilute branches of the coexistence curve respectively.

Figure 2 shows the predicted variation of K_p with temperature (in the $C_{10}E_4$-water two-phase system) for cytochrome c (dotted line), ovalbumin (dashed line), and catalase (full line) corresponding to the $(\phi_t - \phi_b)$ values determined from Figure 1, $R_0 \approx 21$Å, and the R_p values listed above. Note that these predictions are based on Eq. 1, since $C_{10}E_4$ forms cylindrical micelles in aqueous solutions. As can be seen, there is good agreement with the experimentally measured K_p values. In spite of the more pronounced deviations between theory and experiment in the case of catalase, which may reflect the effect of nonuniversal protein-specific characteristics such as surface hydrophobicity and charged residues, the universal, nonspecific excluded-volume description is able to capture the correct order of magnitude of the observed protein partitioning behavior. Presumably, an analysis based on the detailed protein chemical structures may enable a meaningful rationalization of the observed deviations between the theoretical and experimental K_p values. This, however, is beyond the scope of this article.

The dependence of the partition coefficient K_p on protein size R_p can be seen clearly by plotting K_p as a function of the ratio R_p/R_0, at a fixed temperature, or equivalently, at a fixed value of $(\phi_t - \phi_b)$. Specifically, for $C_{10}E_4$ at 21°C, $\phi_t - \phi_b \approx 10\%$ (see Figure 1). Figure 3 shows the predicted variation of K_p as a function of R_p/R_0 (full line), together with the experimental K_p values corresponding to cytochrome c (▲), soybean trypsin inhibitor (◆), ovalbumin (●), bovine serum albumin (▼), and catalase (■). The hydrodynamic radii of soybean trypsin inhibitor and bovine serum albumin are $R_p = 22$Å and 36Å, respectively (Dubin and Principi, 1989). This figure indicates that as R_p increases relative to R_0, the value of K_p decreases and can become vanishingly small for $R_p/R_0 > 5$.

In the case of C_8-lecithin, for illustrative purposes, we selected a temperature of 10°C, at which $(\phi_b - \phi_t) \approx 10\%$. Note that in the C_8-lecithin case, *the bottom phase is micelle-rich while the top phase is micelle-poor.* Accordingly, due to excluded-volume interactions, hydrophilic proteins should partition preferentially into the top micelle-poor phase, namely, the values of K_p should be greater than 1. Figure 4 shows the predicted variation of K_p as a function of R_p/R_0 using Eq. 1, together with the experimental K_p values corresponding to cytochrome c (▲), ovalbumin (●), and catalase (■). This figure shows that, as expected, $K_p > 1$ and increases as R_p/R_0 increases. One can see from Figures 3 and 4 that the agreement between theory and experiment is good for both surfactant systems.

Conclusion

We have presented a comprehensive comparison of the predicted protein partition coefficients, in the context of a recently developed excluded-volume theory with a new broad

set of experimentally measured partition coefficients, of several hydrophilic proteins in two-phase aqueous $C_{10}E_4$ and C_8-lecithin surfactant systems. The good agreement between theory and experiment indicates that excluded-volume interactions between noncharged micelles and proteins are the dominant factor in determining the protein partitioning behavior.

Although there are other methods which exploit excluded-volume effects to separate proteins, including filtration and size-exclusion chromatography, two-phase aqueous surfactant systems exhibit several unique features (see the Introduction section for details), which are not present in the more traditional size-exclusion methods. Needless to say, this liquid-liquid extraction method should be particularly useful in large-scale operations. As such, two-phase aqueous surfactant systems appear very promising from an applications point of view.

Finally, our findings suggest that large hydrophilic particles, such as water-soluble colloids and cells, should exhibit extreme partitioning in this type of two-phase aqueous surfactant systems. Work aimed at examining this interesting possibility is in progress.

Acknowledgments

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