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Protein-Protein Interactions in Concentrated Electrolyte Solutions: Hofmeister-Series Effects

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Abstract

Protein-protein interactions have been measured for ovalbumin and for lysozyme in aqueous salt solutions. Protein-protein interactions are correlated with a proposed potential of mean force equal to the free energy to desolvate the protein surface that is made inaccessible to the solvent due to the protein-protein interaction. This energy is calculated from the surface free energy of the protein that is determined from protein-salt preferential-interaction parameter measurements. In classical salting-out behavior, the protein-salt preferential interaction is unfavorable. Because addition of salt raises the surface free energy of the protein according to the surface-tension increment of the salt, protein-protein attraction increases, leading to a reduction in solubility. When the surface chemistry of proteins is altered by binding of a specific ion, salting-in is observed when the interactions between (kosmotrope) ion-protein complexes are more repulsive than those between the uncomplexed proteins. However, salting-out is observed when interactions between (chaotrope) ion-protein complexes are more attractive than those of the uncomplexed proteins.

Introduction

Salt-induced precipitation/crystallization provides an extensively used method in biotechnology for obtaining high-quality crystals and for separating target proteins from multicomponent protein solutions as the first purification step. However, because protein phase behavior is not well understood, selecting optimum conditions to precipitate a target protein is difficult. Protein solubility is governed by many factors including pH, surface hydrophobicity, surface-charge distribution, size, salt-type, and salt concentration (Rothstein, 1994). The goal of this work is to understand how these factors influence protein solubility. The first step is to determine the interactions between the protein molecules, salt ions, and water molecules; these interactions, described quantitatively, are

then used to predict conditions for optimal separation of a mixture of proteins. However, generating a phase diagram from knowledge of the intermolecular potentials between the proteins, salt, and water is impossible without adopting physically realistic simplifying assumptions. In general, most models of protein precipitation are based on an effective protein-protein interaction (Chiew et al., 1995; Fornaseiro et al., 1999; Malfois et al., 1996; Piazza, 1999; Poon, 1997; Rosenbaum et al., 1996) that is mediated by salt ions and water. At the present time, effective protein-protein interactions in solutions of concentrated electrolytes are not well understood. Because protein solubility usually decreases with rising salt concentration (salting-out) (Cohn, 1943), we similarly expect that as salt concentration rises, the effective protein-protein interactions become more attractive. However, it is not clear whether these interactions are related to solvation forces between proteins or possibly to specific short-ranged forces that stabilize protein crystals, such as van der Waals contacts, salt bridges, or hydrogen bonds (Neal et al., 1999). In this work, we focus on determining these effective protein-protein interactions; from these, we develop specific criteria for choosing conditions favorable for selective protein precipitation or protein crystallization.

In concentrated salt solutions, protein solubility depends on the anion's or cation's position in the lyotropic series (Hofmeister, 1888), but this dependence cannot be explained by considering salt ions as charged hard spheres. It is well known that salting-out effectiveness is related to the water-structure-making or water-structure-breaking ability of the salt (Collins and Washabaugh, 1985). To predict protein solubility, this effect needs to be incorporated into models for effective protein-protein interactions. Presently, the only theories that account for these ion-specific effects are based on the

approach originally proposed by Melander and Horvath (1977) where protein solubility is determined by the preferential interactions between the protein and salt (Arakawa and Timasheff, 1985). To incorporate these theories into a statistical-mechanical model based on effective protein-protein interactions, we need to formulate effective protein-protein interactions in terms of protein-salt interactions.

For this reason, in the next section we summarize the salting-out theory of Melander and Horvath and present a brief discussion of protein-salt interactions. The following section discusses current models for effective protein-protein interactions and proposes a potential of mean force that can account for ion-specific effects.

Protein-Salt Interactions

Salting-Out Theory

The important result of Melander and Horvath (1977) is that protein solubility can be expressed in terms of the solvation free energy of the protein molecule in the equilibrated fluid phase. When the solubility is small, we can neglect fluid-phase protein-protein interactions. The phase-equilibrium criterion (the chemical potential of the protein in the crystal is equal to that in the fluid) at a given salt concentration reduces to

$$\mu_2^s - \mu_2^\theta = RT \ln \frac{S_2}{S_2^\theta} \tag{1a}$$

where μ_2^s and μ_2^θ are the chemical potential of the protein in the solid phase and the infinite-dilution standard-state chemical potential of the protein, respectively, and S_2/S_2^θ is protein solubility relative to a standard-state solubility. The major assumption of the salting-out theory is that the protein crystal is a pure phase; in that event, the salting-out

behavior is determined from the dependence of the standard-state chemical potential on salt concentration. The standard-state chemical potential can be further decomposed into a hypothetical ideal-gas contribution and a contribution from the reversible work to transfer a protein molecule from a hypothetical ideal gas into the aqueous salt solution. This work is the protein solvation free energy; it is given by the product of the surface free energy, σ, and the solvent-accessible surface area of the protein molecule A:

$$\mu_2^{\theta} = \mu_2^{ig} + N_{av} A \sigma \tag{1b}$$

where N_{av} is Avogadro's number (Ben-Naim, 1978). Substituting Equation (1a) into (1b) and taking the derivative with respect to molality of salt, m_3, gives

$$RT \frac{d \ln S_2}{dm_3} = N_{av} A \frac{d\sigma}{dm_3}. \tag{1c}$$

The problem of determining protein solubility as a function of salt concentration is reduced to solving for the surface free energy of the protein molecule. The dependence of the surface free energy of the protein on salt molality can be determined from preferential interaction-parameter measurements (Casassa and Eisenberg, 1964):

$$A \left(\frac{\partial \sigma}{\partial m_3} \right)_{T,p_o}^{o} = - \left(\frac{dm_3}{dm_2} \right)_{T,\mu_1,\mu_3}^{\infty} \left(\frac{d\mu_3}{dm_3} \right)_{T,p_o}^{o} \tag{2}$$

Here, m_3 and m_2 are salt and protein molality and μ_1 and μ_3 are the chemical potential of water and salt, respectively. Superscript o denotes that the property is evaluated at the conditions of a hypothetical outside solution that is in dialysis equilibrium with a solution at the same salt chemical potential and water chemical potential and temperature as those of the protein solution. The partial derivatives of the outside solution are taken at constant temperature and pressure of the outside solution, p_o. Superscript ∞ denotes

infinite dilution of protein. The experimentally-measured preferential-interaction parameter, $\left(\partial m_3/\partial m_2\right)^{\infty}_{T,\mu_1,\mu_3}$ is related to the excess (or deficiency) of salt in the domain of the protein relative to the bulk solution (Lee et al., 1979). If the preferential interaction parameter is positive, the protein-salt interaction is classified as favorable; there is an excess amount of salt in the domain of the protein. According to Equation (2), favorable protein-salt interactions decrease the protein-surface free energy resulting in higher solubility. An unfavorable protein-salt interaction indicates that the salt is excluded from the domain of the protein and protein solubility declines upon the addition of salt (salting-out).

Protein-salt interactions can be divided into three main groups: (1) effect of salt on charged groups, (2) effect of salt on exposed peptide groups, and (3) effect of salt on nonpolar groups (von Hippel and Schleich, 1969; Robinson and Jencks, 1965). At low salt concentrations, there is a salting-in region due to the favorable interaction between the protein-surface charge and the surrounding ion atmosphere. At higher salt concentrations, protein solubility is determined by the balance of unfavorable interactions between the salt and the nonpolar surface of the protein, and by favorable weak ion-binding interactions between the salt and either the charged surface groups or the surface peptide groups of the protein. In most cases, because the unfavorable hydrophobic interactions are greater than the attractive weak ion-binding interactions, salting-out is observed.

Surface-Tension-Increment Effect

The surface free energy of a nonpolar surface in contact with a solvent is related to the surface tension of the solvent. Because all salts increase the surface tension of water, they similarly increase the surface free energy of nonpolar groups. Consequently, the magnitude of the salt-protein interaction is related to the molal surface-tension increment of the salt, δ_3. The molal surface-tension increment of the salt is correlated with the ion's position in the lyotropic series that was originally developed to describe the salting-out effectiveness of various ions for globular proteins (Hofmeister, 1888). For anions, the series in decreasing order of the molal surface-tension increment is $SO_4^{2-} > F^- > Cl^- > Br^- > I^- > NO_3^- > SCN^-$; the corresponding series for cations is $Mg^{2+} > Na^+ > K^+ > Li^+ > NH_4^+ > Cs^+$. High lyotropic-series salts (kosmotropes) are good salting-out agents because they interact strongly with water; water molecules surrounding the salt ions are more structured relative to bulk water. Low lyotropic-series salts (chaotropes) break the structure of water of the surrounding water molecules. Chaotropes are weak salting-out agents due to weak interaction with water (Collins and Washabaugh, 1985).

Preferential Ion-Binding Interactions

Studies on the solubilities of model peptides in salt solutions have shown that there is a salting-in effect due to an electrostatic interaction between the salt ions and the peptide group (Nandi and Robinson, 1972). The interaction between the salt and the peptide group is attributed to the large dipole moment of the peptide group. The peptide amino group carries a partial positive charge and the carbonyl oxygen carries a partial negative charge, suggesting anion binding at or near the nitrogen atom and cation binding

at or near the oxygen atom. The magnitudes of the binding affinities follow the reverse lyotropic series for the anions. In addition, divalent cations also have large binding affinities to the peptide group as indicated by measurements of the retention times of salt on columns containing a stationary phase of polyacrylamide $[-CH_2CH(CO-NH_2)-]_n$ (von Hippel and Schleich, 1969). In these studies, chaotropic anions or divalent cations were retarded relative to water due to interaction with the peptide group, whereas kosmotropic anions were not retarded due to unfavorable interaction with the nonpolar backbone of polyacrylamide.

Anion binding to the positively charged surface groups of protein molecules has been observed in studies concerning stabilization of folded structures of protein molecules at low pH (Goto et al., 1990). The strength of this interaction is related to the ion's position in the electroselectivity series (Gjerde et al., 1980), as measured by the affinity of an ion for an anion-exchange resin. The series depends on the resin employed, but the general trend is in the order $SO_4^{2-} > SCN^- > I^- > Br^- > Cl^-$. For monovalent anions, the electroselectivity series is the inverse of the lyotropic series. It is likely that the higher binding affinities of the chaotropic anions (with either the anion-exchange resin or the polyacrylamide-based resin) reflect weaker unfavorable interactions with the nonpolar backbones of the resins. However, a divalent charge interacts more strongly with the charged resin than a monovalent charge, as is observed with SO_4^{2-}. The importance of anion binding to positively charged surfaces is illustrated by the reverse lyotropic-series dependence of the solubility of basic proteins, for example lysozyme, whose pI is 11.3 (Ries-Kautt and Ducruix, 1989). This reverse solubility dependence is attributed to the formation of insoluble protein-anion complexes; these are formed by

proteins with low-lyotropic-series anions. This observation is counterintuitive because favorable protein-salt interactions should favor solubilization of the protein molecule. However, in addition to protein-salt interactions, changes in protein-protein interactions must also be considered; a decline in solubility occurs if there is an increase in the net attraction between the proteins.

Protein-Protein Interactions

Osmotic Second Virial Coefficient, B_{22}

George and Wilson (1994) have shown the importance of the protein-protein pair potential of mean force for predicting solution conditions favorable for protein crystallization; they have established a B_{22} crystallization window for protein solutions. As a favorable but not sufficient condition for protein crystallization, B_{22} should be in the region -2×10^{-4} and -8×10^{-4} mLmol/g^2. For B_{22} more positive than -2×10^{-4} mLmol/g^2, the protein-protein attraction is usually not sufficiently strong to form stable protein crystals. For solutions where B_{22} is more negative than -8×10^{-4} mLmol/g^2, amorphous precipitation is likely to occur because protein-protein attractions are sufficiently strong that the protein molecules do not have adequate time to orient themselves into a crystal lattice. Furthermore, Guo et al. (1999) have shown that there is a strong correlation between B_{22} and protein solubility. Since the protein solubility is a direct measure of the crystal chemical potential, this result implies that protein-protein interactions related to B_{22} for proteins in dilute solution can be extrapolated qualitatively to protein-protein interactions in the crystal.

Protein-protein interactions can be studied by a variety of methods including membrane osmometry, sedimentation, and static laser-light scattering (SLS or LALLS). All of these methods yield a protein-protein osmotic second virial coefficient that can be related to the sum of the potentials of mean force. The potential of mean force (pmf) is defined such that its negative derivative with respect to distance is the force between two solute molecules at infinite dilution, averaged over all configurations of the solvent molecules (McMillan and Mayer, 1945). Because integration of the average force over distance yields the reversible work, W_{22} is the free energy of two protein molecules as a function of the center-to-center separation, r. If the sum of the potentials-of-mean-force (W_{22}) is spherically symmetric, B_{22} is given by the following volume integral

$$B'_{22} = \frac{1}{2}\int_0^\infty \left[1 - e^{-\beta W_{22}}\right] 4\pi r^2 dr . \tag{3.a}$$

where B'_{22} is related to the experimentally-determined osmotic second virial coefficient B_{22} by

$$B_{22} = \frac{N_{av} B'_{22} 10^{-24}}{M_2^2} \tag{3.b}$$

N_{av} is Avogadro's number, M_2 is protein molecular weight, and $\beta = (k_B T)^{-1}$; here k_B is Boltzmann's constant and T is absolute temperature.

In dilute aqueous electrolyte solutions, the protein-protein pmf has been modeled with DLVO theory (Verwey and Overbeek, 1948), where proteins are modeled as rigid spheres with uniform surface charge immersed in a continuous dielectric medium containing point charges that represent salt ions (Coen et al., 1995; Vilker et al., 1981). Here, the interaction consists of a repulsive electric double-layer potential, an attractive Hamaker dispersion potential, and a hard-sphere repulsive potential. However, because

in concentrated salt solutions the potential of mean force described by DLVO theory is independent of salt concentration, classic salting-out behavior cannot be represented with DLVO theory. Because protein solubility is a strong function of the type of salt following the lyotropic series, and because in DLVO theory all ions are point charges, DLVO theory does not distinguish between different ions of identical charge.

Solvation Potential of Mean Force

Salting-out effects can be predicted using the work of Melander and Horvath (1977) where protein solubility is determined by solvation free energy of the protein in the aqueous salt solution according to Equations (1a) and (1b). Because the authors assume the protein crystal is a pure phase, the change in free energy upon forming protein-protein contacts in the crystal is given by the free-energy change of desolvating the entire protein molecule. However, experimental studies show that a protein crystal contains a significant amount of solvent, hence a more realistic free energy of crystallization would be given by the free energy of desolvating only the protein surface that is inaccessible to the solvent in the protein crystal (Arakawa and Timasheff, 1990).

Phase-equilibrium theories based on a protein-protein potential of mean force are in the context of McMillan-Mayer solution theory. In McMillan-Mayer solution theory, the protein standard state is the same for all phases, thus it cancels out in phase-equilibrium calculations. The effect of burying the protein surface upon crystallization is contained in the potential of mean force at contact. Here we propose an approximate potential of mean force given by the free energy required to desolvate that part of the area of the protein molecule that is inaccessible to the solvent due to protein-protein two-body

interaction. In McMillan-Mayer solution theory, the free energy of forming protein-protein contacts in the crystal is given by the sum of the potential of mean force over the protein-protein pair interactions in the crystal. With the proposed potential of mean force, this free energy of protein crystallization is given by the free energy to desolvate the protein surface that is inaccessible to the solvent in the crystal. Consequently, theories based on the Melander-and-Horvath work and those models based on McMillan-Mayer solution theory give the same free energy of protein crystallization.

Here, we assume that the average size of nonpolar and polar surface patches are significantly less than the area buried upon desolvation. With this approximation the solvation pmf is independent of orientation. The solvation pmf is given by

$$W_{solv}(r) = -A(r)\left(f_a\sigma_a + f_p\sigma_p\right) \qquad \text{for } d_2 < r < d_2 + r_c \tag{4}$$

$$= 0 \qquad \text{for } r > d_2 + r_c$$

where f_a and f_p are the surface fractions of nonpolar and polar groups, σ_a and σ_p are the characteristic surface energies of nonpolar and polar groups in contact with solvent. Based on Equation (4), we classify two types of solvation forces: (1) hydration forces occur when polar surface groups are desolvated. These forces are repulsive because the change in free energy for this process is positive (σ_p is less than zero); work is required to remove water from polar groups. (2) Hydrophobic forces result from desolvating nonpolar groups. These forces are attractive because σ_a is greater than zero. As shown in Figure 1, the surface area that is inaccessible to the solvent because of the protein-protein interaction, A(r) is given by

$$A(r) = \pi d_2^2 - \pi r d_2 + \pi r_c d_2 \tag{5}$$

where r is center-to-center separation. A(r) is given by a monotonically decreasing function that goes to zero at surface-to-surface separation r_c.

In calculating the solvation pmf, it is assumed that (a) the protein surface can be divided into atomic surface groups whose solvation properties are independent of the neighboring groups and (b) the energy of the solvation of the surface groups is determined by the first hydration layer. Both these approximations follow from the additivity approximation where the solvation free energy of the entire protein molecule is given by the sum of the solvation free energies of the surface groups on the protein surface, as suggested by Eisenberg and McLachlan (1986) and Hermann (1972). Eisenhaber (1996) has shown that the additivity approximation is valid for nonpolar groups in aqueous solutions. However, because the interaction between polar groups and the surrounding water molecules is longer-ranged than one solvent layer, the additivity approximation is not necessarily valid for predicting solvation free energies of polar groups (Chalikian et al., 1994; Wang and Ben-Naim, 1997).

Here, we decompose the surface free energy into a hypothetical free energy in salt-free water, σ_o, and a correction term that accounts for the perturbation of the surface free energy from addition of the salt, given by $(d\sigma/dm_3)m_3$.

$$W_{solv}(r) = -A(r)\left[\sigma_o + \left(\frac{d\sigma}{dm_3}\right)m_3\right] = -A(r)\left[\sigma_o + f_a\left(\frac{d\sigma_a}{dm_3}\right)m_3 + f_p\left(\frac{d\sigma_p}{dm_3}\right)m_3\right] \quad (6)$$

Because the surface free energy increment, $(d\sigma/dm_3)$, is proportional to the negative value of the preferential interaction parameter, it follows from Equation (6) that a preferential interaction between salt and water is associated with a repulsive solvation pmf, whereas preferential exclusion is associated with an attractive solvation pmf.

Enhanced attraction results from preferential exclusion of the salt in the domain of the nonpolar surface of the protein. The change in free energy due to the enhanced hydrophobic interaction is given by the second term on the right side of Equation (6) where $(d\sigma_a/dm_3)$ has been correlated with the molal surface-tension increment of the salt. Addition of salt can also increase the repulsive hydration force, given by the last term on the right side of Equation (6). Because the interaction between the peptide group and the salt ions is favorable, more work (free energy) is required to remove the layer of solvent when salt is present.

We have not included any effect of the charged state of the protein in Equation (6). Because the electrostatic interaction between the surface charge and the surrounding ion atmosphere is favorable, we similarly expect a repulsive force due to removing the ion atmospheres surrounding the charged proteins. However, this force is the double-layer overlap force given by DLVO theory. Consequently, we expect the solvation force to be independent of the charged state of the protein. This expectation is strictly true if the hydration forces between the different charged groups are the same. Generally, solvation forces depend on the lyotropicity of the bound charge. Forces between surfaces with bound kosmotropic charges are significantly more repulsive then those with bound chaotropic charges (Israelachvili, 1992). This result follows from the solvation force described by Equation (4). The surface free energy of the ions increases with decreasing order in the lyotropic series. Therefore, surfaces with bound chaotropic charges (chaotropic surfaces) have lower surface free energies than surfaces with bound kosmotropic charges (kosmotropic surfaces). Thus, we expect that forces between chaotropic surfaces are more attractive than those between kosmotropic surfaces.

Attractive interactions between chaotropic surfaces have been observed for solutions of basic proteins dissolved in solutions of chaotropic anions. Interactions between anion-protein complexes are more attractive than those between uncomplexed proteins (Ries-Kautt and Ducruix, 1989).

Potential-of-Mean-Force Model

In classical DLVO theory, the relationship between B_{22} and the potential of mean force model is given by

$$B'_{22} = \frac{2}{3}\pi\left(d_2 + 2\kappa\right)^3 + \frac{1}{2}\int_{d_2+2\kappa}^{\infty}\left(1 - e^{-\beta W_{DLVO}}\right)4\pi r^2 dr \qquad (7)$$

where W_{DLVO} includes the electric double-layer repulsion and Hamaker dispersion potentials (Hamaker, 1937). The first term on the right side of Equation (7) is the contribution to B_{22} from the protein-excluded volume. The parameters in the DLVO theory are the Hamaker constant, H, the protein net charge, z, and the thickness of an impenetratable layer of water, κ. The Hamaker constant determines the magnitude of the protein-protein dispersion interaction. In general, because most proteins have similar densities and compositions, they also have similar Hamaker constants. A good approximation for the Hamaker constant of a protein in aqueous solutions is on the order of 3 - 5 k_BT (Nir, 1976). The hydration-layer thickness, κ, determines the lower limit of integration for the DLVO potentials. B_{22} is very sensitive to this parameter because the dispersion potential goes to negative infinity at surface-to-surface contact. The dispersion potential is based on a continuum approximation for the solvent that is invalid for surface-to-surface separations on the order of a solvent diameter. Consequently, the

binding energy is not given by negative infinity, but a finite value related to the sum of Van-der-Waals interactions between the atoms of the two protein molecules plus a contribution from desolvating the buried surface groups. Including a layer of tightly attached solvent provides an upper bound to B_{22} provided that the hydration force is not significantly longer-ranged than one solvent diameter. Here, instead of using a layer of tightly bound water in the calculation of B_{22}, we include the solvation force $W_{solv}(r)$ to represent the short-ranged forces between protein molecules. With this approximation B_{22} is given by

$$B'_{22} = \frac{2}{3}\pi d_2^3 + \frac{1}{2}\int_{d_2}^{d_2+r_c}\left(1-e^{-\beta W_{solv}}\right)4\pi r^2 dr + \frac{1}{2}\int_{d_2+r_c}^{\infty}\left(1-e^{-\beta W_{DLVO}}\right)4\pi r^2 dr \qquad (8)$$

where r_c is chosen to be the thickness of one solvent layer and W_{solv} is given by Equation (6).

Experimental Methods

Solution Preparation

A bulk dilute protein solution of 100 mL with a concentration between 3 and 5 g/L was prepared by gradually dissolving the protein powder in the salt solution. The strong acid or base of the salt at the same ionic strength as the protein solution was used to adjust pH. If there was any appearance of irreversible precipitation, the protein solution was centrifuged at 20,000 rpm, at 20°C, for 20 min., and the supernatant was carefully removed with a pipet. Five 25 mL protein samples were prepared by diluting the 3 - 5 g/L protein solution with the salt solution in the following volumetric ratios (protein:salt): 1:4, 2:3, 3:2, 4:1, 5:0. Concentrations were measured using a Milton Roy

Spectronic 1201 spectrophotometer. The extinction coefficient is 2.63 (l/g.cm) for lysozyme (Sophianopoulus et al., 1962) and 0.734 (l/g.cm) (Cunningham and Muenke, 1959) for ovalbumin.

Light-Scattering Measurements

Light-scattering measurements were performed using an LDC Milton Roy KMX-6 Low-Angle Laser-Light Scattering (LALLS) photometer, with a 2 mW helium-neon laser at fixed wavelength 633 nm. For each experiment, each of the five protein samples and the solvent were filtered through a 0.1 μm-Millipore filter before analysis. The samples were pumped through the light-scattering cell at 0.3 mL/min using a Sage-Instruments syringe pump. The Rayleigh ratio was measured and determined according to the LDC/Milton Roy KMX 6 Instruction Manual (1986).

For the three-component system water (1), protein (2), salt (3) in dilute protein solutions, the light-scattering equation is given by (Stockmayer, 1950)

$$\frac{Kc_2\left(\partial n/\partial c_2\right)^2_{T,\mu_1,\mu_3}}{\overline{R_\theta}} = \frac{1}{M_2} + 2B_{22}c_2 \qquad (9)$$

where $\overline{R_\theta}$ is the excess Rayleigh scattering of the protein solution over the aqueous salt solution, K is the light-scattering constant, n is refractive index of the protein of the protein solution, c_2 is weight concentration of protein, and M_2 is protein molecular weight. Subsequently, a plot of $Kc_2\left(\partial n/\partial c_2\right)_{T,\mu_1,\mu_3}/\overline{R_\theta}$ vs. c_2 is used to determine M_2 and the osmotic second virial coefficient of the protein, B_{22}.

Determination of Refractive-Index Increment

The difference in refractive index between the sample and the solvent (Δn) was measured using an LDC Milton Roy KMX-16 Laser Differential Refractometer with a 0.5 mW helium-neon laser at fixed wavelength 633 nm. The refractive-index increment at constant salt molality for the protein was determined by plotting $\Delta n/c_2$ for each sample vs. its concentration and extrapolating to zero protein concentration.

$$\left(\frac{\partial n}{\partial c_2}\right)_{T,\mu_1,\mu_3} = \left(\frac{\Delta n}{c_2}\right)_{c_2 \to 0} \tag{10}$$

Refractive-index increments for the protein at constant chemical potentials of water and salt were also measured. To obtain chemical potentials of salt and water in the protein solution equal to those in the pure solvent, the protein solutions were dialyzed overnight in a 2 L salt solution at 4°C using a Spectraphor dialysis membrane with a molecular-weight cut off 5000 to 8000 daltons. The differences in refractive indices between the dialyzates and the respective protein samples were measured. The refractive-index increment for the protein at constant solvent chemical potential was determined by extrapolating the plot of $\Delta n/c_2$ vs. concentration to zero.

Results and Discussion

Overview

Tables 1a and 1b show B_{22} and M_2 obtained from light-scattering experiments for lysozyme and for ovalbumin in several salt solutions over a range of salt concentration and pH.

Lysozyme obtained from Sigma was used in the experiments performed in solutions of ammonium sulfate and sodium chloride. The molecular weights obtained from the experiments in solutions of sodium chloride are approximately 17,500 daltons, larger than the monomer molecular weight of 14,600 daltons indicating that the Sigma lysozyme contains high molecular-weight impurities. This result is consistent with that reported by Haynes et al. (1993), who found molecular weights between 17,800 and 18,100 g/mol for the same commercial lysozyme with a variety of salts and a range of pH. Furthermore, Skouri et al. (1995) reported that the same Sigma lysozyme contains 2% ovalbumin and conalbumin, which interact with the lysozyme to form large aggregates in aqueous salt solutions. However, the B_{22} values obtained with the Sigma lysozyme in sodium chloride solutions are in good agreement with the values reported in literature (Rosenbaum et al., 1996) indicating that B_{22} is insensitive to the amount of aggregation in these solutions.

The molecular weights obtained from the experiments of ovalbumin in magnesium chloride solutions are also greater than the monomer molecular weight of 45,000 daltons. This result is most likely due to the formation of aggregates with the protein impurities (lysozyme and conalbumin) contained in the commercial ovalbumin formulation. The experimental molecular weights for ovalbumin in ammonium-sulfate or potassium-isothiocyanate solutions are in good agreement with the monomer value.

Ovalbumin-Ovalbumin Interactions

Figure 2 shows B_{22} for ovalbumin in solutions of ammonium sulfate at pH 6, magnesium chloride at pH 7, or potassium isothiocyanate at pH 7. The solutions of

ovalbumin in ammonium sulfate and ovalbumin in potassium isothiocyanate follow classical salting-out behavior. As salt molality rises, the protein-protein interaction becomes more attractive and protein solubility decreases. The attraction results from enhancing the hydrophobic interaction; the addition of salt raises the surface free energy of the nonpolar protein surface making it less favorable for their exposure. Because ammonium sulfate has a larger surface-tension increment than potassium isothiocyanate, the interactions in solutions of ammonium sulfate are more attractive than in solutions of potassium isothiocyante.

For ovalbumin in solutions of magnesium chloride, B_{22} remains positive for all salt molalities, consistent with the salting-in behavior of many proteins by magnesium chloride. The salting-in behavior is attributed to preferential interactions between the magnesium ion and either the peptide group or the negatively charged residues on the protein surface. This preferential interaction compensates the preferential exclusion of the magnesium ion from the nonpolar surface of the protein molecule. Arakawa et al. (1990) have measured the preferential interaction parameters for β-lactoglobulin, BSA, and lysozyme in solutions of magnesium chloride. In all cases, the preferential interaction parameter indicates that magnesium chloride is excluded at low salt molalities, but preferentially bound at higher salt molalities. Because there is a minimum in the preferential interaction parameter, it appears that the solvation pmf has a minimum. According to Figure 2, the solvation pmf may go through a minimum at a salt molality of 2 molal in semi-qualitative agreement with the results of Arakawa et al. (1990).

Arakawa and Timasheff (1982) found that at pH 5.6, the preferential interactions of β-lactoglobulin with potassium isothiocyanate are slightly stronger than those with

magnesium chloride, in contrast to our results where the preferential interactions between magnesium chloride and ovalbumin are larger then those between potassium isothiocyanate and ovalbumin. The difference between the results may be related to the differences in net charge between the two proteins. The experimental results for ovalbumin are at pH 7 where ovalbumin has a significant negative charge. Consequently, the high negative-charge density may prevent isothiocyanate ions from interacting with the surface peptide groups of ovalbumin. In addition, the negatively-charged groups provide additional binding sites for magnesium ions. The results for β-lactoglobulin are near the pI of the protein where the preferential interactions should be primarily determined by interactions between magnesium or isothiocyanate ions with the peptide groups. It is likely that these interactions are similar as supported by data from Nandi and Robinson (1972), who showed that the peptide salting-in constant for calcium ion is similar to that of isothiocyanate ion. Because magnesium and calcium have a divalent charge, they have similar interactions with the peptide group.

Figure 3 shows B_{22} as a function of pH for ovalbumin in 1.0 molal solutions of magnesium chloride or ammonium sulfate. For both cases, there is negligible pH dependence as expected from the pmf model; the only pH-dependent potential is the electric double layer repulsion that is screened out at 1.0 molal salt concentration.

Earlier, it was postulated that the preferential interaction of magnesium ion would increase with rising pH due to an increase in negatively charged residues. To examine the effect of increasing the amount of tightly bound solvent, we examined the predictions of the pmf model described by Equation (7) for κ = 1.5 Å and 3 Å. If the effect of magnesium binding is to increase the size of the protein molecule, it is unlikely that

measurements of B_{22} can probe the small change in size as shown in Figure 3, where increasing the hard-sphere protein diameter by 3 Å results in a change of 1.5 x 10^{-4} mLmol/g^2 in B_{22}. However, if binding of the magnesium ion to the surface results in long-ranged hydration forces between protein molecules, then B_{22} would be significantly greater than 1.0 x 10^{-4} mLmol/g^2. Because the measured B_{22} for ovalbumin in magnesium chloride solution do not increase significantly with salt molality, we expect that there are no long-ranged hydration forces between ovalbumin-magnesium complexes.

Lysozyme-Lysozyme Interactions in Solutions of Monovalent Anions

Figure 4 shows B_{22} for lysozyme in 0.1 molal solutions of potassium chloride or potassium isothiocyanate as a function of pH. In both cases, the lysozyme-lysozyme interactions are more attractive with rising pH as a result of lowering the net charge of lysozyme and reducing the electric double-layer repulsion. In addition, the intermolecular interactions depend on the type of salt. The difference in B_{22} is related to the relative anion-binding affinities and the change in the pair potential due to anion binding. Because the isothiocyanate ion is more chaotropic than the chloride ion, isothiocyanate has a higher binding affinity. However, the difference in the pair potential cannot be explained in terms of changes in the net charge of lysozyme due to anion binding (Curtis et al., 1998). To fit the data with DLVO theory, a much larger Hamaker constant is required for the solutions in potassium isothiocyanate than for those in potassium chloride. Consequently, it is likely that there are other specific attractive forces between the lysozyme-isothiocyanate complexes that do not exist between native

lysozyme molecules. The surface chemistry of lysozyme is altered upon isothiocyanate binding because isothiocyanate is strongly chaotropic, whereas the imidazolium-binding sites are marginally chaotropic. Because the surface free energies of the lysozyme-isothiocyanate complexes are higher than those of the uncomplexed form, the forces are more attractive between the complexed forms (Ries-Kautt and Ducruix, 1989)

Isothiocyanate ions can be either strong salting-out agents or salting-in agents. Both effects are attributed to the preferential interaction between isothiocyanate ion and the protein surface. The salting-out occurs because of the formation of an insoluble complex between isothiocyanate ion and the protein. Isothiocyanate ions bind to the positively charged residues and the pair potential for the complexes is more attractive than that for the uncomplexed form, leading to reduced solubility. The salting-in interaction is due to the preferential interaction of isothiocyanate ions with the exposed peptide groups of the protein as described earlier for the interactions between ovalbumin molecules in solutions of magnesium ions. The difference in the salting-in and salting-out behavior of isothiocyanate is related to the isothiocyanate ion binding affinities. If the binding is weak, the two-body force is determined by the work to remove the solvent and the force is more repulsive because the weak interactions decrease the surface free energy. If the binding is strong, the force is determined from the pair potential between the protein-ion complexes. For isothiocyanate, the forces are more attractive between the complexes than those between the uncomplexed forms; this difference is related to making the surface more chaotropic upon binding isothiocyanate ions.

Here, we have identified the strong ion-binding sites as the positively charged residues and the weak-ion binding sites as the exposed peptide groups. However,

Chakrabarti (1993) found for many crystal forms, that protein crystals contain anions whose locations are adjacent to peptide groups in addition to locations near positively charged sites, indicating that a clear delineation of sites does not exist. For example, ovalbumin-ovalbumin interactions follow salting-in behavior for magnesium chloride and weak salting-out behavior for potassium isothiocyanate although the preferential interaction parameters for both systems are similar. The weak-salting out behavior observed for ovalbumin in potassium isothiocyanate may result from the formation of insoluble ovalbumin-isothiocyanate complexes.

Lysozyme-Lysozyme Interactions in Aqueous Ammonium Sulfate and Sodium Chloride

Figure 5 shows B_{22} as a function of salt molality for lysozyme in solutions of ammonium sulfate at pH 4 and 7 and in solutions of sodium chloride at pH 4.5. In all solutions, classical salting-out behavior is observed; as salt molality rises, lysozyme-lysozyme interactions are more attractive. The pair potential between lysozyme molecules is more repulsive in ammonium sulfate solutions at low pH than at high pH for all ionic strengths. The increase in repulsion is attributed to sulfate binding to the positively charged residues of lysozyme at low pH (Curtis et al., 1998); sulfate ion has a strong binding affinity for positively charged residues. Because sulfate is more kosmotropic than the imidazolium binding sites on the lysozyme surface, the surface free energy of the lysozyme-sulfate complex is lower than that of the uncomplexed form; more work is required to remove solvent from the complexed forms than from around the uncomplexed forms. It is also likely that chloride ions form lysozyme-ion complexes. However, because chloride ion is located in a similar position in the lyotropic series as

the imidazolium binding sites, the lysozyme surface chemistry is not significantly altered upon binding chloride ion.

The enhancement of the hydrophobic interactions is observed for those solutions where sulfate binds to the surface of lysozyme in contrast to the behavior observed for ovalbumin in magnesium chloride solution where the protein-protein interaction remains repulsive for all salt molalities. The difference in the two types of behavior is related to the binding sites of the respective ions. Because magnesium ions bind to peptide groups, the preferential interaction occurs uniformly over the entire protein surface counteracting the preferential exclusion. Sulfate ions bind only to positively charged sites that are distributed randomly across the surface at a much lower surface density than the peptide groups. Because these sites are not necessarily adjacent to the nonpolar surface of the protein, salt is preferentially excluded from the nonpolar surfaces and the hydrophobic interaction is enhanced.

Solvation Potential of Mean Force

We fit the potential-of-mean-force model described by Equation (8) by varying the surface free-energy parameter, σ. The Hamaker constant for lysozyme and for ovalbumin is set equal at 3.0 k_BT and the cut-off separation of the solvation force, r_c is set at 3 Å. The fractional nonpolar and polar surface coverage, f_a and f_p, are determined using the Michael Connolly Surface Package (Connolly, 1993) where carbon atoms are classified as nonpolar and all others are classified as polar. For lysozyme, f_a is 0.49 and for ovalbumin f_a is 0.59. Table 2 lists the results of the fit for ovalbumin in potassium isothiocyanate solutions and in ammonium sulfate solutions and the fit for lysozyme in

solutions of ammonium sulfate at pH 4 and pH 7 and for lysozyme in solutions of sodium chloride.

Figure 6 shows the fit values of σ for lysozyme in ammonium sulfate and in sodium chloride solutions. The values extrapolated to zero salt concentration are related to the surface free energy of the protein in salt-free water, σ_o. These values should be independent of the salt and pH as observed in sodium chloride and ammonium sulfate solutions at pH 7. However σ_o is significantly less for ammonium sulfate solutions at pH 4. Because there is significant sulfate binding to lysozyme, the extrapolated surface free energy refers to that of the lysozyme-sulfate complex. This value is most likely less than that of the unbound form due to replacing the imidazolium ions with kosmotropic sulfate ions on the surface of lysozyme.

The surface free-energy increments for lysozyme are determined from the slopes in Figure 6. The values of these slopes can be compared to the results of Arakawa and Timasheff (1982) who determined the surface free-energy increments from preferential interaction-parameter measurements (see Equation 2). For lysozyme in sodium chloride solutions at pH 4.5, the authors report that the ratio of the surface free-energy increment to the sodium chloride surface-tension increment is equal to 0.68, in good agreement with our fit value of 0.53 given in Table 1. We obtain a similar ratio for ammonium-sulfate solutions at pH 7. However, the ratio obtained for ammonium-sulfate solutions at pH 4 is significantly larger indicating that the effect of salt on the protein-protein attraction is more pronounced in ammonium sulfate solutions at low pH. This result may be related to altering the protein-protein interactions by formation of lysozyme-sulfate complex.

Figure 7 shows results for ovalbumin in ammonium sulfate or potassium isothiocyanate solutions. The surface free energies in salt-free water for ovalbumin are smaller than those for lysozyme, indicating that the intermolecular attraction in salt-free water between lysozyme molecules is larger than that between ovalbumin molecules. This result is supported by light-scattering studies of lysozyme where an unreasonable high Hamaker constant is required to fit the lysozyme interaction data using DLVO theory at low salt concentrations (Eberstein et al., 1994; Muschol and Rosenberger, 1995). The ratio of the surface free-energy increment to the surface-tension increment for ovalbumin in ammonium sulfate solution is 0.65; for ovalbumin in potassium isothiocyanate solutions it is also 0.65.

To quantify the effect of salt on the surface free energy of the protein molecule, we decompose the surface free-energy increment according to

$$\left(\frac{d\sigma}{dm_3}\right) = f_a\left(\frac{d\sigma_a}{dm_3}\right) + f_p\left(\frac{d\sigma_p}{dm_3}\right). \tag{11}$$

The nonpolar surface free energy increment is proportional to the molal surface tension increment of the salt, δ_3

$$\left(\frac{d\sigma_a}{dm_3}\right) = \alpha\delta_3 \tag{12}$$

where the proportionality constant α is related to the radius of curvature of the surface interface. For small molecules, α is on the order of 1/3, whereas α is unity for flat interfaces. For ovalbumin or lysozyme in ammonium sulfate solutions, we can neglect the effect of salt on the surface free energy of the polar groups because there is negligible interaction between the kosmotropic sulfate ion and the peptide groups. With this

approximation we obtain α equal to 1.1 for both solutions; it appears that the interface between the protein molecule and the salt solution resembles that of a flat interface. For lysozyme in sodium chloride solutions and for ovalbumin in potassium isothiocyanate solutions, we also obtain the same values for the proportionality constant, indicating that the preferential interactions between the anions and the peptide groups are negligible. From these results, the surface tension increment and the fractional nonpolar coverage of the protein molecule provide a quantitative measure of the protein-protein attraction in concentrated salt solutions. However, for ovalbumin in potassium isothiocyanate solutions, it is not clear whether the salting-out effect is due to enhancement of hydrophobic attraction or to formation of insoluble ovalbumin-isothiocyanate complexes.

Conclusions

We have measured protein-protein interactions for lysozyme and for ovalbumin in concentrated salt solutions. The interactions are explained in terms of the effect of salt on the solvation forces between the protein molecules. The two-body force depends on the extent of ion binding to the protein surface. When ion binding to the protein surface is weak or zero, classical salting-out behavior is observed. Protein-protein interactions become more attractive with addition of salt due to enhancement of hydrophobic attraction. The magnitude of this attraction depends on the nonpolar fraction of the protein surface and on the surface tension of the salt solution. If the surface chemistry of the protein is altered by ion binding, salting-out behavior follows the reverse lyotropic series. In this case, interactions between (chaotrope) ion-protein complexes are more attractive than those of the uncomplexed proteins, whereas interactions between

(kosmotrope) ion-protein complexes are more repulsive than those of the uncomplexed proteins.

Our model is a reformulation of the salting-out theory of Melander and Horvath (1977) and Arakawa and Timasheff (1985) where protein solubility is determined from the dependence of the protein infinite-dilution activity on salt molality. This dependence is related to protein-salt interactions. Our model may be useful for predicting protein phase diagrams within the McMillan-Mayer framework, where the thermodynamic properties of the protein solutions are determined by the solvent-mediated protein-protein potential of mean force. We have shown that solvent-mediated protein-protein interactions are most convincingly explained in terms of protein-salt interactions. This explanation provides the connection between models based on McMillan-Mayer solution theory and those described by Melander and Horvath.

Acknowledgements

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Nomenclature

A	surface area, [Å^2]
B'_{22}	protein experimental osmotic second virial coefficient, [mL-mol/g^2]
B_{22}	protein osmotic second virial coefficent, [Å^3]
c_2	protein weight concentration, [g/mL]
d_2	protein hard-sphere diameter, [Å]
f	fractional surface coverage
H	Hamaker constant, [cal]
k_B	Boltzmann's constant, = 3.30×10^{-24} cal/K
K	light-scattering constant, [cm^2-mol/g^2]
m_i	molality of component i, [molal]
M_2	protein molecular weight, [g/mol]
n	refractive index
N_{av}	Avogadro's number, = 6.022×10^{23}
p	pressure
r	center-to-center separation, [Å]
r_c	surface-to-surface cut-off separation, [Å]
R	gas constant, = 1.987 cal/mol-K
$\bar{R}_\theta$	Rayleigh ratio, [cm^{-1}]
S_2	protein solubility, [mol/L]
T	temperature, [K]
W	two-body potential of mean force, [cal]
z	protein net charge

Greek symbols

β	dimensionless inverse temperature, = $(k_B T)^{-1}$
δ_3	molal surface-tension increment, [cal/(mol-Å^2-molal)]
μ_i	chemical potential of component i, [cal/mol]
σ	surface free energy, [cal/(mol-Å^2)]

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List of Tables:

Table 1a : Molecular weights and osmotic second virial coefficients from LALLS measurements for lysozyme in various aqueous salt solutions at 25°C.

Table 1b : Molecular weights and osmotic second virial coefficients from LALLS measurements for ovalbumin in various aqueous salt solutions at 25°C.

Table 2: Results of fitting the surface free-energy parameter, σ, to the potential of mean force model described by Equation (8). σ_o is obtained from extrapolating σ to zero salt molality. The slopes of the plots in Figures 6 and 7 give the surface free-energy increments, $d\sigma/dm_3$. The last column refers to the ratio of the surface free-energy increment to the surface-tension increment of the salt.

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Figure 1 : The solvation force is described by a surface free energy multiplied by the surface area buried by the interaction. This is approximated by the spherical cap area of the proteins colored in dark grey. The surface-to-surface cut-off separation, r_c, is approximated by a solvent diameter. The combined areas of the spherical cap regions enclosed by the box is given by $A(r) = 2\left[\int_0^{\theta} \frac{\pi d_2^2}{2} \sin\theta' d\theta'\right] = \pi d_2^2 - \pi r d_2 + \pi r_c d_2$ where d_2 is the diameter of the protein, θ is the solid angle corresponding to the boundary of the spherical cap and $\cos\theta = \frac{r - r_c}{2R}$.

Figure 2: Effect of salt molality on B_{22} for lysozyme in magnesium chloride, ammonium sulfate, or potassium isothiocyante solutions at 25°C.

Figure 3: Effect of pH on B_{22} for ovalbumin in magnesium chloride or ammonium sulfate solutions. Dashed lines are predictions of DLVO theory (Equation 7) for two values of of the thickness of the hydration layer, κ. For this calculation, $H = 3.0\ k_BT$ at 25°C.

Figure 4: Effect of pH on B_{22} for lysozyme in potassium chloride or potassium isothiocyanate solutions at 25°C.

Figure 5: Effect of salt molality on B_{22} for lysozyme in ammonium sulfate solutions at pH 4 or 7 or in sodium chloride solutions at pH 4.5 at 25°C.

Figure 6: Surface free energy of lysozyme determined from fitting B_{22} to the potential of mean force model described by Equation (8) with $H = 3.0\ k_BT$, $r_c = 3$ Å at 25°C.

Figure 7: Surface free energy of ovalbumin determined from fitting B_{22} to the potential-of-mean-force model described by Equation (8), with $H = 3.0\ k_BT$, $r_c = 3$ Å at 25°C.

salt molality (molal), pH	B_{22} x 10^4 (mLmol/g^2)	M_2 (kg/mol)
KSCN		
0.1, 3.5	-3.0	15.5
0.1, 4.5	-4.0	14.9
0.1, 5.5	-10.0	14.8
0.1, 6.5	-14.0	15.1
0.1, 7.5	-18.9	15.3
KCl		
0.1, 4.5	-1.2	15.6
0.1, 5.5	0.0	15.4
0.1, 6.5	-1.9	14.9
0.1, 7.2	-4.9	15.3
NaCl		
0.17, 4.5	1.0	-
0.34, 4.5	-3.0	17.4
0.68, 4.5	-4.3	17.6
1.0, 4.5	-5.6	17.6
1.4, 4.5	-6.0	-
1.7, 4.5	-8.4	17.8
$(NH_4)_2SO_4$		
0.3, 4	0.1	17.8
0.3, 7	-5.3	17.8
1.0, 4	-4.1	18.0
1.0, 7	-6.1	17.8
1.7, 4	-15.0	17.8
1.7, 7	-16.7	17.0

Table 1.a

salt molality (molal), pH	B_{22} x 10^4 (mLmol/g^2)	M_2 (kg/mol)
$MgCl_2$		
0.0013, 7	-0.9	62.9
0.03, 7	-1.1	63.5
0.3, 5	0.0	73.0
0.3, 7	-0.4	59.7
0.3, 9	-0.7	59.0
1.0, 7	1.6	62.3
2.0, 7	0.7	63.3
3.0, 7	1.8	48.8
KSCN		
1.0, 8	-1.2	44.3
3.0, 8	-3.0	44.4
$(NH_4)2SO_4$		
0.3, 5	-1.0	43.6
0.3, 6	-0.2	44.7
0.3, 7	0.0	44.7
0.3, 8	0.4	-
1.0, 6	-0.5	42.0
1.3, 6	-1.3	-
1.7, 6	-4.1	44.4
2.0, 6	-16.0	-

Table 1.b

	σ_o (cal/molÅ^2)	$d\sigma/dm_3$ (cal/molÅ^2)(molal)$^{-1}$	ratio
lysozyme			
$(NH_4)_2SO_4$, pH 4	3.2	3.1	1.03
$(NH_4)_2SO_4$, pH 7	6.2	1.6	0.54
NaCl, pH 4.5	5.8	1.3	0.54
ovalbumin			
$(NH_4)_2SO_4$, pH 6	2.6	2.0	0.65
KSCN	4.3	0.43	0.65

Table 2

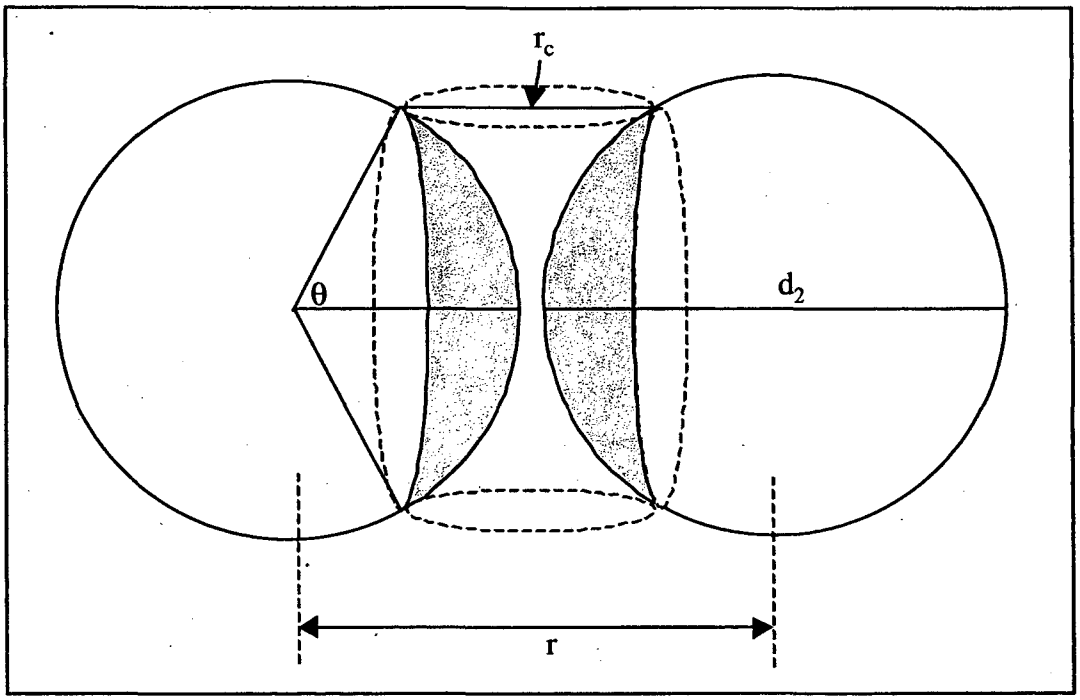

Figure 1

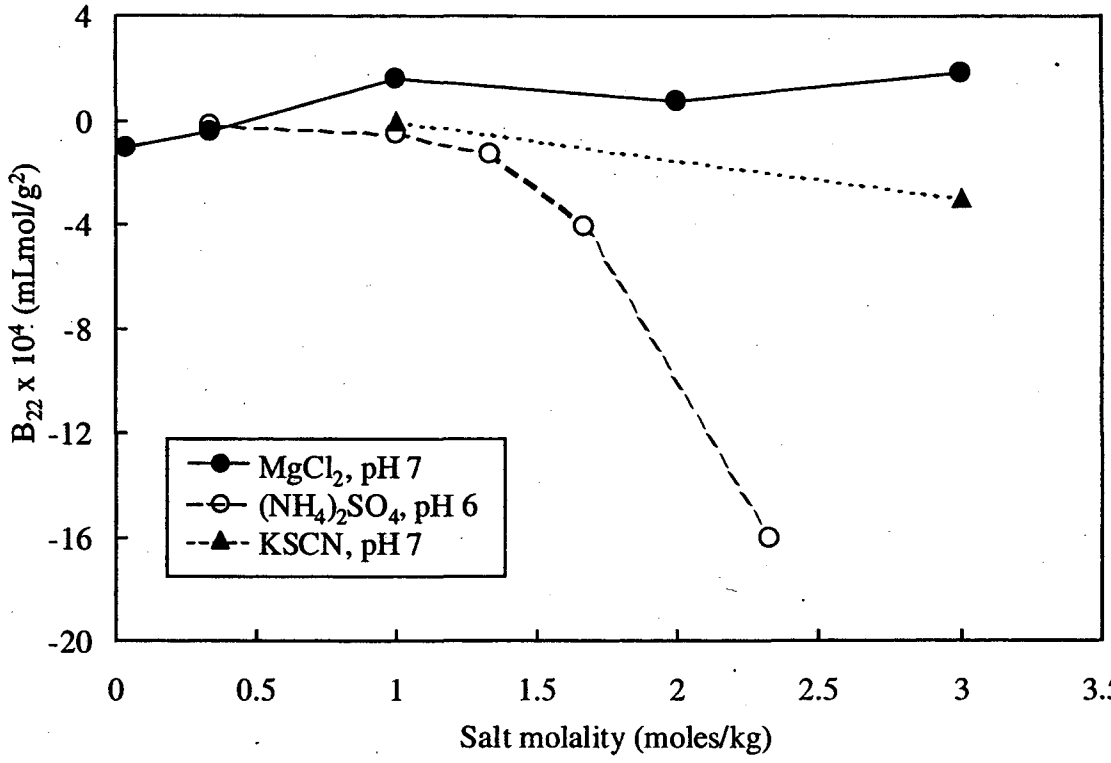
4
0
-4
-8
-12
-16
-20
B_{22} x 10^4 (mLmol/g^2)
0
0.5
1
1.5
2
2.5
3
3.5
Salt molality (moles/kg)
$MgCl_2$, pH 7
$(NH_4)_2SO_4$, pH 6
KSCN, pH 7

Figure 2

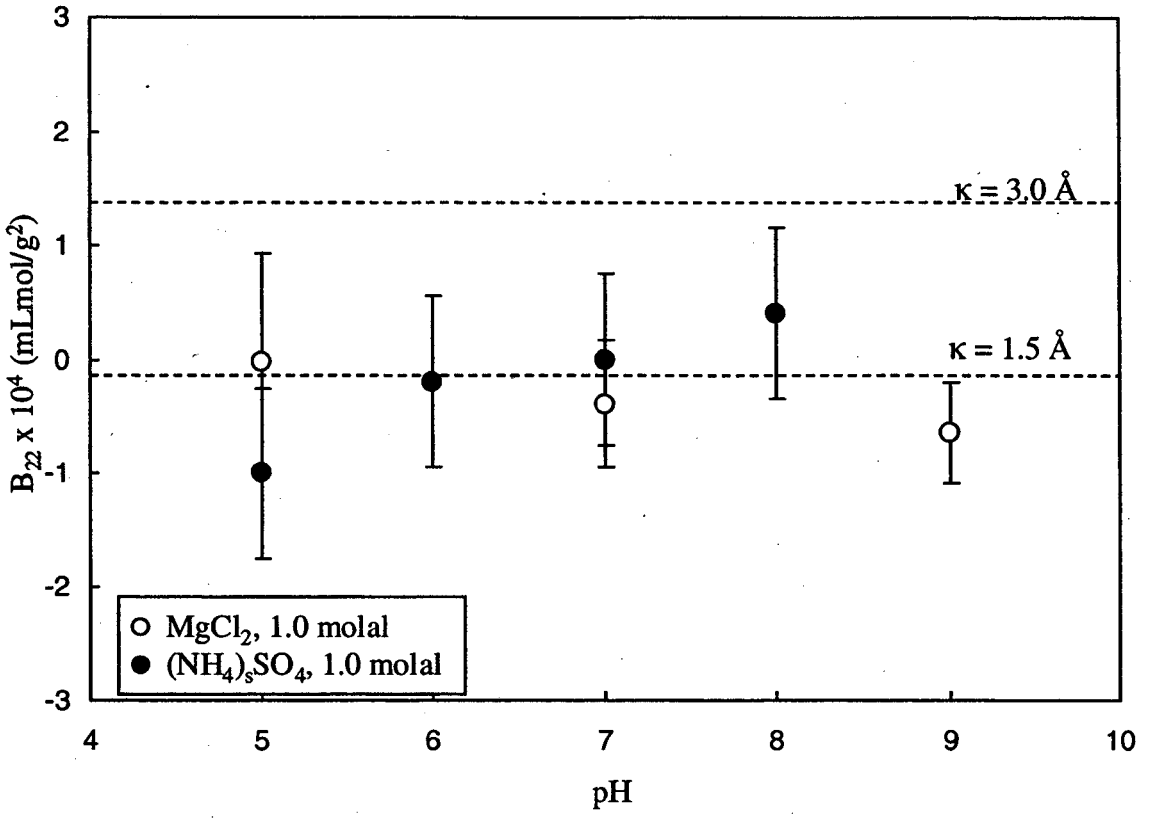
3
2
1
0
-1
-2
-3
B_{22} x 10^4 (mLmol/g^2)
κ = 3.0 Å
κ = 1.5 Å
MgCl$_2$, 1.0 molal
(NH$_4$)$_s$SO$_4$, 1.0 molal
4
5
6
7
8
9
10
pH

Figure 3

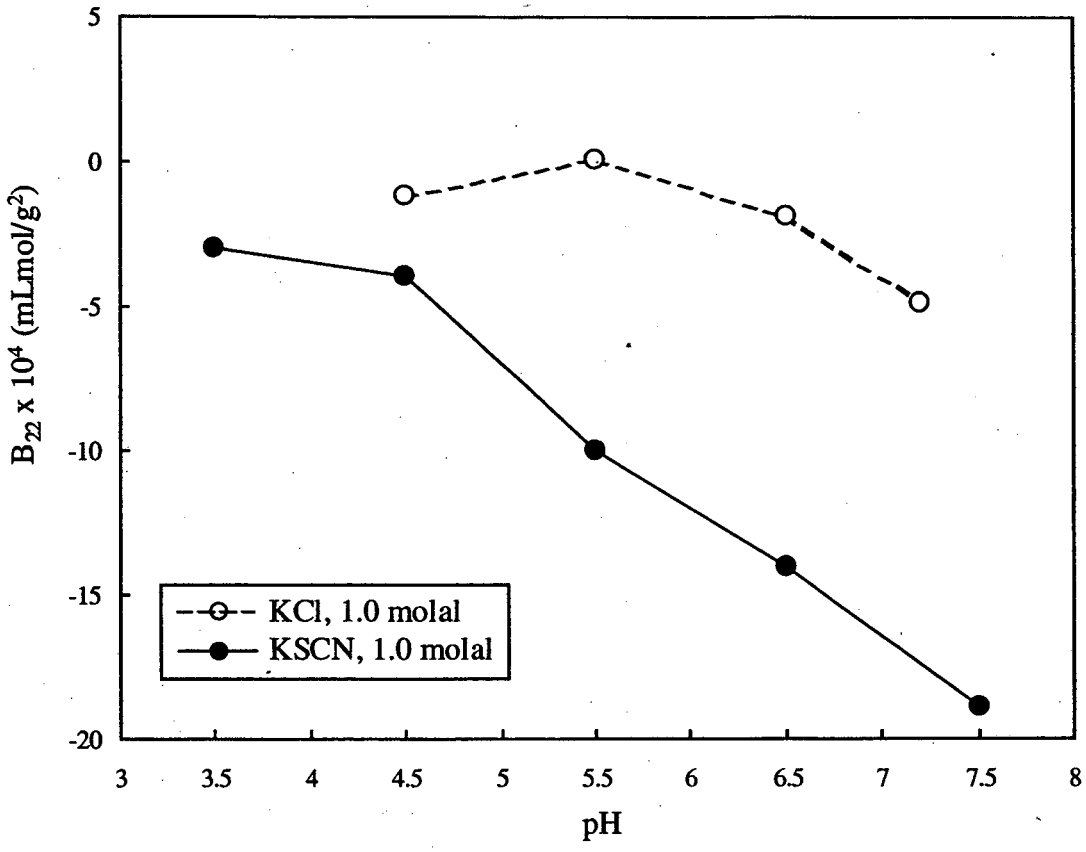
5
0
-5
-10
-15
-20
B_{22} x 10^4 (mLmol/g^2)
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
pH
KCl, 1.0 molal
KSCN, 1.0 molal

Figure 4

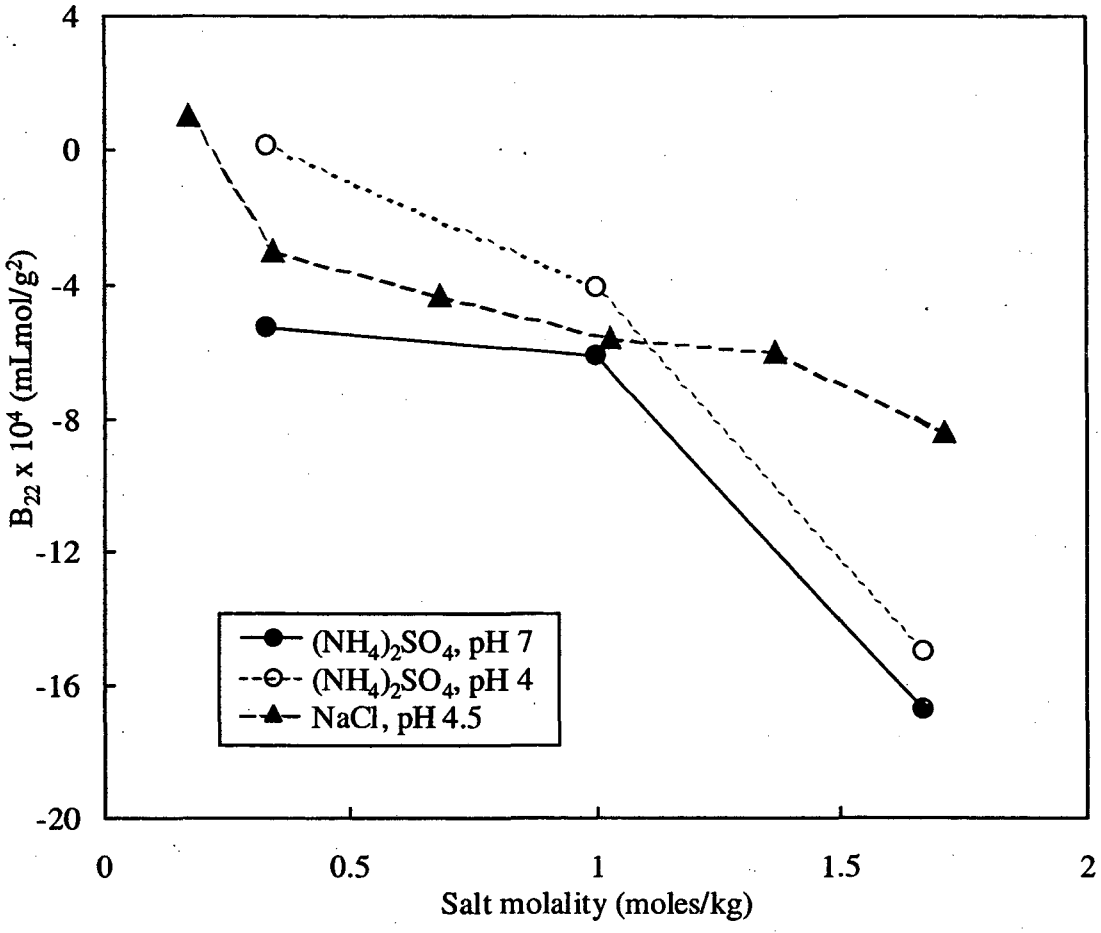
4
0
-4
-8
-12
-16
-20
B_{22} x 10^4 (mLmol/g^2)
0
0.5
1
1.5
2
Salt molality (moles/kg)
$(NH_4)_2SO_4$, pH 7
$(NH_4)_2SO_4$, pH 4
NaCl, pH 4.5

Figure 5

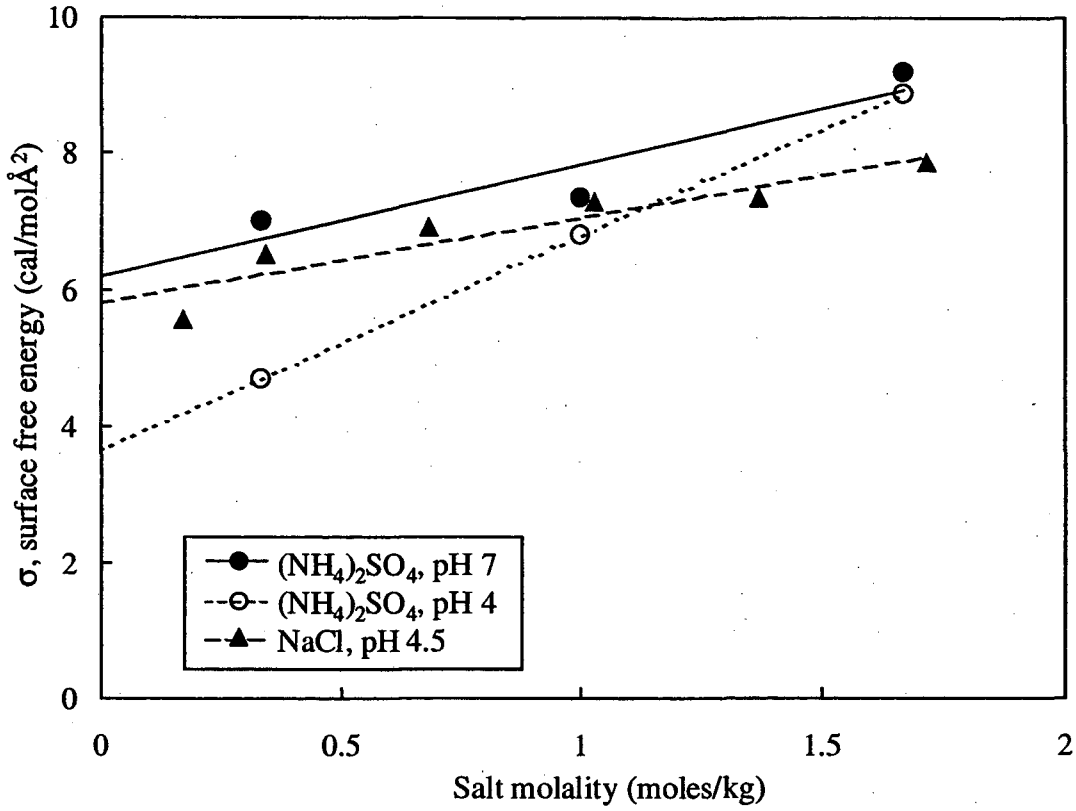
10
8
6
4
2
0
σ, surface free energy (cal/molÅ²)
0
0.5
1
1.5
2
Salt molality (moles/kg)
(NH4)2SO4, pH 7
(NH4)2SO4, pH 4
NaCl, pH 4.5

Figure 6

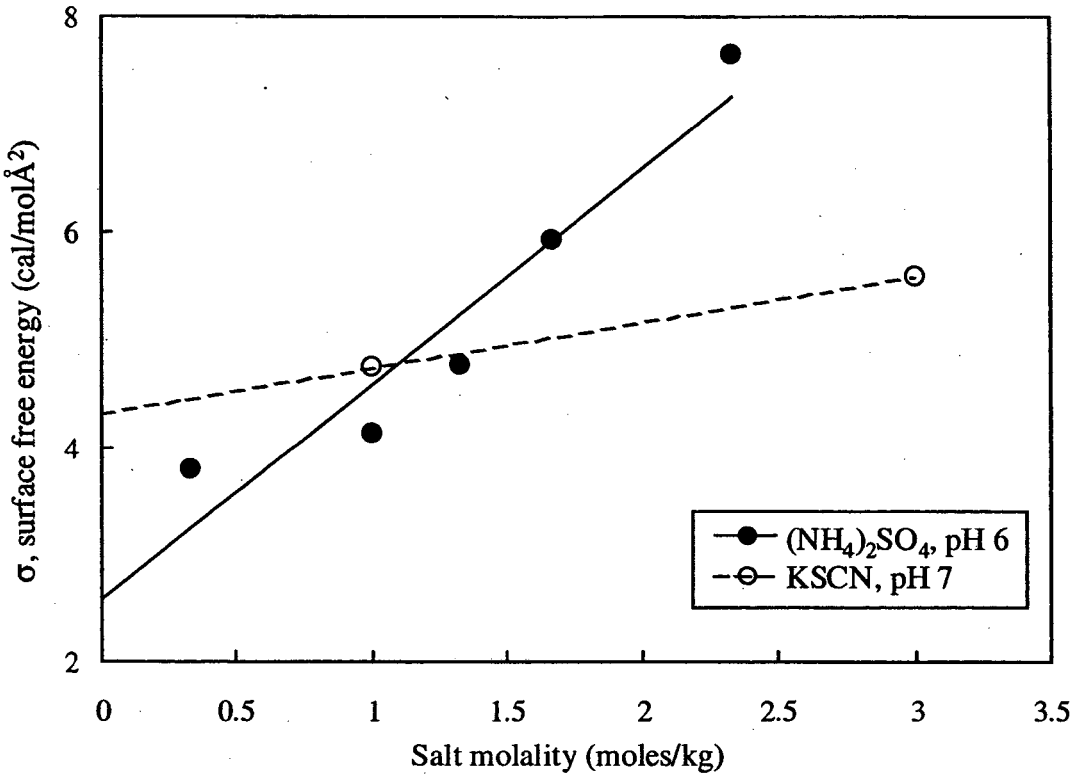
8
6
4
2
σ, surface free energy (cal/molÅ²)
0
0.5
1
1.5
2
2.5
3
3.5
Salt molality (moles/kg)
(NH4)2SO4, pH 6
KSCN, pH 7

Figure 7

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