Self-interaction chromatography: a novel screening method for rational protein crystallization

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The osmotic second virial coefficient, B_{22}, has become the quantity most widely used in developing a rational understanding of protein crystallization. In this work a novel method of measuring B_{22} using self-interaction chromatography (SIC) is presented that is at least an order of magnitude more efficient than traditional characterization methods, such as static light scattering. It is shown that SIC measurements of second virial coefficients for BSA are in quantitative agreement with static light scattering results. The measured virial coefficient for both BSA and myoglobin reveal a surprisingly narrow range of concentrations of ammonium sulfate that promote weakly attractive interactions that are optimal for crystallization. Using the virial coefficient information, myoglobin crystals were obtained by ultracentrifugal crystallization in a rational and rapid manner.

Keywords: protein interactions, static light scattering, ultracentrifugal crystallization, osmotic second virial coefficient

1. Introduction

The rapidly emerging field of proteomics aims to elucidate the complex role of protein molecules in the cellular environment, which will require detailed structural information for thousands of proteins. Most high-resolution protein structural information is currently obtained by X-ray diffraction. The limiting factor in protein structural characterization typically is the determination of solution conditions that promote protein crystallization. The importance of protein structural information has consequently led to hundreds of millions of dollars being directed toward developing high-throughput crystallization technology to perform a vast number of crystallization screens daily (Schulz, 2001). Although empirical crystallization screens have been used to crystallize proteins for decades, many proteins, particularly membrane proteins, have proven difficult to crystallize using this type of approach. It would be of significant value if high-throughput methods could be developed to determine conditions for protein crystallization in a rational manner, reducing the number of crystallization experiments and, therefore, the cost and time required for determining the structure of a protein.

The goal of rationally determining solution conditions conducive to protein crystallization has been the focus of much research in the past decade. One of the most promising findings has been that solution conditions that are conducive to protein crystallization correlate with slightly negative, or moderately attractive, values of the osmotic second virial coefficient (B_{22}), a range referred to as the "crystallization slot" (George & Wilson, 1994). The link between molecular interactions in terms of B_{22}, a thermodynamic parameter characterizing pairwise protein interactions, and protein crystallization offered hope that screening second virial coefficient values may be useful for the predictive crystallization of proteins not crystallized previously. Yet there has been little use of the second virial coefficient for predictive crystallization, largely because the methods for determining virial coefficients, such as static light scattering (SLS) and membrane osmometry, are too expensive in terms of protein and time to allow extensive screening.

We have recently introduced a highly efficient method for measuring the second virial coefficient using self-interaction chromatography (SIC) (Tessier et al., 2002b). SIC was initially proposed as a method for screening excipients for use in drug formulation (Patro & Przybycien, 1996), but we have adapted the method for characterizing the effects of additives that promote protein association and may lead to crystallization. The basis of the method is that protein is covalently immobilized on chromatographic particles that are packed into a chromatography column, and a pulse of the same protein is passed through the column. The retention time reflects the average protein-protein interactions, and this can be related to the second virial coefficient using statistical mechanics, without the need for adjustable parameters (Tessier et al., 2002b). We have previously demonstrated that the virial coefficient values measured by SIC are typically in quantitative agreement with values measured by SLS, yet SIC requires at least an order of magnitude less protein and time.

The efficiency of SIC enables the rapid measurement of virial coefficients at a variety of solution conditions, which can then be compared to protein phase behavior results to validate the applicability of the "crystallization slot" correlation for use in predictive crystallization. However, the second virial coefficient does not provide an adequate indication of the proper initial protein concentration to use for crystallization. Crystallization techniques such as vapor diffusion circumvent the need for solubility information by concentrating the protein by evaporation, but the concentration of the crystallization agents also changes, making it difficult to compare the phase behavior results to thermodynamic measurements. Ultracentrifugal crystallization was used in this work to maintain constant solution conditions during our crystallization experiments (Wyckoff & Corey, 1936; Karpukhina et al., 1975; Barynin & Melik-Adamyan, 1982; Lenhoff et al., 1997; Pjura et al., 2000). This technique involves placing a dilute protein solution in a centrifugal field, which causes the protein concentration to increase at the bottom of the vial due to sedimentation until the solubility and nucleation threshold are exceeded, allowing crystallization to commence. The advantages of ultracentrifugal crystallization are that high protein concentrations can be obtained at the beginning of the experiment, which promotes nucleation, yet due to depletion of protein locally around the growing crystal, the protein concentration is lower in the later stages of the experiment, which promotes the growth of high quality crystals. An additional benefit of ultracentrifugal crystallization is that crystals may appear within hours to days, compared to other methods such as vapor diffusion, which can require weeks to months for crystallization to occur.

We have previously used SIC to characterize ribonuclease A interactions at more than 50 solution conditions, and the virial coefficient information was used to obtain ribonuclease A crystals that diffract to 1.5 Å (Tessier et al., 2002a). In this paper we demonstrate that SIC can be used to measure the second virial coefficient quantitatively for BSA. We then demonstrate how self-interaction chromatography can be used to characterize myoglobin interactions, which are found to be quite different from those normally seen for small globular proteins. We then demonstrate, for myoglobin, that crystallization can be accomplished in a predictive manner despite the unusual virial coefficient trends.

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2. Methods and materials

2.1. Materials

BSA (A-7638), carboxymethyl BSA (A-6285) and horse skeletal muscle myoglobin (M-0630) were obtained from Sigma and used as received. Bis-tris (B-7535), MES (M-8250), bis-tris propane (B-6755), glutaraldehyde (G-5882), and ethanolamine (E-9508) were also purchased from Sigma. Boric acid (ACS grade, A73), potassium phosphate (ACS grade, P288), hydrochloric acid (ACS grade, A114), ammonium sulfate (ACS grade, A702), sodium carbonate (ACS grade, S263) and sodium chloride (ACS grade, 5271) were purchased from Fisher. Nochromix (19-010) was purchased from Godax Laboratories. Micro-BCA assay reagents (23231BP, 23232BP and 23234BP) were obtained from Pierce. AF-Amino-650M chromatography particles (08002) were obtained from Tosoh Biosep.

2.2. Procedures and analysis

2.2.1. Light scattering. The SLS procedures used in this work were essentially the same as those described previously (Velev et al., 1998). Light scattering experiments were typically conducted using 5 protein samples ranging from 2 to 16 mg/ml. The concentration of BSA was determined spectrophotometrically at 280 nm using an extinction coefficient of 0.67 l/g-cm (Sorber, 1970). All SLS experiments were carried out at a temperature of 25 +/- 1°C. The specific refractive index (d*n*/d*c*) for BSA was measured using a C.N. Wood differential refractometer (Model RF 600) at λ = 488 nm. The measured value of d*n*/d*c* for BSA was 0.21 ml/g.

2.2.2 Self-interaction chromatography. Myoglobin, BSA and carboxymethyl BSA were immobilized on Tosoh Biosep AF-Amino-650M particles using glutaraldehyde as a cross-linking agent. Approximately 75 mg of protein was dissolved in 15 g of 1 M potassium phosphate at pH 8.5. After 2 ml of amino particles (settled volume) were activated with glutaraldehyde and washed with 2 liters of water, the particles were added to approximately 11 g of protein solution and allowed to react overnight. The particles were then washed with 1 M potassium phosphate at pH 8.5 and added to 15 g of 1 M ethanolamine at pH 8 for endcapping unreacted glutaraldehyde. After a few hours, the particles were washed in either 1 M potassium phosphate or 1 M sodium chloride and stored at 4°C. The initial protein solution and the wash solutions were analyzed at 280 nm to determine the amount of protein immobilized, and a direct measure of the amount of protein immobilized was also obtained using the micro BCA method (Plant et al., 1991; Tessier et al., 2002b). The immobilization densities were found to be 17.5 +/- 1.6, 19.1 +/- 2.3 and 19.6 +/- 0.2 mg of protein per ml of settled particle volume for carboxymethyl BSA, native BSA, and myoglobin respectively.

The chromatography experiments and analysis were conducted using a Pharmacia FPLC system. The particles were packed into the following glass columns: 3x25 mm column from Cobert Associates for BSA, 3x50 mm column from Cobert Associates for myoglobin, and 5x50 mm column from Waters for carboxymethyl BSA. The packing flow rate was 3 to 5 ml/min, which was continued until the bed height stabilized. The column integrity was analyzed by passing a pulse of acetone through the bed and confirming that the unretained peak was symmetric, which was typically the case in this work. Therefore, we used the peak maximum as the retention volume.

For each virial coefficient measurement, the column was equilibrated with several column volumes of the solution environment of interest. Then 20 or 50 µl of a protein solution at a concentration of 5 to 20 mg/ml was injected into the 3 or 5 mm diameter column respectively (unless otherwise stated). After the protein was eluted from the column, the electrolyte concentration was typically raised to 1 M for several column volumes, and then lowered to a concentration of 5 mM for several column volumes. Finally the column was equilibrated in the solution environment of the next sample. The buffers used for myoglobin were 5 mM bis-tris or MES at pH 6, 5 mM potassium phosphate at pH 7.4 and 5 mM boric acid or bis-tris propane at pH 9. The buffers used for BSA were 50 mM sodium phosphate at pH 6.2 and 5 to 10 mM at pH 7. All SIC experiments were carried out at a temperature of 23 +/- 2°C.

The retention measurements obtained by SIC were used to calculate the retention factor,

$$k' = \frac{V_r - V_o}{V_o} \qquad (1)$$

where V_r is the retention volume, or the volume required to elute a solute from the column, and V_o is the dead volume, or the volume required to elute a non-interacting solute of the same size from the column. The dead volume was evaluated using a column without protein immobilized (Tessier et al., 2002b).

The second virial coefficient can be related to the retention factor by (Tessier et al., 2002b)

$$B_{22} = B_{HS} - \frac{k'}{\rho_s \phi} \qquad (2)$$

where B_{HS} is the excluded volume or hard sphere contribution ($B_{HS}=2/3\pi d^3$ for spheres), ρ_s is the amount of protein immobilized per unit surface area, and ϕ is the phase ratio, or the accessible surface area per mobile phase volume, which is available for a variety of chromatography particles (DePhillips & Lenhoff, 2000). The excluded volume can be calculated using the protein diameter evaluated from the molecular volume (Connolly, 1985; Connolly, 1993), or a reasonable estimate can be found using the protein partial specific volume and molecular weight.

2.2.3. Ultracentrifugal crystallization. Centrifugal crystallization was conducted using a Sorvall Discovery 90 ultracentrifuge with a Beckman SW5 1-50,000 RPM rotor and Beckman centrifuge tubes (344057). Approximately 4 ml of a 10 mg/ml solution of myoglobin was added to each centrifuge tube, and the samples were spun at 50,000 rpm for 48 hours. Afterwards, most of the supernatant was removed and the samples were analyzed by optical microscopy to determine the phase behavior.

3. Results

In order to validate the ability of SIC to measure virial coefficients quantitatively relative to SLS, we first present a comparison of the results obtained for BSA. Figure 1 shows a comparison of SIC and SLS virial coefficient measurements for native BSA in ammonium sulfate at pH 6.2. The virial coefficient dependence on the ionic strength is weak except at very high ammonium sulfate concentrations. The agreement is good at low to moderate ionic strength, while a slight offset is observed at higher ionic strength. Figure 2 shows that carboxymethyl BSA virial coefficients in sodium chloride measured by SIC and SLS are also in good agreement, with the largest offset observed at low ionic strength. The average difference between the virial coefficients measured by SIC and SLS is 0.5×10^{-4} mol-ml/g^2, which is small compared to the size

of the "crystallization slot", $7x10^{-4}$ mol-ml/g^2. Finally, there was no significant dependence of the amount of injected protein (20 µl of protein at 5 to 20 mg/ml) on the virial coefficient values measured by SIC at solution conditions that promote attractive pairwise interactions (e.g., pH 6.2, 1.8 M ammonium sulfate), as was found previously for α-chymotrypsinogen and ribonuclease A (Tessier et al., 2002a; Tessier et al., 2002b). However, we observed a separate aggregate peak that was resolvable from the monomer peak under similar solution conditions, yet it did not interfere with our measurements.

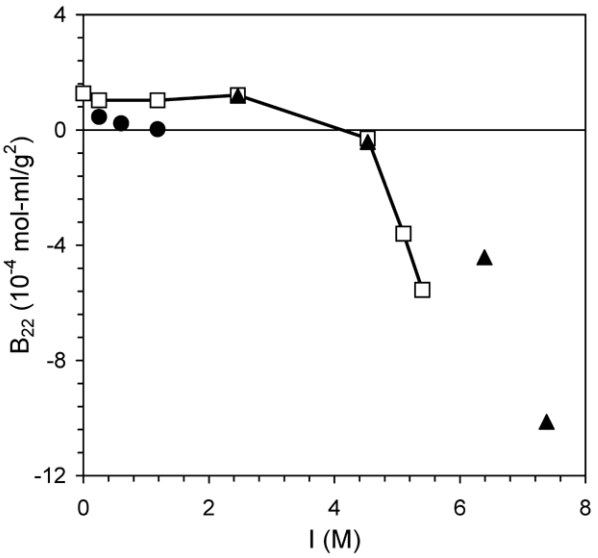

Figure 1

BSA virial coefficients for ammonium sulfate solutions at pH 6.2 as a function of ionic strength measured by SIC (squares) and static light scattering: (filled triangles) (Asanov et al., 1997) and (filled circles) (Kulkarni et al., 2000).

The good agreement between the virial coefficient measurements using SIC and static light scattering for BSA is consistent with earlier observations for lysozyme and chymotrypsinogen (Tessier et al., 2002b). The accurate measurements for BSA provide a basis on which to conduct SIC virial coefficient measurements for myoglobin, despite the general inaccessibility of results by light scattering due to the strong absorbance of the heme group. (This problem can be circumvented using an IR laser, although most light scattering systems are configured with a laser operating in the visible region.) The results of the myoglobin measurements are shown in Figure 3. The virial coefficients for myoglobin in sodium chloride at pH 7.4 show little variation as a function of ionic strength up to 5 M, while the virial coefficients in potassium phosphate at the same pH show a significant reduction and pass sharply through the"crystallization slot" at an ionic strength of approximately 4 M. We also measured myoglobin virial coefficients as a function of ammonium sulfate concentration at two pH values, and in both cases found a sharp decrease at an ionic strength of approximately 6 to 7 M. The virial coefficients for myoglobin measured at pH 9 become negative (attractive) at a lower ionic strength than at pH 6.

The elution volume for myoglobin was dependent on the amount of injected protein for solution conditions that led to attractive protein interactions at a protein concentration of 5 mg/ml and an injection volume of 20 µl. However, at higher injection concentrations (20 to 30 mg/ml) and a larger injection volume (50 µl), the elution volume became independent of the injection consistent with our previous results for lysozyme (Tessier et al., 2002b). Therefore, we used an injection concentration of 5 mg/ml and an injection volume of 20 µl for virial coefficients that were repulsive, and 20 mg/ml and 50 µl for virial coefficient measurements that were attractive.

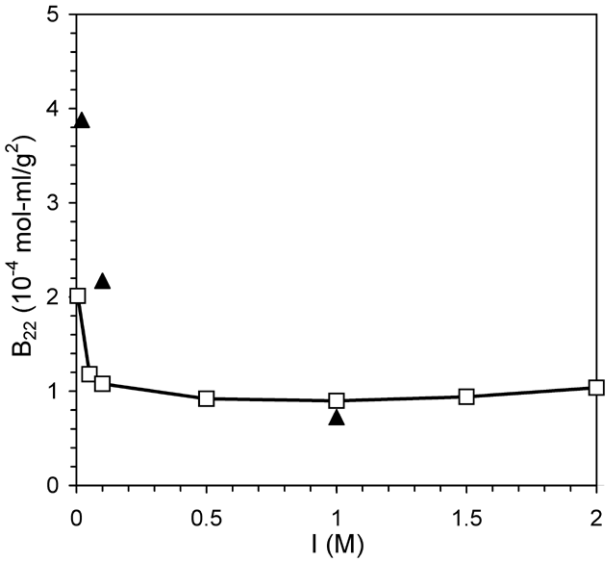

Figure 2

BSA virial coefficients for sodium chloride solutions at pH 7 as a function of ionic strength measured by SIC (squares) and static light scattering (filled triangles).

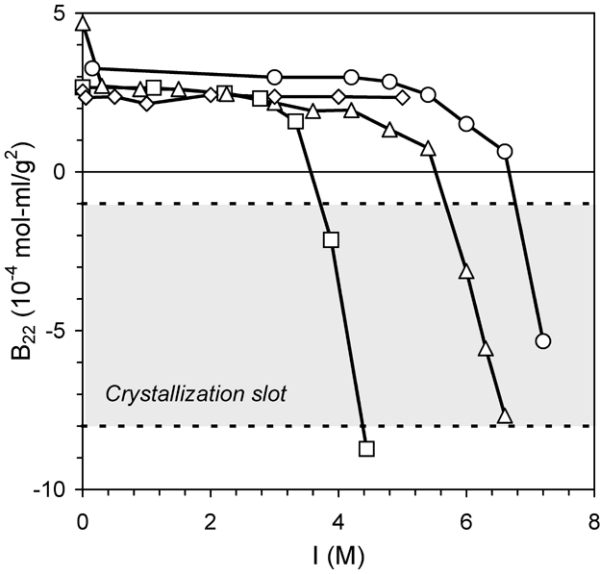

Figure 3

Myoglobin virial coefficients measured by self-interaction chromatography at pH 7.4 in potassium phosphate (squares), pH 6 (circles) and 9 (triangles) in ammonium sulfate, and at pH 7.4 in sodium chloride (diamonds).

Table 1

Summary of virial coefficient measurements for myoglobin as a function of pH and the corresponding phase behavior determined by ultracentrifugal crystallization. The location of the "crystallization slot" is denoted by the bold lines.

pH	Crystallization additives	Phase separation	B_{22} (10^{-4} mol-ml/g^2)
6	1 M ammonium sulfate	none	3.0
7.4	3 M sodium chloride	none	2.4
7.4	5 M sodium chloride	none	2.3
9	1 M ammonium sulfate	none	2.2
9	1.8 M ammonium sulfate	none	0.8
6	2.2 M ammonium sulfate	none	0.6
7.4	1.75 M potassium phosphate	phosphate sep.	-2.1
9	2.0 M ammonium sulfate	crystal	-3.1
6	2.4 M ammonium sulfate	crystal	-5.3
9	2.1 M ammonium sulfate	crystal	-5.6
9	2.2 M ammonium sulfate	precip. & crystal	-7.7
7.4	2 M potassium phosphate	phosphate sep.	-8.7

To determine the relationship between the virial coefficient measurements and the myoglobin phase behavior, ultracentrifugal crystallization experiments were conducted at the corresponding solution conditions (Table 1). It can be seen that the "crystallization slot" (-1 to -8 x10^{-4} mol-ml/g^2) is a good predictor of protein phase behavior. The conditions at which crystals or precipitate formed correspond to virial coefficient values within the "crystallization slot", while conditions where no phase separation was observed correspond to virial coefficient values above the "crystallization slot". The solution conditions corresponding to the most attractive conditions at pH 9 (2.2 M ammonium sulfate) produced a mixture of crystals and precipitate, while the other two conditions with negative virial coefficients at pH 9 correspond to samples that were primarily crystalline. The two phase behavior points at pH 7.4 with negative virial coefficient values correspond to potassium phosphate samples (1.75 and 2 M) that phase separated during centrifugation. Myoglobin partitioned into the top phase, while a clear liquid was observed in the bottom phase. Finally, the virial coefficient at pH 6 that fell within the "crystallization slot" corresponds to a phase with only a few very small protein crystals relative to those at the three crystallization conditions at pH 9.

4. Discussion

The development of SIC as a rapid screening method for measuring the second virial coefficient has been reported only recently and needs to be further validated (Tessier et al., 2002a; Tessier et al., 2002b). Previous work has shown that quantitative agreement between SIC and SLS can be obtained for lysozyme and α-chymotrypsinogen (Tessier et al., 2002b), and this paper demonstrates that accurate BSA virial coefficient values can also be measured by SIC. An important advantage is that the agreement between SIC and SLS is obtained without the use of adjustable parameters, which is particularly important if SIC is to find use as a tool for predictive crystallization.

The minor deviation between the SIC and SLS virial coefficient measurements for BSA in ammonium sulfate above an ionic strength of 5 M may be due to immobilized BSA partially unfolding on the surface of the chromatography particles, causing the exposure of hydrophobic residues (Sarkar & Chattoraj, 1994). However, this seems to be a small effect since a difference between the SIC and SLS measurements is seen only at high concentrations of ammonium sulfate. For carboxymethyl BSA, the virial coefficients measured by SIC at pH 7 and low ionic strength ($\leq$ 0.1 M) are more attractive than the corresponding light scattering values, which was also observed previously for lysozyme at low ionic strength (Tessier et al., 2002b). SIC cannot accurately characterize highly repulsive interactions since the free protein that passes through a column containing immobilized protein will be excluded from a significant fraction of the pore space, causing the retention to depend weakly on the amount of immobilized protein. However, accurately characterizing repulsive forces is of little interest when searching for conditions under which proteins will favorably associate and crystallize.

It is interesting that the BSA and myoglobin virial coefficients show similar trends. Myoglobin and BSA both display little intrinsic affinity to self-associate, even at high concentrations of sodium chloride. We have conducted BSA virial coefficient measurements in sodium chloride up to 5 M (data not shown), and confirmed that the values show little variation as a function of salt concentration above 0.1 M, which is similar to the behavior seen here for myoglobin. The repulsive protein interactions for BSA and myoglobin at high concentrations of sodium chloride agree with the high solubility of BSA and myoglobin reported previously (Cohn & Edsall, 1943), and are consistent with the behavior of halophilic enzymes, which are stable and maintain activity at concentrations of sodium chloride exceeding 4 M (Hecht & Jaenicke, 1989). It has also been shown that for the large oligomeric proteins aspartate transcarbamylase (306 kD) (Budayova et al., 1999), urate oxidase (128 kD) (Bonneté et al., 2001) and α-crystallin (800 kD) (Finet, 1999), the pairwise interactions remain repulsive above an ionic strength of 1 M. It has been speculated that the lack of attraction may be attributed to the size of the protein (Tardieu et al., 2001), yet our results for myoglobin suggest that this type of behavior can also be manifested for relatively small proteins.

The virial coefficient behavior of BSA and myoglobin, as well as aspartate transcarbamylase, urate oxidase and α-crystallin, can be generalized into a class of proteins that display little self-association affinity at low to moderate salt concentrations (< 1 M sodium chloride). There are at least two other patterns of virial coefficient behavior previously observed in which attractive protein interactions are observed at low to moderate salt concentrations. The first classification is for proteins that display virial coefficient behavior similar to that described by the DLVO theory over a wide range of pH values, such as the behavior observed for lysozyme (Rosenbaum & Zukoski, 1996; Gripon et al., 1997; Curtis et al., 1998; Velev et al., 1998; Bonneté et al., 1999; Piazza & Pierno, 2000). Lysozyme virial coefficients decrease monotonically with increasing salt concentration and increasing pH, up to the isoelectric point, consistent with electrostatic interactions dominated by the net charge on the protein. The other major classification is for proteins that display attractive electrostatic interactions at low ionic strength even when their net change is non-zero. This behavior has been observed for chymotrypsinogen (Velev et al., 1998), ribonuclease A (Tessier et al., 2002a), and staphylococcal nuclease (unpublished results). This type of behavior appears to be caused by the complex orientational dependence of protein interactions, with a relatively small number of high-affinity configurations being especially significant (Neal at al, 1998).

Myoglobin and BSA also show similar behavior in ammonium sulfate: both proteins show little change in their virial coefficient values at low concentrations of ammonium sulfate, and a sharp reduction in their virial coefficient values at high concentrations of ammonium sulfate. It has been estimated that sulfate, a strong kosmotrope, has 14 water molecules bound in its hydration layer (Lovrien et al., 1994). At ammonium sulfate concentrations of 2 to 3 M (I = 6 to 9 M), the total amount of water bound by sulfate would be at least 28 to 42 M, which is more than half of the total available water. Interestingly, the water activity of ammonium sulfate

solutions is a weak function of salt concentration at high ionic strengths relative to the dramatic change observed for the virial coefficient measurements. Therefore, we speculate that a reduction in the hydration of BSA and myoglobin leads to the sharp reduction in the virial coefficient values at high ionic strengths, although the precise mechanism is not known.

Structures of myoglobin obtained by X-ray diffraction from crystals grown in ammonium sulfate contain at least one sulfate ion bound in a crystal contact (Evans & Brayer, 1988). Since the virial coefficient is highly sensitive to the pairwise configurations that are most attractive, among which are often the crystal contact configurations, it is possible that sulfate ions bound in crystal contacts may provide a repulsive barrier to self-association due to their highly hydrated nature. This is consistent with the results in this work since the interactions became attractive at higher ammonium sulfate concentrations at pH 6 than at pH 9. The fact that sulfate is bound in a crystal contact also suggests that sulfate may be involved in ion bridging during the crystallization process. However, if this is the case, it appears that high concentrations of ammonium sulfate are required to dehydrate the ion to promote attractive interactions that are conducive to crystallization. It should also be noted that although depletion forces may become important at high salt concentrations, the virial coefficient trends for myoglobin and BSA do not follow a classical logarithmic dependence characteristic of depletion forces (Asakura & Oosawa, 1954), indicating that other intermolecular forces, such as hydration forces, contribute more significantly than depletion forces to the value of the virial coefficient.

The myoglobin phase behavior results confirm that the "crystallization slot" is a useful guide for crystallization, and strengthen the idea of using this type of methodology for predictive crystallization. It is interesting to note that for myoglobin and BSA in ammonium sulfate, there is only a narrow window of salt concentrations that correspond to virial coefficients within the "crystallization slot". It would be difficult to determine this narrow range of salt concentrations using empirical crystallization screens since many crystallization experiments would be required, which reinforces the advantage of approaching protein crystallization in a rational manner. In fact a recent study was unsuccessful in its attempt to crystallize myoglobin at pH 7.2 with ammonium sulfate at 6 different ionic strengths ranging from approximately 3 to 15 M (McPherson, 2001); it may have been that the small window of suitable salt concentrations for crystallization was missed.

5. Conclusions

Self-interaction chromatography was used to measure the second virial coefficient for BSA, and the results were shown to be in quantitative agreement with those from static light scattering. Self-interaction chromatography revealed a sharp reduction in the virial coefficient values at high concentrations of ammonium sulfate for both BSA and myoglobin, indicating that hydration forces may be important. This sharp variation of virial coefficient values results in a narrow range of electrolyte concentrations that fall within the "crystallization slot", unlike the more gradual variations seen for lysozyme and α-chymotrypsinogen. The unexpected pattern of virial coefficient behavior for myoglobin and BSA emphasizes the importance of measuring protein interactions to determine conditions for protein crystallization. The most important conclusion of this work is that self-interaction chromatography appears to be a powerful method for the predictive crystallization of proteins, and should be readily amenable for proteins that have not been crystallized previously.

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