Interactions of Proteins in Aqueous Ammonium-Sulfate Solutions: Mixtures of Lysozyme and Ovalbumin

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Abstract

Fluorescence anisotropy was used to determine lysozyme-ovalbumin interactions in 20-mM sodium-phosphate buffer at pH 7 and 25°C over an ammonium-sulfate ionic-strength range 0.0 to 7.5 M. At pH 7, the net charge on lysozyme is +8 while that on ovalbumin is –12. The association constant calculated from fluorescence-anisotropy data in salt-free buffer solution was 1.0×10^5 M^{-1}, in agreement with literature values. The lysozyme-ovalbumin potential of mean force (PMF) was calculated from these association constants and compared to the PMF for lysozyme-lysozyme and ovalbumin-ovalbumin interactions. The lysozyme-ovalbumin interaction is more attractive than the self-interactions in buffer solution. In salt-free buffer solutions, association is governed by attractive electrostatic interactions between lysozyme and

ovalbumin. The lysozyme-ovalbumin PMF decreases at intermediate ionic strength due to screening of electrostatic attraction. The cross-interaction is intermediate between the two self-interactions at these moderate ionic strengths. At high ionic strength, the cross- and self-interactions are greater because of increasing hydrophobic attraction, and both self-interactions are more attractive than the cross-interaction of lysozyme and ovalbumin.

Introduction

Protein-protein interactions can be selectively enhanced to precipitate target proteins from fermentation processes and to crystallize proteins for characterization by X-ray diffraction. Because of the structural complexity of proteins, it is often difficult rationally to design a protein-purification process. It is useful to develop a molecular-thermodynamic model for predicting optimal conditions for selective precipitation. Generally, phase separation is enhanced when protein-protein interactions are attractive because the free energy of the dense phase decreases due to formation of favorable intermolecular contacts between protein molecules. George and Wilson (1994) have shown that the osmotic second virial coefficient, a measure of the interactions between proteins, can be used as a diagnostic to determine whether crystallization is favored over amorphous precipitation at given solution conditions. Thus, quantitative understanding of protein-protein interactions can provide a first estimate of protein solubility (Curtis et al., 2001).

While much information is available in the literature on protein-protein interactions in single-protein systems, there are few data concerning such interactions in mixtures of proteins. Such information is needed for the prediction of selective precipitation from protein mixtures. Hen egg-white lysozyme and ovalbumin exist together in egg whites. Approximately 4% of hen

egg-white protein is lysozyme and 60% is ovalbumin (Hegg, 1979; Awadé et al., 1994). These two proteins have been studied extensively in connection with the processing of eggs in the food-processing industry (Rossi and Schiraldi, 1992) under conditions (e.g., high temperature) that denature proteins. Judge et al. (1995) crystallized ovalbumin to a purity of 99% from aqueous ammonium sulfate containing a mixture of ovalbumin, conalbumin and lysozyme. Lysozyme has been selectively precipitated from lysozyme-ovalbumin mixtures at high pH and ammonium sulfate ionic strength (Coen et al., 1997). At a mole ratio of 3:1 (lysozyme:ovalbumin), Jeffrey et al. (1979) found a maximum in solution turbidity for low ionic strength near neutral pH. The authors postulated that a complex containing three lysozymes for each ovalbumin would give the lowest solubility. This postulate may correspond to either a 3:1 ratio of associated lysozyme:ovalbumin (leading to a highly insoluble complex), or to an indefinite association of lysozyme and ovalbumin, where four lysozymes are coordinated with each ovalbumin and lysozyme can bind to two ovalbumin molecules.

Fluorescence anisotropy and ultracentrifugation results have shown that the equilibrium constant for a 1:1 lysozyme-ovalbumin association ranges from 0.3 to 1.1 $\times$ 10^5 M^{-1} in 20-mM sodium-phosphate buffer at pH 6.8 (Clarke and Howlett, 1979; Nakai and Kason, 1974). Nakai and Kason (1974) showed that the association between lysozyme and ovalbumin was highly dependent on pH and ionic strength up to 0.3 M; this suggests that the association at low ionic strength is primarily driven by the electrostatic interactions of the oppositely charged lysozyme and ovalbumin. These electrostatic attractions are screened at moderate ionic strengths.

In this work, we measure the fluorescence anisotropy as a function of ammonium-sulfate ionic strength for mixtures of lysozyme and ovalbumin at 25^{o}C and pH 7 to obtain the lysozyme-ovalbumin cross association constants. The effective lysozyme-ovalbumin potential of mean

force (PMF) is calculated from these association constants and compared to lysozyme-lysozyme and ovalbumin-ovalbumin PMFs. We find that DLVO (Derjaguin-Landau-Verwey-Overbeek) theory (Verwey and Overbeek, 1948) only captures some of the physics of the intermolecular protein-protein interactions in concentrated salt solutions. Our PMF contains contributions from the DLVO potentials (hard-sphere repulsion, electric double-layer interactions, and dispersion attraction) and from a square-well potential that accounts for short-range attraction such as hydrophobic interactions.

Materials and methods

Materials

Hen egg-white lysozyme (cat# 837 059) was obtained from Boehringer Mannheim GmbH (Germany) and fluorescein-labeled ovalbumin (cat# O-835, lot# 7103-1) was from Molecular Probes, Inc. (Eugene, OR). ACS-grade ammonium sulfate, sodium phosphate monobasic monohydrate, and sodium phosphate dibasic heptahydrate were obtained from Fisher Scientific Company (Fair Lawn, NJ). A Barnstead-Nanopure water-purification system was used to purify water in all experiments.

Sample preparation

Solutions were prepared containing 20-mM sodium-phosphate buffer and the appropriate concentration of ammonium sulfate at pH 7. The salt solution was filtered with 0.1-μm Millex syringe filters (cat# SLVV 025 LS) from Millipore Corporation (Bedford, MA) to remove particulates. Lysozyme solutions were prepared in the filtered salt solutions at a concentration of 3 mM and filtered with 0.2-μm Acrodisc syringe filters (cat # 4652) from Pall Corporation (Ann

Arbor, MI) to remove any precipitates. Because lysozyme and ovalbumin do not have significant intrinsic fluorescence, we use ovalbumin labeled with fluorescein, an extrinsic fluorophore. Labeled ovalbumin was dissolved in filtered salt solutions at a concentration of 4 μM and filtered with 0.02-μm Anotop 10 syringe filters (cat# 6809 1002) from Whatman International Ltd. (England). The pH was adjusted to 7 using ammonium hydroxide and sulfuric acid of the same ionic strength as that of the protein solution.

The total concentration of lysozyme, $[L]_T$, was measured using a Beckman DU-6 Spectrophotometer (Beckman Instruments Inc.) at 280 nm. The extinction coefficient is 2.635 L-g^{-1}-cm^{-1} (Sophianopoulos et al., 1962). The total concentration of labeled ovalbumin, $[O]_T$, was determined from the absorbance at 497 nm, the wavelength of maximum absorbance for fluorescein conjugated to ovalbumin. There are approximately three fluorescein molecules conjugated to each ovalbumin molecule. Thus, $[O]_T$ is calculated by:

$$[O]_T = \frac{A_{497}}{3 \cdot \varepsilon_F \cdot l} \qquad (1)$$

where A_{497} is the absorbance at 497 nm, l is the pathlength of the cuvette, and ε_F = 68,000 M^{-1}-cm^{-1} is the extinction coefficient of the fluorescein label (personal communication, Molecular Probes, Inc.). Lysozyme solutions were diluted with the appropriate salt solution to obtain twenty 1-ml samples ranging from 1 nM to 3 mM. Ovalbumin solutions were diluted with the corresponding salt solution to a concentration of approximately 6 nM; 100 μL was added to each of the lysozyme solutions. This final ovalbumin concentration of approximately 0.6 nM was used to ensure that there was sufficient fluorescence signal for measurement (total intensity between 50 and 200), while minimizing fluorescence transfer. The concentration of ovalbumin was kept

constant for all measurements as confirmed by measuring the total fluorescence intensity of the fluorescein-ovalbumin conjugate (see Fig. 1).

Fluorescence-anisotropy measurements

In the fluorescence-anisotropy technique, a fluorophore is excited with vertically polarized light at a given wavelength (Bentley et al., 1985; Lakowicz, 1999). When the polarized light impinges on the sample, it can be depolarized in several ways; here, we focus on depolarization by rotation of ovalbumin molecules. The fluorescence intensity of light emitted from the fluorophore is measured at a higher wavelength in two directions: parallel to and perpendicular to the direction of polarization of the incident light. The difference gives a measure of depolarization. The anisotropy is defined by (Perrin, 1926):

$$A = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}} \tag{2}$$

where I_{II} is the intensity of emitted light measured in the direction parallel to excitation and $I_{\perp}$ is the intensity of light measured in the direction perpendicular to excitation. The sum in the denominator is the total fluorescence intensity.

With fluorescence anisotropy, changes in the association state of molecules are reflected by changes in rotational Brownian motion. The anisotropy depends on temperature, viscosity, the fluorescence lifetime of the fluorophore, and the molar volume of the peptide (Lundblad et al., 1996). Association of molecules leads to an increase in effective molar volume. This increase results in slower rotation of the molecules, as well as a higher intensity of light emitted in the direction parallel to the incident light. Thus, anisotropy increases with the degree of association.

Fluorescence anisotropy was measured at 25°C on a Beacon® 2000 Fluorescence Polarization System from Panvera Corporation using filters for fluorescein with maximum transmissions at 488 ± 2 nm and 535 ± 3 nm. Because the solution viscosity increases with rising ammonium-sulfate ionic strength (Wolf et. al., 1986), the anisotropy was corrected as described previously (Anderson et al., 2000).

Association constant

The association constant for the lysozyme-ovalbumin pair is determined by measuring the anisotropy of fluorescein-labeled ovalbumin (*O*) as a function of lysozyme (*L*) concentration. The concentration of ovalbumin is sufficiently low (~0.6 nM) compared to that of lysozyme (~1 nM to 3 mM) such that ovalbumin self-association can be neglected. Thus, ovalbumin is only involved in cross-interactions with lysozyme. Because lysozyme-lysozyme interactions affect the cross-interactions, it would be computationally intensive to compute the ovalbumin-lysozyme association constant using the indefinite-association model of Jeffrey et al. (1979). We use instead the alternate model of a 3:1 ratio for the lysozyme:ovalbumin association. Assuming that all three lysozyme molecules bind to ovalbumin with the same energy:

$$\begin{aligned} O + L &\overset{K_{OL}}{\Leftrightarrow} OL \\ OL + L &\overset{K_{OL}}{\Leftrightarrow} OL_2 \\ OL_2 + L &\overset{K_{OL}}{\Leftrightarrow} OL_3 . \end{aligned} \tag{3}$$

Self-association of lysozyme is described by:

$$L + L \overset{K_{LL}}{\Leftrightarrow} L_2 . \tag{4}$$

The association constant for lysozyme-lysozyme interactions is calculated from osmotic second virial coefficients of lysozyme at the appropriate solution conditions, obtained from Curtis et al. (1998, 2000).

At equilibrium the association constants in Eqs. 3 and 4 are given by:

$$K_{OL} = \frac{[OL]}{[O][L]} = \frac{[OL_2]}{[OL][L]} = \frac{[OL_3]}{[OL_2][L]}$$

$$K_{LL} = \frac{[L_2]}{[L]^2}. \tag{5}$$

The measured anisotropy is the sum of the individual anisotropies of labeled species in the system (Weber, 1952). Since only ovalbumin molecules are labeled, the total anisotropy is:

$$A = \frac{[O]}{[O]_T} A_O + \frac{[OL]}{[O]_T} A_{OL} + \frac{[OL_2]}{[O]_T} A_{OL_2} + \frac{[OL_3]}{[O]_T} A_{OL_3} \tag{6}$$

where A_i is the anisotropy of species i and $[O]_T$ is the total concentration of ovalbumin. The mass balance equations for this system are:

$$[O]_T = [O] + [OL] + [OL_2] + [OL_3] \tag{7}$$

$$[L]_T = [L] + 2[L_2] + [OL] + 2[OL_2] + 3[OL_3] \tag{8}$$

where $[L]_T$ is the total concentration of lysozyme. Combination of Eqs. 5-8 leads to two coupled equations:

$$A = \frac{A_O + K_{OL}[L]A_{OL} + K_{OL}^2[L]^2 A_{OL_2} + K_{OL}^3[L]^3 A_{OL_3}}{1 + K_{OL}[L] + K_{OL}^2[L]^2 + K_{OL}^3[L]^3} \tag{9}$$

$$[L]_T = [L] + 2K_{LL}[L]^2 + [O]_T \left\{ \frac{K_{OL}[L] + 2K_{OL}^2[L]^2 + 3K_{OL}^3[L]^3}{1 + K_{OL}[L] + K_{OL}^2[L]^2 + K_{OL}^3[L]^3} \right\}. \tag{10}$$

$[O]_T$ and $[L]_T$ are measured spectroscopically. The anisotropies of the various species are obtained from a plot of experimental anisotropy versus total lysozyme concentration, $[L]_T$, as shown in Fig. 1. K_{LL} is calculated as described in the *Results* section. Equations 9 and 10 are solved simultaneously to obtain the association constant for the ovalbumin-lysozyme interaction, K_{OL}.

Potential of mean force

The association constant can be related to the potential of mean force, $W_{ij}(r)$, defined such that its negative derivative with respect to distance is the force between two solute molecules, *i* and *j*, at infinite dilution, averaged over all configurations of the solvent molecules (McMillan and Mayer, 1945). For a spherically-symmetric potential of mean force, the association constant is given by the volume integral (Chandler, 1987):

$$K_{ij} = \frac{1}{s_{ij}} \int_{\sigma_{ij}}^{r_{ij,c}} \exp\left[-W_{ij}(r)/kT\right] \cdot 4\pi r^2 dr \qquad (11)$$

where *k* is Boltzmann's constant, *T* is absolute temperature, and s_{ij} is the symmetry number for the interacting pair (s_{ij} = 1 when $i = j$, and s_{ij} = 2 when $i \neq j$). For interactions of a single globular protein, diameter σ_{ii} is determined from crystal-structure dimensions obtained by X-ray diffraction. Cross-interactions between dissimilar proteins *i* and *j* are determined from the arithmetic mean of σ_{ii} and σ_{jj}, denoted as the diameter σ_{ij}. The cutoff distance $r_{ij,c}$, described below, refers to the specific-interaction potential.

The potential of mean force is given as the sum of four spherically symmetric pairwise potentials:

$$W_{ij}(r) = W_{ij}^{hs}(r) + W_{ij}^{elec}(r) + W_{ij}^{disp}(r) + W_{ij}^{spec}(r) \tag{12}$$

where $W_{ij}^{hs}(r)$ is the hard-sphere potential, $W_{ij}^{elec}(r)$ is the electric double-layer potential, and $W_{ij}^{disp}(r)$ is the dispersion potential. $W_{ij}^{spec}(r)$ is a site-specific square-well potential that accounts for intermolecular attraction due to hydrophobic forces, hydrogen-bond formation, or short-range attractive electrostatic forces (Curtis et al., 1998). The first three terms of Eq. 12 comprise the potential of mean force in Derjaguin-Landau-Verwey-Overbeek (DLVO) theory (Verwey and Overbeek, 1948). The DLVO potentials are given in Anderson et al. (2000) where σ is replaced with σ_{ij} and z^2 is replaced with $z_i z_j$. (z_i is the net charge on protein *i*.)

In concentrated salt solutions, interactions between proteins are more attractive due to enhancement of short-range attraction between apolar surface groups. These hydrophobic interactions are incorporated through the specific-interaction potential, given here by the square-well model:

$$W_{ij}^{spec}(r) = \begin{cases} -\varepsilon_{ij}^{spec} & for \quad \sigma_{ij} < r < r_{ij,c} \\ 0 & for \quad r > r_{ij,c} \end{cases} \tag{13}$$

where $r_{ij,c}$ is the cutoff distance of this attractive potential. Ten Wolde and Frenkel (1997) have shown that the range of attraction between protein molecules must be set at less than 25% of the protein diameter to approximate the experimental phase diagram of aqueous proteins. Here, $r_{ij,c}$ is set equal to $1.2\sigma_{ij}$. In this work, the depth of the square well for the cross-interaction, ε_{OL}^{spec}, is a fitting parameter calculated from experimental values of K_{OL}.

Results

Fluorescence anisotropy

Anisotropy was measured in 20-mM sodium-phosphate buffer at pH 7 and 25°C as a function of ammonium-sulfate ionic strength. Figs. 2 and 3 show the anisotropy as a function of ionic strength. In salt-free buffer solution (0.0-M ammonium sulfate, 20-mM sodium phosphate), the anisotropy increases sharply with rising lysozyme concentration, indicating that the cross-interaction is highly attractive. At intermediate ionic strengths, the anisotropy is a weaker function of lysozyme concentration because there is less attraction between lysozyme and ovalbumin. However, at high ionic strengths, the anisotropy is again strongly dependent on the total concentration of lysozyme due to increasing attraction between the two proteins.

Association constant and potential of mean force

The lysozyme-lysozyme association constant, K_{LL}, was calculated from the experimental osmotic second virial coefficients for lysozyme, B_{LL}, measured by Curtis and coworkers (1998, 2000). Well depths for lysozyme-lysozyme interactions, ε_{LL}^{spec}, are obtained from Eqs. 12 and 13 using:

$$B_{LL} = -\frac{N_A}{2M_L^2}\int_0^\infty \left[e^{-W_{LL}(r)/kT} - 1\right]\cdot 4\pi r^2 dr \,. \qquad (14)$$

The protein net charge is estimated from hydrogen-ion titration data. In 1.0-M potassium-chloride solution at pH 7, the net charge is +8 for lysozyme (Kuehner et al., 1999). The molecular weight for lysozyme, M_L, is 14,300. The effective spherical diameter of lysozyme can be calculated from crystal-structure dimensions. Lysozyme has dimensions of 45 X 30 X 30 Å (Blake et al., 1965) giving an effective spherical diameter of 34.4 Å. The Hamaker constant for all interactions was assumed to be 5 *kT*. For those ionic strengths where no virial coefficient

data are available, ε_{LL}^{spec} values are extrapolated. The ε_{LL}^{spec} value in salt-free buffer, however, was calculated from a maximum possible K_{LL} of 286 M^{-1}, measured by Jeffrey et al. (1979) in pH 6.8, 20-mM sodium-phosphate buffer. K_{LL}, shown in Fig. 4, is then computed using Eq. 11.

Association constants for the ovalbumin-lysozyme interaction, K_{OL}, are calculated using Eqs. 9 and 10, assuming a 3:1 (lysozyme:ovalbumin) binding ratio. Parameters A_O, A_{OL}, A_{OL_2}, and A_{OL_3} are estimated by plotting the experimental anisotropy, A, versus $[L]_T$ as shown in Fig. 1. The best-fit curves are shown in Figs. 2 and 3. Fig. 4 shows K_{OL} as a function of ammonium-sulfate ionic strength. The association constant in salt-free buffer solution, 1.0×10^5 M^{-1}, is not shown, as it is two orders of magnitude larger than that at higher ionic strength. (When a 1:1 binding ratio is used for the salt-free buffer case, the same K_{OL} is obtained.)

The potential of mean force for ovalbumin-lysozyme interactions is calculated using Eqs. 11-13. The net charge on ovalbumin is –12 at pH 7 (Edsall, 1943). The molecular weight is 45,000. The crystal structure dimensions, 70 X 45 X 50 Å (Stein et al., 1990) yield an effective spherical diameter of 50 Å for ovalbumin. Fitted values of the well depths for the cross-interactions are shown in Fig. 5. In salt-free buffer solutions, the well depth ε_{OL}^{spec} was estimated to be 3.3 *kT*. When the ionic strength of ammonium sulfate was raised to 0.5 M, the well depth estimate was 1.1 *kT* and remained constant at ammonium-sulfate ionic strengths up to 5.3 M. It increased to 2.5 *kT* in the ionic-strength interval 5.3 to 6.8 M. The well depth decreased again as the ionic strength rises to 7.5 M.

Fitted well depths for ovalbumin-ovalbumin interactions, ε_{OO}^{spec}, were calculated in the same manner as that described for ε_{LL}^{spec}. Osmotic second virial coefficients at pH 7 were not available in the literature for high ammonium-sulfate ionic strengths. However, Curtis et al.

(1998) have shown that B_{OO} is essentially independent of pH at 1.0-M ionic strength. Thus, we use the pH 6 virial-coefficient data of Curtis et al. (1998) to calculate ε_{OO}^{spec}. For those ammonium-sulfate ionic strengths where no virial-coefficient data are available, ε_{OO}^{spec} is interpolated or extrapolated. Well depths for the ovalbumin-ovalbumin interactions, ε_{OO}^{spec} are compared to ε_{OL}^{spec} and ε_{LL}^{spec} in Fig. 5.

Discussion

Nature of interactions

Results for K_{LL} at ionic strengths in the range 0.5 to 5.0 M compare favorably with literature values. Banerjee et al. (1975) found a monomer-dimer association constant for lysozyme of 345 M^{-1} in 0.1-M sodium-chloride solution at pH 7 and 30°C. This association constant is somewhat higher than those obtained here. However, because lysozyme has been shown to follow the reverse lyotropic series, we expect its intermolecular interactions in sodium-chloride solutions to be more attractive than those in ammonium-sulfate solutions. The higher K_{LL} reported by Banerjee and coworkers may also be due to a temperature dependence of the protein-protein interactions. This temperature dependence suggests that, in moderate to high concentrations of salt, the interactions are affected by hydrophobic attraction (Claesson et al., 1986).

The solvent-accessible surface area of the hydrophobic residues for lysozyme was calculated using the Brookhaven Protein Data Bank (pdb) file 1LYZ in conjunction with the Molecular Surface Package described by Connolly (1993). Of the surface of lysozyme, 23% is covered with hydrophobic residues (Gly, Ala, Val, Leu, Ile, Phe, Trp). As the ionic strength of

ammonium sulfate increases, the surface tension of water rises, leading to unfavorable interactions between water and the exposed hydrophobic surfaces (Melander and Horváth, 1977). Thus, attractive interactions are enhanced between apolar groups on the protein surfaces.

Fig. 5 shows that when no ammonium sulfate is present, ε_{LL}^{spec} and ε_{OL}^{spec} are unusually large. It is evident that DLVO theory does not capture the physics of these interactions. In this work, additional attraction is attributed to hydrophobic interactions. However, the cross-interaction is essentially independent of temperature at this low ionic strength (Nakai and Kason, 1974). Because hydrophobic attraction increases with temperature (Claesson et al., 1986), hydrophobic interactions are not indicated at these solution conditions. Therefore, it is likely that other short-range forces cause this strong attraction. There may be significant dipole-dipole and charge-dipole interactions that are not accounted for in our PMF model.

At intermediate ionic strengths (0.5 to 3.0 M), the well depth for the cross-interaction is intermediate between those of the two self-interactions. This well depth is essentially given by the geometric mean of ε_{LL}^{spec} and ε_{OO}^{spec}. Under these solution conditions, the cross-interaction appears to be related to a protein surface with averaged properties. Similar behavior has been observed for bovine serum albumin (BSA)-lysozyme interactions at moderate ionic strengths (Moon et al., 2000). Specific interactions in this ionic-strength region may be due to hydrophobic interactions. The solvent-accessible hydrophobic surface area of ovalbumin, calculated using the pdb file 1OVA and the Molecular Surface Package of Connolly (1993), is 19%. This is comparable to the hydrophobic surface coverage of lysozyme.

In highly concentrated salt solutions, the cross-interaction due to short-range forces is less attractive than the corresponding self-interactions. Thus, it appears that there may be additional short-range forces at high ionic strength that are not accounted for in our PMF model.

For example, Banerjee et al. (1975) determined that, in 0.1 M sodium-chloride solution at pH 7, lysozyme shows considerable head-to-tail self-association at residues Glu-35 and Trp-62. This interaction would be screened at intermediate ionic strengths, but may become important at high ionic strength. This increase in lysozyme-lysozyme attraction would result in the observed diminished association of lysozyme-ovalbumin pairs.

Accuracy of measurements

The accuracy of the ovalbumin-lysozyme association constant decreases with rising ammonium-sulfate ionic strength due to a sharp decrease in lysozyme solubility. For accurate measurement of K_{OL} = 1070 M^{-1}, the concentration of lysozyme should be varied from 47 μM to 4.7 mM (Burke et al., 1996). However, the solubility of lysozyme is only 37 μM in 7.5-M ammonium-sulfate solution. Accuracy may also be reduced because of the extrapolation of ε_{LL}^{spec} from virial-coefficient data in the range 1.0 to 5.0 M ammonium sulfate. Accuracy would be improved if ε_{LL}^{spec} were obtained from virial coefficients at the appropriate ionic strength. Measuring the virial coefficient at very high ionic strength may prove difficult, however, because of low lysozyme solubility: static light-scattering results are generally obtained with lysozyme concentrations near 0.3 mM.

Some error may be introduced by assuming a 3:1 (lysozyme:ovalbumin) binding ratio rather than the indefinite-association model suggested by Jeffrey et al. (1979). Our assumption may explain the shape of the anisotropy versus $[L]_T$ curve in buffer solution. The calculated anisotropy rises more sharply with concentration than does the experimental anisotropy. Jeffrey and coworkers (1979) showed that the assumption of a 3:1 cross association leads to an insoluble complex, implying a highly irreversible association. Such irreversible association would lead to

the behavior seen in our calculated curve. Because our model does not accurately describe the solution behavior of this protein mixture, there may be a discrepancy between the experimental and calculated anisotropy.

As the ionic strength rises, the anisotropy of free ovalbumin, A_O, decreases. This lower anisotropy implies that the free ovalbumin molecule rotates more rapidly at these high salt concentrations. Faster rotation may result from a salt-induced stabilization of the molecule, leading to a more compact shape. Kosmotropic anions such as sulfate have been shown to stabilize the structure of proteins and to aid in the folding of proteins and peptides (Collins and Washabaugh, 1985; Dennison and Lovrien, 1997; Jelesarov et al., 1998).

Conclusions

This work describes interactions between lysozyme-lysozyme, ovalbumin-ovalbumin, and lysozyme-ovalbumin pairs in aqueous solution. In buffer solutions, we observe significant attractive interactions beyond those from DLVO theory. The cross-protein interaction becomes intermediate between the corresponding protein self-interactions at moderate ionic strengths due to screening of electrostatic attraction. Hydrophobic interactions are important at these solution conditions. At high ionic strength, these hydrophobic interactions are enhanced, leading to larger well depths in the potential of mean force for all three pairs. These solution conditions result in a cross-interaction that is less attractive than those for the self-interactions, contrary to the result expected if the specific interaction were due solely to hydrophobic attraction. Additional short-range interactions contribute to the overall potential of mean force. These additional forces must be better understood to develop an accurate representation of protein-protein interactions in concentrated electrolyte solution.

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Figure legends

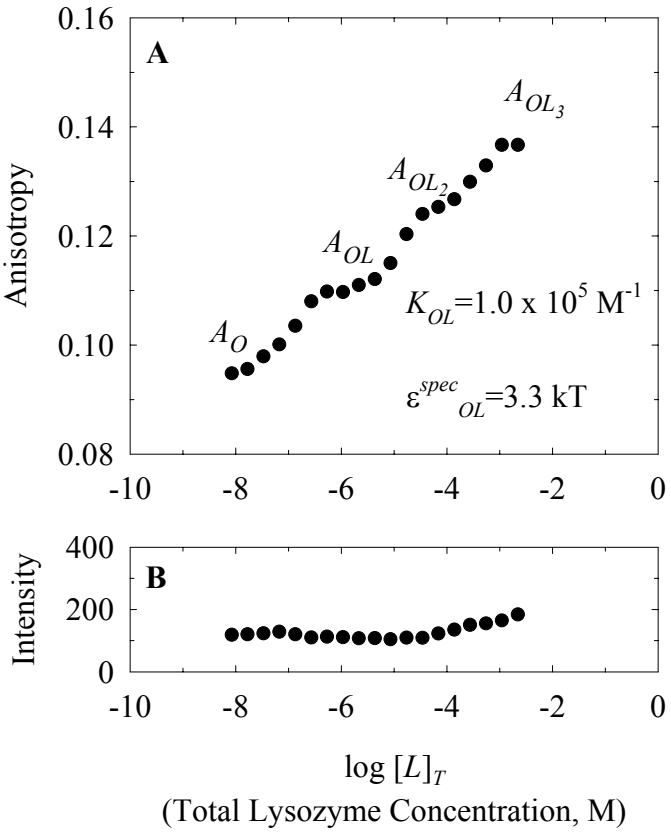
Figure 1: (*A*) Anisotropy of ovalbumin as a function of lysozyme concentration in 20-mM sodium-phosphate buffer at pH 7 and 25°C. (*B*) Total fluorescence intensity as a function of lysozyme concentration.

Figure 2: Anisotropy of ovalbumin in 20-mM sodium phosphate buffer at pH 7 and 25°C as a function of lysozyme concentration and low ammonium-sulfate ionic strength, $[O]_T \approx 0.6$ nM; (*A*) 0.0 M $(NH_4)_2SO_4$, (*B*) 0.5 M $(NH_4)_2SO_4$, (*C*) 1.0 M $(NH_4)_2SO_4$, (*D*) 3.0 M $(NH_4)_2SO_4$. Solid lines represent the best-fit curves using Eqs. 9 and 10.

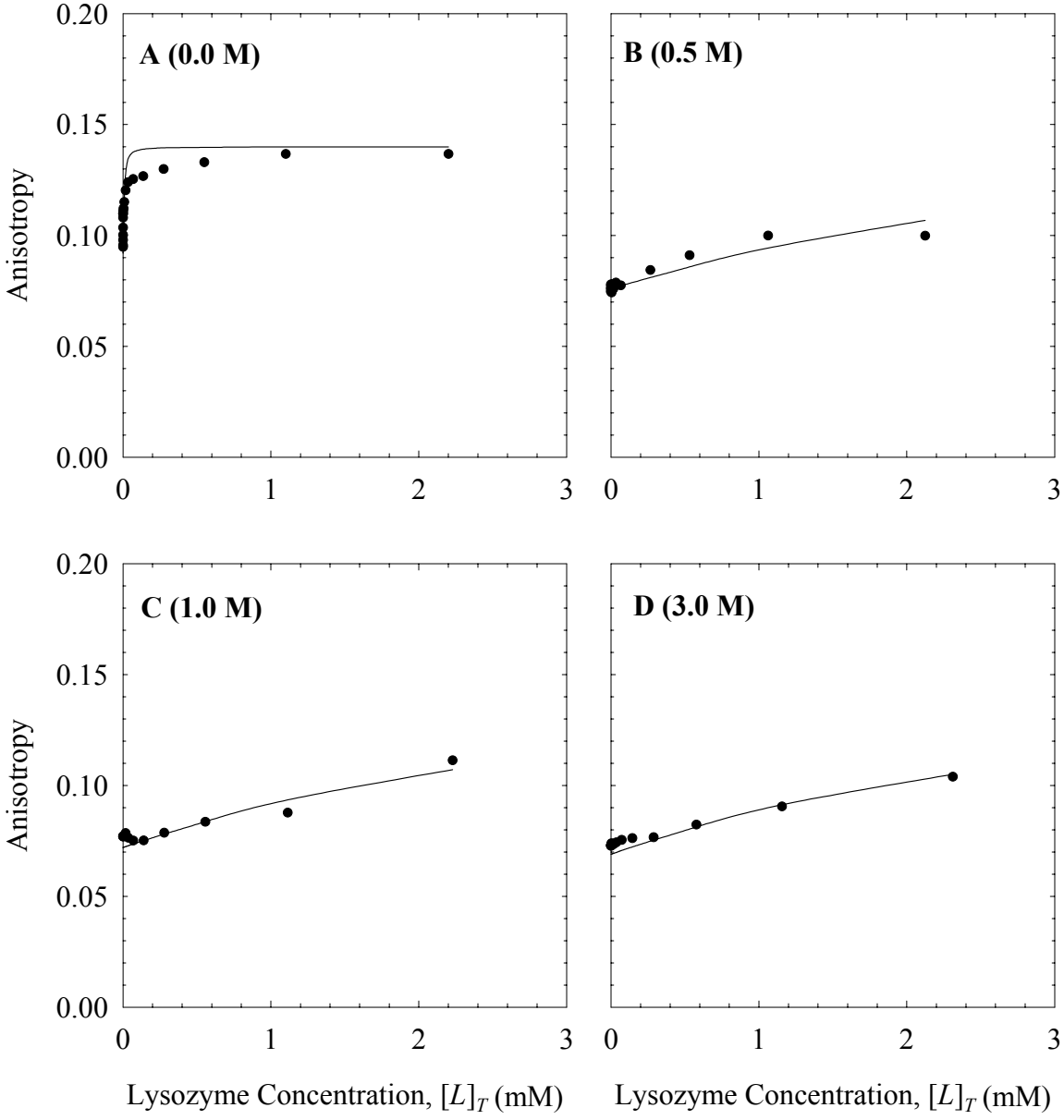
Figure 3: Anisotropy of ovalbumin in 20-mM sodium phosphate buffer at pH 7 and 25°C as a function of lysozyme concentration and high ammonium-sulfate ionic strength, $[O]_T \approx 0.6$ nM; (*A*) 5.3 M $(NH_4)_2SO_4$, (*B*) 6.1 M $(NH_4)_2SO_4$, (*C*) 6.8 M $(NH_4)_2SO_4$, (*D*) 7.5 M $(NH_4)_2SO_4$. Solid lines represent the best-fit curves using Eqs. 9 and 10.

Figure 4: Association constants at pH 7 as a function of ammonium-sulfate ionic strength; (A) lysozyme-lysozyme interactions, and (B) for ovalbumin-lysozyme interactions. For the self-interactions, closed circles represent values obtained from experimental B_{LL} of Curtis et al. (1998, 2000). Open circles represent values calculated using extrapolated well depths ε_{LL}^{spec}. Gray circle represents value from Jeffrey et al. (1979).

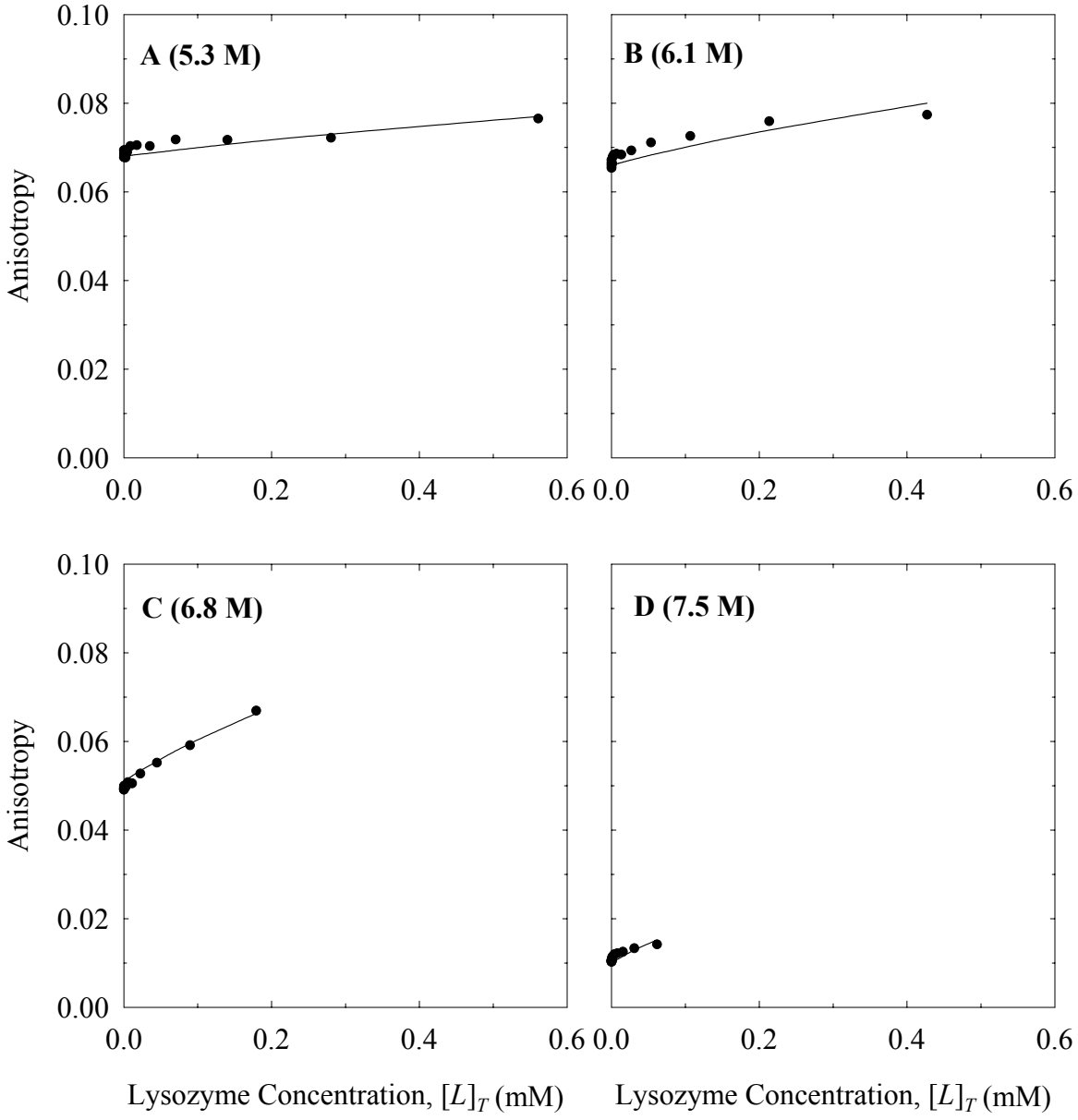
Figure 5: Reduced square-well depths, $\varepsilon_{ij}^{spec}/kT$, as a function of ammonium-sulfate ionic strength for (A) lysozyme-lysozyme interactions at pH 7, (B) ovalbumin-ovalbumin interactions at pH 6, and (C) ovalbumin-lysozyme interactions at pH 7. For self-interactions, closed symbols represent values obtained from experimental B_{LL} and B_{OO} of Curtis et al. (1998, 2000). Open symbols represent extrapolated/interpolated values. Gray symbol represents value obtained from experimental K_{LL} of Jeffrey et al. (1979).



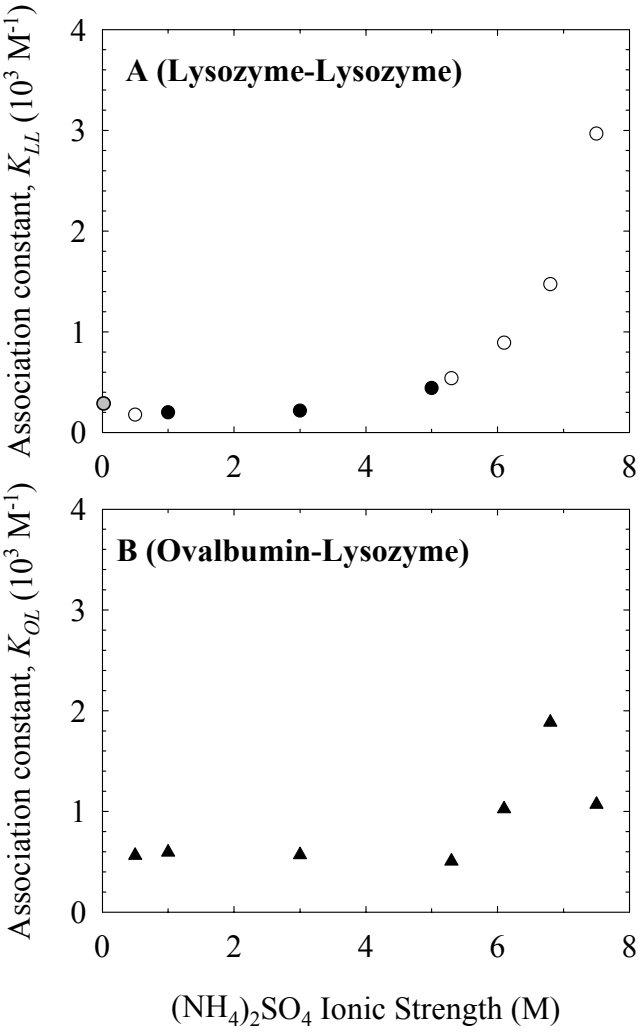
Anderson et al., Figure 1



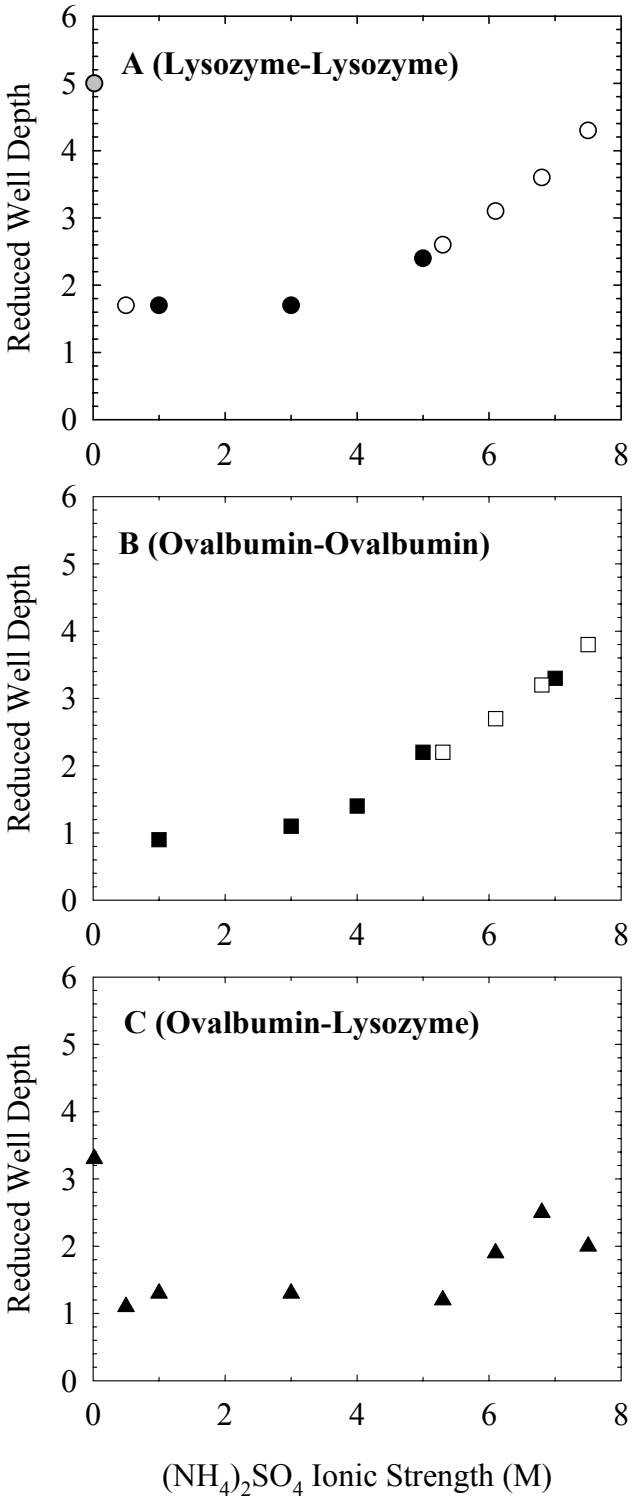
Anderson et al., Figure 2



Anderson et al., Figure 3



Anderson et al., Figure 4



Anderson et al., Figure 5