

# Protein–Protein Interactions in Concentrated Electrolyte Solutions

## Hofmeister-Series Effects

R. A. Curtis,<sup>1</sup> J. Ulrich,<sup>3</sup> A. Montaser,<sup>1</sup> J. M. Prausnitz,<sup>1,2</sup> H. W. Blanch<sup>1\*</sup>

<sup>1</sup>Chemical Engineering Department, University of California, Berkeley, California 94720; telephone: (510) 642-1387; fax: (510) 643-1228; e-mail: blanch@socrates.berkeley.edu

<sup>2</sup>Chemical Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720

<sup>3</sup>Automatic Control Laboratory, Swiss Federal Institute of Technology, ETH-Z, ETL I 22, CH-8092 Zurich, Switzerland

Received 12 November 2000; accepted 4 July 2001

DOI: 10.1002/bit.10342

**Abstract:** Protein–protein interactions were 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. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* 79: 367–380, 2002.

**Keywords:** Hofmeister-series; protein interactions; hydrophobic effect; protein salting-out

## 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; 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-range forces that stabilize protein crystals, such as van der Waals contacts, salt bridges, or hydrogen bonds (Neal et al., 1999; Asthagiri 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

\*Correspondence to: H. W. Blanch

Contract grant sponsors: the Office for Basic Energy Sciences of the U.S. Department of Energy, the National Science Foundation

Contract grant number: CTS-9530793

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 Washbaugh, 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, 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 simple form for the potential of mean force that can be used in protein phase equilibrium models for predicting protein solubility and for determining conditions favorable for protein crystallization. Ion-specific effects are included in the model by relating the protein–protein potential of mean force to the experimentally determinable protein–salt preferential interaction parameter.

## 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:

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

where  $\mu_2^s$  and  $\mu_2^\theta$  are the chemical potential of the protein in the solid phase and the infinite-dilution standard-state chemical potential of the protein, respectively, and  $S_2/S_2^\theta$  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,  $\sigma$ , and the solvent-accessible surface area of the protein molecule  $A$ :

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

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

$$RT \frac{d \ln S_2}{d m_3} = N_{av} A \frac{d \sigma}{d m_3}. \quad (1c)$$

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):

$$A \left( \frac{\partial \sigma}{\partial m_3} \right)_{T, p_o}^o = - \left( \frac{\partial m_3}{\partial m_2} \right)_{T, \mu_1, \mu_3}^\infty \left( \frac{\partial \mu_3}{\partial m_3} \right)_{T, p_o}^o \quad (2)$$

Here,  $m_3$  and  $m_2$  are salt and protein molality and  $\mu_1$  and  $\mu_3$  are the chemical potential of water and salt, respectively. Superscript o denotes that the property is

avorable 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,  $\delta_3$ . (The molal surface tension increment is equal to the derivative of the surface tension of the aqueous salt solution with respect to salt molality.) 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  $\text{SO}_4^{2-} > \text{F}^- > \text{Cl}^- > \text{Br}^- > \text{I}^- > \text{NO}_3^- > \text{SCN}^-$ ; the corresponding series for cations is  $\text{Mg}^{2+} > \text{Na}^+ > \text{K}^+ > \text{Li}^+ > \text{NH}_4^+ > \text{Cs}^+$ . 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 Washbaugh, 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  $[-\text{CH}_2\text{CH}(\text{CO}-\text{NH}_2)-]_n$  (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  $\text{SO}_4^{2-} > \text{SCN}^- > \text{I}^- > \text{Br}^- > \text{Cl}^-$ . 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  $\text{SO}_4^{2-}$ . 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  $-2 \times 10^{-4}$  and  $-8 \times 10^{-4}$  mLmol/g<sup>2</sup>. For  $B_{22}$  more positive than  $-2 \times 10^{-4}$  mLmol/g<sup>2</sup>, the protein-protein attraction is usually not sufficiently strong to form stable protein crystals. For solutions where  $B_{22}$  is more negative than  $-8 \times 10^{-4}$  mLmol/g<sup>2</sup>, 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,  $B'_{22}$  (noting  $B_{22} = N_{av}B'_{22}/M_2^2$ ) is given by the following volume integral:  $\beta_{22}$

$$B'_{22} = \frac{1}{2} \int_0^\infty [1 - e^{\beta W_{22}}] 4\pi r^2 dr. \quad (3)$$

where  $\beta = (k_b T)^{-1}$ ; 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 Eqs. (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-Horvath approach and those models based on McMillan-Mayer solution theory give the same free energy of protein crystallization.

Here we assume that the average sizes 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:

$$W_{\text{solv}}(r) = \begin{cases} -A(r)(f_a\sigma_a + f_p\sigma_p) & \text{for } d_2 < r < d_2 + r_c \\ = 0 & \text{for } r > d_2 + r_c \end{cases} \quad (4)$$

where  $f_a$  and  $f_p$  are the surface fractions of nonpolar and polar groups,  $\sigma_a$  and  $\sigma_p$  are the characteristic surface energies of nonpolar and polar groups in contact with solvent. Because protein crystals are stabilized by highly anisotropic interactions, it is also believed that the contributions to  $B_{22}$  arise from an anisotropic two-body potential of mean force. For instance, Neal et al. (1999) have shown that the potential of mean force in dilute electrolyte solutions is determined by a small percentage of the orientation space available to the protein molecules. When protein molecules are crystallized from concentrated electrolyte solutions, the majority of the surface involved in protein-protein contacts is nonpolar (Iyer et al., 2000). Consequently, we expect that  $B'_{22}$  is given by an orientational average of interactions between clusters of nonpolar groups on the protein surfaces. Equation (4) provides a first approximation for the interaction between nonpolar surfaces. Here we have assumed a form for the potential of mean force that is independent of protein

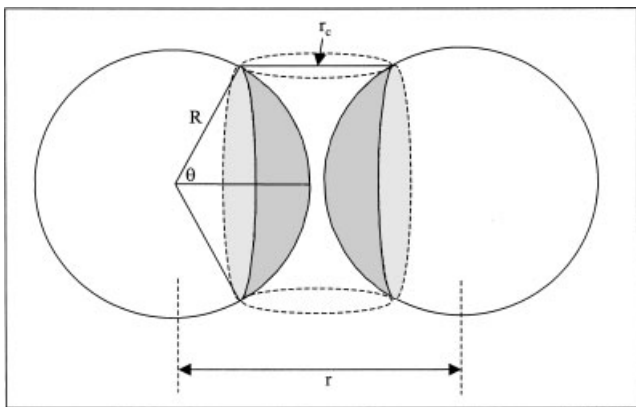
orientation with the advantage that the potential can be used in protein phase equilibrium models, which generally require non-anisotropic potentials.

Based on Eq. (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 ( $\sigma_p$  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  $\sigma_a$  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:

$$A(r) = \pi d_2^2 - \pi r d_2 + \pi r_c d_2 \quad (5)$$

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  $r_c$ . Elcock and McCammon (2001) use a similar potential of mean force for the nonpolar interaction between protein molecules given by the total protein surface area inaccessible to solvent multiplied by a scaling factor fit to  $B_{22}$  data.

In calculating the solvation pmf, it is assumed that 1) the protein surface can be divided into atomic surface groups whose solvation properties are independent of the neighboring groups, and 2) 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



**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 gray. 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  $A(r) = 2 \left[ \int_0^\theta 2^2 \sin \theta' d\theta' \right] = 4\pi R^2 - 2\pi r R + 2\pi r_c R$  where  $R$  is the radius of the protein,  $\theta$  is the solid angle corresponding to the boundary of the spherical cap, and  $\cos \theta = \frac{r-r_c}{2R}$ .

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). There is also evidence that the interaction between surfaces with small hydrophobic patches is different from that with large hydrophobic patches (Lum et al., 1999), where the latter interaction is not additive (Kokkoli and Zukoski, 2001). The crossover between the two types of interactions occurs at a length scale of approximately 1 nm. However, because the additivity approximation has been used successfully for describing intramolecular interactions in protein folding, we expect the approximation is also valid for describing intermolecular interactions between proteins.

Here, we decompose the surface free energy into a hypothetical free energy in salt-free water,  $\sigma_o$ , and a correction term that accounts for the perturbation of the surface free energy from addition of the salt, given by:

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

Because the surface free energy increment,  $(d\sigma/dm_3)$ , is proportional to the negative value of the preferential interaction parameter, it follows from Eq. (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 Eq. (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 Eq. (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 Eq. (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 atmosphere 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 than those with bound chaotropic charges (Israelachvili, 1992). This result follows from the solvation force described by Eq. (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 (Verwey and Overbeek, 1948), proteins are modeled as rigid spheres with uniform surface charge immersed in a continuous dielectric continuum containing point charges depicting salt ions. With these approximations, the potential of mean force is given by

$$W_{\text{DLVO}}(r) = W_{\text{hs}}(r) + W_{\text{elec}}(r) + W_{\text{disp}}(r) \quad (7a)$$

Here,  $W_{\text{hs}}$  is the protein hard-sphere potential,  $W_{\text{elec}}$  is the electric double-layer overlap force, and  $W_{\text{disp}}$  is the Hamaker dispersion potential (Hamaker, 1937).

The simplest form of the hard-sphere potential is given by:

$$W_{\text{hs}}(r) = \begin{cases} \infty & \text{for } r \leq d_2 \\ 0 & \text{for } r > d_2 \end{cases} \quad (7b)$$

where the effective spherical diameter,  $d_2$ , gives the distance of closest approach of two protein molecules. Here  $d_2$  is calculated from the protein crystal structure dimensions to give values of 34.4 Å for lysozyme (Blake et al., 1965) and 54 Å for ovalbumin (Stein et al., 1990). Modeling the proteins as smooth spheres is an approximation which has been investigated by Neal and Lenhoff (1995). They showed that surface roughness may increase the excluded volume of the protein by a factor of 1.7.

The electric double layer force between two similarly charged proteins is repulsive according to DLVO theory. It is given by:

$$W_{\text{elec}}(r) = \frac{z^2 e^2 \exp[-\gamma(r - d_2)]}{4\pi\epsilon_0\epsilon_r r (1 + \gamma d_2/2)^2} \quad \text{for } r > d_2 + 2\kappa \quad (7c)$$

where  $z$  is the charge of the protein,  $e$  is the elementary charge,  $4\pi\epsilon_0$  is the dielectric permittivity of free space in SI units (C/Jm),  $\epsilon_r$  is the dielectric permittivity of water, and  $\gamma$  is the inverse of the Debye length. The approximation that the salt ions behave as point charges is poor at ionic strengths greater than 0.1 molar. However, the electric double-layer repulsion is small at these salt concentrations and the assumption is of little significance in calculating the electrostatic contribution to  $B'_{22}$ .  $\kappa$  is a parameter describing the hydration-layer thickness of the protein that determines the lower limit of integration of the electric double layer and dispersion potential discussed below. The net charge of the protein is determined from titration data. Here, we use titration data of ovalbumin in 1.0 M potassium chloride solution (Cohn, 1943) and of lysozyme in 1.0 M potassium chloride solution (Kuehner et al., 1999). The net charge depends on ion binding to the protein; however, at salt concentrations studied here this effect is screened out.

The attractive Hamaker dispersion interaction is given by (Hamaker, 1937):

$$W_{\text{disp}}(r) = -\frac{H}{12} \left\{ \frac{d_2^2}{r^2 - d_2^2} + \frac{d_2^2}{r^2} + 2 \ln \left( 1 - \frac{d_2^2}{r^2} \right) \right\} \quad (7d)$$

for  $r > d_2 + 2\kappa$

where  $H$  is the effective Hamaker constant that 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  $k_B T$  (Nir, 1976). The hydration-layer thickness,  $\kappa$ , 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  $W_{\text{solv}}(r)$  to represent the short-ranged forces between protein molecules. With this approximation  $B'_{22}$  is given by:

$$B'_{22} = \frac{2}{3} \pi d_2^3 + \frac{1}{2} \int_{d_2}^{d_2+\Gamma_c} (1 - e^{-\beta W_{\text{solv}}}) 4\pi r^2 dr \quad (8)$$

$$+ \frac{1}{2} \int_{d_2+\Gamma_c}^{\infty} (1 - e^{-\beta W_{\text{DLVO}}}) 4\pi r^2 dr$$

$$\frac{Kc_2(\partial n/\partial c_2)_{T,\mu_1,\mu_3}}{\bar{R}_0} = \frac{1}{M_2} + 2B_{22}c_2 \quad (9)$$

where  $r_c$  is chosen to be on the order of a solvent diameter and  $W_{\text{solv}}$  is given by Eq. (6). The arbitrary separation we use has been criticized by Neal et al. (1998), who claim that the dispersion potential and the solvation potential are not independent. For instance, the free energy of displacing solvent between the protein surfaces includes a contribution from the van der Waals interactions between the desolvated atoms. Consequently, we expect that the surface free energy parameter defined by Eq. (4) has energetic contributions from interactions other than the free energy of dehydrating the protein surface.

## 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 pipette. Five to 10 25 mL protein samples were prepared by diluting the 3–5 g/L protein solution. The solutions were dialyzed overnight in a 2-L salt solution at 4°C using a Spectraphor dialysis membrane with a molecular-weight cutoff 5,000–8,000 daltons. Concentrations were measured using a Milton Roy Spectronic 1201 spectrophotometer. The extinction coefficient is 2.63 (l/g · cm) for lysozyme (Sophianopoulos 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):

where  $\bar{R}_0$  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 solution,  $c_2$  is weight concentration of protein, and  $M_2$  is protein molecular weight. Subsequently, a plot of  $Kc_2(\partial n/\partial c_2)_{T,\mu_1,\mu_3}/\bar{R}_0$  vs.  $c_2$  is used to determine  $M_2$  and the osmotic second virial coefficient of the protein,  $B_{22}$ . All measurements of  $B_{22}$  were repeated at least twice. Additional measurements were made if the values differed by greater than  $1.0 \times 10^4$  mLmol/g<sup>2</sup>. Furthermore, if the standard deviation of the slope of the light-scattering data was greater than 1.0 mLmol/g<sup>2</sup>, the measurement was thrown out. For lysozyme, the error in the molecular weight determination corresponding to the standard deviation of the intercept is on the order of 400 g/mol. For ovalbumin, this error is on the order of 1,200 g/mol.

### Determination of Refractive-Index Increment

The difference in refractive index between the sample and the solvent ( $\Delta n$ ) 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:

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

Refractive-index increments for the protein at constant chemical potentials of water and salt were also measured. Here, 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

Table Ia,b shows  $B_{22}$  and  $M_2$  obtained from light-scattering experiments for lysozyme and for ovalbumin in several salt solutions over a range of salt concentrations and pH.

Lysozyme obtained from Sigma (St. Louis, MO) 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

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

Salt molality (molal), pH	$B_{22} \times 10^4$ (mLmol/g <sup>2</sup> )	$M_2$ (kg/mol)
<b>KSCN</b>		
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
<b>KCl</b>		
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
<b>NaCl</b>		
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
<b>(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub></b>		
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

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 the 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. However, the experimental molecular weights for ovalbumin in ammonium-sulfate or potassium-isothiocyanate solutions are in good agreement with the monomer value.

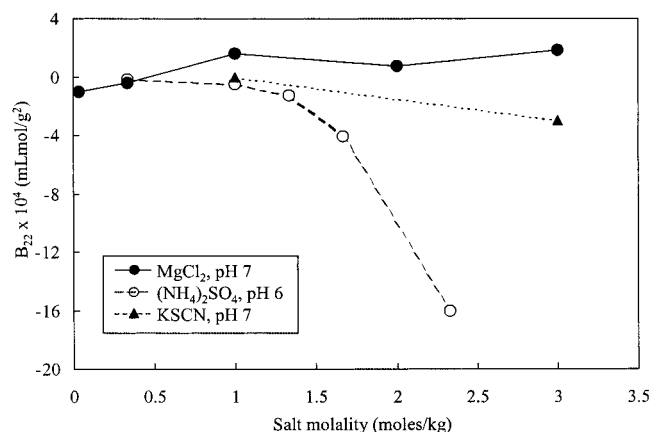
### Ovalbumin–Ovalbumin Interactions

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

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

Salt molality (molal), pH	$B_{22} \times 10^4$ (mLmol/g <sup>2</sup> )	$M_2$ (kg/mol)
<b>MgCl<sub>2</sub></b>		
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
<b>KSCN</b>		
1.0, 8	-1.2	44.3
3.0, 8	-3.0	44.4
<b>(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub></b>		
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	—

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 isothiocyanate.



**Figure 2.** Effect of salt molality on  $B_{22}$  for ovalbumin in magnesium chloride, ammonium sulfate, or potassium isothiocyanate solutions at 25°C.

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) measured the preferential interaction parameters for  $\beta$ -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 semiquantitative agreement with the results of Arakawa et al. (1990).

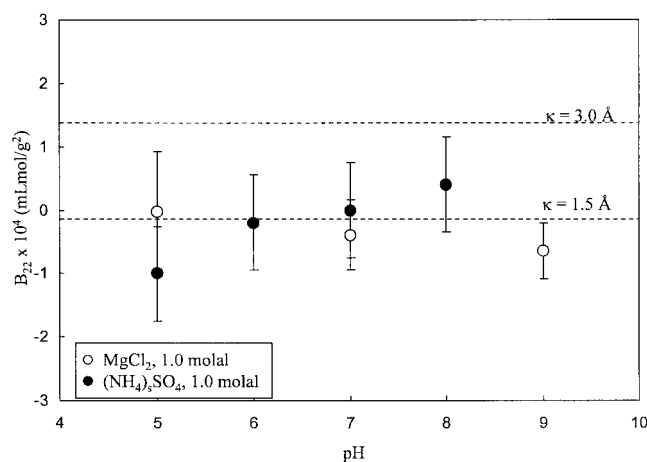
The measured molecular weights for ovalbumin in solutions of magnesium chloride are greater than the monomer value. It is unlikely that the high molecular weights result from protein impurities in the ovalbumin formulation because the same formulation was used for the experiments of ovalbumin in solutions of potassium isothiocyanate, where the measured molecular weights correspond to the monomer value. Furthermore, protein impurities were not observed using SDS-page gel and native gel electrophoresis. Consequently, it is likely that ovalbumin self-aggregates in the presence of magnesium ion. However, this aggregation occurs irreversibly with respect to protein concentration because the measured  $B_{22}$  is always positive. It is possible that magnesium ion stabilizes a partially unfolded state of ovalbumin through specific interactions with the surface exposed during unfolding. The partial unfolding may subsequently result in irreversible aggregation.

Arakawa and Timasheff (1982) found that at pH 5.6 the preferential interactions of  $\beta$ -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 than 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  $\beta$ -lactoglobulin are near the pI of the protein, where the preferential in-

teractions 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 Eqs. (7a–7d) for  $\kappa = 1.5 \text{ \AA}$  and  $3 \text{ \AA}$ . 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  $3 \text{ \AA}$  results in a change of  $1.5 \times 10^{-4} \text{ mLmol/g}^2$  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  $1.0 \times 10^{-4} \text{ mLmol/g}^2$ . 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.



**Figure 3.** Effect of pH on  $B_{22}$  for ovalbumin in magnesium chloride or ammonium sulfate solutions. Dashed lines are predictions of DLVO theory (Eq. [7]) for two values of the thickness of the hydration layer,  $\kappa$ . For this calculation,  $H = 3.0 k_B T$  at  $25^\circ\text{C}$ .

## 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).

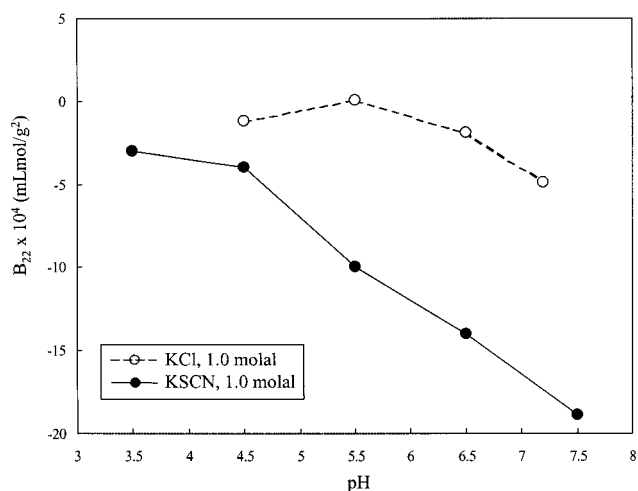
Isothiocyanate ions can be either strong salting-out agents or salting-in agents. Both effects are attributed to the preferential interaction between isothiocyanate ion and the protein surface. The salting-out occurs because of the formation of an insoluble complex between isothiocyanate ion and the 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.

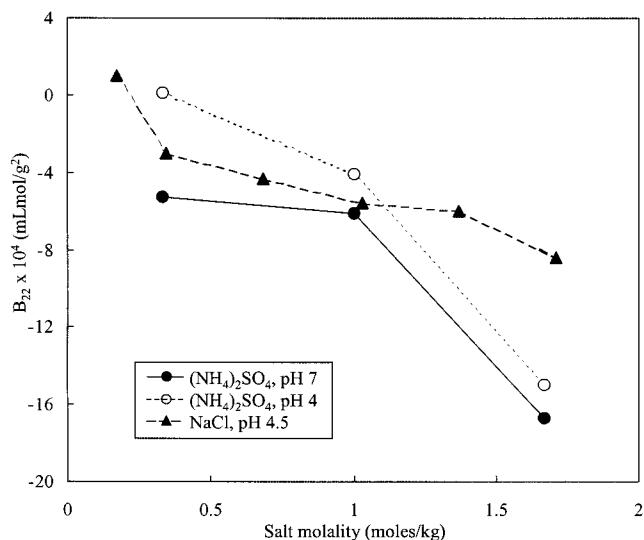
Here, we have identified the strong ion-binding sites as the positively charged residues and the weak-ion binding sites as the exposed peptide groups. However, Chakrabarti (1993) found for many crystal forms that protein crystals contain anions whose locations are adjacent to peptide groups in addition 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. This is in agreement with Moretti et al. (2000), who showed that crystalline lysozyme is less soluble in ammonium sulfate solutions at pH 8 than in solutions of the same ionic strength at pH 4. The increase in repulsion is attributed to sulfate binding to the positively charged residues of lysozyme at low pH (Goto et al., 1980; Curtis et al., 1998); sulfate ion has a strong binding affinity for positively charged residues, as represented by its position in the electroselectivity series (Gjerde et al., 1980). Because sulfate is more kosmo-



**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.

tropic 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 the 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

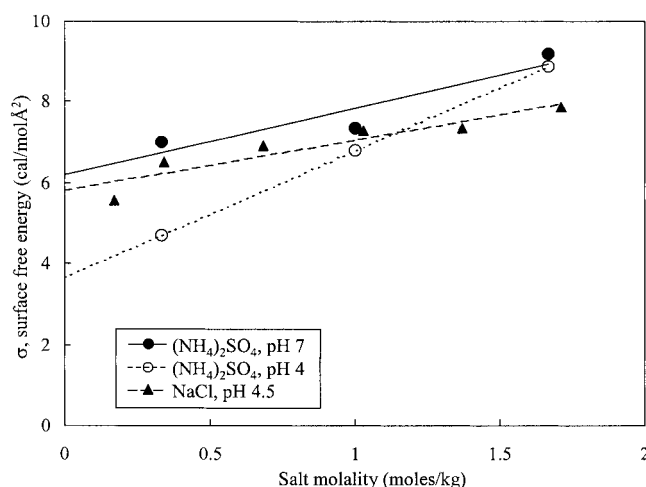
We fit the potential-of-mean-force model described by Eq. [8] by varying the surface free-energy parameter,  $\sigma$ . The Hamaker constant for lysozyme and for ovalbumin is set equal at 3.0  $k_B T$  and the cut-off separation of the

**Table II.** Results of fitting the surface free-energy parameter,  $\sigma$ , to the potential of mean force model described by Eq. (8)  $\sigma_0$  is obtained from extrapolating  $\sigma$  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.

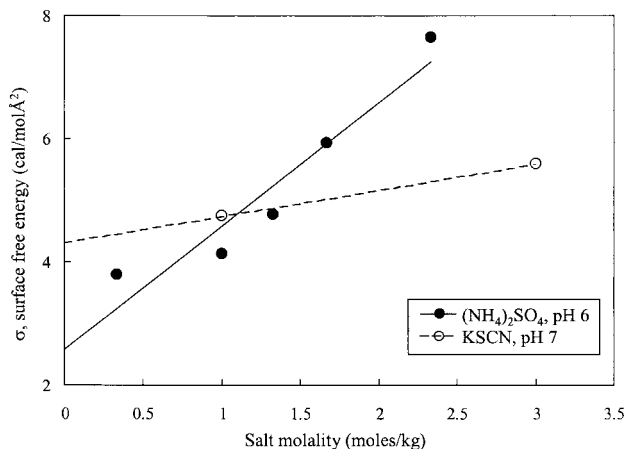
	$\sigma_0$ (cal/mol $\text{\AA}^2$ )	$d\sigma/dm_3$ (cal/mol $\text{\AA}^2$ ) (molal) <sup>-1</sup>	Ratio
Lysozyme			
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , pH 4	3.2	3.1	1.03
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , pH 7	6.2	1.6	0.54
NaCl, pH 4.5	5.8	1.3	0.54
Ovalbumin			
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , pH 6	2.6	2.0	0.65
KSCN	4.3	0.43	0.65

solvation force,  $r_c$  is set at 3  $\text{\AA}$ . 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 II 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  $\sigma$  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,  $\sigma_0$ . These values should be independent of the salt and pH, as observed in sodium chloride and ammonium sulfate solutions at pH 7. However  $\sigma_0$  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



**Figure 6.** Surface free energy of lysozyme determined from fitting  $B_{22}$  to the potential of mean force model described by Eq. (8) with  $H = 3.0 k_B T$ ,  $r_c = 3 \text{\AA}$  at 25°C.



**Figure 7.** Surface free energy of ovalbumin determined from fitting  $B_{22}$  to the potential-of-mean-force model described by Eq. (8), with  $H = 3.0 k_B T$ ,  $r_c = 3 \text{ \AA}$  at  $25^\circ\text{C}$ .

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 Eq. [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 I. 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 unreasonably 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:

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

The nonpolar surface free energy increment is proportional to the molal surface tension increment of the salt,  $\delta_3$ :

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

where the proportionality constant  $\alpha$  is related to the radius of curvature of the surface interface. For small molecules,  $\alpha$  is on the order of  $1/3$ , whereas  $\alpha$  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  $\alpha$  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.

## References

- Arakawa T, Timasheff SN. 1982. Preferential interactions of proteins with salts in concentrated solutions. *Biochemistry* 21:6545–6552.
- Arakawa T, Timasheff SN. 1985. Theory of protein solubility. *Meth Enzymol* 114:49–74.
- Arakawa T, Bhat R, Timasheff SN. 1990. Preferential interactions determine protein solubility in three-component solutions: the MgCl<sub>2</sub> system. *Biochemistry* 29:1914–1923.
- Asthagiri D, Neal BL, Lenhoff AM. 1999. Calculation of short-range interactions between proteins. *Biophys Chem* 78:219–231.
- Ben-Naim A. 1978. Standard thermodynamics of transfer, uses and misuses. *J Phys Chem* 82:792–803.
- Blake CC, Koenig DF, Mair GA, North ACT, Phillips DC, Sarma VR. 1965. Structure of hen-egg white lysozyme. *Nature* 206:757–770.
- Casassa EF, Eisenberg H. 1964. Thermodynamic analysis of multi-component solutions. *Adv Protein Chem* 19:287–393.
- Chakrabarti A. 1993. Anion binding sites in protein structures. *J Mol Biol* 234:463–482.
- Chalikian TV, Sarvazyan AP, Breslauer KJ. 1994. Hydration and partial compressibility of biological compounds. *Biophys Chem* 51:89–109.
- Chiew Y, Kuehner D, Blanch H, Prausnitz J. 1995. Molecular thermodynamics for salt-induced protein precipitation. *AIChE J* 41:2150–2159.
- Coen CJ, Blanch HW, Prausnitz JM. 1995. Salting-out of aqueous proteins: phase equilibria and intermolecular potentials. *AIChE J* 41:996–1004.
- Cohn EJ. 1943. The solubility of proteins. In: Cohn EJ, Edsall JT, editors. *Proteins, amino acids, and peptides*. New York: Reinhold. p 586–622.
- Collins KD, Washabaugh MW. 1985. The Hofmeister effect and the behavior of water at interfaces. *Q Rev Biophys* 18:323–421.
- Connolly M. 1993. The molecular surface package. *J Mol Graph* 11:139–143.
- Cunningham JC, Muenke BJ. 1959. Physical and chemical studies of a limited reaction of iodine with proteins. *J Biol Chem* 234:1447–1451.
- Curtis RA, Montaser A, Prausnitz JM, Blanch HW. 1998. Protein–protein and protein–salt interactions in aqueous protein solutions containing concentrated electrolytes. *Biotechnol Bioeng* 57:11–21.
- Eberstein W, Georgalis Y, Saenger W. 1994. Molecular interactions in crystallizing lysozyme solutions studied by photon correlation spectroscopy. *J Cryst Growth* 143:71–78.
- Eisenberg D, McLachlan AD. 1986. Solvation energy in protein folding and binding. *Nature* 319:199–203.
- Eisenhaber F. 1996. Hydrophobic regions on protein surfaces, derivation of the solvation energy from their area distribution in crystallographic protein surfaces. *Protein Sci* 5:1676–1686.
- Elcock AH, McCammon JA. 2001. Calculation of weak protein–protein interactions: the pH dependence of the second virial coefficient. *Biophys J* 80:613–625.
- Fornaseiro F, Ulrich J, Prausnitz JM. 1999. Molecular thermodynamics of precipitation. *Chem Eng Proc* 38:463–475.
- George A, Wilson WW. 1994. Predicting protein crystallization from a dilute solution property. *Acta Crystallogr D* 51:361–365.
- Gjerde DT, Schmuckler G, Fritz JS. 1980. Anion chromatography with low conductivity eluents II. *J Chrom* 187:35–45.
- Goto Y, Takahashi N, Fink AL. 1990. Mechanism of acid-induced folding of proteins. *Biochemistry* 29:3480–3488.
- Guo B, Kao S, McDonald H, Asanov A, Combs LL, Wilson WW. 1999. Correlation of second virial coefficients and solubilities useful in protein crystal growth. *J Cryst Growth* 196:424–433.
- Hamaker HC. 1937. The London van-der-Waals attraction between spherical particles. *Physica* 4:1058–1072.
- Haynes CA, Benítez FJ, Blanch HW, Prausnitz JM. 1993. Application of integral-equation theory to aqueous two-phase partitioning systems. *AIChE J* 39:1539–1557.
- Hermann RB. 1972. Theory of hydrophobic bonding. II. The correlation of hydrocarbon solubility in water with solvent cavity surface area. *J Phys Chem* 76:2754–2759.
- Hofmeister F. 1888. *Zur Lehre von der Wirkung der Salze*. *Arch Exp Pathol Pharmacol* 24:247–260.
- Israelachvili J. 1992. *Intermolecular and surface forces: with applications to colloidal and biological systems*, 2nd ed. London: Academic Press.
- Iyer GH, Dasgupta S, Bell JA. 2000. Ionic strength and intermolecular contacts in protein crystals. *J Cryst Growth* 217:429–440.
- Kokkoli E, Zukoski CF. 2001. Surface pattern recognition by a colloidal particle. *Langmuir* 17:369–376.
- Kuehner DE, Engmann J, Fergg F, Wernick M, Blanch HW, Prausnitz JM. 1999. Lysozyme net charge and ion binding in concentrated aqueous electrolyte solutions. *J Phys Chem B* 103:1368–1374.
- Lee JC, Gekko K, Timasheff SN. 1979. Measurements of preferential solvent interactions by densimetric techniques. *Meth Enzymol* 61:26–49.
- Lum K, Chandler D, Weeks JD. 1999. Hydrophobicity at small and large length scales. *J Phys Chem B* 103:4570–4577.
- Malfois M, Bonnete F, Belloni L, Tardieu A. 1996. A model of attractive interactions to account for fluid–fluid phase separation of protein solutions. *J Chem Phys* 105:3290–3300.
- McMillan WG, Mayer JE. 1945. The statistical thermodynamics of multicomponent systems. *J Chem Phys* 13:276–305.
- Melander W, Horvath C. 1977. Salt effects on hydrophobic interactions in precipitation and chromatography of proteins: an interpretation of the lyotropic series. *Arch Biochem Biophys* 183:200–215.
- Moretti JJ, Sandler SI, Lenhoff AM. 2000. Phase equilibria in the lysozyme–ammonium sulfate–water system. *Biotechnol Bioeng* 70:498–506.
- Muschol M, Rosenberger F. 1995. Interactions in undersaturated and supersaturated lysozyme solutions: static and dynamic light scattering results. *J Chem Phys* 103:10424–10432.
- Nandi PK, Robinson DR. 1972. The effects of salts on the free energy of the peptide group. *J Am Chem Soc* 94:1299–1307.
- Neal BL, Lenhoff AM. 1995. Excluded volume contribution to the osmotic second virial coefficient for proteins. *AIChE J* 41:1010–1014.

- Neal BL, Asthagiri D, Velev OD, Lenhoff AM, Kaler EW. 1999. Why is the osmotic second virial coefficient related to protein crystallization. *J Cryst Growth* 196:377–387.
- Nir S. 1976. Van-der-Waals interactions between surfaces of biological interest. *Prog Surf Sci* 8:1–58.
- Piazza R. 1999. Interactions in protein solutions near crystallization: a colloid physics approach. *J Cryst Growth* 196:415–423.
- Poon WCK. 1997. Crystallization of globular proteins. *Phys Rev E* 55:3762–3764.
- Ries-Kautt MA, Ducruix AF. 1989. Relative effectiveness of various ions on the solubility and crystal growth of proteins. *J Biol Chem* 264:745–750.
- Robinson DR, Jencks WP. 1965. The effect of concentrated salt solutions on the activity coefficient of acetyltetraglycine ethyl ester. *J Am Chem Soc* 87:2470–2479.
- Rosenbaum D, Zamora PC, Zukoski CF. 1996. Phase behavior of small attractive colloidal particles. *Phys Rev Lett* 76:150–153.
- Rothstein F. 1994. Differential precipitation of proteins. In: Harrison RG, editor. *Protein purification process engineering*. New York: Marcel Dekker. p 115–208.
- Skouri M, Lorber B, Giege R, Munch JP, Candau JS. 1995. Effect of macromolecular impurities on lysozyme solubility and crystallizability: dynamic light scattering, phase diagram, and crystal growth studies. *J Cryst Growth* 152:209–220.
- Sophianopoulos AJ, Rhodes CK, Holcomb DN, van Holde KE. 1962. Physical studies of lysozyme. I. Characterization. *J Biol Chem* 237:1107–1112.
- Stein PE, Leslie GW, Finch JT, McLaughlin DJ, Carrell RW. 1990. Crystal structure of ovalbumin as a model for the reactive centre of serpins. *Nature* 347:99–102.
- Stockmayer WH. 1950. Light scattering in multi-component solutions. *J Chem Phys* 18:58–61.
- Verwey EJW, Overbeek JTK. 1948. *Theory of stability of lyophobic colloids*. Amsterdam: Elsevier.
- Vilker VL, Colton CK, Smith KA. 1981. Osmotic pressure of concentrated protein solutions: the effect of concentration and pH in saline solutions of bovine serum albumin. *J Colloid Int Sci* 79:548–566.
- von Hippel PH, Schleich T. 1969. Ion effects on the solution structure of biological macromolecules. *Acc Chem Res* 2:257–265.
- Wang H, Ben-Naim A. 1997. Solvation and solubility of globular proteins. *J Phys Chem B* 101:1077–1086.