Study of Interactions of Bovine Serum Albumin in Aqueous $(NH_4)_2SO_4$ Solution at 25 °C by Osmotic Pressure Measurements

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We have made some improvements to the osmometer described previously (*Colloid Interface Sci.* **1981**, *79*, 548−566). Using the improved osmometer, we measured the osmotic pressures of aqueous bovine serum albumin (BSA) solutions at four pH values (4.5, 4.8, 5.4, and 7.4) and at four ammonium sulfate concentrations [(0.15, 0.50, 1.00, and 1.50) mol·L^{-1}]. The osmotic second virial coefficients of BSA were calculated from the osmotic-pressure data. According to the molecular thermodynamic model (*Fluid Phase Equilib.* **2000**, *168*, 229−239), the electrostatic repulsion potential, attractive dispersion potential, and ion-excluded-volume potential have been calculated. The dependence of the three potentials on solution pH and on ionic strength is discussed.

Introduction

Salting-out is one of the important methods of separating and purifying protein in the laboratory and biological industries. There are a number of factors that affect the salting-out behavior of the protein. These factors include the nature of the protein, the nature and concentration of salt, and the pH and temperature of the solution.[1] Usually, the best salting-out conditions are obtained by experiments. The salting-out behavior of a protein is dependent on the interactions between protein molecules and on the effect of salt. Through the study of interactions taking place in the aqueous solution of protein and salt by thermodynamics, some light can be thrown on the salting-out behavior of protein.

The interactions between proteins in an aqueous solution can be indirectly measured with light scattering and osmotic pressure.[2,3] Several researchers have studied the osmotic pressure of bovine serum albumin (BSA) under different solution conditions. Vilker et al.[4] measured the osmotic pressures of BSA in 0.15 mol·L^{-1} sodium chloride aqueous solutions at pH = 4.5, 5.4, and 7.4. The concentrations of BSA were from 84 g·L^{-1} to 475 g·L^{-1}. Wu and Prausnitz[5] measured the osmotic pressure of BSA in aqueous solutions of three sodium chloride concentrations [(1, 3, and 5) mol·L^{-1}] and three pH values (4.5, 5.4, and 7.4). The concentrations of BSA were from 12 g·L^{-1} to 150 g·L^{-1}.

In this study, we measured the osmotic pressures of BSA in aqueous solutions of four ammonium sulfate concentrations [(0.15, 0.50, 1.00, and 1.50) mol·L^{-1}] and four pH values (4.5, 4.8, 5.4, and 7.4). The concentration of the protein solution is usually kept between 10 g·L^{-1} and 50 g·L^{-1} in crystallization. In order to throw some light on the salting-out behavior of protein, we keep the protein concentration from 7.8 g·L^{-1} to 67.2 g·L^{-1}. Ammonium sulfate is used as the salting-out agent because it is widely used in the crystallization of proteins.

Theory Correlation

The crystallization/precipitation process of protein by adding precipitant is a molecule gathering process, which is caused by the interactions between protein molecules.[6] According to the solution theory of McMillan and Mayer,[7] the osmotic virial equation can be expressed as

$$\pi = RT\left(\frac{w_2}{\bar{M}} + B_2 {w_2}^2 + B_3 {w_2}^3 + ...\right) \quad (1)$$

where R is the universal gas constant, T is absolute temperature, M is average molecular weight of the protein, w_2 is the concentration of protein (grams per liter), and B_2 and B_3 are the second and third osmotic virial coefficients. For spherical molecules, the second osmotic virial coefficient is related to the potential of mean force $W_{22}(r)$:

$$B_2 = -\frac{N_A}{2\bar{M}\bar{M}}\int_0^\infty [e^{-W_{22}(r)/kT} - 1]4\pi r^2\, dr \quad (2)$$

where N_A is Avogadro's number and r is the center-to-center distance between two protein molecules. According to the molecular thermodynamic model presented by Prausnitz and co-workers,[2] the overall potential of mean force, $W_{22}(r)$, between protein molecules is given by the sum of four potentials of mean force:

$$W_{22}(r) = W_{hs}(r) + W_{elec}(r) + W_{disp}(r) + W_{osmotic}(r) \quad (3)$$

where $W_{hs}(r)$ is the protein hard-sphere potential, $W_{elec}(r)$ is the electrostatic repulsion potential, $W_{disp}(r)$ is the attractive dispersion potential, and $W_{osmotic}(r)$ is an attractive interaction due to the excluded-volume effect of the salt ions. These four terms are spherically symmetric potentials of mean force that depend on the center-to-center distance between proteins, on the properties of the protein molecules, and on the solvent chemistry (e.g., pH and ionic strength).

The hard-sphere potential of mean force between proteins, $W_{hs}(r)$, is represented by

$$W_{hs}(r) = \begin{cases} \infty & \text{for } r \le \sigma \\ 0 & \text{for } r > \sigma \end{cases} \quad (4)$$

where σ is the diameter of the protein. For BSA, σ is $6.26 \cdot 10^{-9}$ m.[5]

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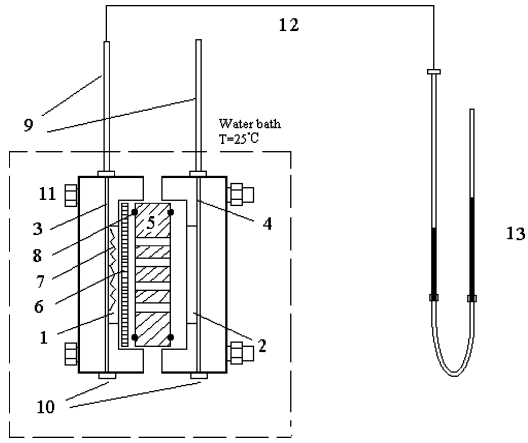

Figure 1. Experimental osmotic pressure apparatus: 1, solution chamber; 2, solvent chamber; 3, 4, and 5, channels; 6, membranes; 7, metal mesh supporter; 8, O-ring seal; 9, glass capillary; 10, filling channels; 11, sealing bolt; 12, rubber pipe; 13, water manometer.

According to the DLVO theory of colloidal stability,[8] the electrostatic repulsion potential, $W_{elec}(r)$, is represented by

$$W_{elec}(r) = \frac{Z_p{}^2 e^2 \exp[-\kappa(r-\sigma)]}{4\pi\varepsilon_0\varepsilon_r r(1+\kappa\sigma/2)^2} \qquad \text{for } r > \sigma \quad (5)$$

where Z_p is the protein valence and e is the elementary unit charge, ε_0 is the dielectric permittivity of vacuum, ε_r is the ionic-strength-dependent dielectric constant, and κ is the Debye screening parameter, given by

$$\kappa^2 = 2e^2 N_A I/\varepsilon_0\varepsilon_r k_B T \quad (6)$$

where I is ionic strength and k_B is the Boltzmann constant.

The attractive dispersion potential, $W_{disp}(r)$, is given by[9]

$$W_{disp}(r) = -\frac{H}{12}\left(\frac{\sigma^2}{r^2-\sigma^2} + \frac{\sigma^2}{r^2} + 2\ln\frac{r^2-\sigma}{r^2}\right) \qquad \text{for } r > \sigma \quad (7)$$

where H is the Hamaker constant of the protein.

The ion-excluded-volume potential, $W_{osmotic}(r)$, is given by[10]

$$W_{osmotic}(r) = \begin{cases} \infty & \text{for } r < \sigma \\ -\frac{4\pi}{3}\sigma_{ps}{}^3\Pi_{osm}\left(1 - \frac{3r}{4\sigma_{ps}} + \frac{r^3}{16\sigma_{ps}{}^3}\right) & \text{for } \sigma < r < 2\sigma_{ps} \\ 0 & \text{for } r \geq 2\sigma_{ps} \end{cases} \quad (8)$$

where $\sigma_{ps} = (\sigma + \sigma_{ion})/2$ and the composition-weighted average ion diameter $\sigma_{ion} = (\upsilon_{cat}\sigma_{cat} + \upsilon_{an}\sigma_{an})/\upsilon$, where υ_{cat} and υ_{an} are the stoichiometric coefficients of the cation and anion, respectively, and $\upsilon = \upsilon_{cat} + \upsilon_{an}$.

Experimental Section

Materials. Bovine serum albumin (BSA, purity > 98 %, molecular weight 68 000) was purchased from Sigma. Cellulose membrane (10 000 molecular weight cutoff) was purchased from Millipore.

Apparatus. Figure 1 shows schematically the experimental apparatus for osmotic pressure measurements. In this apparatus some improvements have been made on the basis of that used by Vilker et al.[4] The solution chamber is linked to its channel directly. In this way, the osmosis of solvent can be carried out more quickly. A Plexiglas plate with some holes is used to support the membrane, instead of the metal screen and the porous frit support.

Preparation of Solvents and Solutions. Ammonium sulfate aqueous solutions of (0.15, 0.50, 1.00, and 1.50) mol·L^{-1} were prepared by adding certain amounts of ammonium sulfate into deionized water, with the corresponding pH values being 3.93, 3.68, 3.40, and 3.26, respectively. Since ammonium sulfate is a salt of a strong acid and weak alkali, the pH will decrease with increasing concentration of ammonium sulfate. We regulated the pH to 4.5, 4.8, 5.4, and 7.4 by using $NH_3 \cdot H_2O$ (and H_2SO_4 if too much $NH_3 \cdot H_2O$ was added). The values of pH were measured on a model PHS-2C pH meter (Shanghai Instrument Co. Ltd.). In the experiment, we take the ammonium sulfate aqueous solutions as solvents. The BSA solutions were prepared by dissolving albumin crystals in the solvents, and the final solution pH was adjusted to the value of the solvent by adding a little 0.1 mol·L^{-1} $NH_3 \cdot H_2O$ or H_2SO_4. During pH adjustment, vigorous vortex mixing was applied to prevent local protein denaturation. The densities of BSA solutions were measured by using a specific gravity bottle of 5 mL. The densities were used to calculate the volumetric molar concentrations of BSA solutions.

Measurement of Osmotic Pressure. The osmometer cell was assembled by sandwiching the cellulose membrane between two Plexiglas chambers, The solvent and the protein solution were simultaneously injected into the corresponding chambers until the liquid levels in both capillaries reached about $^2/_3$ of full length. Then the osmometer was settled in a water bath that was controlled at (25 ± 0.01) °C. Pressure was applied gradually to the solution side such that there was no net matter flow between the two chambers. Equilibrium was attained when the liquid levels in both capillaries kept the same height for 3 h at a given applied pressure. The applied pressure is the osmotic pressure of the protein solution, which can be read from the water manometer.

Table 1 shows the measured osmotic pressures of BSA solutions at different pH and salt concentrations.

Results and Discussion

In aqueous solutions with low concentrations of protein, the truncated form of eq 1 for osmotic pressure can be used:

$$\pi = RT\left(\frac{w_2}{\bar{M}} + B_2 w_2{}^2\right) \quad (9)$$

In our experiments, the largest concentration of BSA is less than $1 \cdot 10^{-3}$ mol·L^{-1}. According to Moon et al.,[2] we can use eq 9 to calculate the osmotic second virial coefficient and the average molecular weight with a nonlinear fitting method. The results are given in Table 2 and shown in Figure 2.

George and Wilson[11] have shown the importance of the pair potential of mean force for predicting solution conditions favorable for protein crystallization; they have shown a B_2 crystallization window for protein solutions. As a necessary (but not sufficient) condition for protein crystallization, B_2 should lie between $-2 \cdot 10^{-4}$ and $-8 \cdot 10^{-4}$ mL·mol·g^2. For B_2 less negative than $-2 \cdot 10^{-4}$ mL·mol·g^2, the protein−protein attraction is usually not sufficiently strong to form stable protein crystals. For solutions where B_2 is more negative than $-8 \cdot 10^{-4}$ mL·mol·g^2, amorphous precipitation is likely to occur because protein−protein attractions are sufficiently strong that the protein molecules do not have adequate time to orient themselves into

Table 1. Measured Osmotic Pressure of Ternary Unsaturated BSA−Ammonium Sulfate−Water Solutions at 25 °C[a]

$(NH_4)_2SO_4$							
0.15 mol·L^{-1}		0.50 mol·L^{-1}		1.00 mol·L^{-1}		1.50 mol·L^{-1}	
C / g·L^{-1}	π / mmH$_2$O	C / g·L^{-1}	π / mmH$_2$O	C / g·L^{-1}	π / mmH$_2$O	C / g·L^{-1}	π / mmH$_2$O
			pH 4.5				
8.3	30	8.5	32	8.6	29	10.1	35
15.8	55	13.6	49	13.6	47	18.1	57
26.5	90	25.5	90	27.3	90	22.3	72
40.9	150	36.0	125	37.7	127	33.5	104
50.1	165	44.2	160	48.2	170	42.3	120
59.4	198	52.7	200	57.7	195	50.1	140
			pH 4.8				
8.7	32	8.2	30	8.1	26	9.6	32
13.5	53	13.0	48	14.3	45	14.7	48
26.7	97	26.3	90	25.3	78	24.6	75
38.3	135	38.6	135	36.1	104	31.8	86
50.0	165	48.7	170	42.9	125	41	8100
55.3	187	56.5	195	50.5	140	45.3	113
			pH 5.4				
8.1	30	8.7	33	8.9	33	8.7	34
15.2	58	14.2	53	14.3	49	14.0	49
28.3	112	27.4	105	29.6	105	26.1	89
41.7	143	40.4	149	41.3	142	38.3	133
55.5	201	52.5	197	54.4	198	46.3	158
59.8	214	60.0	220	61.3	220	54.1	171
			pH 7.4				
7.8	29	9.5	35	8.2	34	8.5	36
15.5	55	13.8	50	14.2	50	13.3	51
28.3	98	29.2	108	29.9	117	27.3	95
42.7	157	41.7	156	40.5	148	37.6	125
55.4	210	54.4	217	53.0	203	50.2	165
63.0	228	67.2	272	65.1	240	60.4	205

[a] C is the concentration of BSA in aqueous ammonium sulfate, and π is the osmotic pressure for a solution of BSA in aqueous ammonium sulfate.

Table 2. Osmotic Second Virial Coefficients and Average Molecular Weight of BSA in Aqueous Ammonium Sulfate at 25 °C[a]

C / mol·L^{-1}	$B_2 \cdot 10^4$ / mL·mol·g^2	M	R^2
	pH 4.5		
0.15	−0.2596	68 000	0.9945
0.50	−0.0363	68 000	0.9938
1.00	−0.2386	68 000	0.9951
1.50	−0.7654	68 000	0.9961
	pH 4.8		
0.15	−0.2631	68 000	0.9945
0.50	−0.1985	68 000	0.9981
1.00	−0.7791	68 000	0.9933
1.50	−1.1589	68 000	0.9896
	pH 5.4		
0.15	−0.0948	67 998	0.9947
0.50	−0.1065	68 000	0.9992
1.00	−0.1009	68 006	0.9968
1.50	−0.3470	68 000	0.9960
	pH 7.4		
0.15	−0.0310	68 000	0.9970
0.50	0.1723	68 000	0.9982
1.00	0.0105	68 000	0.9971
1.50	−0.0270	68 000	0.9949

[a] C is the concentration of $(NH_4)_2SO_4$, B_2 is the osmotic second virial coefficient, and M is the average molecular weight of BSA.

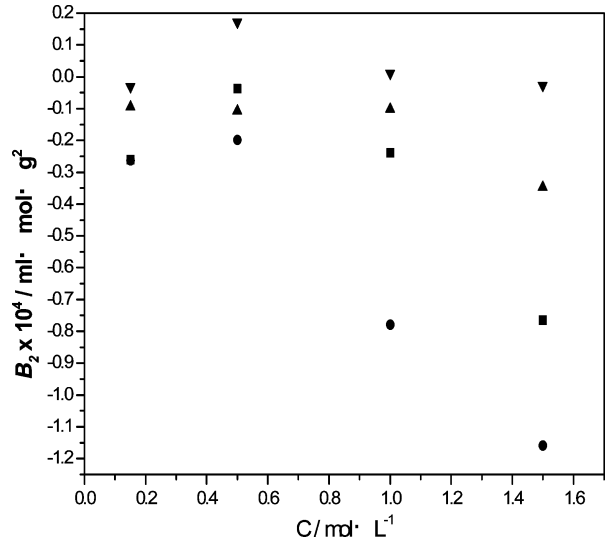

Figure 2. Effect of $(NH_4)_2SO_4$ concentration (C) on the osmotic second virial coefficient (B_2) at different pH values at 25 °C: ■, pH = 4.5; ●, pH = 4.8; ▲, pH = 5.4; ▼, pH = 7.4.

a crystal lattice. From Figure 2, we can see that the suitable pH value for BSA crystallization in ammonium sulfate aqueous solution should be close to its isoelectric point (pH = 4.8). This is because when the pH value deviates from 4.8, whether larger or smaller, the values of B_2 increase. The concentration of ammonium sulfate should be greater than 1.5 mol·L^{-1}, because when the concentration of ammonium sulfate is smaller than 1.5 mol·L^{-1}, the value of B_2 is greater.

It is obvious that keeping the pH value at the isoelectric point (pH = 4.8) and increasing the concentration of ammonium sulfate can make the second virial coefficient close to the salting-out point, so it is important to analyze the interactions between BSA molecules at the conditions of the isoelectric point and the concentrations of ammonium sulfate studied. According to the molecular thermodynamic model of Prausnitz and co-workers[2] mentioned in the Theory Correlation section, we

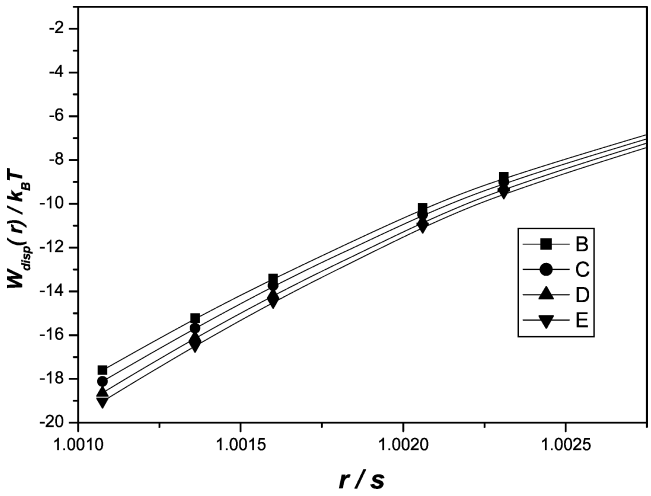

Figure 3. Dispersion potential of mean force as a function of the ratio of BSA center-to-center distance to BSA diameter at different ionic strengths at 25 °C: ■, ionic strength 0.50; ●, ionic strength 0.15; ▲, ionic strength 1.00; ▼, ionic strength 1.50.

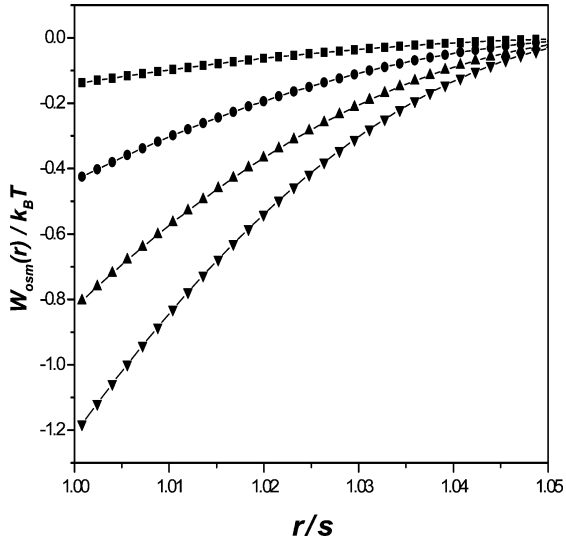

Figure 4. Ion-excluded-volume potential of mean force as a function of the ratio of BSA center-to-center distance to BSA diameter at different ionic strengths at 25 °C: ■, ionic strength 0.15; ●, ionic strength 0.50; ▲, ionic strength 1.00; ▼, ionic strength 1.50.

calculated the various kinds of potentials and the total potential. The hard-sphere potential is independent of the intensity of ion and pH value. At the isoelectric point, the net charge on the protein is zero; according to eq 5, the electrostatic repulsion potential is also zero. We show only the dispersion potential (Figure 3), ion-excluded-volume potential (Figure 4), and total potential (Figure 5).

From Figures 3 and 5, we can see that the attractive dispersion potential is the main part of the total potential between protein molecules. Equation 7 shows that the attractive dispersion potential has no explicit dependence on ionic strength. The effect of ionic strength is contained within the effective Hamaker constant. From the osmotic second virial coefficients in Table 2, the Hamaker constant, as an adjustable model parameter, was calculated. When the concentration of $(NH_4)_2SO_4$ is (0.15, 0.50, 1.00, and 1.50) mol·L^{-1}, respectively, the corresponding H/k_BT results are 0.400, 0.390, 0.412, and 0.421. As the concentration of $(NH_4)_2SO_4$ increased from (0.15 to 1.5) mol·L^{-1}, the values of the Hamaker constant changed only a little. This is because the intrinsic frequency of the electronic fluctuations, which give

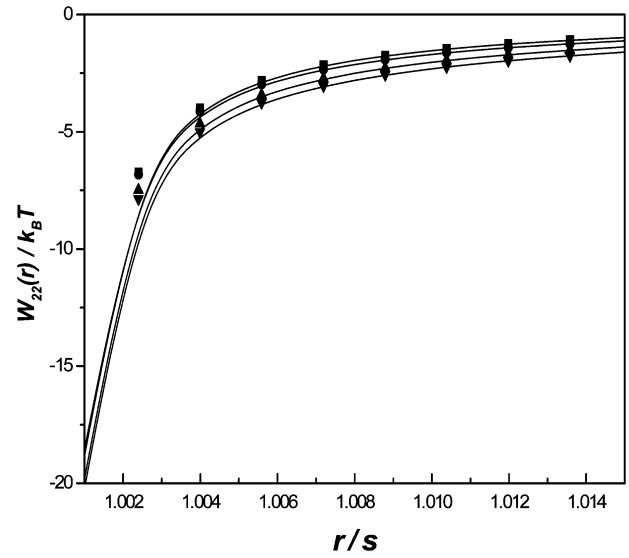

Figure 5. Total potential of mean force as a function of the ratio of BSA center-to-center distance to BSA diameter at different ionic strengths at 25 °C: ■, ionic strength 0.15; ●, ionic strength 0.50; ▲, ionic strength 1.00; ▼, ionic strength 1.50.

rise to the dispersion force, is much greater than the time constant for rearrangement of ions in the double layer between the protein molecules. Therefore, dielectric screening of the dispersion attraction is very small.

Equation 8 was used to calculate the ion-excluded-volume potential. In eq 8, the osmotic pressure of the protein-free salt solution can be obtained,[12] in terms of the osmotic coefficient φ_{osm}, by $\pi_{osm} = \varphi_{osm}\pi_{id}$. Here $\pi_{id} = \rho_s k_B T$, where ρ_s is given in terms of the molar salt concentration, C_s, by $\rho_s = C_s N_A v$. In this investigation, the ion diameters are[13] $\sigma_{NH_4} = 2.86 \cdot 10^{-10}$ m and $\sigma_{SO_4} = 4.60 \cdot 10^{-10}$ m. The concentrations of $(NH_4)_2SO_4$ in water we controlled are (0.15, 0.50, 1.00, and 1.50) mol·L^{-1} and the corresponding φ_{osm} are (0.734, 0.679, 0.643, and 0.630), respectively.[14] Using these values of φ_{osm} will make a small error. This is because we used $NH_3 \cdot H_2O$ and H_2SO_4 as pH regulators, which will have some effect on φ_{osm}. The largest concentration of either pH regulator used is (0.0026, 0.0075, 0.012, and 0.015) mol·L^{-1} for the concentration of $(NH_4)_2SO_4$ at m = (0.15, 0.5, 1, and 1.5) mol·L^{-1}, respectively. Since no other ions are introduced to the system by pH regulating, we think that the effect of the pH regulator for φ_{osm} is caused only by the increase in total concentration of electrolyte. The increase in total concentration of electrolyte is relatively small, so the effect on φ_{osm} is small. When the approximation[12] of eq 8 and the uncertainties of BSA diameter and ion diameters are considered, it is reasonable to neglect the effect of pH regulator on φ_{osm}.

From Figure 4, it is seen that the ion-excluded-volume potential, which is a short-range attractive potential, increases with increasing ionic strength. As two protein molecules approach each other, ions are excluded from a region between the protein particles. The resulting imbalance in the local osmotic pressure exerted by the ions on the proteins gives rise to a short-range attractive force. This effect increases with increasing ionic strength.

By keeping the concentration of ammonium sulfate at 1.50 mol·L^{-1}, we can also make B_2 close to the salting-out point by changing the pH value of the solution. The interactions between BSA molecules under the condition used should also be discussed. Electrostatic repulsion potential is dependent on the net charge of the protein molecule, and the net charge of protein

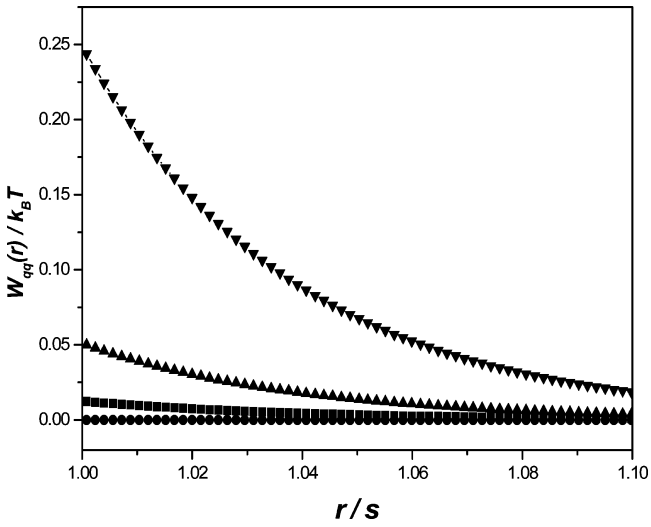

Figure 6. Electrostatic repulsion potential of mean force as a function of the ratio of BSA center-to-center distance to BSA diameter at different pH values at 25 °C: ■, pH = 4.5; ●, pH = 4.8; ▲, pH = 5.4; ▼, pH = 7.4.

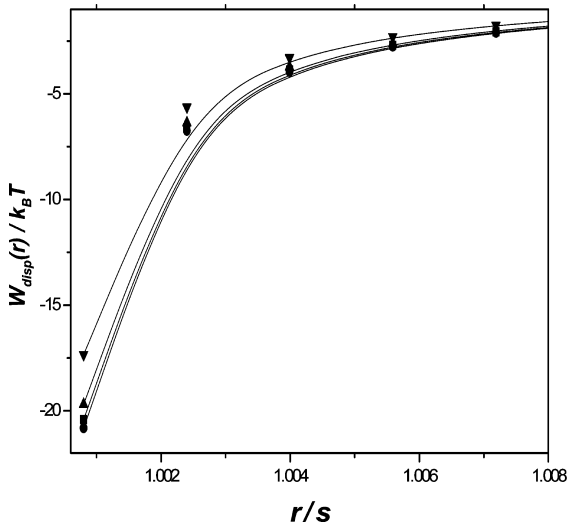

Figure 7. Dispersion potential of mean force as a function of the ratio of BSA center-to-center distance to BSA diameter at different pH values at 25 °C: ■, pH = 4.5; ●, pH = 4.8; ▲, pH = 5.4; ▼, pH = 7.4.

is related to the pH value of the solution. The net charge of BSA we used was obtained by Lin et al.[15] According to their data, when the pH value is 7.4, 5.4, and 4.5, respectively, the corresponding net charge is −20.4, −9.1, and +4.51. For pH = 4.8 (isoelectric point), we take zero as the net charge of BSA. In the calculation of the attractive dispersion potential, we obtained Hamaker constants $H/(k_BT)$ of 0.346, 0.392, 0.421, and 0.407 for pH = 7.4, 5.4, 4.8, and 4.5, respectively.

Since the ionic strength is fixed, the ion-excluded-volume potential is also fixed. So we show only the electrostatic repulsion potential (Figure 6), dispersion potential (Figure 7), and total potential (Figure 8).

At the isoelectric pH, the average net molecular charge of the protein is zero. Therefore, the electrostatic repulsion potential between BSA molecules is zero at pH = 4.8 (Figure 6). The average net molecular charge will be positive when pH < 4.8 and negative when pH > 4.8.[4] No matter whether the average net molecular charge is positive or negative, the electrostatic repulsion potential always increases with the degree of the pH deviation from isoelectric pH. When the dispersion potential (Figure 7) and total potential (Figure 8) are compared, the

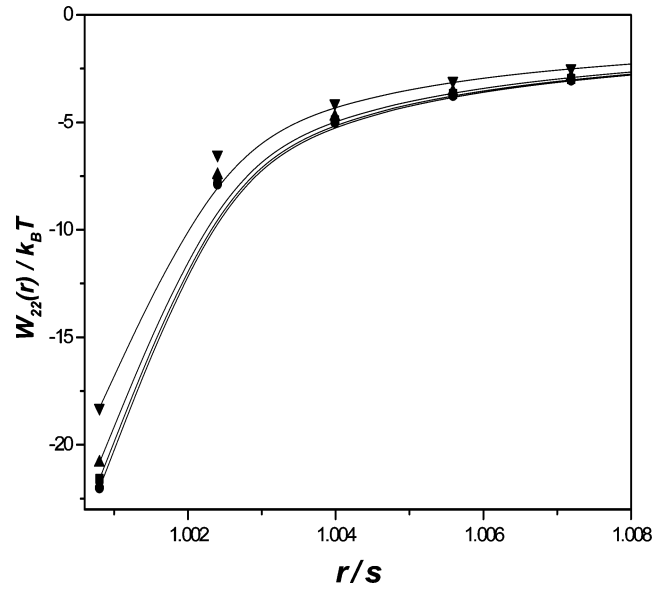

Figure 8. Total potential of mean force as a function of the ratio of BSA center-to-center distance to BSA diameter at different pH values at 25 °C: ■, pH = 4.5; ●, pH = 4.8; ▲, pH = 5.4; ▼, pH = 7.4.

electrostatic repulsion potential is small in absolute value. The reason is that there is a large effect of dielectric screening of ions on the electrostatic repulsion potential in higher salt concentrations. As mentioned above, the effect of dielectric screening of ions on the dispersion potential is very small. Therefore, the dispersion potential is also the main part of total potential.

Conclusions

By using the osmotic pressure data of bovine serum albumin in $(NH_4)_2SO_4$ aqueous solution, we calculated the osmotic second virial coefficients. As the pH of the solution approaches the isoelectric point and the concentration of salt increases, the value of the osmotic second virial coefficient decreases. According to the molecular thermodynamics model, various potentials of mean force between the protein molecules were calculated. The results show that the attractive dispersion potential is the main part of the overall potential. When the pH of solution is equal to the isoelectric point, the absolute value of the dispersion potential and the ion-excluded-volume potential increase with increasing concentration of salt. When the concentration of $(NH_4)_2SO_4$ is fixed at 1.50 mol·L^{-1}, the positive value of electrostatic repulsion potential and the negative value of attractive dispersion potential increase with increasing departure of pH from the isoelectric point.

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