Protein Partitioning in Two-Phase Aqueous Polymer Systems. 5. Decoupling of the Effects of Protein Concentration, Salt Type, and Polymer Molecular Weight

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ABSTRACT: Measurements of the partitioning behavior of ovalbumin in the poly(ethylene oxide) (PEO)–dextran aqueous two-phase system reveal that experimental conditions can be found at which the effects of protein concentration, salt type, and PEO molecular weight can be decoupled. Specifically, the change in the logarithm of the ovalbumin partition coefficient, $\Delta(\ln K_p)$ (where K_p is the ratio of the protein concentrations in the top PEO-rich phase and the bottom dextran-rich phase), that accompanies a change in PEO molecular weight over the range 5000 to 35 000 Da was measured to be independent of the ovalbumin concentration and the type of salt (NaCl and Na_2SO_4) present in the system. This observation is used to simplify a general thermodynamic framework that serves as the basis for molecular-level descriptions of protein partitioning in two-phase aqueous polymer systems.

1. Introduction

Interest in the partitioning of proteins in two-phase aqueous polymer systems stems from the unique ability of these polymer solutions to provide water-based, yet immiscible, liquid phases for the purification of proteins using liquid–liquid extraction techniques.[1-7] Because each of the two coexisting phases contains predominantly water, water-soluble proteins maintain their native conformations and biological activity when purified in these systems.

In general, it is well known that the partitioning of proteins in two-phase aqueous polymer systems reflects a large number of factors, such as polymer concentration and molecular weight, pH, and salt type and concentration, which are often coupled.[8-12] Indeed, the coupling of these factors has made elucidating the underlying molecular-level mechanisms of protein partitioning a formidable task. In order to help make this task more tractable, in this paper we demonstrate with a simple experiment that, under judiciously chosen experimental conditions, a useful decoupling of some of these factors is possible. Specifically, in the poly(ethylene oxide) (PEO)–dextran aqueous two-phase system, we have measured the partition coefficient of ovalbumin, K_p, defined as the ratio of the protein concentrations in the top PEO-rich phase and the bottom dextran-rich phase, as a function of PEO molecular weight in the presence of two different salts (NaCl and Na_2SO_4). Note that these salts were used because they are known to have very different effects on the partitioning behavior of charged proteins.[1,2] We have found that, in these systems, the *change* in the logarithm of the protein partition coefficient, $\Delta(\ln K_p)$, is indepedent of the type of salt used, as well as of the protein concentration. This realization is useful because it allows us to study the influence of the polymer solution structure on protein partitioning in two-phase aqueous polymer systems without the obscuring (and not fully understood) effects of salts, as well as at solution pH's that differ from that corresponding to the isoelectric point of the protein.[13] An alternative experiment approach to decoupling the electrostatic effects from the influence of polymer molecular weight could be to measure the influence of polymer molecular weight on protein partitioning as a function of the pH of the two-phase system (cross partitioning).[1,2] However, this approach can be complicated by the fact that the conformation of a protein can change in response to changes in solution pH, thus modifying the nature of the protein by exposing different surface residues. A second alternative would be to partition proteins in a two-phase aqueous polymer system having a very small (or zero) difference in the electrical potential between the two coexisting phases.[1,2]

In this paper, we show how decoupling the effects of polymer molecular weight, salts, and protein concentration can be used to simplify a general thermodynamic framework that has been utilized as the basis for molecular-level descriptions of protein partitioning in two-phase aqueous polymer systems.[13-16] In particular, we provide experimental evidence that the general thermodynamic framework that relates the measurable protein partition coefficient, K_p, to the relevant molecular-thermodynamic characteristics of the system, namely,[1,2]

$$\ln K_p = \ln\left(\frac{c_{p,t}}{c_{p,b}}\right) = \ln\left(\frac{\gamma_{p,b}}{\gamma_{p,t}}\right) + \left(\frac{\mu^\circ_{p,b} - \mu^\circ_{p,t}}{kT}\right) + \left(\frac{z_p(\psi_b - \psi_t)}{kT}\right) \quad (1)$$

can be simplified. Here $c_{p,i}$, $\gamma_{p,i}$, and $\mu^\circ_{p,i}$ are the protein concentration, activity coefficient, and standard-state chemical potential, respectively, in phase i (t or b), z_p is the net charge of the protein molecule (assumed to be independent of the polymer solution phase), ψ_i is the electrical potential of phase i, k is the Boltzmann constant, and T is the absolute temperature.

Equation 1, which is of a phenomenological character, is obtained[1,2] be defining the chemical potential of a protein molecule in a polymer solution phase to be comprised of three additive contributions: (1) a contribution reflecting the presence of an isolated protein molecule in a reference state that is characterized by a vanishing protein concentration and electrical potential (the term $\mu^\circ_{p,i}$ in eq 1), (2) a contribution due to protein–protein interactions which becomes increasingly important as the concentration of protein increases (reflected in the term $\gamma_{p,i}$ in eq 1), and (3) a contribution due to an electrical potential difference between the two coexisting polymer solution phases, which

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acts on the charges of the proteins (reflected in the term $z_p(\psi_b - \psi_t)$ in eq 1).

Under judiciously chosen experimental conditions (see sections 3 and 4), eq 1 can be simplified to yield[13]

$$\Delta(\ln K_p) \approx -(\Delta\mu^\circ_{p,t}/kT) \quad (2)$$

where the symbol Δ denotes changes in $\ln K_p$ and $\mu^\circ_{p,t}$ which accompany changes in the molecular weight of PEO present in the two-phase system.

The remainder of this paper is organized as follows. Section 2 describes the materials and experimental methods used in this study. Section 3 presents the results of our experimental measurements which support the simplification of eq 1 to eq 2. Finally, section 4 concludes with a discussion of these results.

2. Materials and Experimental Methods

A. Materials. Poly(ethylene oxide) (PEO) having molecular weights of (Da, according to the specifications of the manufacturers) 5000 (M_w/M_n = 1.05), 9000 (M_w/M_n < 1.10), 11 000 (M_w/M_n = 1.10), 15 000 (M_w/M_n = 1.2), and 22 000 (M_w/M_n = 1.07), where M_w and M_n are the weight-average and number-average molecular weights, respectively, was purchased from Polysciences Inc. (Warrington, PA). PEO having a molecular weight of 35 000 Da was purchased from Fluka (Switzerland). Dextran (M_w = 300 000 Da) was purchased from Polysciences Inc. (Warrington, PA). Chicken egg albumin (ovalbumin) was purchased from Sigma Chemicals (St. Louis, MO).

B. Experimental Methods. All two-phase systems contained 6.0% w/w PEO and 7.9% w/w dextran, and were prepared in aqueous buffered solutions containing 10 mM sodium phosphate to control the pH at 7.0, 1.5 mM sodium azide to prevent bacterial growth in the samples, and either 0.1 M NaCl or 0.05 M Na_2SO_4. All polymer solutions were prepared by weight since the high viscosities of the solutions prevented the accurate measurement of the volumes of the solutions. First, a stock solution of dextran was prepared in the aqueous buffer with a concentration of dextran equal to that desired in the two-phase system. The stock solution was then divided into 3-g aliquots, into which the PEO was weighed. The resulting two-phase system was then thoroughly mixed. All two-phase systems were prepared in duplicate. Into one of the resulting two-phase systems, 60 μL of protein solution was added, and to the other solution the same volume of buffered solution was added. The protein-free two-phase system served as the reference solution in the spectrophotometer for the measurement of the protein concentrations. The resulting polymer solutions were gently centrifuged at 1000 rpm (Centra 4, International Centrifuge) for 5 min, and then equilibrated for 12–18 h in a temperature-controlled water bath (Magni Whirl, Blue M) at 25 °C.

In order to determine the concentrations of proteins in each of the coexisting phases, samples from each solution phase were aspirated using a syringe. First, without disturbing the fragile liquid–liquid interface between the two phases, a sample of the top PEO-rich solution phase was carefully collected. Following the collection of the top-phase sample, the remainder of the top phase was sucked from the interfacial region using a Pasteur pipet. The interfacial sample, which typically contained a mixture of the top and bottom phases, was then discarded. The remaining solution, namely, the bottom dextran-rich phase, was withdrawn from the test tube using a syringe and then prepared for the measurement of protein concentration as described next. Due to the high viscosities of the polymer solutions which were withdrawn from each of the phases, it was necessary to dilute the samples prior to measurement of the protein absorbance. If the samples were not diluted, streaks appeared in the polymer solutions as they were pipetted into the spectrophotometer cuvettes, which in turn scattered light during the measurement of the protein absorbance. The absorbance of ovalbumin was measured at 280 nm using a Perkin-Elmer Lambda 3B UV–vis spectrophotometer, utilizing the corresponding protein-free two-phase system as a reference.

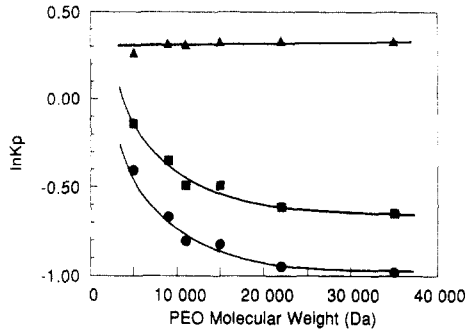

Figure 1. Logarithm of the partition coefficient of ovalbumin, $\ln K_p$, as a function of PEO molecular weight in a two-phase aqueous PEO–dextran system with either 0.1 M NaCl (circles) or 0.05 M Na_2SO_4 (squares). Also shown is $\ln K_p(Na_2SO_4) - \ln K_p(NaCl)$ (triangles).

3. Results

The partition coefficients of ovalbumin as a function of the overall ovalbumin concentration were measured in two two-phase systems, one containing PEO having a molecular weight of 5000 Da and the other containing PEO having a molecular weight of 10 000 Da. Both systems contained 0.05 M Na_2SO_4 in addition to the buffer. Over the range of protein concentrations 0.2–2 g/L, a *constant* protein partition coefficient was measured for each PEO molecular weight ($\ln K_p$(5000Da) = −0.13 ± 0.02, and $\ln K_p$(10000Da) = −0.6 ± 0.01). As discussed in section 4, these observations support our hypothesis that protein–protein interactions do not influence the protein partition coefficient over the range of protein concentrations investigated.

In Figure 1, partition coefficients of ovalbumin are shown as a function of the molecular weight of PEO for two different salts: 0.05 M Na_2SO_4 and 0.1 M NaCl. The logarithms of the partition coefficients of ovalbumin with Na_2SO_4 are less negative (squares) than those with NaCl (circles), and in each case, with an increase in the molecular weight of PEO there is a decrease in the measured $\ln K_p$ values. However, an inspection of Figure 1 reveals that the *changes* in $\ln K_p$ which accompany *changes* in PEO molecular weight are, in fact, independent of the type of salt. In other words, $\ln K_p(Na_2SO_4) - \ln K_p(NaCl)$ is *insensitive to changes in PEO molecular weight*. This supports the assumption leading to eq 2 that $\psi_b - \psi_t$ is independent of PEO molecular weight over the range of molecular weights studied (see section 4). Note that, in Figure 1, the value of $\ln K_p(Na_2SO_4) - \ln K_p(NaCl)$ for the PEO molecular weight of 5000 Da (approximately 0.25) is slightly lower than the values measured at higher PEO molecular weights (approximately 0.30). This small difference arises from the weak influence of the PEO molecular weight on the compositions of the coexisting phases (see Figure 3 of ref 13), and the associated dependence of the electrical potential difference on the phase compositions, over the range of polymer compositions and molecular weights studied.

4. Discussion and Conclusions

The activity coefficient term $\gamma_{p,b}/\gamma_{p,t}$ in eq 1 accounts for the influence of protein–protein interactions on the protein partition coefficient. The elimination of this term, used to obtain eq 2, is only valid under conditions where $\gamma_{p,b}/\gamma_{p,t} \approx 1$. An experimentally accessible condition that can satisfy this requirement is the limit of vanishing protein concentration, where protein–protein interactions become sufficiently infrequent such that they do not influence

the observed partitioning behavior of the proteins. In this case, both $\gamma_{p,b}$ and $\gamma_{p,t}$ approach unity.

The results reported in section 3 clearly indicate that the protein partition coefficient of ovalbumin is independent of protein concentration over the range 0.2–2 g/L. In view of the relatively low protein concentration (the average distance between protein molecules is about 1000 Å (300 Å) at protein concentrations of 0.2 g/L (2 g/L)), and the presence of salt (0.05 M Na_2SO_4) to screen electrostatic interactions, it appears very reasonable that the protein partition coefficient is not sensitive to protein–protein interactions. In contrast, it appears somewhat remarkable that the partition coefficient of human serum albumin has been reported to be independent of protein concentration up to concentrations of 50 g/L.[1] This is particularly surprising because small-angle neutron scattering studies of aqueous solutions of bovine serum albumin, at similar concentrations and salinity, show some evidence of protein–protein interactions.[17-19] One possible explanation for the apparent insensitivity of K_p to the protein concentration may be that only the ratio of the protein activity coefficients, $\gamma_{p,b}/\gamma_{p,t}$, enters the prediction of the protein partition coefficient, rather than their individual values (see eq 1). Therefore, if protein–protein interactions are similar in each of the coexisting phases, the contributions of protein–protein interactions to the protein partition coefficient will tend to balance each other, and thus the protein partition coefficient will be observed to be insensitive to the protein concentration.

The second step in simplifying eq 1 to obtain eq 2 was to assert that the standard-state chemical potential of the protein in the bottom dextran-rich phase is invariant to changes in PEO molecular weight; that is, $\Delta\mu^\circ_{p,b} \approx 0$. This assertion is justified if (i) the weight fractions of the polymers in the coexisting polymer solution phases are insensitive to changes in PEO molecular weight, and (ii) a negligible concentration of PEO exists in the bottom dextran-rich phase. The conditions under which the above requirements are satisfied need clarification. While, in general, the phase equilibrium of two coexisting polymer solution phases is a function of the molecular weights of the "phase-forming polymers", the molecular-weight effect can be minimized by (i) making the dextran molecular weight in the two-phase system as high as possible and (ii) selecting the concentrations of both PEO and dextran in such a way that the resulting two-phase aqueous system is far inside the two-phase region of the equilibrium phase diagram.[1] Under these experimental conditions, the compositions of the coexisting phases can be quite insensitive to PEO molecular weights that are greater than 4000 Da.[1,13,20] While small changes in the polymer concentrations in the coexisting phases certainly occur with increasing molecular weight of PEO,[13] the results of this investigation provide additional support for the assertion that these changes are indeed very small. That is, had the phase compositions changed significantly in response to changes in PEO molecular weight, then, through the influence of the phase compositions on the electrical potential difference, $\psi_b - \psi_t$, one would expect a dependence of $\Delta(\ln K_p)$ on the type of salt present in the two-phase system, contrary to the finding reported in Figure 1. It is also relevant to point out that the same experimental conditions that yield PEO–dextran two-phase aqueous systems having phase compositions that are insensitive to the PEO molecular weight also provide dextran-rich phases which contain only very small concentrations (less than about 1% w/w) of PEO.[1,13,20]

The third simplification made in arriving at eq 2 was based on the hypothesis that the electrical potential difference between the two coexisting polymer solution phases is independent of PEO molecular weight. In section 3, we reported experimental measurements of the change in the partition coefficient of ovalbumin as a function of PEO molecular weight in the presence of two different salts, and showed that this change is, in fact, independent of the salt type (see Figure 1). This finding supports the assertion that, when considering how changes in PEO molecular weight affect changes in K_p, the effect of the electrical potential difference, $\psi_b - \psi_t$, in eq 1 can be neglected. These observations are consistent with the intuitive reasoning that the interactions between salts and PEO are short-ranged as compared to the polymer-coil sizes. In other words, salt is assumed to interact with PEO at the length scale of the polymer segments rather than the polymer-coil size, and therefore, salt effects should only be a function of the polymer-segment concentration (weight fraction of polymer). In view of the previous measurements[1,20] of the compositions of the coexisting polymer solution phases, which found them to be essentially invariant over the range of PEO molecular weights for which the protein partition coefficients were measured (see the discussion above), it appears reasonable that, in studying the *changes* in the protein partition coefficient, salt effects can be eliminated.

In conclusion, the results of this paper emphasize the utility of selecting experimental conditions under which the influences of protein concentration, polymer molecular weight, and specific salt type on the protein partitioning behavior can be decoupled. This decoupling allows the general thermodynamic framework that describes protein partitioning in these systems to be simplified considerably, a feature that has been exploited by us to facilitate the development of a molecular-level description of the influence of the structure of the polymer solution on the protein partitioning behavior in two-phase aqueous polymer systems.[13-16] We stress that while other researchers have been successful in illustrating a variety of different protein partitioning behaviors, they conducted their experimental investigations over a range of experimental conditions where the protein partitioning behavior reflected the coupling of numerous partitioning mechanisms.[21,22] As such, until *all* the mechanisms of protein partitioning are understood, the interpretation of these measurements will be very difficult. Our approach has been different, in that we have carefully selected the experimental conditions with the aim of differentiating between the various contributions to the protein partitioning problem. Indeed, this has motivated our choice of two-phase systems at experimental conditions that enabled us to focus on the contribution of the standard-state protein chemical potential in the PEO-rich phase to the protein partition coefficient, as illustrated in eq 2.

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