Temperature-Dependent Protein Partitioning in Two-Phase Aqueous Polymer Systems

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ABSTRACT: The partitioning of ribonuclease A and myoglobin in two different two-phase aqueous polymer systems was investigated experimentally and theoretically. Systems used were poly(ethylene oxide)/dextran/water and Ucon/dextran/water [Ucon being a random poly(ethylene oxide)-poly(propylene oxide) copolymer]. The experiments involved the determination of the temperature and polymer concentration dependence on the partitioning. The theoretical results were obtained through a self-consistent mean-field lattice theory for multicomponent mixtures of copolymers with internal states in heterogeneous systems. Comparisons between theoretical and experimental findings show a qualitative agreement regarding the increased preference for the dextran-rich phase at increased polymer concentrations and the different temperature response displayed by the two systems. Furthermore, details about the temperature response, obtained from the model calculations, are discussed. The overall protein-polymer interaction was found to be repulsive and it is dominated by an entropic repulsion due to the restriction of possible polymer conformations for polymers close to the protein surface. The energetic contribution to the overall interaction is smaller in magnitude. It was repulsive for poly(ethylene oxide) and Ucon, whereas it was attractive for dextran for both proteins.

Introduction

Biomaterials tend to partition unevenly in two-phase aqueous polymer systems. This was discovered by Albertson[1] in the 1950s, and a number of applications have appeared during the last decades on separation and purification of proteins, cell organelles, cells, etc.[2] However, not until recently has a fundamental understanding of biomaterial partitioning between the two phases emerged. In the approach by Baskir et al., the lattice theory for polymers in heterogeneous systems, developed by Scheutjens and Fleer,[3,4] is used to evaluate the free energy of placing a protein in the two polymer phases, and thus being able to model the partitioning. Although the approach includes a number of simplifications, the theory captures essential aspects of partitioning.[5,6] The influence of model parameters, such as the size of the protein and the length and bulk concentration of the polymer, was explored, as well as the response to variation of the interaction (adsorption) parameters. Comparisons of results from the model calculations with experimental partition coefficients showed that qualitative trends in the variation of the partition coefficients with polymer length and concentration could be reasonably accounted for with adequate choices of interaction parameters. For an overview of this as well as other theories, see ref 7. Recently, a scaling thermodynamic formalism has been used to describe protein partition in two-phase aqueous polymer systems.[8]

Among the polymers used for partitioning of biomaterials, poly(ethylene oxide) (PEO) is one of the most frequent. Indeed, it was in two-phase systems with PEO as one of the components that much of the early exploration of these phenomena was done,[1] and its widespread use has lasted ever since. The behavior of PEO in aqueous solution is somewhat peculiar, as such solutions are known to exhibit "clouding", a separation into two phases when the temperature is raised.[9] In a broader context, the most conspicuous feature of PEO as well as of other polymers and surfactants containing ethylene oxide groups is the strong inverse temperature dependence of a wide range of properties, of which the clouding is one.[10] Consequently, much effort has been directed to the understanding of the mechanisms underlying this anomalous behavior. Over the years, three different models have emerged, discussing the reversed temperature dependence in terms of solute–solute,[11] solute–solvent,[12] and solvent–solvent[13] interactions, respectively. We have adopted the first of these models, since it has the largest predictive capacity.

In this PEO model the ethylene oxide groups can be in either of two states, one polar and the other less polar. At low temperatures, the former state dominates, and the interaction between the polymer and the solvent is favorable, whereas at higher temperatures, the latter state dominates for entropic reasons, rendering the solute–solvent interaction less favorable. This model was used to describe the temperature dependence of the PEO/dextran/water system.[14] We have previously extended the lattice theory for heterogeneous systems with this two-state model[15,16] and successfully applied the theory for describing the adsorption of Pluronic polymers [PEO–PPO–PEO triblock copolymer, PPO being poly(propylene oxide)] to solid surfaces,[17,18] as well as modeling the micellization of Pluronic polymers in aqueous solution.[19]

The aim of the present work is to utilize this theory for reverse temperature dependence of PEO and related polymers to explain the partitioning of proteins in two-phase aqueous polymer systems containing PEO or Ucon (a random copolymer of ethylene oxide and propylene oxide). We have therefore performed measurements of partition coefficients of two well-characterized proteins, ribonuclease A and myoglobin, at room temperature and at an elevated temperature in PEO/dextran/water and Ucon/dextran/water systems. The reason for our interest in Ucon is that aqueous solutions of Ucon display the same type of clouding as aqueous PEO solutions do, but at much lower temperature. The clouding phenomenon, now at a temperature of 50 °C, has then the potential of facilitating the recycling of polymers in technical applications.[20] The partitioning at elevated temperature in the Ucon/dextran/water system was made at 45 °C in order to be close to the Ucon cloud point.

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Table I
Protein Data

property	ribonuclease A	myoglobin
mol wt[a]	13 700	17 000
partial specific vol (mL g^{-1})[a]	0.692	0.743
radius of equiv sphere (Å)	15.5	17.0
isoelectric pH[b]	8.2–9.6	7.0

[a] Reference 30, p 336. [b] Reference 29.

The experimental results are accompanied by model calculations employing the statistical mechanical mean-field lattice theory for multicomponent mixtures of copolymers with internal degrees of freedom in heterogeneous systems recently presented by us.[16] Since the investigations by Baskir et al.[5,6] and by us employ the same lattice field theory, our models are closely related. The main difference is that we consider the temperature dependence of the partitioning, and therefore have to invoke a model describing the reverse temperature behavior of aqueous solutions of PEO and related polymers. Moreover, we describe the bulk phases as three-component solutions, which makes it possible to take changes in phase compositions with temperature more adequately into account, the latter in distinction to earlier works where only the majority polymer and solvent were included (two-component system).

Experimental Section

Materials. Poly(ethylene oxide) 4000, average molecular weight = 4000 (latter referred to as PEO only), was purchased from Merck, Darmstadt, Germany, whereas Ucon 50-HB-5100, average molecular weight = 4000 [50% ethylene oxide and 50% propylene oxide random copolymer, latter referred to as Ucon only], was a kind gift from Union Carbide. Dextran T500, M_w = 500 000 (latter referred to as dextran only) was obtained from Pharmacia, Uppsala, Sweden. The dextran polydispersity (M_w/M_n) was 3.0. The PEO and dextran samples used for the optical rotation and refractive index standards were dried over phosphorus pentoxide before using. The Ucon was ultrafiltered (filter cutoff = 1 kDa, Filtron, NJ) and freeze-dried before drying over phosphorus pentoxide. This removed most of an impurity with a light absorption maximum at 290 nm which was present in the original Ucon. Ribonuclease A (bovine pancreas) and myoglobin (horse skeletal muscle) were purchased from Sigma, St. Louis, MO, and used as received. Data of the proteins are given in Table I.

Phase Diagrams. In order to determine the phase diagrams, various proportions of polymer stock solutions, thermally equilibrated at the appropriate temperature (22 and 50 °C for PEO and dextran and 22 and 45 °C for Ucon and dextran), were mixed in test tubes during more than 1 min before being returned to the water bath to separate for at least 45 min. Samples were withdrawn from the separated phases for analysis of composition by measurement of optical rotation and refractive index (after suitable dilution). The values of the measured quantities were translated to solution compositions by means of separately determined standard curves for the refractive index of PEO/water, Ucon/water, and dextran/water solutions, and a standard curve for the optical rotation of dextran/water solutions.

Partition Coefficients. For the determination of the protein partition coefficients, the appropriate proportions (by weight) of polymer stock solutions were put into test tubes and let come to thermal equilibrium with a water bath. The protein stock solution was added and the components were thoroughly, though gently, mixed for at least 1 min, and the systems were left to separate for 10–30 min in a water bath. Samples were withdrawn from the separated phases, and after dilution the protein content in each phase was determined by measurement of light absorption at 280 nm. As blanks were used equally diluted samples from identical phase systems without protein, which had been prepared in parallel. The protein content was 2–5 mg per 10 g of system. The systems were kept at constant pH by means of a 50 mmol/L sodium phosphate buffer, with pH = 8.2 for ribonuclease A and pH = 7.0 for the myoglobin (corresponds to their isoelectrical pH). Partition coefficients were determined at three different total compositions for each of the PEO–dextran and Ucon–dextran polymer combinations. The total polymer contents and the phase compositions are given in Table II.

Theoretical Modeling

Background. The Flory–Huggins lattice theory of homogeneous solutions[21] can be extended to describe the adsorption of polymers at surfaces.[3,4] In a heterogeneous system, the solution close to a surface is divided into layers parallel to the surface, where the thickness of the layers corresponds to the size of the solute (or of the polymer segment). Within each layer the densities are constant as a consequence of the mean-field approximation. Density gradients perpendicular to the surface are created by allowing the volume fraction of the components to vary among the layers.

An important step was taken by Evers, Scheutjens, and Fleer,[22] who extended the theory to be valid for a general multicomponent system. This formulation has furthermore been generalized to the case where the copolymer segments possess internal degrees of freedom.[16] The extension provides a means of modeling effective segment–segment interaction parameters which are temperature as well as density dependent. This polymer model was originally devised to describe the existence of a lower consolute point in homogeneous aqueous PEO solutions,[11] but has been extended to heterogeneous systems of homopolymers.[15]

The basis of the PEO model is that the distribution of conformations of a segment depends on temperature, and that different conformations interact differently with adjacent polymer segments and solvent molecules. From quantum mechanical calculations,[23] the conformations of the –OCCO– segment were divided into two classes or states, one being polar and having a low energy and a low statistical weight, and one being less polar or nonpolar and having the higher energy and a higher statistical weight. At low temperature the former state is dominating, and thus a more favorable polymer–water interaction is obtained, whereas at elevated temperatures the latter state becomes progressively more important which results in a more unfavorable polymer–water interaction. In the following paragraphs we will give a brief description of the model we have used, concentrating on the concepts and physical picture, while the detailed formal derivations may be found elsewhere.[16]

Homogeneous System. The extension of the Flory–Huggins theory to allow for internal degrees of freedom, as well as several types of segments in each component, leads[16] to the following expression for the chemical potential of a component x in a multicomponent solution:

$$\beta(\mu_x - \mu^*_x) = r_x \sum_A \sum_B \phi^*_{Ax} \left\{ P_{AB} \left[\beta U_{AB} + \ln \frac{P_{AB}}{g_{AB}} \right] - P^*_{AB} \left[\beta U_{AB} + \ln \frac{P^*_{AB}}{g_{AB}} \right] \right\} + \ln \phi_x + 1 - r_x \sum_{x'} \frac{\phi_{x'}}{r_{x'}} - \frac{r_x}{2} \sum_A \sum_{A'} \sum_B \sum_{B'} [(\phi_A - \phi^*_{Ax}) P_{AB} \chi_{BB'} P_{A'B'} (\phi_{A'} - \phi^*_{A'x}) + \phi^*_{Ax} \phi^*_{A'x} \chi_{BB'} (P^*_{AB} P^*_{A'B'} - P_{AB} P_{A'B'})] \quad (1)$$

Here indices A and B run over monomer species and species' states respectively (with the primed states' indices referring to states of species referred to by primed species' indices), r_x is the number of segments in component x,

Table II
Composition of the Two-Phase Systems[a]

		22 °C				50 °C			
total comp		dextran-rich phase		PEO-rich phase		dextran-rich phase		PEO-rich phase	
dextran	PEO	dextran	PEO	dextran	PEO	dextran	PEO	dextran	PEO
5.7	6.0	12.9	2.6	0.7	8.4	10.1	3.7	1.6	8.0
7.2	6.5	16.4	1.8	0.3	9.9	14.8	2.6	0.5	10.0
9.0	7.1	19.6	1.4	0.1	11.7	18.8	1.9	0.1	11.8
		22 °C				45 °C			
total comp		dextran-rich phase		Ucon-rich phase		dextran-rich phase		Ucon-rich phase	
dextran	Ucon	dextran	Ucon	dextran	Ucon	dextran	Ucon	dextran	Ucon
5.1	4.4	10.4	1.2	0.9	6.9	8.9	1.4	0.2	8.3
9.2	4.6	15.3	0.9	0.4	10.1	13.3	1.0	0.1	12.3
11.9	6.1	20.3	0.5	0.3	13.4	17.2	0.7	0.0	17.2

[a] In weight percent.

$\chi_{BB'}$ is the Flory–Huggins interaction parameter between species A in state B and species A' in state B', $\beta = 1/kT$, and stars refer to the reference state of pure amorphous components. In particular, ϕ^*_{Ax} denotes the volume fraction of species A in pure component x (=0 or 1 for homopolymers as in our case). P_{AB} is the fraction of species A that is in state B (with relative degeneration g_{AB} and internal energy U_{AB}), and is at equilibrium given by

$$P_{AB} = X_{AB}/\sum_B X_{AB}$$

$$X_{AB} \equiv g_{AB} \exp[-\beta U_{AB} - \sum_{A'}\sum_{B'} \chi_{BB'} P_{A'B'} \phi_{A'}] \qquad (2)$$

Heterogeneous System. Here we consider a polymer solution in the close vicinity of an impenetrable surface, which in this case has the shape of a sphere. A lattice of M concentrical shells (layers) is placed around the surface, the ith shell having a number L_i of sites. These numbers L_i are given by $L_i = V_i - V_{i-1}$, where V_i is the volume of a sphere with radius $(R + i)$ and all lengths are measured in lattice units. Let the fraction of nearest neighbors of a segment in layer i that are in layer j be denoted λ_{ij}, with $\sum_j \lambda_{ij} = 1$, and $\lambda_{ij} = 0$ if $|i-j| > 1$. Since the relation of being nearest neighbor is a reflexive one, these fractions characterizing the lattice must satisfy the flux constraint

$$L_i \lambda_{ij} = L_j \lambda_{ji}, \qquad i, j = 0, 1, ..., M \qquad (3)$$

A natural choice[15] is $\lambda_{i,i+1} = tS_i/L_i$, $\lambda_{i,i-1} = tS_{i-1}/L_i$, where t is a constant describing the planar lattice and S_i is the surface area of a sphere with radius $(R + i)$. Another definition of these fractions used in a study of protein partitioning[5] does not satisfy the flux constraint.

We will now have to get an expression for how the (Helmholtz) free energy depends on the system composition. To achieve this we divide the free energy into a sum of three terms:

$$\beta(A - A^*) = \beta(A_{\text{int}} - A^*_{\text{int}}) - \ln \frac{\Omega}{\Omega^*} + \beta(U - U^*) \qquad (4)$$

where A_{int} denotes the contribution from internal degrees of freedom, $\ln(\Omega/\Omega^*)$ is the mixing entropy from the configurational degeneration, and U denotes the total interaction energy.

The presence of a surface introduces a unique direction (*the normal*) in the system, and hence the complete directional degeneration of segment spatial distribution in the homogeneous system is lifted; various conformations of the polymer chains can be distinguished according to the different ordering of their segments with respect to the layer numbers. Formally a *conformation* c of component x is defined as a sequence $k(x,s,c)$; $s = 1,r$ of layer numbers, $k(x,s,c)$ being the layer number where the sth segment resides of a chain of component x in conformation c. With this definition, and neglecting at first self-exclusion, the degeneration of a conformation c of component x becomes $\exp((r_x - 1)\ln z + \ln \omega_{xc})$, where z is the total number of nearest neighbors of a site in the lattice, and

$$\omega_{xc} = L_{k(x,c,1)} \prod_{s=2}^{r_x} \lambda_{k(x,c,s-1),k(x,c,s)} \qquad (5)$$

When the self-exclusion on a mean-field level is taken into account, and the contributions from all conformations of all the components are included, the total conformational degeneration relative to the amorphous reference state, constituting the mixing entropy of the system, is given simply by[16]

$$\ln \frac{\Omega}{\Omega^*} = -\sum_x \sum_c n_{xc} \ln \frac{n_{xc} r_x}{\omega_{xc}} \qquad (6)$$

where n_{xc} denotes the number of chains of component x in conformation c.

The interaction energy, secondly, is obtained by summing all the nearest-neighbor interactions between the segments but taking into account now that the volume fractions of the segments may vary between the layers. The resulting expression becomes[16]

$$\beta U = \frac{1}{2}\sum_{i=0}^{M} L_i \sum_A^s \sum_{A'}^s \sum_B \sum_{B'} \phi_{Ai} P_{ABi} \chi_{BB'} \langle P_{A'B'i} \phi_{A'i} \rangle \qquad (7)$$

where $\langle x_i \rangle = \sum_j \lambda_{ij} x_j$, $\phi_{Ai} = n_{Ai}/L_i$, and n_{Ai} is the number of segments of species A located in layer i. The notation $\sum^s$ implies that the sum includes the surface species as well.

Finally, the expression for the internal free energy[16] is obtained by summing over all the layers, each layer contributing a term of the same form as in the homogeneous solution, but with the bulk values of the variables replaced by layer dependent ones:

$$\beta A_{\text{int}} = \sum_{i=1}^{M} \sum_A n_{Ai} \sum_B P_{ABi} \left[\beta U_{AB} + \ln \frac{P_{ABi}}{g_{AB}} \right] \qquad (8)$$

Now, the equilibrium distributions $\{n_{xc}\}$ of component conformations and $\{P_{ABi}\}$ of species states are those that minimize the free energy as given by eqs 4–8 above, under

Table III
Internal-State Parameters (U_{AB} and g_{AB}) and Flory-Huggins Interaction Parameters ($\chi_{BB'}$) (Energy in kJ mol^{-1})

species	state	U_{AB}	g_{AB}
water		0	1
EO	polar	0[a]	1[a]
	nonpolar	5.086[a]	8[a]
Ucon	polar	0[b]	1[b]
	nonpolar	8.750[b]	19.3[b]
dextran		0	1

	$RT\chi_{BB'}$				
state	EO, polar	EO, nonpolar	Ucon, polar	Ucon, nonpolar	dextran
water	0.6508[a]	5.568[a]	1.175[c]	7.035[c]	1.096[d]
EO,polar		1.266[a]			0.360[e]
EO,nonpolar					1.542[e]
Ucon,polar				1.333[c]	0.520[f]
Ucon,nonpolar					1.700[f]

[a] From fit to experimental PEO/water phase diagram.[11,15] [b] From fit to experimental Ucon/water phase diagram. Data from ref 24. [c] Mean of PEO–water and PEO[11,15] and PPO–water and PPO[16] interaction parameters. [d] Reference 14. [e] From fit to experimental PEO/dextran/water phase diagram at 22 °C (Figure 1). [f] From fit to experimental Ucon/dextran/water phase diagram 22 °C (Figure 2).

the constraints of $\sum_A \phi_{Ai} = 1$ in each layer i and $\sum_B P_{ABi} = 1$ for all species A in each layer i. This minimization problem may be solved numerically using a computational procedure presented in ref 16.

Model Calculations. On the basis of the molecular weight PEO 4000 is modeled as polymer with 91 EO units, whereas Ucon 50-HB-5100 is described as a homopolymer with 78 segments that have two internal states with interaction with water intermediate between PEO and poly(propylene oxide) (PPO). The internal energy and statistical weight of the nonpolar state were obtained from a fit to the experimental Ucon–water phase diagram.[24] The simplification of having only one type of segment is reasonable since the phase diagrams of aqueous PEO and aqueous PPO solutions are similar; it is merely the extent and the location of the phases which differ. This simplification is further supported by the fact that the internal energy and statistical weight obtained for Ucon are very close to the arithmetic and geometric means, respectively, of the PEO/water and PPO/water systems (cf. Table I of ref 18 and Table III). As far as Dextran T500 is concerned, it should be modeled as a polymer with 3080 segments. However, the number of segments were reduced to 200 with the constraint of constant segment density. This was done in order to avoid excessively long computation times, and affected the partition coefficient, typically, by less than 1% (cf. also Figures 8 and 9 of ref 5).

The interaction parameters for the two-phase aqueous polymer systems are given in Table III. They are obtained by fitting calculated phase diagrams, employing eq 1, to experimental ones. In particular, the internal PEO and Ucon as well as the PEO/water parameters are obtained by fitting to experimental binary phase diagrams, whereas the dextran parameters are obtained by fitting to the experimental three-component phase diagrams (see Table III for further details).

The mean-field treatment of the solution imposes the protein to be modeled as an object with a homogeneous surface. We have further simplified the model by treating the protein as a hard sphere where the surface interacts with the solute and polymer segments according to the Flory–Huggins theory. On the basis of the molecular weight of the proteins and a lattice size of 4 Å (cf. refs 17–19) a protein radius of four lattice sides was used. The protein interaction parameters are difficult to obtain from independent experiments (however, cf. ref 6). They have thus been fitted to the experimental partitioning, and the protein interaction parameters obtained are compiled in Table IV. In order to reduce the number of independent parameters, the difference between protein-polar and protein-nonpolar interaction parameters of 3.2 kJ/mol was held fixed. Moreover, since only the difference between the protein interaction parameters are significant, the scale was chosen by setting the protein–water values to zero.

Table IV
Protein Interaction Parameters $RT\chi_{B,\text{protein}}$ (in kJ mol^{-1})[a,b]

species	state	ribonuclease A	myoglobin
water		0	0
EO	polar	0.0 ± 0.5	1.5 ± 1.0
	nonpolar	3.2 ± 0.5	4.7 ● 1.0
Ucon	polar	1.0 ± 1.5	5.0 ± 3.0
	nonpolar	4.2 ± 1.5	8.2 ± 3.0
dextran		−1.2 ● 0.15	−1.2 ± 0.2

[a] The adsorption parameter χ_s is related to the interaction parameters by $\chi_s = -\lambda_{10}(\chi_{\text{segment,protein}} - \chi_{\text{solvent,protein}})$, where $\lambda_{10} = 1/4$ (hexagonal lattice). See also ref 16. [b] The confidence limits of the polymer–protein interaction parameters were estimated as follows. The rms difference between the calculated and experimental partition coefficients were constructed according to $f = (\sum_{i=1}^{N}[K_i^{\text{calc}}(\chi_1,\chi_2,\chi_3) - K_i^{\text{exp}}]^2/N)^{1/2}$, where $\chi_1 \equiv \chi_{\text{EO,polar;protein}}$, $\chi_2 \equiv \chi_{\text{Ucon,polar;protein}}$, $\chi_3 \equiv \chi_{\text{dextran;protein}}$, and the sum extends over $N = 12$ points (two systems each at two temperatures and three concentrations). The difference $\chi_{\text{EO,polar;protein}} - \chi_{\text{EO,nonpolar;protein}}$ was kept fixed at 3.2 kJ mol^{-1} and similarly for Ucon. The polar and nonpolar interaction coefficients are nearly linear dependent since it is their weighted average which governs the interaction with the protein. The ellipsoidal confidence region of f around the minimum $f = f_0$ at $\chi_{1,0},\chi_{2,0},\chi_{3,0}$ was investigated by calculating f on a three-dimensional grid about $\chi_{1,0},\chi_{2,0},\chi_{3,0}$. The confidence limit given for each χ_k was obtained by taking the projection of the long axis of the ellipsoidal confidence region on the χ_k axis. The values given were obtained for $f = 1.2f_0$ with $f_0 = 0.13$ for ribonuclease A and 0.10 for myoglobin.

It should be admitted that the protein model is very crude and the interaction parameters obtained have to describe a number of interactions (see ref 6 for a further discussion). Moreover, since the partition coefficients measured have been used to fit the protein interaction parameters, the qualitative agreement obtained (see below) is maybe not unexpected. However, since the protein parameters obtained are reasonable, we believe that the model has a physical meaning and the conclusions made from the theoretical results provide an additional insight in the partitioning, in particular in the temperature dependence.

From the model, the partitioning was obtained by separate calculations of the free energy of placing a protein in either of the two polymer phases. Both phases were modeled as three-component systems and the experimental compositions given in Table II were used as bulk values. The computations were performed using a spherical lattice, where a single protein was placed in the center. The calculation involves a self-consistent determination of the volume fractions of all species (EO or Ucon, dextran segments, and water molecules) and state distributions of the EO or Ucon segments in each layer of the heterogeneous system in layers outside the protein until the bulk values are reached.[16] Given the distributions of volume fractions and states, the free energy $A^{\text{exc}}(0)$ of placing the protein at a fixed position may be calculated.[25] From the thermodynamics of ideal solutions it follows (see Appen-

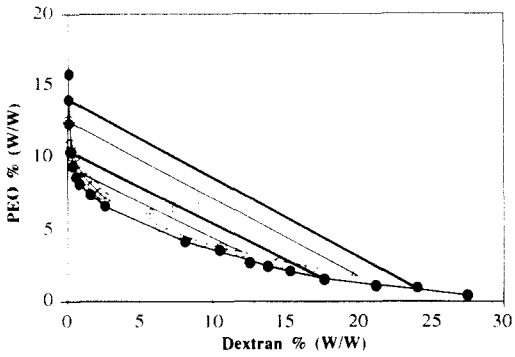

Figure 1. Experimental phase diagram for PEO 4000/Dextran T500/water at 22 °C (filled circles) and 50 °C (open circles). Some of the tie lines are shown (22 °C, bold style; 50 °C, plain style). The compositions used in the partition experiments are indicated (crossed circles).

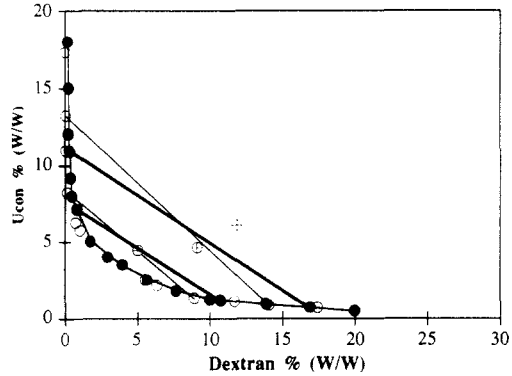

Figure 2. Experimental phase diagram for Ucon 50-HB-5100/Dextran T500/water at 22 °C (filled circles) and 45 °C (open circles). The data for 22 °C are taken from ref 20 and were measured in our laboratory on the same batches of the polymers as were used here. Some of the tie lines are shown (22 °C, bold style; 45 °C, plain style). The compositions used in the partition experiments are indicated (crossed circles).

dix) that the partition coefficient is given by

$$K = \exp[-\beta(A^{\mathrm{exc},1}(0) - A^{\mathrm{exc},2}(0))]$$

where indices 1 and 2 refer to the two phases. In as follows 1 refers to the PEO- or Ucon-rich phase and 2 to the dextran-rich phase.

Results and Discussion

Phase Diagram. Prior to the partitioning study, the phase diagrams of the two polymer systems were determined. Figure 1 shows the phase diagram of the PEO/dextran/water system at 22 and 50 °C, while Figure 2 shows the phase diagram of the Ucon/dextran/water system at 22 and 45 °C. In the PEO/dextran/water system, a slight shift in the position of the binodal curve can be observed, whereas the tie lines at the two temperatures remain parallel. This implies that a system of a given total composition separates into phases with rather similar contents at the two temperatures. The Ucon/dextran/water system, on the other hand, shows an almost invariant binodal under the temperature change from 22 to 45 °C, but here the slope of the tie lines shifts considerably as the temperature rises. Consequently, for such a system of given total composition, the differences are appreciable between the compositions of the corresponding phases at the lower and the higher temperature. This will be important for the understanding of the different temperature behavior of the partitioning displayed by the two polymer systems.

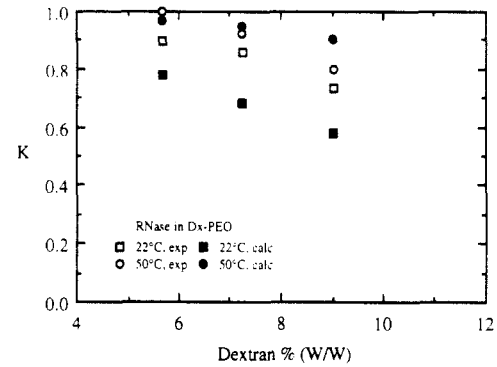

Figure 3. Partition coefficients for ribonuclease A in the PEO 4000/Dextran T500/water system at 22 °C (squares) and 50 °C (circles). Open symbols refer to experimental data and filled symbols to calculated ones. Phase compositions are given in Table II.

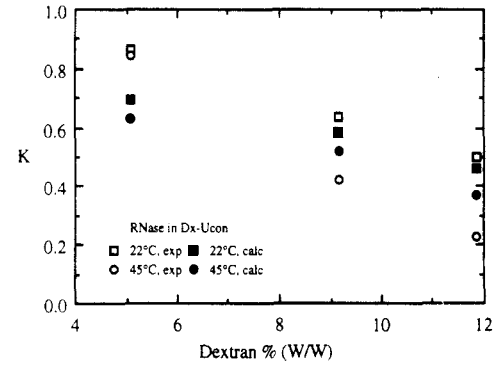

Figure 4. Partition coefficients for ribonuclease A in the Ucon 50-HB-5100/Dextran T500/water system at 22 °C (squares) and 45 °C (circles). Open symbols refer to experimental data and filled symbols to calculated ones. Phase compositions are given in Table II.

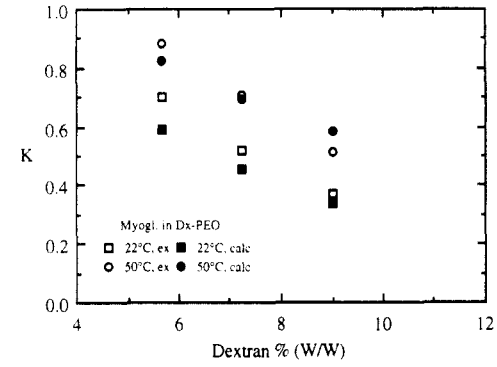

Figure 5. Partition coefficients for myoglobin in the PEO 4000/Dextran T500/water system at 22 °C (squares) and 50 °C (circles). Open symbols refer to experimental data and filled symbols to calculated ones. Phase compositions are given in Table II.

Partitioning. The partitioning of ribonuclease A in the PEO/dextran/water and Ucon/dextran/water systems, each at two temperatures and three polymer concentrations, is shown in Figures 3 and 4. The corresponding partitioning of myoglobin is displayed in Figures 5 and 6. From the experimental findings in Figures 3–6 (open symbols) we can readily state the following: (i) the partition coefficient K is less than one (i.e., the dextran-rich phase is preferred over the PEO- or Ucon-rich phase in all cases (with one exception where $K \approx 1$), independent of the protein, polymer system, polymer concentration, and temperature); (ii) myoglobin is more unevenly distributed [smaller K (<1)] as compared with ribonuclease A in all corresponding cases (i.e., for the same polymer system, polymer concentration, and temperature); (iii) the par-

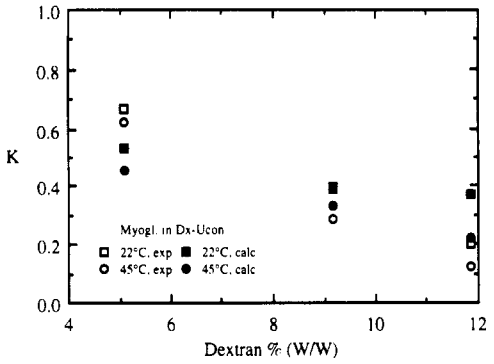

Figure 6. Partition coefficients for myoglobin in the Ucon 50-HB-5100/Dextran T500/water system at 22 °C (squares) and 45 °C (circles). Open symbols refer to experimental data and filled symbols to calculated ones. Phase compositions are given in Table II.

titioning becomes more uneven at increasing polymer concentration in all cases; (iv) the partitioning becomes more uneven in the case of the Ucon/dextran/water system as compared to the PEO/dextran/water system in all corresponding cases (i.e., for the same protein, polymer concentration, and temperature); (v) at increasing temperature the distribution of the proteins between the two phases becomes more *even* in the PEO/dextran/water system, whereas it becomes more *uneven* in the Ucon/dextran/water system in all corresponding cases (i.e., for the same protein, polymer system, and polymer concentration).

It is interesting to relate the preference of the dextran-rich phase and the difference in the partitioning of ribonuclease A and myoglobin with what is known form other studies of these proteins. Experimental data describing the surface properties of ribonuclease A, myoglobin, and three other proteins have been collected by Wei et al.[26] Parameters describing the effective surface hydrophobicity were retention time on hydrophobic interaction chromatography, binding of the fluorescent hydrophobic probe *cis*-parinaric acid, percent accessible area of negatively charged oxygen atoms (inversely correlated with the effective hydrophobicity of proteins), and the rate constant of surface tension kinetics. The parameter values indicate a more hydrophilic character of the myoglobin surface relative to ribonuclease A (which nonetheless also should be labeled overall hydrophilic, being readily soluble in water). Since PEO and Ucon are more hydrophobic compared with dextran our finding in the partitioning experiments, that myoglobin exhibits a stronger preference for the dextran-rich phase relative to ribonuclease A (Figures 3–6), is in agreement with the other studies; i.e., myoglobin is the most hydrophilic of the two proteins studied.

The reduction of the partition coefficient with increasing polymer concentration is in accordance with the general finding that proteins (and particles) tend to partition more unequally (partition coefficient more different from unity) as the composition of the system gets further away from the critical point.[1,6]

The finding that the proteins are more unevenly distributed in the Ucon/dextran/water system is at least partly due to that the points used in the Ucon/dextran/water phase diagram lie further away from the critical point as compared with the PEO/dextran/water system (cf. Figures 1 and 2). Thus, it is probably not a strong effect on the partition whether PEO or Ucon is used, provided similar points (with respect to the critical point) in the phase diagram are used. However, *given* the same total polymer composition, the protein partition will be more uneven in the Ucon system than in the PEO system since the Ucon/dextran/water system displays the smallest one-phase region.

Moreover, there is a qualitative difference in the temperature dependence of the partitioning in the two systems: while it becomes more *even* in the PEO/dextran/water case it becomes more *uneven* in the Ucon/dextran/water case when the temperature is raised. The trends observed in the PEO/dextran/water system are in concordance with results recently reported by Forciniti et al.[27] on the partitioning of a few other proteins in similar systems.

Model Calculations. Figures 3–6 show that all experimentally observed trends are qualitatively captured in the model calculations, although the agreement is not quite generally quantitative. Turning to the surface interaction parameters (Table IV), we may note that the interaction with the dextran segments is equal for both the proteins, the differences in partitioning behavior depending in the model only on the interactions with PEO and Ucon. The protein interaction parameters for PEO and Ucon suggest the following hydrophobic–hydrophilic order: Ucon, PEO, ribonuclease A, and myoglobin, with Ucon being most hydrophobic, where the relative protein ordering is in agreement with the previous discussion. The equal protein–dextran interaction parameters are, on the other hand, not rationalized by such a simple picture.

The net attractive energetic interactions found between the dextran and the protein surfaces (the difference $\chi_{dextran,protein} - \chi_{water,protein}$ being negative) agree with the conclusions made by Forciniti et al.,[27] even if arrived at from a different approach. On the other hand, net repulsive energetic interactions between PEO and the protein surfaces are found to give better representation of data, in distinction to Baskir et al.[6] and Abbot et al.,[8] who found attractive energetic interactions also with PEO for some proteins (different from those in this study). Their analyses were based on experimental data at pH different from the isoelectric pH of the proteins considered, whereas the present study is performed at the isoelectric pH. Thus, in our case the role of the electrostatic interaction on the polymer–protein interaction should be different (smaller). Moreover, parameters were inferred by Baskir et al.[6] from fits to partition data at one single temperature, whereas in this study there is the additional demand to reproduce the observed direction of change of the partition coefficients with temperature as well as with polymer concentration in two systems with one of the polymers (i.e., dextran) in common. In fact, if the signs (at least) of $\ln K$, $\Delta(\ln K)/\Delta T$, and $\Delta(\ln K)/\Delta c_{polymer}$ are all to come out correct simultaneously for both of the two interrelated systems, this imposes rather more severe constraints on the signs and magnitudes allowable for the interaction parameters than in the one temperature–one system case. The sensitivity of the fit to changes in the protein interaction parameters around the best values is indicated in Table IV.

Another consideration that also has a bearing on these matters concerns how much the calculated partition coefficients are affected by treating the three-component phases as two-component systems (neglecting the minority component), as was done in the earlier studies.[5-7] In Table V we compare some of the partition coefficients calculated for the three-component system with those obtained setting the concentration of the minority polymer in each phase to zero, keeping everything else the same. Evidently these effects are of the same order of magnitude as the

Table V
Comparison of Calculated Partition Coefficients with and without Minority Component[a]

total composition		actual phase compositions: dextran-rich phase		actual phase compositions: PEO-rich phase		calculated partition coefficients (K_{calc})	
dextran	PEO	dextran	PEO	dextran	PEO	for actual phases	for $c_{minority\ comp} = 0$
5.7	6.0	12.9	2.6	0.7	8.4	0.59	0.33
9.0	7.1	19.6	1.4	0.1	11.7	0.34	0.24

[a] For myoglobin at 22 °C.

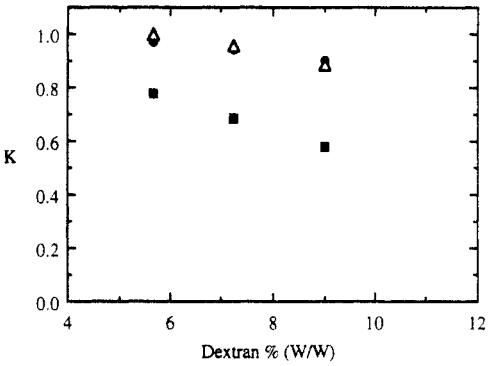

Figure 7. Calculated partition coefficients for ribonuclease A in the PEO/dextran/water system at 22 °C (squares) and 50 °C (circles). Open triangles refer to values calculated for a temperature of 50 °C but with the phase compositions of 22 °C.

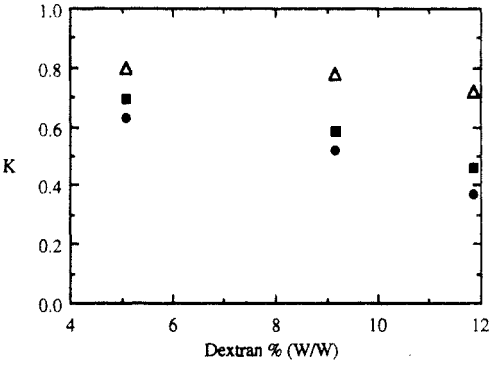

Figure 8. Calculated partition coefficients for ribonuclease A in the Ucon/dextran/water system at 22 °C (squares) and 45 °C (circles). Open triangles refer to values calculated for a temperature of 45 °C but with the phase compositions of 22 °C.

concentration and temperature effects, implying that the influence of the minority polymer should not be ignored.

To address the issue whether it is the change in phase composition or the intrinsic change in the phase polymer solution that is the dominant factor in the temperature effects, we compare the partition coefficients calculated for ribonuclease A in the PEO/dextran/water and Ucon/dextran/water systems at the two temperatures to the hypothetical partition coefficients the model gives for the elevated temperatures calculated with the *low* temperature phase compositions. The results, shown in Figures 7 and 8, show that for the PEO/dextran/water system the change in composition (which is small) is of negligible influence, while in the Ucon/dextran/water system it is this change in composition that comes into prominence and determines the sign of the change in partition coefficients. Hence, it seems that the different partition properties with respect to temperature changes in the two systems are consequences of the differences in how the phase diagrams change with temperature. It may, however, also be observed that a change in phase composition is not a necessary condition for a change in partition coefficient with temperature, according to both the experimental PEO/dextran/water data and the model calculations. Thus,

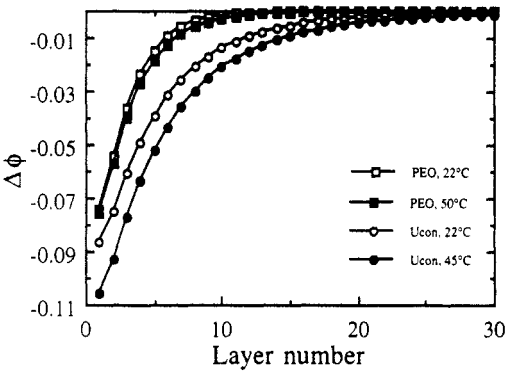

Figure 9. Calculated segment volume fraction of PEO and Ucon near the surface of myoglobin. $\Delta\phi = \phi(N) - \phi(\text{bulk})$, where $\phi(N)$ is the volume fraction in layer number N, and $\phi(\text{bulk})$ is the volume fraction in the bulk solution far away from the surface. Symbols refer to PEO, 22 °C (open squares); PEO, 50 °C (filled squares); Ucon, 22 °C (open circles); and Ucon, 45 °C (filled circles).

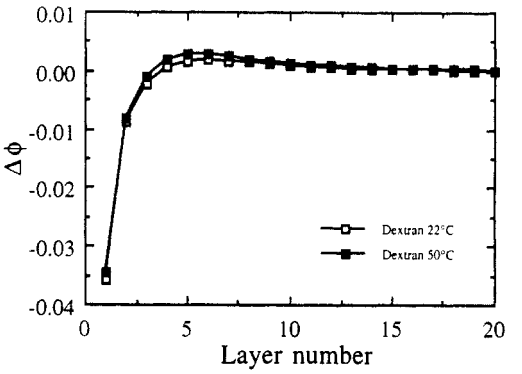

Figure 10. Calculated segment volume fraction of dextran near the surface of myoglobin. $\Delta\phi = \phi(N) - \phi(\text{bulk})$, where $\phi(N)$ is the volume fraction in layer number N, and $\phi(\text{bulk})$ is the volume fraction in the bulk solution far away from the surface. Open squares refer to 22 °C, and filled ones to 50 °C.

it seems that there is not one single factor which dominates in all systems, but rather a delicate interplay between how changes in solution properties of the phase polymers with temperature manifest themselves partly as changes in the phase diagram and partly as changes in "solvent quality" for the solute proteins.

In addition to the results concerning such directly measurable properties as partition coefficients, the calculations in the model also yield information about concentration profiles of the solution components in the vicinity of the protein surface. Those calculated for myoglobin are shown in Figures 9 and 10. In all cases there is a depletion of polymers in the layers closest to the protein surface, and hence the total protein–polymer interaction is repulsive. This is further corroborated by the fact that the calculated free energy of transferring the protein from pure water to one of the polymer phases is in all cases positive (ca. 1–2 kT). The main contribution to the unfavorable overall protein–polymer interaction is entropic. The physical picture is that, as the polymer approaches the protein surface, the number of chain

conformations is restricted, which is entropically costly. The second contribution to the overall protein–polymer interaction, the energetic interaction expressed by the χ parameters, is less dominating. In the case of PEO and Ucon, the repulsive energetic interaction increases the depletion and makes the total protein–polymer interaction even more repulsive. The more repulsive energetic interaction of Ucon as compared to PEO is reflected in a greater depletion effect (Figure 9). The fact that the higher temperature is much closer to the clouding temperature of Ucon than to that of PEO seems also to be reflected in the greater temperature change in the concentration profiles between the two temperatures, as Figure 9 shows. On the other hand, the attractive energetic dextran–protein interaction results in a smaller depletion (although the total dextran–protein interaction is repulsive), and there is even a small positive concentration difference some 5–10 layers away from the surface (Figure 10).

Concentration Regime. Recently, an alternative description of the PEO-rich phases in aqueous two-phase polymer systems has been presented.[8] There, phases of low molecular weight PEO (M less than about 10 kDa) are considered from the dilute solution point of view, where the polymers exist mainly as separate coils rather than entangled in a more or less homogeneous web, which is the physical picture behind the Flory–Huggins type of model that we are using. Based on the separate-coil notion scaling laws are derived, relating changes in protein partition coefficients to PEO chain length and protein size. The modeling of the PEO-rich phases as composed of separate coils is motivated by comparison with the corresponding PEO–water two-component solutions: the estimated overlap concentration in these two-component solutions is considerably higher than the actual PEO concentration in the PEO-rich phases. It seems thus reasonable that in a two-component PEO/water solution with the same PEO concentration as in the PEO-rich three-component phases, the PEO will exist as separate coils. However, there is one important difference between the two-component solutions and the three-component phases: the latter lie on the binodal in the three-component phase diagram. This means that any however minute addition of, say, the minority polymer to the phase will cause phase separation. Now it is a rather established view that segregative phase separation in two-polymer systems occurs only when the polymer domains have come into contact, and so the short-range unfavorable effective interactions between the unlike segments have come into play. In the dilute two-polymer solution, with mean distances between polymers larger than coil sizes, miscibility is expected. From this point of view then, the very fact that the compositions of the PEO-rich phases correspond to points on the binodal in itself is an indication that overlap is at hand. This effect of the minority polymer is perhaps not quite as astonishing as it might look in view of the small weight fraction involved, if we consider the usually high degree of polymerization of this polymer (dextran in this case). For a 500-kDa dextran a crude estimate gives an overlap concentration in water of about 2%, and hence already concentrations below 1% in the phases would be expected to occupy a substantial fraction of the volume. On these grounds the physical picture of the PEO-rich phases as being in the semidilute, entangled concentration regime seems reasonable.

Conclusions

The present model is capable of qualitatively reproducing a number of experimental findings on the partitioning of two proteins in PEO- and Ucon-containing two-phase aqueous polymer systems. These include the preference of the dextran-rich phase, the more uneven partitioning in the Ucon-containing system, and the different temperature dependence displayed by the PEO- and Ucon-containing polymer systems. Furthermore, the model directly provides information on the details of the protein solvation, e.g., polymer concentration and conformational state profiles near the protein surface. The model contains a number of parameters, of which those describing the polymer systems were obtained separately from binary and three-component phase diagrams. These values of the parameters have successfully been used in previous studies of the adsorption[17,18] of Pluronic block copolymers to solid surfaces and micellization[19] of Pluronic block copolymers in aqueous solution. The probably weakest part of the present model is the description of the protein–polymer interaction, although the interaction parameters obtained are reasonable.

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Appendix

Expression for the Partition Coefficient. Assuming that there are N particles (spherical surfaces) in a phase of volume V, at such large average separation (low concentration) that the interaction between them can be neglected, and that hence each interacts independently with the polymer solution, the total excess free energy due to interaction with the polymer solution is simply $NA^{exc}(0)$. Since the particles are assumed noninteracting the only further contribution to the free energy is the ideal free energy of N particles confined in a volume V, which is given by[28] $A^{id} = NkT \ln (N/V) + Nf(T)$, where $f(T)$ does not depend on N, V, nor the properties of the other components in the phase. In total then, $A^{exc}(N) = NA^{exc}(0) + NkT \ln (N/V) + Nf(T)$. Consider a system of two phases in equilibrium, with volumes V_1 and V_2, and the problem of distributing N particles between them in a way that minimizes the total excess free energy, $A^{exc,tot} = N_1A^{exc,1}(0) + N_1kT \ln (N_1/V_1) + N_1f(T) + N_2A^{exc,2}(0) + N_2kT \ln (N_2/V_2) + N_2f(T)$, with $N_1 + N_2 = N$. Using the criterion for a minimum, $dA^{exc,tot}/dN_1 = 0$, noting that $dN_2/dN_1 = -1$, we get the equilibrium distribution

$$\frac{N_1/V_1}{N_2/V_2} = \exp((A^{exc,2}(0) - A^{exc,1}(0))/kT)$$

The left-hand side of this equation is precisely the definition of the partition coefficient.

References and Notes

(1) Albertsson, P. A. *Partition of Cell Particles and Macromolecules*; Wiley: New York, 1986.
(2) Albertsson, P. A.; Johansson, G.; Tjerneld, F. In *Separation Processes in Biotechnology*; Marcel Dekker: New York, 1990; p 287.
(3) Scheutjens, J. M. H. M.; Fleer, G. J. *J. Phys. Chem.* **1979**, *83*, 1619.
(4) Scheutjens, J. M. H. M.; Fleer, G. J. *J. Phys. Chem.* **1980**, *84*, 178.
(5) Baskir, J. N.; Hatton, T. A.; Suter, U. W. *Macromolecules* **1987**, *20*, 1300.
(6) Baskir, J. N.; Hatton, T. A.; Suter, U. W. *J. Phys. Chem.* **1989**, *93*, 2111.
(7) Baskir, J. N.; Hatton, T. A.; Suter, U. W. *Biotechnol. Bioeng.* **1989**, *34*, 541.

(8) Abbot, N. L.; Blankschtein, D.; Hatton, A. T. *Macromolecules* **1991**, *24*, 4334; **1992**, *25*, 3917; **1992**, *25*, 3932.
(9) Saeki, S.; Kuwahara, N.; Nakata, M.; Kaneko, M. *Polymer* **1976**, *17*, 685.
(10) Lindman, B.; Carlsson, A.; Karlström, G.; Malmsten, M. *Adv. Colloid Interface Sci.* **1990**, *32*, 183.
(11) Karlström, G. *J. Phys. Chem.* **1985**, *89*, 4962.
(12) Goldstein, R. E. *J. Chem. Phys.* **1984**, *80*, 5340.
(13) Kjellander, R.; Florin, E. *J. Chem. Soc., Faraday Trans. 1* **1981**, *77*, 2053.
(14) Sjöberg, A.; Karlström, G. *Macromolecules* **1989**, *22*, 1325.
(15) Björling, M.; Linse, P.; Karlström, G. *J. Phys. Chem.* **1990**, *94*, 471.
(16) Linse, P.; Björling, M. *Macromolecules* **1991**, *24*, 6700.
(17) Tiberg, F.; Malmsten, M.; Linse, P.; Lindman, B. *Langmuir* **1991**, *7*, 2733.
(18) Malmsten, M.; Linse, P.; Cosgrove, T. *Macromolecules* **1992**, *25*, 2474.
(19) Linse, P.; Malmsten, M. *Macromolecules* **1992**, *25*, 5434.
(20) Harris, P. A.; Karlström, G.; Tjerneld, F. *Bioseparation* **1991**, *2*, 237.
(21) Flory, P. J. *Principles of Polymer Chemistry*; Cornell University Press: Ithaca, NY, 1953.
(22) Evers, O. A.; Scheutjens, J. M. H. M.; Fleer, G. J. *Macromolecules* **1990**, *23*, 5221.
(23) Anderson, M.; Karlström, G. *J. Phys. Chem.* **1985**, *89*, 4957.
(24) Johansson, H.-O.; Karlström, G.; Tjerneld, F., submitted for publication in *Macromolecules*.
(25) Equation A.8.4 of ref 16.
(26) Wei, A.-P.; Herron, J. N.; Andrade, J. D. *Science*, submitted.
(27) Forciniti, D.; Hall, C. K.; Kula, M. R. *Bioseparation* **1991**, *2*, 115.
(28) Hill, T. L. *An Introduction to Statistical Thermodynamics*; Dover: New York, 1986.
(29) Albertsson, P.-A.; Sasakawa, S.; Walter, H. *Nature* **1970**, *228*, 1329.
(30) Kuntz, I.; Kauzmann, W. In *Advances in Protein Chemistry*; Academic Press: New York, 1974; Vol. 28, p 239.