ALDOLASE IN CONCENTRATED FICOLL 70 SOLUTION: ANALYSIS WITH SCALED PARTICLE THEORY

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ABSTRACT

The apparent average buoyant molar mass, M^*_{app}, of Ficoll 70 and 2 x 10^{-3} g/cm^3 aldolase in Ficoll 70 were measured as a function of Ficoll 70 concentrations at concentration up to 0.16 g/cm^3 in 0.15 M NaCl, pH 7.4, using tracer sedimentation equilibrium at 25^{o}C and 37^{o}C. The dependences were quantitatively accounted for by scaled particle theory of fluid mixtures using a simple model in which a weak attractive interaction between Ficoll 70 and aldolase was assumed, in addition to the excluded volume between the component mixtures. The self interaction between Ficoll 70 was repulsive and independent of temperature in the range of this experiment. The excluded volume between Ficoll 70 and aldolase was significantly reduced by the attractive interaction, which was dependent on temperature.

Keywords: Sedimentation Equilibrium, Crowder, FITC, Interaction, Aldolase, Ficoll 70

INTRODUCTION

The functions of proteins are related to their states of association, conformation and aggregation, which are in turn determined by the total concentration of the medium (Minton, 2000; Perhama *et al.*, 2007). Attempts at understanding the effect of the presence of diverse or single macromolecule at high total concentration in cells have led to the use of proteins and carbohydrate polymers (Ren *et al.*, 2003; Monterroso and Minton, 2007) to mimic the presence of the crowded environment encountered in living cells (Ellis, 2001). A number of such experimental and theoretical observations, particularly those involving single globular proteins, have been successfully accounted for in terms of the excluded volume theory (Minton, 1981). In general, macromolecular crowding is predicted (Hong, 2010; Tsao and Dokholyan, 2010) and found to favour compaction (Wang *et al.*, 2010), shifting denatured and intermediate ensembles in a folding reaction away from more extended states. Ficoll 70, an inert, synthetic carbohydrate polymer, has been used by many investigators to produce a semblance of the high total concentrations that are encountered in the cytoplasm (Ren *et al.*, 2003; Monterroso and Minton, 2007). Recently, Jiao *et al.* (2010) reported the presence of weak attractive interactions in addition to the excluded volume interaction between protein and polymer. Fodeke and Minton (2011) made similar observations for the interaction of binary mixtures of superoxide dismutase, bovine serum albumin (BSA) and ovomucoid. Results from Brownian dynamic simulations of the *Escherichia coli* cytoplasm also showed that steric and electrostatic potential alone could not account for experimental results on either protein stability in the cell or diffusion rates, and that the inclusion of short range attractive hydrophobic interactions produced much better fits (Christiansen *et al.*, 2010). Additionally, calculations by these authors showed that the impact of crowding depends on the properties of a given protein and the size differential between it and the surrounding molecules, as was also concluded by McGuffee and Elcock, (2010). Previously, non-ideal tracer sedimentation equilibrium, the method adopted in this experiment, had been used for quantifying BSA self-interaction and BSA interaction with Ficoll 70 at up to a Ficoll concentration of 100 mg/cm^3 between 5^{o}C and 37^{o}C. The temperature effect on both interactions was found to be negligible. In this experiment, the effect of Ficoll 70, an uncharged carbohydrate polymer (at high

concentration), on the free energy of interaction of globular protein, aldolase was studied. Any observed attractive interaction should be hydrophobic. It is hoped that the results from this experiment will contribute to a body of data which provide intriguing information from the use of inert polymer to mimic *in vitro* conditions resulting from high total concentration of inert molecules. The experimental data were analysed using (i) model-free thermodynamic virial relations between the activity coefficient and concentration, and (ii) a simple model of the component mixture based on Scaled Particle Theory (SPT). The effect of temperature on the free energy of heterointeraction was also determined and used to propose the mechanism of the crowding.

MATERIALS AND METHODS

Materials

Rabbit muscle aldolase and Ficoll 70 were purchased from Sigma-Aldrich (St Louis, MO). The dialysis cassettes (10,000 MWCO) and fluorescence isothiocyanate (FITC) were products of Thermo Scientific. X10 phosphate buffer, pH 7.4 (0.15 M NaCl), was a product of KD chemicals. The concentration of Ficoll 70 was determined refractometrically.

Methods

The labeling of aldolase was carried out as described previously (Fodeke and Minton, 2010). All samples were dialyzed extensively in phosphate buffer pH 7.4 using a Slide- A- lyzer dialysis cassette. An $A^{1\%}$ 1 cm value of 0.938 at 280 nm was used for estimating the aldolase concentration. Size exclusion chromatorgraphy (SEC), coupled with an on-line light scattering detector, was used to compare both FITC labeled and unlabeled aldolase. The similarity of the elution peak of the labeled aldolase to that of the unlabeled protein in the SEC column showed that FITC-labeled aldolase was a bonafide tracer for the experiment. The degree of labeling of aldolase was 12 FITC/aldolase. The tracer sedimentation equilibrium experiment was carried out on Ficoll 70 and on mixtures of aldolase and Ficoll 70 in 2 mm inner diameter centrifuge tubes at 25°C and 37°C according to the method described earlier (Fodeke and Minton, 2010), with modifications needed to give good gradients. The rotor speed was between 6,000 and 10,000 rpm, depending on the concentration of Ficoll 70 in the equilibrium mixture. Upon the attainment of sedimentation equilibrium, the contents of the centrifuge tubes were fractionated using a Brandel Fractionator. The apparent bouyant molar masses of aldolase and Ficoll 70 in the equilibrium mixture were calculated from the plot of the dependence of concentration (determined from the absorbance, in the case of aldolase, and from the refractive index, in the case of Ficoll 70) on the square of the radial distance of the fractions collected from the axis of rotation in the centrifuge. The dependence of apparent average buoyant weight molar mass of Ficoll 70, $M^*_{app,fic}$ on concentration of Ficoll 70, w_{fic} was obtained at different Ficoll 70 concentration up to 0.16 g/cm^3. The apparent molar masses of Ficoll 70 and aldolase were then plotted as a function of the total concentration of Ficoll 70 in the equilibrium mixture.

Methods of Analysis

The apparent average buoyant molar mass, M^*_{app}, of a sedimenting species on the attainment of sedimentation equilibrium is related to the radial position, r, and concentration, c, according to the

$$M^*_{app} \quad \frac{\ln c}{r^2} \frac{2RT}{2} \qquad (1)$$

R, T and are the gas constant, the temperature in Kelvin and the angular velocity of rotation respectively. The value of Mapp is calculated from the equation:

$$M_{app} \quad \frac{M^*_{app}}{\overline{w}}$$

In which $\overline{w}$ is the density increment of the protein or polymer measured under conditions in which it is held constant at dialysis equilibrium. The value used for aldolase was approximately 0.27, the average reported for globular proteins. For Ficoll 70, approximately 0.38 was used (Durschlag, 1986).

Thermodynamic Non-model Virial Analysis

Interacting species under sedimentation

equilibrium are related thermodynamically by the equation:

$$M^*_{j,app} \quad M^*_j \quad \sum_i w_i \frac{\ln \gamma_j}{w_i} M^*_{i,app} \qquad (2)$$

where $M^*_{j,app}$ is the average buoyant apparent molar mass of the species j, $M^*_{i,app}$ is the average buoyant apparent molar mass of species i, w_i is the concentration of species i whose interaction with species j affects the $M^*_{j,app}$ of species j (Rivas *et al.*, 1999).

The activity coefficient γ_j of a dilute macromolecule j is related to the concentration of species i by equation:

$$\ln \gamma_j \quad B_{ji} w_i \quad B_{jii} w_i^2 \quad B_{jiii} w_i^3 \quad \ldots \qquad (3)$$

species i is present in solution at a concentration that is sufficiently high to affect the thermodynamic properties of species j (Minton, 1998). In equation (3) B_{ji}, B_{jii}, B_{jiii} represent the coefficients of two, three and four body interactions respectively of species i with j, whose activity coefficient depends on the concentration w_i of species i.

A special case in which j = i yields equation (4) below from equation (3)

$$\ln \gamma_i \quad B_{ii} w_i \quad B_{iii} w_i^2 \quad B_{iiii} w_i^3 \quad \ldots \qquad (4)$$

Considering that only one species i, is present in sufficient concentration to affect $M^*_{j,app}$ and truncating equations (3) and (4) at the quadratic term reduces equation (2) to:

$$M^*_{i,app} \quad \frac{M^*_i}{1 \quad B_{ii} w_i \quad 2B_{iii} w_i^2} \qquad (5)$$

and

$$M^*_{j,app} \quad M^*_j \quad (B_{ji} w_i \quad 2B_{jii} w_i^2) \frac{M^*_i}{1 \quad B_{ii} w_i \quad 2B_{iii} w_i^2} \qquad (6)$$

It is therefore possible to obtain the fitting parameters M^*_i, M^*_j B_{ji}, B_{jii}, B_{ii} and B_{iii} by simultaneously plotting the dependence of $M^*_{i,app}$ on w_i and the dependence of $M^*_{j,app}$ on wi using equations (5) and (6).

Analysis Based on Statistical Model

According to the scaled particle theory of hard convex particle mixtures, for a case in which only a single species of macromolecule is present at high concentration (Rivas *et al.*, 1999), the general theoretical expression for the derivative of activity coefficient (Labowitz, 1965; Boublik, 1974) is given as:

$$\ln \gamma_j \quad \frac{V_1}{(1 \quad \eta_1 V_1)} \quad H_j S_1 \quad S_j H_1 \quad V_j \frac{1}{1 \quad \eta_1 V_1^2} \quad \ldots$$

$$H_1^2 S_1^2 \quad 2V_j H_1 S_1 \frac{1}{1 \quad \eta_1 V_1^3} \quad V_j H_1^2 S_1^2 \frac{1^2}{1 \quad \eta_1 V_1^4} \qquad (7)$$

Where η_1 is number density of species 1 (proportional to w_1) and each species of particle is characterized by three shape parameters: The mean radius of curvature H_j, the surface area S_j, and the volume of equivalent convex particle V_j (details of which have been described elsewhere) (Labowitz *et al.,* 1965). V_j is the specific exclusion volume Veff, obtained in this work.

The derivative of the logarithm of the activity coefficient of the tracer species with respect to the concentration in g/cm^3 of the crowder can then be obtained in terms of the number density in cm^{-3}. Equation (7) is used together with equation (2) in fitting the structural model.

In formulating a model for the interaction of aldolase with Ficoll 70, it is posited that a weak heterocomplex, TC, is formed due to a weak attractive interaction between the crowder and the tracer providing that the fractional conversion of aldolase to the heterocomplex was small. The equilibrium concentration of TC is given by the relation:

$$c_{TC} = K_{TC} c_T c_A \qquad (8)$$

where K_{TC} is the equilibrium constant for the formation of the aldolase-Ficoll 70 complex dependent upon the composition of the solution as shown below.

$$K_{TC} \quad K^0_{TC} \frac{C \quad T}{TC} \qquad (9)$$

denotes the association equilibrium constant in the limit of low total Ficoll 70 concentration (Minton 1983). The activity coefficients, , of all species are calculated using the scaled particle theory. The undetermined parameters of this approximate model that was used with equations (2) and (7) for calculating the fit of the dependence of M^*_{app} on concentration of w_{fic} are $M^*_{Aldo,}$ $M^*_{fic,}$ $V_{eff,C,}$ $V_{eff,T}$ and $K_{TC.}$

RESULTS

Figures 1A - C show sample plots of the logarithm of concentration against the square of the radial position of each fraction from the axis of rotation during centrifugation according to equ. (1). M*app values were calculated from each of these plots and other similar plots using equation (1).

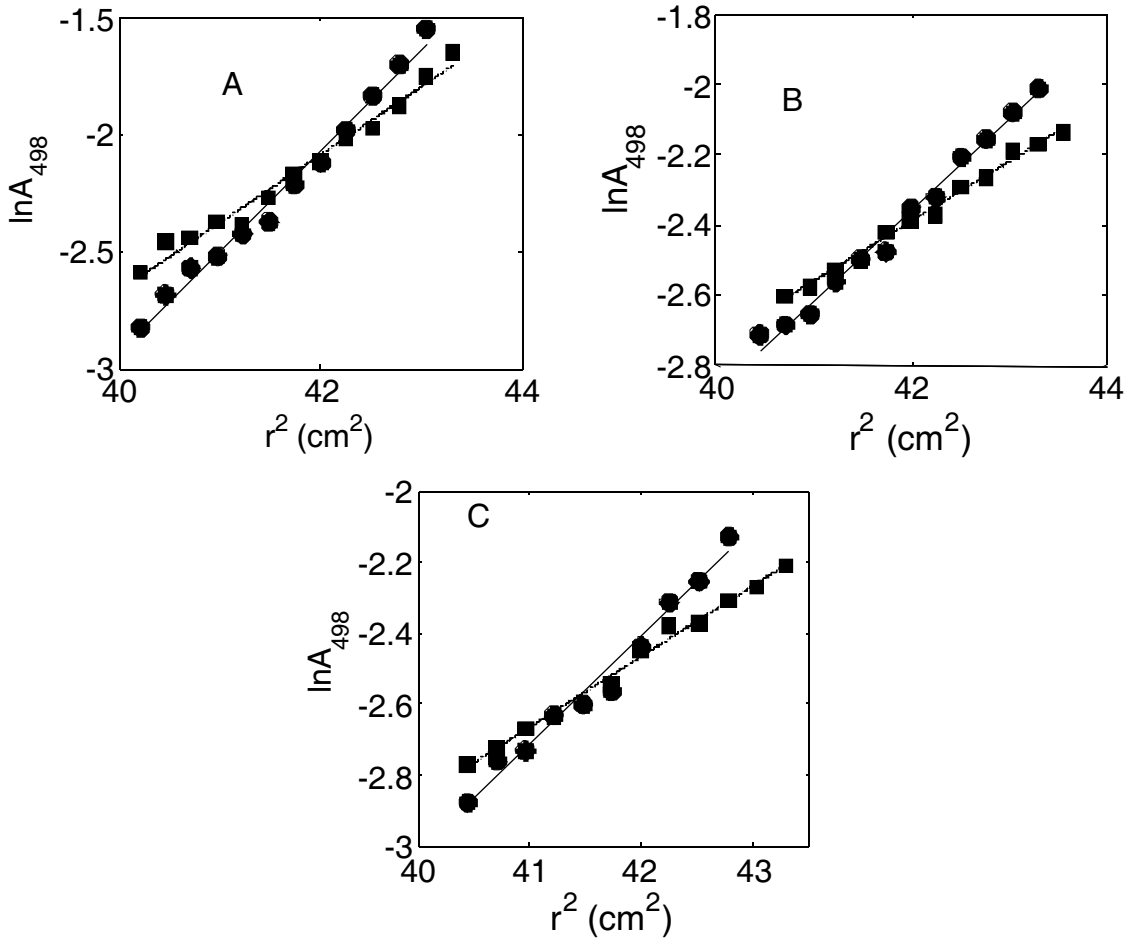

Figure 1: The dependence of logarithm of the concentration of fractions of aldolase in Ficoll 70 on the radial positions in the centrifuge at equilibrium centrifugation. (Panel A (at 7,000 rpm); aldolase centrifuged in 0.02 g/cm^3 Ficoll 70 (circles) and 0.08 g/cm^3 Ficoll 70 (squares) at 25^{o}C. Panel B (at 6,000 rpm); aldolase centrifuged in 0.01 g/cm^3 Ficoll 70 (circles) and 0.06 g/cm^3 Ficoll 70 (squares) at 37^{o}C. Panel C (at 10,000 rpm); aldolase centrifuged in 0.14 g/cm^3 Ficoll 70 (circles) and 0.16 g/cm^3 Ficoll 70 (squares) at 37^{o}C).

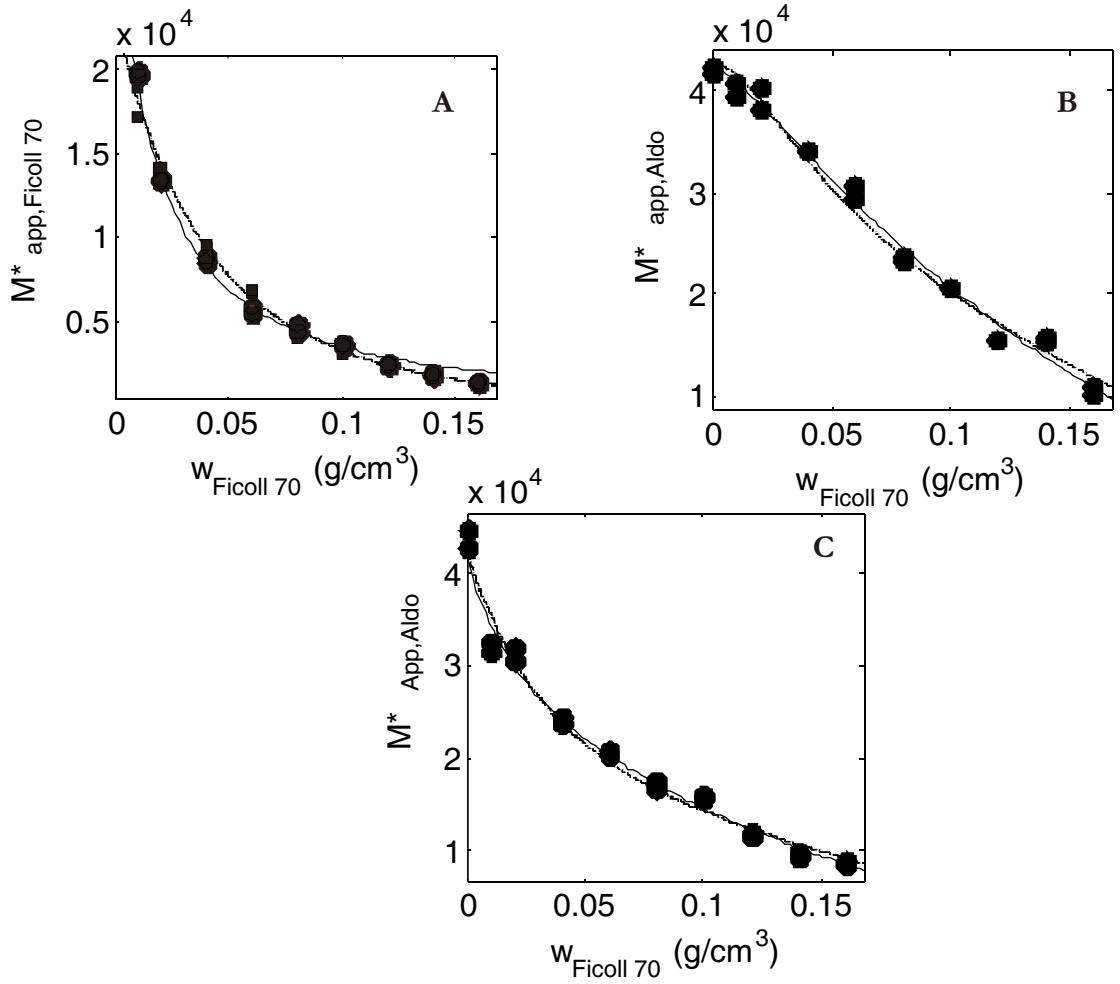

Figure 2: Apparent average buoyant molar mass of X as a function of w/v concentration of Ficoll 70: panel A (X = Ficoll 70 at 25°C (filled circle) and 37°C (square), Panel B (X = FITC-aldolase at 25°C) and Panel C (X = FITC-aldolase at 37°C). (Solid line is calculated using equation (6) with best-fit parameter values in Table 1 column 1. Broken line is calculated using equations (2) and (7) with best fit parameter in Table 1 column 3).

Data set 1, plotted in Figure 2A, describes the dependence of $M^*_{app,fic}$ on w_{fic} at 25°C and 37°C . The dependence is independent of temperature over the range 25-37°C. The negative slope of the curve shows that Ficoll 70 self-interactions are essentially repulsive over the entire concentration range of the experiment. Data set 2 (Figure 2B) describes the dependence of $M^*_{app,aldo}$ on w_{fic} at 25°C. Data set 3 (Figure 2C) describes the dependence of $M^*_{app,aldo}$ on w_{fic} at 37°C. In data sets 2 and 3, aldolase is a tracer species j, interacting with species i (Ficoll 70) at 25°C and at 37°C respectively. For fitting the data with non-model thermodynamic equation with virial expansion of the concentration of solute whose concentration affect the interaction of the component species (full line), truncating equation 5 and 6 at the quadratic term of the concentration was found to satisfactorily fit the data. The broken line is the theoretical fit of the data using simple structural model based on hard particle theory. Table 1 shows the fitting parameters of equations (6) and (7) to the three sets of data.

Column 1 of the table contains the fitting parameters of the data to equation (6) truncated at the quadratic term. WSSR indicates the weighted sum of squared residuals of each fitting result. The slope of the curve of the profiles of the dependence of $M^*_{app,aldo}$ on Ficoll 70 concentration are both negative but not identical at both temperatures. This showed that the interactions between the two interacting species were essentially repulsive, and dependent on temperature.

Table 1: Fitting Parameters of the combined data of Ficoll 70 and Aldolase Binary Mixture using virial expansion and Statistical Thermodynamic Model Base on Hard Particle Theory B_{ii}, B_{ij}, and B_{iii} are in cm^3/g, cm^6/g^2 and cm^9/g^3 respectively. $Veff_T$ and $Veff_C$ are in cm^3/g and M_{io} are in g/mol. Subscript i represents ficoll 70, j is aldolase.

Thermodynamic Expansion	Statistical Structural Model[1]	Statistical Structural model[2]
$M^*_{fic} = 27000$ $M^*_{aldo} = 42200$ $B_{ii} = 47.30$ $B_{iii} = 101.51$ $B_{ji}(25°C) = 0.06$ $B_{jii}(25°C) = 305.83$ $B_{ji}(37°C) = 35.41$ $B_{jii}(37°C) = 215.44$ WSSR = 1.21×10^8	$M^*_{fic} = 21400$ $M^*_{aldo} = 42500$ $Veff_T = 4.04$ $Veff_C = 1.17$ $\log K^0_{TC}$ (25°C) = 2.93 $\log K^0_{TC}$ (37°C) = 1.74 WSSR = 2.27×10^8	$M^*_{fic} = 23200$ $M^*_{aldo} = 42300$ $Veff_T = 2.86$ $Veff_C = 1.37$ $\log K^0_{TC}$ (25°C) = 2.97 $\log K^0_{TC}$ (37°C) = 1.13 WSSR = 1.48×10^8

(1 - Spherocylindrical geometry assumed for Ficoll 70 and Spherical for Aldolase and heterocomplex. 2 - Spherocylindrical geometry for Ficoll 70 and heterocomplex, and spherical for Aldolase).

Data set 1 contains twice the number of data points in each of the data sets 2 and 3. All three sets of data were modeled simultaneously to each of equations (6) and (7) to obtain the best fit of the model equations by minimizing the total sum of squared residuals for the three data sets with respect to the variation of single set of fitting parameters in Table 1.

In modeling the data using the simplified structural model, the effective specific volumes of Ficoll 70 and aldolase are v_{effC} and v_{effT} respectively. The equilibrium constant of the weak heterocomplex CT formed by the interaction between C and T is K_{TC}. The fitting parameters of the data sets 1 - 3 using the simplified model assuming that the heterocomplex formed is a sphere is presented in Table 1 column 2.

The data with the structural model was first fitted with equations (2) and (7) using the parameters in Table 1 column 2. This fitting was based on assumption that aldolase, a globular protein, is spherical - based on known structure of globular proteins. Ficoll 70 was modeled as a spherocylinder with length of cylindrical portion to diameter ratio of 6 and spherical geometery was assumed for the heterocomplex on the assumption of a higher probability of formation of the weak complex through the interaction of Ficoll 70 with aldolase via the lateral surface. This model however, did not give sufficiently good fit to the data (compare WSSR of Table 1 column 1 with column 2). The data was however, well fitted with a spherocylindrical geometry of length of the cylindrical portion to diameter ratio of 3 for the hetecomplex as seen by comparing WSSR values of Table 1, column 1 and column 3.

Plots in Figure 3 are calculated from the fitting parameters of the virial coefficients in Table 1 using equation (4) (solid line) or (3) (broken and dotted lines). It is seen that at 37°C, the free energy of solvation of aldolase in Ficoll 70 is higher than that at 25°C at the same Ficoll 70 concentration over the entire concentration of the experiment.

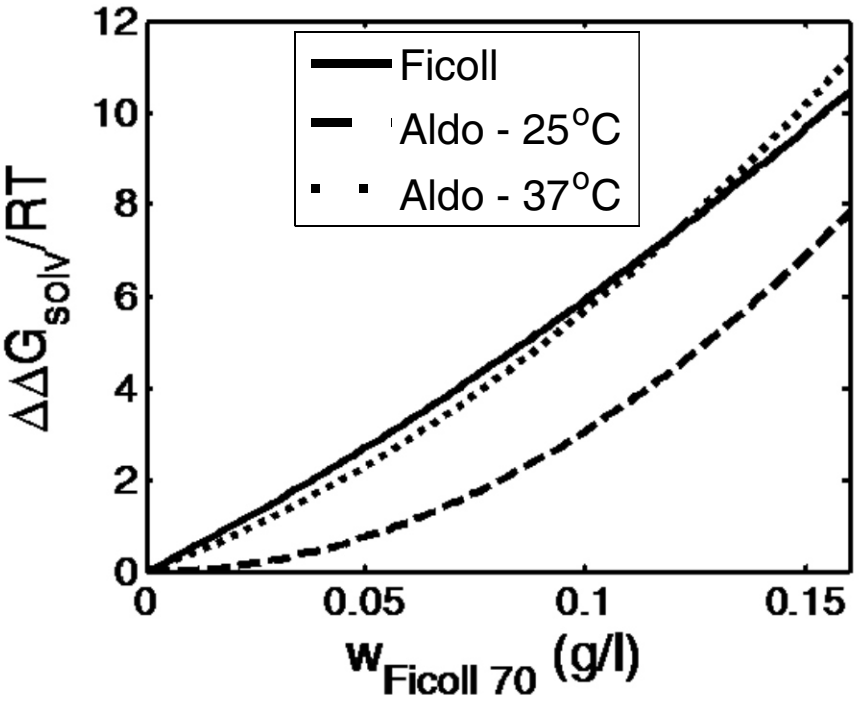

Figure 3: Calculated dependence of free energy of interaction X as a function of Ficoll 70 concentration. X = Ficoll 70 (full line), X = FITC-aldolase at 25°C (broken line) FITC-aldolase at 37°C (dotted line). (The full line was obtained using equation (4) with best fit parameters in Table 1. The dash and dotted lines were calculated using equation (3) with best fit parameters in Table 1).

DISCUSSION

One major difference between data set 1 and earlier reported experiment (Fodeke and Minton, 2010) is in the higher concentration limit of Ficoll 70 (0.16 g/cm^3) compared with 0.1 g/cm^3 in the earlier experiment. It was found that this difference resulted in no requirement to increase the number of virial terms needed to fit the experimental data. The earlier data (Fodeke and Minton, 2010) was fitted with equation (5) truncated at the quadratic term requiring six undetermined fitting parameters apart from the buoyant molar masses of the crowder and tracer. In fitting this experimental data the virial equation was truncated at the quadratic term since it gave satisfactory fit to the data. The virial coefficients of the nonstructural thermodynamic model were used to calculate the free energy of interaction of each component species in solution as a function of concentration. This method had previously been used to account for the free energy of interaction of Ficoll 70 and bovine serum albumin (BSA) with Ficoll 70 (Fodeke and Minton, 2010) and the interaction of superoxide dismutase (SOD) with BSA and also with ovmucoid at concentrations up to 0.1 g/cm^3 (Fodeke and Minton, 2011).

It is important to note that effective volume of Ficoll 70 obtained in this work is in good agreement with that reported earlier despite almost doubling the concentration of Ficoll 70 in this experiment. This is an indication that hard convex particle model does not break down for Ficoll 70 throughout the concentration of this experiment. The result of the structural model also indicates the importance of geometrical configuration for each species in the solution for the simplified model to correctly fit experimental data of this nature. The ratio of the length to diameter of the heterocomplex necessary to fit the data indicates that the interaction between Ficoll 70 and aldolase must indeed be a weak one.

The effective diameter calculated from $veff_T$ of aldolase obtained from the fit of structural model, based on molecular interaction, to this experimental data was 11.25 nm. This result is in agreement with the value of 10.01 nm obtained assuming a spherical geometry for the rabbit muscle aldolase tetramer determined using x-ray crystallography (Sygusch *et al.* 1985) from the

overall dimension of 9 x 9 x 6.5 nm. The slight discrepancy between the present result and that of (Sygusch *et al.* 1985) may have arisen from the divergence between spherical geometry assumed for aldolase in the present experiment and the geometry determined by x-ray crystallography.

The dependence of the free energy of interaction of aldolase with Ficoll 70 on the concentration of Ficoll 70 (Fig. 3) indicates that the free energy of interaction of Ficoll 70 with aldolase at 25°C is less than that at 37°C for the same concentration of Ficoll 70 over the concentration range of the experiment. A probable explanation for this observation is that low temperature significantly enhances the attractive interaction. The consequence of this is a greater reduction in the contribution of excluded volume to free energy of interaction at the lower temperature. This assertion is corroborated by the fact that, up to about 0.12 g/cm^3 of Ficoll 70, the free energy of interaction of aldolase with Ficoll 70 at 25°C is less than even the free energy of interaction of Ficoll 70, at the same crowder concentration. This could be an indication that, in addition to the excluded volume interaction, there was an attractive interaction, which increases with increasing concentration of crowder, between aldolase and Ficoll 70. That the free energy of a Ficoll 70 interaction with aldolase at 37°C, at the same weight to volume concentration is greater than that of Ficoll 70 interactions throughout the concentration range of the experiment is an indication that the attractive interaction was weak enough to be overcome by increasing the temperature of the polymer protein mixture from 25°C to 37°C. The small value of K_{TC}, and hence the small fraction of heterocomplex formed between Ficoll and aldolase at both experimental temperatures is also an additional piece of evidence for the observed differences.

This result could be an indication that the interplay of forces of temperature and crowder concentration changes could have significant consequences on the nature of the observed changes under different physiological conditions. The result in this project lends further credence to an earlier observation (Fodeke and Minton, 2011) that temperature can qualitatively and quantitatively affect the nature of the observed effect of the crowder on dilute solute species. It is, however, too early to generalize the observation in this study. It will be necessary to carry out similar studies on other types of proteins and, where possible, to work at higher crowder concentrations, particularly since concentrations in the cellular milieu, though often less, could be as high as a third of a gram per cm^3.

CONCLUSION

Attractive interactions can become important in the limit of high concentrations of crowding agent, resulting in quantitative and or qualitative differences in the kinetics and equilibria of reactions they affect. This effect can become very significant, with lowered temperature of the system depending on the nature of the interacting surfaces. The agreement between the non-model thermodynamic fit, requiring a total of eight undetermined fitting parameters, and the simple statistical structural model based on the nature of interaction, and requiring only six undetermined fitting parameters, is an indication that the model is robust for the experimental data. Its ability to account for similar interactions at concentrations up to 0.3 g/cm^3 remains to be tested.

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