Salting Out of Aqueous Proteins: Phase Equilibria and Intermolecular Potentials

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Salting-out phase equilibria are reported for lysozyme and α-chymotrypsin from concentrated ammonium-sulfate solutions. Supernatant and dense-phase protein concentrations and the resulting protein partition coefficients are given as a function of solution pH and ionic strength. Phase equilibria with a trivalent salt (sodium citrate) confirm that ionic strength, rather than salt concentration, is the appropriate variable describing phase equilibria. The salting-out behavior of a mixture of an aqueous lysozyme and α-chymotrypsin is independent of the presence of the other protein.

Parameters for a molecular-thermodynamic description of salting-out behavior are obtained from low-angle laser-light scattering (LALLS). Osmotic second virial coefficients from LALLS are reported over a range of pH for dilute chymotrypsin concentrations in aqueous electrolyte solutions at 0.01 and 1.0 M ionic strengths. Effective Hamaker constants, regressed from experimental osmotic second virial coefficients, are determined for models of the protein–protein potential of mean force. In addition to excluded volume, dispersion, and shielded charge–charge potentials, the description of protein–protein interactions includes attractive charge–dipole and dipole–dipole potentials as well as an osmotic-attraction potential that becomes important at high salt concentrations. Protein dipole–dipole potentials are required to account for the observed pH dependence of osmotic second virial coefficients, especially at low ionic strength.

Introduction

Separation and purification of a protein from aqueous solution is an increasingly important operation in biotechnology as proteins are produced on larger scales. Rothstein (1994), Scopes (1994), and Wheelwright (1991) have given instructive reviews of protein purification. Protein salting-out remains one of the simplest methods for crude protein separations since it was employed to separate blood proteins into distinct fractions in the mid 1850s (Green, 1931).

A molecular-thermodynamic model for salting out is desirable to predict protein separation phase equilibria over a wide range of conditions (such as pH and ionic strength). To date, most studies have focused on simple aqueous systems containing salt and only one protein. A commonly used correlation for protein salting-out data (Cohn and Edsall, 1943) is

$$\log S = \beta - K_s I \tag{1}$$

where S is the protein solubility, I is the ionic strength, and β and K_s are empirical parameters fit to experimental supernatant-phase protein-concentration data as a function of ionic strength. Melander and Horvâth (1977) developed a model for salting out based on electrostatic repulsion and the energy of forming a cavity in the solvent for the protein. This model includes effects of salt type (such as the lyotropic series), protein dipole moment, and the hydrophobic surface area of the protein; however, it is not satisfactory for predicting experimental phase equilibria due to the presence of additional intermolecular forces not included in the model (Przybycien and Bailey, 1989a). To develop a useful model based on molecular thermodynamics, we require a comprehensive database of protein salting-out phase equilibria. For a range of pH and ionic strength, this work presents salting-out phase equilibria for two model proteins in addition to pertinent light-scattering data for characterizing protein–protein interactions.

In previous studies (for example, Bell et al., 1983; Arakawa and Timasheff, 1985), protein salting-out phase equilibrium is described in terms of a saturated protein solution in equilibrium with a pure protein solid phase. Recent experiments have shown that significant amounts of water and salt are present in the precipitate phase (Shih et al., 1992). Therefore, it may be preferable to represent salting out of proteins as a liquid–liquid phase transition characterized by the protein partition coefficient (Shih et al., 1992):

$$K_2 = \frac{c_{2,dp}}{c_{2,sp}} \tag{2}$$

where $c_{2,dp}$ is the concentration of the protein in the dense phase and $c_{2,sp}$ is the concentration of the protein in the supernatant phase. Experimental results presented here give the protein concentrations in both phases and the protein partition coefficient.

For the purification of proteins it is useful to predict the selectivity of a salting-out process for a target protein. Toward that end, Richardson et al. (1990) developed an empirical method to salt out selectively alcohol dehydrogenase from yeast extract. They generalized this procedure, showing how to estimate the maximum achievable selectivity by salting out. An excellent overall review of selective precipitation is given by Niederauer and Glatz (1992). Both studies indicate the empirical nature of selective salting-out separations, requiring extensive salting-out experiments to optimize the selective precipitation of a target protein.

A comprehensive molecular-thermodynamic description of protein salting-out behavior requires an understanding of intermolecular forces in solution. The theory of colloidal solutions, based on potentials of mean force, serves as the basis for several models of protein solution thermodynamics (Vilker et al., 1981; Mahadevan and Hall, 1990; Haynes et al., 1992; Mahadevan and Hall, 1992a,b; Vlachy and Prausnitz, 1992; Vlachy et al., 1993; Chiew et al., 1994). The DLVO model (Verwey and Overbeek, 1948) describes the interactions of spherical colloids of uniform surface properties with attractive dispersion forces and repulsive coulombic forces in a continuum solvent where the influence of salt ions is described solely through electrostatic screening. The DLVO model assumes pairwise additivity of two potentials of mean force:

$$W_{\mathrm{DLVO}}(r) = W_{\mathrm{disp}}(r) + W_{q\text{-}q}(r), \qquad r > d_2 \tag{3}$$

where r is the center-to-center distance between two spherical particles with hard-sphere diameter d_2, $W_{\mathrm{disp}}(r)$ is the dispersion potential of Hamaker, and $W_{q\text{-}q}(r)$ is the screened repulsive charge–charge potential of mean force. The DLVO potential can predict (Vlachy and Prausnitz, 1992) protein–solution osmotic pressures (Vilker et al., 1981) up to high protein concentrations at low salt concentrations. However, Vlachy et al. (1993) observed that the DLVO model does not adequately describe the thermodynamic properties of aqueous protein solutions at high salt concentrations. For such solutions, Vlachy et al. (1993) suggested use of the osmotic-attraction potential of Asakura and Oosawa (1954, 1958) to describe the effect of concentrated electrolytes on protein phase separation. When two large particles are close to each other, an osmotic attraction between them is due to depletion of a second, smaller solute in the narrow region between these two particles. The Asakura and Oosawa potential has been successfully used to model the phase separation of colloids and proteins resulting from the addition of nonadsorbing polymers (De Hek and Vrij, 1981; Gast et al., 1983; Mahadevan and Hall, 1990; Mahadevan and Hall, 1992a; Vlachy and Prausnitz, 1992; Vlachy et al., 1993). When the osmotic-attraction potential is added to Eq. 3, salting-out phase equilibria calculations (Vlachy et al., 1993) yield protein partition coefficients similar to those observed by Shih et al. (1992).

The DLVO potential of mean force does not account for all protein electrostatic interactions. Protein dipole moments, often on the order of several hundred debye (Tanford, 1961), contribute significant attractive protein–protein potentials (Vilker et al., 1981; Haynes et al., 1992). Such electrostatic interactions may be necessary to describe protein–protein interactions at low ionic strengths; however, because of screening, they are less important at high salt concentrations (Phillies, 1974). These potentials are briefly discussed later.

Haynes et al. (1992, 1993) showed that experimental protein–protein osmotic second virial coefficients can be used to examine protein intermolecular potentials. These coefficients are obtained from low-angle laser-light scattering (LALLS) and from membrane osmometry. Here we report LALLS osmotic second virial coefficients, which are used to estimate parameters in potential-of-mean-force expressions.

For solutions containing either lysozyme or chymotrypsin, protein partition coefficients are reported as functions of ionic strength and pH. For aqueous lysozyme–chymotrypsin mixtures, salting-out phase equilibria are similar to those for single-protein solutions. To obtain a suitable protein–protein interaction potential model for understanding salting-out equilibria, LALLS data are reported for chymotrypsin solutions for a range of pH at low and high ionic strengths.

Experimental

Materials

Bovine α-chymotrypsin (C-4129), hen-egg-white lysozyme (L-6876), and phenyl-methyl-sulfonyl fluoride (PMSF) were purchased from Sigma, St. Louis, MO. A.C.S.-grade salts were used. Distilled water was deionized and filtered (0.20 μm) by a NANOpure system prior to use.

Methods

The following experiments were performed: (1) single-protein and two-protein salting-out phase equilibria, and (2) LALLS, as described below. Lysozyme and chymotrypsin concentrations were determined by absorbance at 280 nm using a Milton-Roy 1201 Spectrophotometer. Extinction coefficients are 2.04 L/(g·cm) for chymotrypsin (Fasman, 1989) and 2.635 L/(g·cm) for lysozyme (Sophianopoulos et al., 1962). pH was measured using a Corning General-Purpose Combination electrode with a Sargent-Welch model LSX pH meter. All experiments were carried out at 25 ± 0.1°C.

Protein salting-out phase equilibria

The procedure of Shih et al. (1992) was followed for salting-out phase separations. Protein solutions were prepared by dissolution in pure water. To prevent autolysis, α-chymotrypsin solutions were inhibited according to the method of Fahrney and Gold (1963) with 10% molar excess PMSF from a stock solution of 0.11-M PMSF in 2-propanol. Concentrated salt solution was added a drop at a time and pH was adjusted using dilute solutions of the appropriate conjugate acid or base [for example, NH_4OH and H_2SO_4 for experiments with $(NH_4)_2SO_4$]. No buffers were required. Samples were equilibrated for 4 hours under mild agitation and then centrifuged in a Jouan CT422 temperature-controlled centrifuge for 2 hours at 5,000 g to separate the supernatant phase from the dense phase. At significantly higher centrifugal fields, for example, 30,000 g, $c_{2,sp}$ remains constant but the volume of the dense phase may be reduced, increasing $c_{2,dp}$ and K_2. For this work, we choose to adapt Shih's methodology of centrifugation at 5,000 g. Additional details are provided by Shih et al. (1992).

The two equilibrated phases were then separated and analyzed. Samples of the supernatant and dense phases were diluted and analyzed by UV absorbance (280 nm) to determine protein concentration in mg protein/g water. The ammonium-sulfate concentration of each phase was measured by a titration method (Fritz and Schenk, 1974). The water content of the dense phase was measured by weight loss after freeze-drying for 40 hours at 40 mtorr. Mass balances on the phases closed to within ±5%, which is less than the experimental uncertainty of ±10% in the partition coefficient.

For phase separation of mixtures of lysozyme and chymotrypsin, protein concentrations of both phases were determined by ion-exchange HPLC with UV detection at 280 nm. A Hewlett-Packard Series II 1090 HPLC was employed with an HRLC MA7S, 1-mL capacity, cation exchange column (Bio-Rad, Richmond, CA). All elution buffers contained 0.02 M tris-HCl buffer at pH 7.3 and 0.05% (w/v) sodium azide as a preservative. We used 0.1 M NaCl to elute chymotrypsin and 0.5 M NaCl to elute lysozyme. To achieve linear detector response, dense-phase samples were diluted up to 100-fold. To remove excess salt that interferes with protein binding to the ion-exchange column, supernatant samples were passed through Bio-Spin 6 size-exclusion columns that contain Bio-Gel P polyacrylamide with a 6,000 Dalton molecular-weight cutoff (Bio-Rad, Richmond, CA). Bio-Spin columns were centrifuged at 1,100 g for 3.5 min. Salt and water contents were measured as discussed earlier.

Low-angle laser-light scattering

Static light-scattering experiments were conducted with a KMX-6 LALLS photometer from Chromatix/Milton Roy with a 2-mW helium-neon laser at 633 nm. Measurements were made using the 6–7° annulus. An average of Rayleigh ratios was taken from three field stops (0.3, 0.2 and 0.15 mm). Refractive indices were measured with a KMX-16 Laser Differential Refractometer with a 0.5-mW helium-neon laser at 633 nm.

Stock solutions of α-chymotrypsin at 5 g/L were prepared and dialyzed (8000 Dalton cutoff tubing, Spectropor #132660) overnight against the desired salt solution to remove the 2-propanol and excess PMSF, and to maintain constant ionic strength and pH among all samples after diluting to the desired protein concentration with the dialysate. The pH was adjusted with the conjugate acid or base as necessary. No buffer was used with K_2SO_4 solutions, limiting the pH range to pH < 9. Ionic strengths of sodium-phosphate solutions (NaH_2PO_4 plus NaOH) were maintained constant at different pH's by appropriately adjusting the phosphate concentrations. Samples were filtered inline using a Millipore 0.22-μm-syringe-tip filter. Solutions were pumped through the light-scattering cell at 0.2 mL/min.

To determine osmotic second virial coefficients using LALLS, measurements were made of the reduced Rayleigh ratio, $\overline{R}_\theta = (R_{\theta,\text{solution}} - R_{\theta,\text{solvent}})$ at several protein concentrations. The protein osmotic second virial coefficient, B_2, and weight-average molecular weight $M_{w,2}$ were determined from (Tanford, 1961)

$$\frac{Kc_2}{\overline{R}_\theta} = \frac{1}{M_{w,2}} + 2B_2c_2 \tag{4}$$

$$K = \frac{2\pi n_0^2 (dn/dc_2)^2}{N_A \lambda^4} \tag{5}$$

where c_2 is the protein concentration (g/mL) and K is the optical constant that depends on the refractive index of the solvent, n_0; the refractive-index increment with respect to protein concentration, dn/dc_2; and the wavelength of light λ. Since the solvent is an aqueous salt solution, the regressed second virial coefficient for the protein is only valid in the particular solvent in which the experiment was conducted. The uncertainty in $M_{w,2}$ is approximately ±5% and the estimated uncertainty in B_2 is approximately $\pm 2 \times 10^{-4}$ mL·mol/g^2.

Results and Discussion

Salting-out phase equilibria

Reversible phase equilibrium has been demonstrated for protein salting-out systems (Shih et al., 1992). This reversibility was confirmed for the salts and proteins studied here.

Tables 1 and 2 show phase equilibria for lysozyme and chymotrypsin in aqueous ammonium-sulfate solutions over a range of pH and ionic strength. Experiments with ammonium sulfate were limited to pH < 9 to prevent evolution of gaseous ammonia. Partitioning of salt between the dense and supernatant phases was uniform for all experiments reported here. Tables 1 and 2 show that the protein concentrations in the dense and supernatant phases generally decrease with increasing ionic strength.

Figure 1 shows protein partition coefficients (K_2) for lysozyme in aqueous ammonium sulfate as a function of pH and ionic strength. Initial protein concentrations (that is, protein concentrations before phase separation) varied from 20

Table 1. Phase Equilibrium Measurements for Lysozyme in Ammonium-Sulfate Solutions

I (Molal)	$c_{2,sp}$ (mg/g Water)	$c_{2,dp}$ (mg/g Water)	K_2
pH 4			
5	4.05	316	78.1
6	2.16	210	97.5
7	1.11	160	143
8	0.626	154	247
9	0.292	149	511
pH 5			
5	21.5	132	6.14
6	7.13	126	17.7
7	2.99	160	53.5
8	1.20	150	125
9	0.562	147	261
pH 6			
5	40.3	255	6.34
6	11.6	138	11.9
7	3.82	131	34.4
8	1.38	118	85.7
9	0.576	120	208
pH 7			
5	34.9	228	6.55
6	10.9	142	13.1
7	3.65	125	34.2
8	1.29	110	85.0
9	0.460	93.0	202
pH 8			
5	30.9	196	6.33
6	10.8	145	13.5
7	3.28	114	34.8
8	1.15	97.6	84.6
9	0.418	96.4	231

Table 2. Phase Equilibrium Measurements for Chymotrypsin in Ammonium-Sulfate Solutions

I (Molal)	$c_{2,sp}$ (mg/g Water)	$c_{2,dp}$ (mg/g Water)	K_2
pH 4			
7.5	14.1	202	14.3
8.1	5.18	183	35.3
8.7	2.36	155	65.7
9.3	1.17	147	126
9.9	0.678	156	230
pH 5.5			
7.5	26.1	219	8.39
8.1	10.4	133	12.8
8.7	4.15	142	34.2
9.3	2.20	148	67.3
9.9	1.22	151	124
pH 7			
7.5	23.4	281	12.0
8.1	9.06	185	20.4
8.7	4.08	159	39.0
9.3	2.11	154	73.0
9.9	1.29	144	112
pH 8.3			
8.11	10.2	148	14.5
8.84	4.00	123	30.8
9.58	2.12	113	53.3
10.33	1.40	108	77.1

to 50 mg/g water with no effect on lysozyme partitioning (Shih et al., 1992). As ionic strength rises, larger partition coefficients are observed, as expected. For the pH range 8 to 6, K_2 is independent of pH. However, K_2 increases steadily as pH approaches 4.

Figure 2 shows protein partition coefficients for chymotrypsin in ammonium sulfate as a function of pH and ionic strength. The initial protein concentration was 30 mg/g water. Results for the stated ionic strengths at pH 8.3 were interpolated from data at other ionic strengths in Table 2. Larger partition coefficients are observed with increasing ionic strength. As a function of pH, chymotrypsin exhibits partitioning behavior similar to that of lysozyme. At its isoelectric point ($pI = 8.3$), where the net protein charge is zero, a maximum in partitioning might be expected. However, chymotrypsin's supernatant concentration (often referred to as solubility) is not a minimum at pH 8.3. Protein partition coefficients indicate that chymotrypsin is more likely to partition to the dense phase at low pH than at its (salt-free) isoelectric point.

The effect of pH on phase equilibria for lysozyme and chymotrypsin cannot be described simply by electrostatic screening of charged proteins. Because DLVO theory predicts complete screening of electrostatics at the high ionic strengths necessary for salting out, DLVO theory implies a pH-independent partition coefficient whenever the salt concentration is large. At low pH, protein denaturation could account for the increased partitioning because denatured proteins phase separate more readily than native proteins (De Young et al., 1993); however, both lysozyme and chymotrypsin retain their native forms over the pH range investigated (Biltonen and Lumry, 1969; Privalov and Khechinashvili, 1974; Przybycien and Bailey, 1989b). Increased partitioning observed at low pH may follow from increased anion binding to the protein due to the more positive protein charge at low pH (Carr, 1955). Ion binding may alter salting-out behavior through specific protein–ion interactions (Arakawa and Timasheff, 1985).

To explore the salting-out effect of a different salt, chymotrypsin was also salted out with sodium citrate. Experiments were carried out at pH 9 where citrate is trivalent. Based on observed equipartitioning of ammonium sulfate and

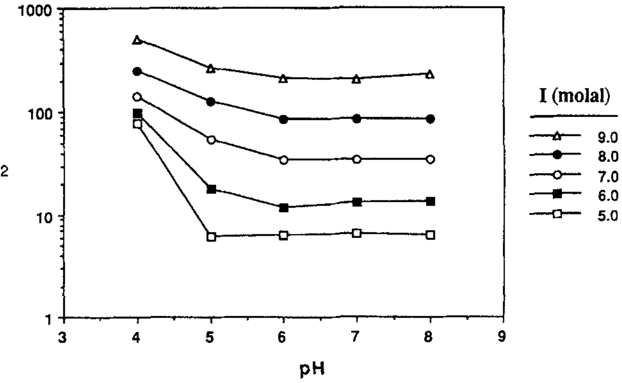

Figure 1. Phase partitioning of lysozyme in ammonium-sulfate solutions.

K_2 is defined by Eq. 2. Ionic strength varies from 5.0 to 9.0 molal.

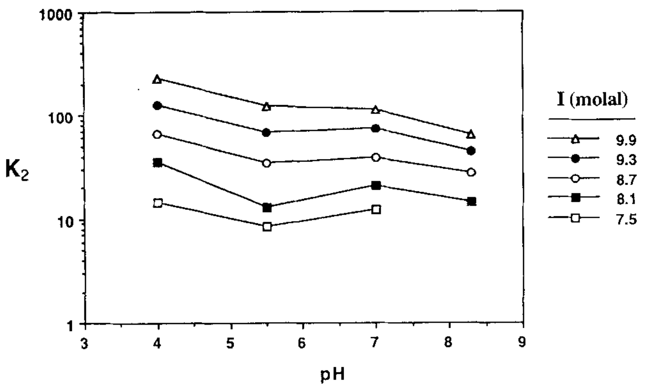

Figure 2. Phase partitioning of chymotrypsin in ammonium-sulfate solutions.

K_2 is defined by Eq. 2. Ionic strength varies from 7.5 to 9.9 molal.

other salts, sodium citrate was assumed to partition uniformly between both phases. Figure 3 shows that the partition coefficients for chymotrypsin with sodium citrate at pH 9 are similar to those with ammonium sulfate at pH 8.3 when plotted against ionic strength (Figure 3a), but they differ significantly when plotted as a function of salt concentration (Figure 3b). Figure 3 confirms that ionic strength (rather than salt concentration) is the better variable for describing the effect of salt. The greater partition coefficients obtained with sodium citrate are consistent with the higher position of sodium citrate in the Hofmeister series relative to ammonium sulfate. A salt's position in the Hofmeister series is directly correlated with its ability to salt out proteins (Melander and Horváth, 1977).

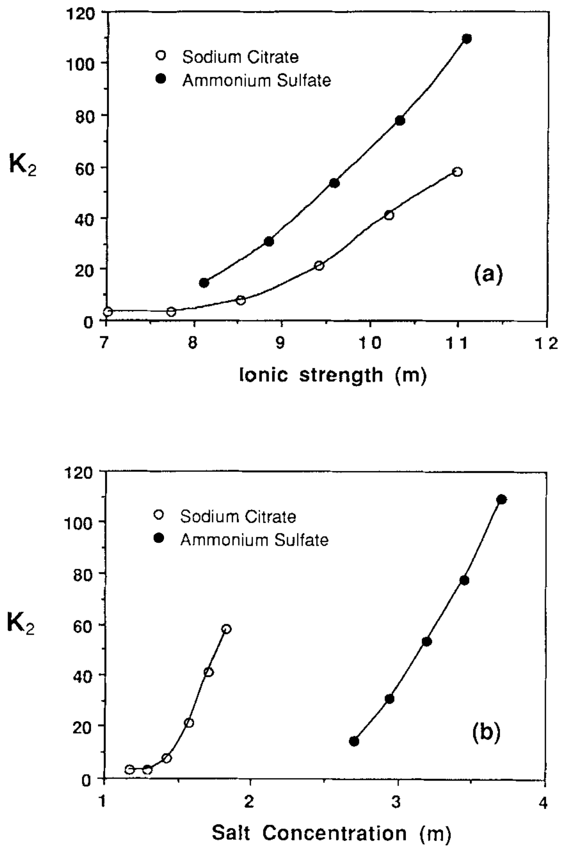

Figure 3. Phase partitioning of chymotrypsin in sodium citrate (pH 9) and in ammonium sulfate (pH 8.3) as functions of salt ionic strength (a) and salt concentration (b).

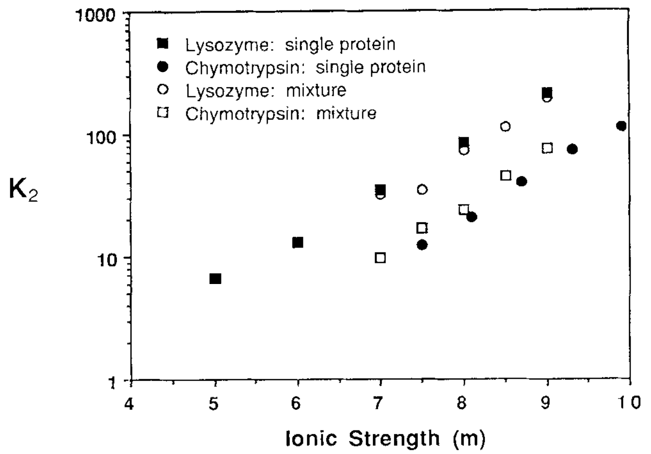

Figure 4. Partition coefficients for lysozyme and for chymotrypsin in aqueous two-protein (open symbols) and one-protein (filled symbols) systems at pH 7 using ammonium sulfate.

K_2 for each protein is defined by Eq. 2.

Two-protein phase equilibrium measurements

Phase equilibria were determined for concentrated aqueous salt systems containing both lysozyme and chymotrypsin. Figure 4 shows partition coefficients for lysozyme and for chymotrypsin with ammonium sulfate at pH 7 as a function of ionic strength. Partition coefficients are also shown for single-protein phase equilibria obtained under the same solution conditions (filled symbols). Initial protein concentrations were 20 mg/g water for both proteins. Figure 4 shows that lysozyme and chymotrypsin appear to salt out independently of each other. For the conditions studied here, specific interactions between lysozyme and chymotrypsin are not important, especially at the high salt concentrations studied where electrostatic screening prevails. The independence of these two proteins' phase behavior suggests that it may be possible to extend single-protein salting-out data to predict multiprotein salting-out phase behavior.

LALLS measurements to determine intermolecular potentials of mean force

LALLS data were obtained for dilute chymotrypsin solutions (1–5 g/L). Measurements were made for aqueous sodium-phosphate and potassium-sulfate solutions at 0.01 and 1.0 M ionic strengths over a range of pH. Results are shown in Table 3. Weight-average molecular weights reported in Table 3 show the self-association of chymotrypsin that occurs at pH > 4 at low ionic strengths (Aune and Timasheff, 1971;

Table 3. LALLS Osmotic Second Virial Coefficients for Chymotrypsin in Salt Solutions*

pH	$B_2 \times 10^4$ (mL·mol/g²)		
	$B_{2,\mathrm{expt}}$	$B^*_{2,MM}$	$M_{w,2}$
$I = 0.01$ M Sodium Phosphates			
4.2	3.64	4.30	25,100
4.9	−1.45	−0.793	30,000
6	−6.67	−6.01	40,700
7	−15.1	−14.4	44,100
8.3	−34.3	−33.6	55,700
$I = 1.0$ M Sodium Phosphates			
5	−3.08	−2.43	28,700
7	−1.43	−0.776	24,900
8.3	−1.10	−0.452	31,300
$I = 0.01$ M Potassium Sulfate			
3	3.27	3.92	29,500
4	0.692	1.35	26,400
5	−5.50	−4.84	28,700
5.8	−10.6	−9.94	33,700
$I = 1.0$ M Potassium Sulfate			
3	−1.21	−0.555	30,600
4	−1.23	−0.576	28,400
5.2	−3.37	−2.72	28,300
6.6	−1.66	−1.01	27,100

*$B_{2,\mathrm{expt}}$ are experimentally observed values and $B^*_{2,MM}$ are calculated from $B_{2,\mathrm{expt}}$ based on work by Hill (1959) and by Cabezas and O'Connell (1993).

Tellam and Winzor, 1977). At low pH or high ionic strength, where chymotrypsin does not self-associate, observed molecular weights are consistent with those for monomeric chymotrypsin (25,000 g/mol).

Figure 5 shows experimental osmotic second virial coefficients, determined from Eq. 4, plotted as a function of pH with ionic strength as parameter. Since no buffer was used, the accessible pH range for K_2SO_4 solutions was limited by carbon dioxide dissolution. Second virial coefficients for 1.0-M ionic-strength solutions, represented by triangles, do not vary with the net protein charge, presumably due to electrostatic screening. These virial coefficients are negative, indicating attractive forces that are not pH-dependent.

On the other hand, virial coefficients for the 0.01-M ionic-strength solutions, represented by circles, decrease with increasing pH. The net charge on α-chymotrypsin is +14.2 at pH 3 and approaches zero at pH 8.3 (Haynes et al., 1992). Therefore, coulombic repulsion, which is not well screened at $I = 0.01$ M, decreases with rising pH. At pH 3, coulombic forces are strongest, producing a positive (repulsive) experimental second virial coefficient. Attractive forces dominate at higher pH's, yielding negative second virial coefficients. The negative (attractive) second virial coefficients at higher pH's suggest the influence of electrostatically screened attractive forces, since they are not observed at $I = 1.0$ M. As suggested in previous studies (Vilker et al., 1981; Haynes et al., 1992), these forces are likely to be dipole forces (charge–dipole, and dipole–dipole forces) that are both attractive and electrostatically screened (Phillies, 1974). The next section discusses the contribution of dipole potentials to osmotic second virial coefficients.

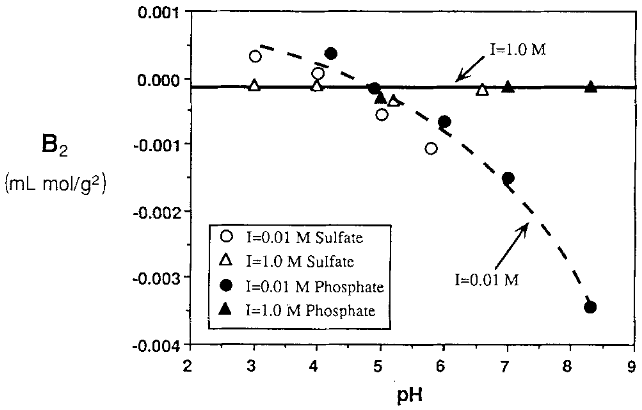

Figure 5. Osmotic second virial coefficients ($B_{2,\mathrm{expt}}$) obtained by LALLS for chymotrypsin in aqueous potassium sulfate and sodium phosphate solutions at 0.01 and 1.0 M ionic strengths.

Lines drawn to indicate ionic strength trends.

Determination of the effective Hamaker constant

Experimental osmotic second virial coefficients may be used to study the potentials of mean force for aqueous protein–protein interactions. The osmotic second virial coefficient, B_2, is related to the potential of mean force, W, by (Hill, 1960)

$$B_2(a_1^0, T) = B_2^{hs} - \frac{N_A}{2}\int_{d_2+3\text{Å}}^{\infty}\{\exp[-W(r, a_1^0, T)/kT] - 1\}4\pi r^2\, dr \quad (6)$$

where a_1^0 is the activity of pure solvent, $B_2^{hs} = (2\pi d_2^3)/3$ is the hard-sphere contribution to the second virial coefficient and d_2 is the protein diameter. Similar to a correction suggested by Vilker et al. (1981), the lower integration limit is taken as $d_2 + 3\text{Å}$ to account for a layer of water bound to the protein. In Eq. 6, the potential-of-mean-force expression is

$$W(r) = W_{\mathrm{disp}}(r) + W_{q\text{-}q}(r) + W_{q\text{-}\mu}(r) + W_{\mu\text{-}\mu}(r) + W_{OA}(r) \quad (7)$$

where $W_{\mathrm{disp}}(r)$ is the dispersion potential of Hamaker; $W_{q\text{-}q}(r)$ is the screened charge–charge repulsion; $W_{q\text{-}\mu}$ is the screened charge–dipole attraction; $W_{\mu\text{-}\mu}$ is the screened dipole–dipole attraction; and $W_{OA}(r)$ is the osmotic-attraction potential of mean force. Table 4 provides expressions for the various potentials of mean force.

Osmotic second virial coefficients obtained by LALLS are in the Lewis-Randall framework (obtained at constant T, pressure, and number of particles) but Eq. 6 is in the McMillan-Mayer framework (constant T, volume, and solvent chemical potential) (Hill, 1959; Cabezas and O'Connell, 1993). Calculations based on the work of Hill and that of Cabezas (see the Appendix) show that the conversion of LALLS data for chymotrypsin to the McMillan-Mayer framework requires the addition of approximately 7×10^{-5} mL·mol/g² to all ex-

Table 4. Contributions to the Potential of Mean Force for Proteins in Aqueous Electrolyte Solutions, $W_{p\text{-}p}(r)$*

Potential, $W(r)$	Screening Parameter, $\zeta(r)$	Reference
$W_{\text{disp}}(r) = -\frac{H}{12}\left[\frac{d_2^2}{r^2} + \frac{d_2^2}{r^2 - d_2^2} + 2\ln\left(1 - \frac{d_2^2}{r^2}\right)\right]$		(1)
$W_{q\text{-}q}(r) = \frac{(z_2 e)^2 \zeta_{q\text{-}q}(r)}{\epsilon r}$	$\zeta_{q\text{-}q}(r) = \frac{\exp[-\kappa(r - d_2)]}{(1 + \kappa d_2/2)^2}$	(1)
$W_{q\text{-}\mu}(r) = -\frac{2}{3}\frac{(z_2 e)^2 \mu^2 \zeta_{q\text{-}\mu}(r)}{\epsilon^2 kTr^4}$	$\zeta_{q\text{-}\mu}(r) = \left\{\frac{3(1+\kappa r)\exp[-\kappa(r - d_2)]}{(1 + \kappa d_2/2)[2 + \kappa d_2 + (\kappa d_2/2)^2 + (1 + \kappa d_2/2)\epsilon_s/\epsilon]}\right\}^2$	(2)
$W_{\mu\text{-}\mu}(r) = -\frac{9}{2}\frac{\mu^4 \zeta_{\mu\text{-}\mu}(r)}{\epsilon^2 kTr^6}$	$\zeta_{\mu\text{-}\mu}(r) = \frac{[(2 + 2\kappa r + (\kappa r)^2)^2 + 2(1 + \kappa r)^2]\exp[-2\kappa(r - d_2)]}{(2 + \kappa d_2 + (\kappa d_2/2)^2 + (1 + \kappa d_2/2)\epsilon_s/\epsilon)^4}$	(2)
$W_{OA} = -\frac{4}{3}\pi d_{23}^3 \rho_3 kT\left[1 - \frac{3r}{4d_{23}} + \frac{r^3}{16 d_{23}^3}\right]$	for $d_2 < r < 2d_{23}$	(3)
$W_{OA} = 0$	for $r > 2d_{23}$	

*Definition of symbols: disp = dispersion, q-q = charge–charge, q-μ = charge–dipole, μ-μ = dipole–dipole, and OA = osmotic attraction potentials; H = Hamaker constant, r = radial distance, d_2 = protein diameter, d_3 is the mean ionic salt diameter, $d_{23} = (d_2 + d_3)/2$, ρ_3 = ion number density, z_2 = net protein charge, μ = protein dipole moment, $\zeta_{i\text{-}j}(r)$ = screening parameter, κ^{-1} = Debye screening length, e = electron charge, $\epsilon = 4\pi\epsilon_0\epsilon_r$, where ϵ_0 = vacuum permittivity and ϵ_r = relative permittivity, ϵ_s = permittivity at protein surface ($\epsilon_s = 4$ (Phillies, 1974)), k = Boltzmann's constant, and T = temperature. An earlier version of this table was presented by Vilker et al. (1981). A similar table (Haynes et al., 1992) contained a misprint in the $W_{\text{disp}}(r)$ equation. References: (1) Verwey and Overbeek (1948), (2) Phillies (1974), and (3) Vlachy et al., (1993).

perimental LALLS virial coefficients ($B_{2,\text{expt}}$). The McMillan-Mayer virial coefficients ($B_{2,MM}^*$) are shown in Table 3. This correction significantly affects the smaller observed virial coefficients.

We consider three potential-of-mean-force models, each based on Eq. 7. They are (1) the DLVO model (Eq. 3); (2) DLVO plus dipole interactions (Eq. 7 without osmotic attraction); and (3) Eq. 7. Figure 6 shows effective Hamaker constants regressed for the three potential-of-mean-force models using LALLS experimental osmotic second virial coefficients, $B_{2,MM}^*$, for chymotrypsin in K_2SO_4 at low and high ionic strengths. The following parameters were used in the calculations: $T = 298$ K; $d_2 = 43.4$ Å (Stryer, 1988); mean ionic diameter $d_3 = 5$ Å; protein charge z_2(pH) and dipole moment μ(pH) from Haynes et al. (1992). Results using Model 1 show that the effective Hamaker constants at low ionic strength are pH-dependent while Hamaker constants at high ionic strength are pH-independent. Hamaker constants should be invariant with respect to solution conditions provided the conformation of chymotrypsin does not change significantly. The effective Hamaker constants are an order of magnitude larger than those calculated for another globular protein, bovine serum albumin, in aqueous solution (1–2 kT) (Nir, 1976). [All globular proteins should have similar Hamaker constants since the Hamaker constant is directly proportional to protein density (Nir, 1976), which is similar for most proteins (Cantor and Schimmel, 1980).] These larger effective Hamaker constants must include attractive forces not accounted for in the DLVO model (Model 1).

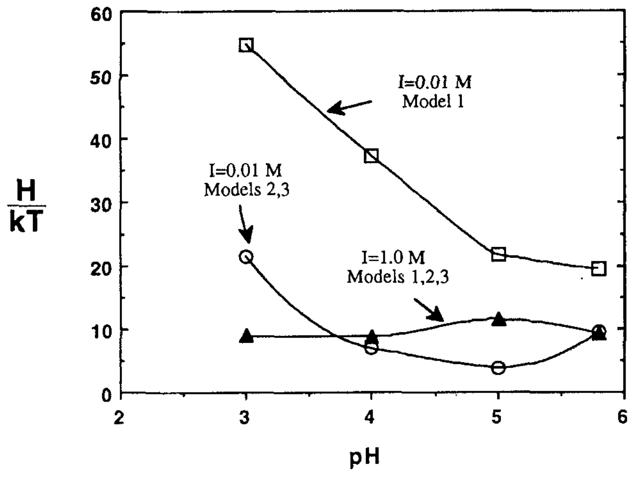

Figure 6. Reduced Hamaker constants regressed from osmotic second virial coefficients ($B_{2,\text{MM}}$) obtained by LALLS for three potential-of-mean-force models.

(1) Equation 3: DLVO potential; (2) Eq. 7 neglecting osmotic attraction: DLVO and charge–dipole and dipole–dipole potentials; and (3) Eq. 7: DLVO, charge–dipole, dipole–dipole, and osmotic-attraction potentials.

In Model 2, screened dipole–dipole and charge–dipole potentials are introduced. These dipole forces are attractive and are pH-dependent because chymotrypsin's dipole moment is a strong function of pH (Haynes et al., 1992). Figure 6 shows that the addition of dipole interactions significantly reduces the pH dependence of the effective Hamaker constants at low ionic strength, while the effective Hamaker constants regressed from high-ionic-strength virial coefficients are unaffected by dipole interactions. The magnitudes of the effective Hamaker constants regressed from low- and high-ionic-strength virial coefficients using Model 2 are comparable, indicating that the dipole interactions account for the more negative virial coefficients at low ionic strength. Effective Hamaker constants regressed using Model 2 are similar to those regressed from membrane osmometry data for chymotrypsin at 0.1-M ionic strength using a similar model (Haynes et al., 1992).

In Model 3, we include the osmotic-attraction potential. However, that inclusion does not significantly affect the regressed Hamaker constants because the experimental salt concentrations (0.0033 and 0.33 M) are not large enough for significant osmotic attraction. Experimental osmotic second virial coefficients at significantly higher salt concentrations are needed to study the effect of the osmotic-attraction potential.

After accounting for various potentials, we still observe larger effective Hamaker constants than those reported in the literature for proteins (Nir, 1976). Additional attractive interactions are indicated. These attractive interactions, independent of pH and ionic strength, may be due to hydrophobic interactions. Unfortunately, available theoretical models for hydrophobic interactions are not readily adaptable to analytical potential-of-mean-force expressions (Pratt and Chandler, 1977; Lazaridis and Paulaitis, 1992). However, regression of LALLS data to obtain model parameters, such as effective Hamaker constants, permits empirical modeling of intermolecular forces that are not explicitly identified.

In contrast to the pH-dependent salting-out data, Hamaker constants from experimental osmotic second virial coefficients at 1.0-M ionic strength are independent of pH. However, the osmotic second virial coefficients were measured by LALLS at protein and salt concentrations significantly lower than those in the salting-out measurements. Thus, the LALLS data presented here cannot probe protein interactions that depend on large salt concentrations (such as osmotic attraction) or on high protein concentrations where the specific protein–charge distribution may need to be considered (Phillies, 1974; Roush et al., 1994). While LALLS is limited to low protein concentrations, LALLS conducted at higher salt concentrations may prove useful in understanding effects such as ion-binding and osmotic attraction.

Conclusions

Salting-out phase equilibrium measurements are reported for lysozyme and for chymotrypsin in concentrated ammonium-sulfate solutions. Protein partition coefficients indicate that pH-dependent interactions are present at high ionic strength. For the systems studied here, two-protein partition coefficients show behavior similar to that for one-protein partition coefficients. Osmotic second virial coefficients measured by LALLS for chymotrypsin solutions are used to examine expressions for the intermolecular potential of mean force. Effective Hamaker constants are regressed from experimental osmotic second virial coefficients obtained from LALLS. Results indicate that pH-independent attractive interactions are present in addition to the attractive potentials accounted for by dispersion, charge–dipole and dipole–dipole potentials.

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Notation

n = refractive index of solution
N_A = Avogadro's number, mol^{-1}
ϵ = absolute permittivity, $C^2 \cdot J^{-1} \cdot m^{-1}$

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Appendix

According to Hill (1959), LALLS measurements yield unitless osmotic second virial coefficients in the Lewis-Randall framework, $\tilde{B}_{2,LR}$:

$$\frac{Kc_2}{\bar{R}_\theta} = \frac{1}{M_{w,2}} + 2c_2\left(\frac{\tilde{B}_{2,LR}V_1}{M_{w,2}^2} - \frac{\bar{V}_2^0}{M_{w,2}^2} - \frac{dn/dc_2}{n_0 M_{w,2}}\right) \quad \text{(A1)}$$

where V_1 is the molar volume of pure solvent and $\bar{V}_2^0$ is the partial molar volume of the solute at infinite dilution. Comparing the righthand sides of Eqs. 4 and A1, we relate the experimentally observed virial coefficient, $B_{2,\text{expt}}$, to $\tilde{B}_{2,LR}$ by

$$B_{2,\text{expt}} = \frac{\tilde{B}_{2,LR}V_1}{M_{w,2}^2} - \frac{\bar{V}_2^0}{M_{w,2}^2} - \frac{dn/dc_2}{n_0 M_{w,2}}. \quad \text{(A2)}$$

The McMillan-Mayer and Lewis-Randall virial coefficients are related by (Hill, 1959; Cabezas and O'Connell, 1993)

$$B_{2,MM} = \tilde{B}_{2,LR}V_1 + \bar{V}_2^0 \quad \text{(A3)}$$

where an isothermal compressibility term has been omitted because it is several orders of magnitude smaller than the terms shown. Substituting A3 into A2 and dividing by the square of the molecular weight yields

$$B_{2,MM}^* = \frac{B_{2,MM}}{M_{w,2}^2} = B_{2,\text{expt}} + \frac{2\bar{V}_2^0}{M_{w,2}^2} + \frac{dn/dc_2}{n_0 M_{w,2}} \quad \text{(A4)}$$

where $B_{2,MM}^*$ is the McMillan-Mayer osmotic second virial coefficient in units of mL mol/g^2. We use $\bar{V}_2^0 = 1.88 \times 10^4$ mL/mol for chymotrypsin based on 0.75 mL/g as a typical value for the protein partial specific volume (Cantor and Schimmel, 1980).

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