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Explicit Incorporation of Hard and Soft Protein–Protein Interactions into Models for Crowding Effects in Protein Mixtures. 2. Effects of Varying Hard and Soft Interactions upon Prototypical Chemical Equilibria

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Abstract

Previously derived approximate analytical relations for the activity coefficient of each solute in a mixture of up to three spherical solutes in a highly nonideal solution interacting via square well potentials of mean force (Hoppe, T.; Minton, A. P. *J Phys Chem B*. **2016**, *120*, 11866–11872) were used to explore the effect of heterogeneity in volume occupancy and intermolecular interactions upon prototypical schemes representing solubility, partitioning, conformational isomerization, and self-association in crowded solutions. Results generally indicate that all of the equilibria explored are exquisitely sensitive to variations in both volume occupancy and intermolecular interaction and have important implications for the design and execution of more detailed simulations of complex media.

Graphical Abstract

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Notes

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INTRODUCTION

A solution containing a high total weight/volume concentration or volume fraction of one or more species of macromolecule is referred to as “crowded”. The influence of nonspecific repulsive and attractive intermolecular interactions in crowded solutions on the thermodynamic and transport properties of all species of macromolecule in a crowded solution has been the subject of extensive theoretical and experimental research over the past 45 years, and a consensus view of such effects has emerged: net repulsive interactions between macromolecules tend to facilitate reactions that reduce the overall ratio of macromolecular surface to volume such as protein folding and association, while net attractive interactions tend to inhibit such reactions.¹⁻⁷ Recent studies of crowding phenomena^{1,7,8} tend to fall into one of two categories: (1) Experimental, theoretical, and simulation studies of the effect of high concentrations of an individual species of an “inert” macromolecule, assumed to neither self-associate nor heteroassociate with other macromolecules in the solution upon the colligative properties of the solution or upon the reactivity or transport properties of one or more species of dilute tracer macromolecule. (2) Experimental and simulation studies of the reactivity or transport properties of one or more species of labeled tracer macromolecules introduced into the interior of a real or simulated intact cell. It is clear that the latter system is far more complex than the former for a variety of reasons, the most prominent of which being the presence of a very large variety of macromolecules (proteins, nucleic acids, and polysaccharides) in abundances that vary widely from point to point within the interior of an individual cell and between cell types.

Although knowledge of the effect of multiple crowding species upon the properties of a dilute probe macromolecule would seem to be prerequisite to an understanding of the behavior of such a probe in a medium as complex and heterogeneous as cytoplasm, only a small number of experimental and theoretical studies of crowding in well-defined mixtures have appeared in the literature.^{9–15} Results of the few experimental studies suggest that the effects of significant concentrations of more than one crowding species upon the free energies of solvation of each solute species may be far from additive, suggesting a significant contribution arising from long-range or “soft” interactions between the various solute species. Prior theoretical studies of crowding in mixtures have generally focused on excluded volume or steric interactions between solutes and with few exceptions^{4,13} have not explored the role of long-range or soft interactions between crowders and between crowders and tracer species. In the present work, we explore the influence of long-range as well as steric interactions in heterogeneous systems upon several prototypical chemical equilibria involving one or two species of tracer reactant.

Consider the general reversible reaction involving n_R reactant species and n_P product species:

where X_i denotes the i th reactant or product species and n_i the reaction stoichiometry of that species with a forward equilibrium constant given at constant temperature and pressure by

$$K = \frac{\prod_{i=n_R+1}^{n_R+n_P} c_i^{n_i}}{\prod_{i=1}^{n_R} c_i^{n_i}} \quad (2)$$

where c_i denotes the molar concentration of the i th species of reactant or product. The effect of crowding (or solute–solute interaction in general) upon this equilibrium constant may be generally written as

$$K(\{c\}) = K^\circ \Gamma(\{c\}) \quad (3)$$

where $\{c\}$ denotes the concentrations of all solutes present, not just those participating in the designated reaction. K° denotes the value of K at the same temperature and pressure in the ideal limit (all solute species dilute), and Γ denotes the crowding or cosolute factor¹⁶

$$\Gamma(\{c\}) = \frac{\prod_{i=1}^{n_R} \gamma_i^{n_i}}{\prod_{i=n_R}^{n_R+n_P} \gamma_i^{n_i}} \quad (4)$$

where γ_i denotes the thermodynamic activity coefficient of the i th species, which is a function of the excess chemical potential, or free energy of solvation, of that solute species:

$$\ln \gamma_i = \frac{[\mu_i(\{c\}) - \mu_i^0]}{kT} = \frac{[\mu_i^{\text{excess}}(\{c\})]}{kT} \quad (5)$$

$\mu_i(\{c\})$ denotes the chemical potential of the i th species in a solution of arbitrary solute composition, μ_i^0 denotes the chemical potential in the ideal limit, k denotes Boltzmann's constant, and T denotes the absolute temperature. It follows from eqs 2–5 that to quantitate the effect of crowding, or cosolutes in general, upon the equilibrium constant for reaction 1, all γ_i must be evaluated as a function of solute composition.

In principle, one can estimate the excess chemical potential of an individual solute species in a solution containing multiple solutes interacting via arbitrary potentials of mean force via Monte Carlo simulation and Widom insertion,¹⁸ and crowding simulations obtained in this fashion have been presented by Zhou and Qin.^{19,20} However, this method is highly compute-intensive and not suitable for systematic investigation of the effect of variation in solution composition and solute–solute interaction. In the first report of this series,²¹ hereafter referred to as Part 1, we recently presented approximate analytical calculations of the excess chemical potential of each solute species in binary and ternary mixtures of spherical particles interacting with each other via square well potentials and showed that the results of the analytical calculations are in good agreement with the results of Monte Carlo/Widom insertion calculations up to total particle volume fractions of ca. 0.2. We adopt this system as a highly coarse-grained model of a solution in which multiple macromolecular solutes interact with each other via potentials of mean force comprising both hard core steric interactions and attractive or repulsive longer ranged soft interactions. Because it is analytically soluble, this model is uniquely suited to a semiquantitative study of the effect of variation in a variety of solution compositions and solute–solute interaction parameters upon the excess chemical potential of each solute species and the equilibrium constants of selected reactions.

As pointed out above, the effect of heterogeneity in a crowded medium has not been systematically studied. In the present work, we utilize the thermodynamic relations and the model system described above, together with the analytical relations presented in Part 1, to explore the effect of crowding on equilibrium reactions taking place in two types of heterogeneous systems: (1) a system in which there are multiple microenvironments varying

in solute volume occupancy and (2) a system in which there are multiple microenvironments varying in solute composition at constant volume occupancy.

METHODS

Calculation of Thermodynamic Activity Coefficients.

The model system consists of a fluid containing three species of spherical particles with radii denoted by r_i and number density denoted by ρ_i , interacting with each other via spherically symmetric square well potentials characterized by well depth ε_{ij} (in units of kT) and range Δ_{ij} . A square well potential of mean force is given by

$$U_{ij}(r_{ij}) = \begin{cases} \infty & r_{ij} < r_i + r_j \\ \varepsilon_{ij} & r_i + r_j \leq r_{ij} < r_i + r_j + \Delta_{ij} \\ 0 & r_i + r_j + \Delta_{ij} \leq r_{ij} \end{cases} \quad (6)$$

The justification for using square well potentials to model the actual potential of mean force acting between macromolecules in solution is presented in the Discussion below. Species 1 and 2, referred to as crowding species or crowders, are present at arbitrary volume fractions ϕ_1 and ϕ_2 , the sum of which is the total fraction of volume occupied by macromolecules ϕ_{tot} . Species 3 is present in the dilute limit $\rho_3 \rightarrow 0$ and is referred to as tracer or probe species. The number densities of species 1 and 2 are related to the volume and mass fractions of these species by

$$\rho_i = \frac{\phi_i}{4\pi r_i^3/3} \quad (7)$$

For the present purpose, we assume that the two crowder species are of equal density so that the volume and mass fractions of the two crowders are identical. The volume fractions of each species may then be described as functions of the total volume occupancy $\phi_{\text{tot}} = \phi_1 + \phi_2$ and the mass (or volume) fraction f_2 of crowder species 2:

$$\begin{aligned} \phi_1 &= (1 - f_2)\phi_{\text{tot}} \\ \phi_2 &= f_2\phi_{\text{tot}} \end{aligned} \quad (8)$$

The thermodynamic activity coefficients of tracer and both crowder species in the model reaction schemes described below are calculated as functions of the sizes and number densities of the species and the square well potentials of mean force acting between them using analytical expressions obtained for the Kihara/SPT hybrid model presented in the appendix of Part 1. The range of square well depths and volume occupancies is limited to those ranges over which results of the analytical hybrid model were shown to agree well with the results of numerical simulation, typically total volume occupancies not greater than 0.2 and square well depths no greater than $-2 kT$. For brevity, we subsequently refer to the

impenetrability of two particles ($r_{ij} = r_i + r_j$) as the “hard” interaction and the interaction between two solute particles within the range of the square well ($r_i + r_j > r_{ij} > r_i + r_j - ij$) as the “soft” interaction.

Effect of Varying Crowder Composition upon Protein Solubility.

Consider a monomeric tracer species T in equilibrium with a single condensed phase that contains only T. The measured solubility of T then is given by

$$c_{\text{sol}} = \frac{c_{\text{sol}}^0}{\gamma_T} \quad (9)$$

where c_{sol}^0 denotes the solubility of T in the absence of added crowder(s).

Distribution of a Tracer Macromolecule among Compositionally Distinguishable Compartments.

Let us denote the mass, concentration, activity, and activity coefficient of the tracer species in compartment i by m_i , c_i , a_i , and γ_i , respectively, and the volume of the compartment by v_i . Assuming that the tracer is free to redistribute between compartments at equilibrium $a_i = a_j$ for all i and j . It follows from conservation of mass that the mass fraction of tracer in the i th compartment is given by

$$f_i \equiv \frac{m_i}{\sum_j m_j} = \frac{v_i \gamma_i}{\sum_j v_j \gamma_j} \quad (10)$$

Crowder composition is specified either as the fractional volume occupancy of each crowding species or the total volume occupancy of both species and the mole fraction of species 1. These two specifications are interconvertible. Given the crowder composition in each compartment, γ_i is calculated for each compartment using the hybrid model presented in Part 1, given the relative sizes of each solute species and interaction potentials acting between them specified in the Results section. Then, eq 10 is solved for all f_i .

Effect of Varying Crowder Composition upon Conformational Equilibria.

Many proteins are known to undergo significant conformational changes in the course of performing their biological functions (see Chapter 1 of ref 22). We model such changes as a reversible interconversion between two conformational states that we call open (O) and closed (C), where the open state is defined to be the state that excludes more volume to the crowding species.

Independent of the details of the actual conformational change leading to an increase in volume exclusion, the increase in volume exclusion may be parametrized as a difference between the relative sizes of equivalent spherical representations of the actual conformations excluding the same volumes to crowders, i.e., $r_O > r_C$. It follows from eqs 3 and 4 that

$$K_{OC} \equiv \frac{c_C}{c_O} = K_{OC}^0 \Gamma_{OC} = \frac{K_{OC}^0 \gamma_O}{\gamma_C} \quad (11)$$

Effect of Varying Crowder Composition upon Tendency of a Protein to Self-Associate.

Consider a dilute protein in a solution of two crowding species that can self-associate to form a single oligomer comprised of n monomers:

It follows from eqs 3 and 4 that

$$K_n \equiv \frac{c_n}{c_1^n} = K_n^0 \Gamma_n = \frac{K_n^0 \gamma_1^n}{\gamma_n} \quad (12)$$

RESULTS

Solubility.

The solubility of dilute tracer species 3 in a mixture of crowding species 1 and 2 was calculated as a function of f_2 , the mass fraction of crowder 2 in a mixture occupying a constant fraction of total volume. Results plotted in Figures 1 and 2 were obtained using interaction parameters specified in Table 1. In Figure 1, results are presented for mixtures of two crowders with size equal to each other and to tracer. In panel A, the two crowders interact with tracer via hard interactions exclusively. In panel B, crowder 1 interacts repulsively, and crowder 2 interacts attractively with tracer. Variations in interactions between the two crowding species are explored in both panels. In Figure 2, results are presented for mixtures of two crowders of unequal size, where crowder 1 has a mass one-third that of the tracer and crowder 2 has a mass three times that of the tracer. In panel A, the two crowders interact with tracer by hard interactions exclusively; in panel B, the smaller crowder 1 interacts repulsively, and the larger crowder 2 interacts attractively with tracer. In panel C, the smaller crowder 1 interacts attractively, and the larger crowder 2 interacts repulsively with tracer. The effect of variation in crowder–crowder interaction is explored in all three panels.

Qualitatively significant observations are summarized below:

1. Hard tracer–crowder interactions (excluded volume) reduce tracer solubility by as much as several orders of magnitude, confirming earlier theoretical and experimental results.^{17,23,24}
2. Crowders that are smaller than tracer have an excluded volume effect on the behavior of tracer much larger than that of crowders that are larger than tracer, confirming earlier theoretical and experimental results.¹⁷
3. Attractive tracer–crowder interactions can compensate for excluded volume effects and increase the solubility of tracer above that predicted on the basis of excluded volume alone and, when sufficiently strong, can actually lead to a net increase in solubility (Figure 1B, $f_2 \sim 1$).
4. crowder–crowder interactions modulate the effect of crowders on tracer. In particular, when crowders self-attract (blue curves in Figures 1B, 2A, and 2B), crowding effects are diminished, in some cases substantially. This is because self-attracting crowder molecules lie on average closer to each other than in the absence of attractive interactions, reducing the volume jointly excluded to tracer. In the extreme limit of self-attraction, crowder molecules may form complexes that have an excluded volume effect substantially reduced relative to that of the individual molecules.²⁵
5. Although excluded volume effects generally predominate, particularly when the crowder is smaller than tracer, when the smaller crowder attracts tracer and the larger crowder repels tracer (Figure 2C), compensating effects may lead to a reversal of the dependence of solubility upon crowder composition expected in the absence of soft interactions.

Tracer Distribution.

Consider a system containing five immiscible compartments of equal volume with different amounts of a single macromolecular crowding species of radius r_C occupying volume fractions ϕ_i ($i = 1-5$) of 0, 0.05, 0.1, 0.15, and 0.2. We assume that to maintain osmotic equilibrium, differences in osmolarity between the compartments due to differences in macromolecular concentration are compensated by differences in the concentration of freely distributing electrolytes. Let us further assume that an added tracer macromolecule of radius r_T is free to redistribute between the several compartments. The crowder interacts with itself via steric interaction only, and the tracer interacts with crowder via a square well potential with a range equal to $0.1 r_C$ and a variable well depth/height ϵ_{TC} .

In Figure 3, the distribution of tracer between compartments is plotted as a function of compartment number and the logarithm of the ratio of the mass of tracer to the mass of crowder for three different values of ϵ_{TC} . Qualitatively significant observations are summarized below.

1. The tendency of tracer to distribute preferentially into the compartments with the lowest concentrations of crowder increases markedly as the ratio of tracer to crowder size increases. This is true even when the net soft interaction between tracer and crowder is attractive (red symbols).

2. The tendency of tracer to distribute preferentially into the least crowded compartments decreases markedly as the soft interaction between tracer and crowder becomes more attractive. A sufficiently attractive tracer–crowder interaction can compensate for the excluded volume effect and, in addition, when the tracer becomes small relative to crowder, tracer may distribute preferentially into the more crowded compartments.
3. In contrast, a soft repulsive interaction between tracer and crowder does not seem to enhance the propensity of a tracer to preferentially distribute into less crowded compartments beyond that observed in the presence of steric repulsion alone.

Two-State Conformational Equilibrium.

The effect of crowding upon the equilibrium constant for the conformational equilibrium $O \rightleftharpoons C$ was calculated as a function of f_2 , the mass fraction of crowder 2 in a mixture occupying a constant fraction of total volume $\phi_{\text{tot}} = 0.2$. Results plotted in Figure 4 were calculated for a system with both crowdors having the same mass (size) as tracer, and results plotted in Figure 5 were calculated for a system with crowder 1 having one-third the mass and crowder 2 having three times the mass of tracer. In both figures, results were obtained with parameter values given in the figure captions and Table 1. Calculations were performed for different values of f_{exp} , and the results resemble those presented in Figures 4 and 5 except for a scaling factor, so results are shown only for $f_{\text{exp}} = 1.5$, a value selected because it was recently found to account for the experimentally measured effect of saccharide crowdors on the thermal stability of a protein.²⁶ Qualitatively significant observations are summarized below.

1. Hard (excluded volume) tracer–crowder interactions enhance the equilibrium constant for formation of the compact species, confirming the results of earlier theoretical arguments and experimental observations.^{16,17}
2. Soft interactions between crowdors modulate the excluded volume interaction between tracer and crowder. When attractive crowder–crowder interactions lead to “clustering” of crowder molecules, the volume jointly excluded by crowdors to tracer will be reduced, as noted above, with respect to results obtained for crowding effects on solubility.
3. An attractive soft interaction between tracer and crowder can partially compensate for the excluded volume effect on the conformational equilibrium. In contrast, a repulsive soft interaction between tracer and crowder does not significantly enhance the overall crowding effect. Although the maximally attractive tracer–crowder interaction presented here does not reverse the crowding effect, leading to a crowding-induced expansion of tracer, it may be possible that a further increase in tracer–crowder attraction could produce such an effect. However, the approximate analytical method of calculation described in Part I limits the range of attractive tracer–crowder interaction for which semiquantitatively accurate results may be obtained. As expected, crowding-induced compaction becomes much stronger as the tracer species increases in size relative to that of the crowder.

4. Comparison of results obtained for different values of f_{exp} (not shown) and 1.5 reveals that the magnitude of $\log \Gamma_{\text{OC}}$ is proportional to the value of $(f_{\text{exp}} - 1)$.
5. Depending upon their strength, attractive tracer–crowder interactions can diminish and even eliminate crowding effects arising from excluded volume, confirming earlier experimental and theoretical findings.^{2,6,27}

Monomer- n -mer Self-Association.

The effect of crowding upon the equilibrium constant for the equilibrium self-association $nP \rightleftharpoons P_n$ was calculated as a function of f_2 at constant $\phi_{\text{tot}} = 0.2$ for different combinations of ϵ_{TC} representing soft interactions between tracer and each species of crowder and two different values of n (2 and 4). It is assumed that the actual volume of protein does not change upon self-association, so that the radius of the spherical n -mer $r_n = n^{1/3}r_1$. Results plotted in Figure 6 were calculated for a system with both crowders having the same mass (size) as tracer, and results plotted in Figure 7 were calculated for a system with crowder 1 having one-third the mass and crowder 2 having three times the mass of tracer. In both figures, results were obtained with parameter values given in the figure captions and Table 1. Qualitatively significant observations are summarized below.

1. Excluded volume effects alone result in an enhancement of self-association (Figures 6A and A' and 7A and A'), in agreement with results of prior analysis.^{16,17}
2. While a repulsive soft tracer–crowder interaction provides a relatively small additional enhancement of self-association, an attractive soft interaction between tracer and crowder can substantially reduce the value of Γ_{1n} relative to that calculated for the same value of ϕ in the absence of soft interaction and, if sufficiently attractive, can result in crowding-induced inhibition of self-association rather than enhancement, as has been observed experimentally.³
3. The magnitude of $\log \Gamma_{1n}$ for a given crowder composition appears to be approximately proportional to the value of n , and the constant of proportionality is only slightly sensitive to the magnitude and sign of tracer–crowder interactions.

DISCUSSION

The results of the calculations presented here provide a first general (as opposed to specific case-oriented) analysis of the effect of heterogeneity in volume occupancy, tracer–crowder, and crowder–crowder interactions on the magnitude of crowding effects on several prototypical chemical equilibria. The ability to assess effects arising from changes in many of the variables characterizing these systems is achieved by extreme simplification, in particular the representation of macrosolutes in solution as spherical particles interacting via square well potentials of mean force. Thus, it is essential to inquire whether these simplifications may result in qualitatively incorrect conclusions.

The representation of macromolecules in solution by equivalent hard spherical particles has provided accurate quantitative and semiquantitative descriptions of many colligative

properties of compact globular proteins and other macromolecules in highly nonideal solutions.^{7,17,25,28} In the presence of significant soft interactions, the equivalent particle description may be generalized as follows. Over a finite range of concentration, the self-interaction between two like molecules may be described exactly by two-body and three-body coefficients denoted by B_2 and B_3 :

$$\frac{\Delta G_{\text{interaction}}}{kT} = B_2 c + B_3 c^2 + \dots \quad (13)$$

The values of both B_2 and B_3 may be measured experimentally via careful measurement of the concentration dependence of any of several colligative properties of macromolecular solutions (see Wu and Minton²⁹). The values of both coefficients may be calculated precisely as functions of the square well parameters r , ϵ , and ϵ defined in eq 6, as described by Kihara^{30,31} and Part 1. The value of r , the radius of the equivalent spherical representation of a rigid globular protein, may be calculated independently from experimentally measured values of molar mass M and partial specific volume $\bar{v}$:²⁹

$$r = \left(\frac{3M\bar{v}}{4\pi N_A} \right)^{1/3} \quad (14)$$

The concentration dependence of the free energy of interaction between two identical protein molecules may then be exactly described over a limited range of concentration by a square well potential of mean force with appropriate values of r and ϵ . It will be shown elsewhere (manuscript in preparation) that the same is approximately true for binary mixtures of two unlike proteins interacting via square well potentials of mean force defined by ϵ_{11} , ϵ_{12} , and ϵ_{22} and ϵ_{11} , ϵ_{12} , and ϵ_{22} . It follows that the representation of long-range interactions, either attractive or repulsive, between two rigid, globular molecules as a square well potential of mean force acting between equivalent spheres provides a simple yet physically reasonable approximation to the actual potential of mean force acting between the macromolecules in solution and provides a semiquantitative description of effective macromolecular interactions over the range of total concentrations (volume fractions) treated in the present work.

One feature common to the various results presented here is that the magnitude of crowding effects increases rapidly as the size of tracer species increases relative to the size of crowding species. This is due to the increased contribution of excluded volume to the total crowding effect, the size dependence of which was recognized long ago.^{16,17} Conversely, as the size of tracer species becomes smaller than that of the crowding species, the excluded volume contribution to total crowding effect diminishes, and the contribution of soft tracer-crowder interactions to the total crowding effect becomes dominant. Recent simulations and experiments aimed at quantifying protein stability in cytoplasm-like environments^{32,33} have focused on the stability of small peptides and proteins in the presence of high concentrations of proteins and other macromolecules significantly larger than the peptide or protein of interest. The authors of these studies assert that crowding in a cytoplasm-like environment

generally destabilizes rather than stabilizes proteins, in conflict with excluded volume theory. The results presented here indicate that in the absence of more comprehensive data obtained in the presence of crowders that are of dimension equal to or smaller than the protein or peptide of interest, the generality of such assertions must be regarded as unsubstantiated.

The calculations of the dependence of tracer solubility upon crowder composition are relevant to an understanding of phenomena such as the formation of amorphous aggregates or protein fibrils that have been shown to be thermodynamically analogous to first-order condensation.^{34,35} This analogy implies that a crowding-induced reduction in solubility parallels an increased tendency to form aggregates or fibrils under a particular set of conditions. Thus, the results shown in Figures 1 and 2 suggest that the tendency of proteins to aggregate or fibrillate depends markedly upon the composition of crowding species, as observed experimentally.³⁶ When the aggregating protein is sufficiently small relative to crowders, it is possible for attractive protein–crowder interactions to suppress fibrillation (black curve in leftmost panel of Figure 1). An extreme example of attractive tracer–crowder interaction would be a side reaction leading to complexation of the tracer with one or more crowding species in a fashion that inhibits self-association of the tracer. However, when the tracer protein is sufficiently large relative to dominant crowding species, exemplified in the rightmost panel, nonspecific attractive interactions between tracer and crowder are likely to be insufficient to overcome the magnitude of the excluded volume effect, promoting condensation-like aggregation or fibrillation, as observed by Groen et al.²

The model calculations for distribution of a tracer between compartments containing different volume fractions of crowder may be relevant to a situation when intracellular compartments are not separated by membranes but are created by phase immiscibility as, for example, in the separation of nucleoid from DNA-free cytoplasm in prokaryotes³⁷ or the formation of P granules in germ cells.^{38–45} Attractive tracer–crowder interactions reduce the tendency of tracer to distribute preferentially into the least crowded compartment, and when tracer is sufficiently small, may even lead to a tendency to partition into a more crowded compartment. However, when the tracer is significantly larger than the dominant crowding species, attractive tracer–crowder interaction is insufficient to compensate for the excluded volume effect driving tracer toward the least crowded compartments. These considerations must be taken into account when attempts are made to interpret the properties of a tracer species introduced into an intact cell when no other information about the distribution of the tracer within the highly heterogeneous cell is obtainable.⁸ The results presented here indicate that the whole-cell average behavior of the tracer may primarily reflect its properties in the least crowded and/or most attractive microenvironment(s) accessible to it.

Upon inspection of the black curves in Figures 1, 2, and 4–7, it appears that in the absence of significant crowder–crowder interaction, dependence of the logarithms of solubility and the equilibrium constants for isomerization and self-association equilibrium vary approximately linearly with f_2 , suggesting that a reasonable first-order estimate of the magnitude of crowding effects in a mixture of crowders jointly occupying a volume fraction ϕ_{tot} might be obtained by assuming a mass (or volume) average of the crowding effects of

each crowding species measured individually at $\phi = \phi_{\text{tot}}$. Letting y represent the logarithm of c_{sol} , Γ_{OC} , or Γ_{In} , this approximate relation may be expressed as

$$y(f_2, \phi_{\text{tot}}) \cong (1 - f_2)y_1(\phi_{\text{tot}}) + f_2y_2(\phi_{\text{tot}}) \quad (15)$$

where y_i denotes the value of y measured in a solution containing a single crowder of species i at a volume fraction of ϕ_{tot} . We note that the applicability of eqn 15 to all three of the excluded volume effects here is not coincidental. It derives from their common relationship to the dependence of $\log \gamma_{\text{tracer}}$ upon the composition of crowders, which also approximately obeys eqn 15 because, according to eqn 8, $\log \gamma_{\text{tracer}} = -\log c_{\text{sol}}$, and both $\log \Gamma_{\text{OC}}$ and $\log \Gamma_{\text{In}}$ are linear combinations of the logarithms of the activity coefficients of various tracer species.

The approximation expressed by eq 15 agrees with that presented earlier by Mittal, Best, and Kim^{12,13} based upon Monte Carlo simulations of isomerization and heteroassociation in hard sphere mixtures. The present work indicates that this rule of thumb also applies to systems in which crowders interact with tracer species via soft as well as hard sphere interactions. However, it must be kept in mind that the approximate validity of eqn 15 depends upon the assumption that attractive soft self- or heterointeractions between crowder molecules are not significant. It follows that an experimental test of this assumption must precede any experimental test of the validity of eqn 15.

A general result of the calculations presented here, approximate as they may be, is that both the direction and magnitude of the effect of crowding upon chemical equilibria of dilute species in a medium containing a mixture of crowders is exquisitely dependent upon not only the volume occupied by each of the crowding species but also the details of the potentials of mean force acting between each crowding species and each reactant/product species and, in many cases, upon the potentials of mean force acting between all of the various crowding species as well. This finding has important consequences for the design and execution of more detailed coarse-grained or atomistic models of complex media²⁰ and models for cytoplasm in particular.¹⁻³ These models have been described as “realistic”,⁴ presumably in contradistinction to simplified models such as those presented here. Yet, these simplified models have been found to account well for the composition dependence of colligative properties of a variety of highly nonideal solutions of one and two macromolecular solutes.⁵⁻¹¹ It behooves those asserting the realistic nature of complex simulations to demonstrate that their various models and simulation methodologies, together with the intermolecular potential functions they have chosen, can indeed reproduce the composition-dependent colligative properties of the simpler systems that are documented in the literature.

CONCLUSION

The direction and magnitude of crowding effects on macromolecular reactions of several types in the presence of multiple crowding species depends sensitively upon the composition

of crowders and the nature and strength of both hard and soft interactions between the crowder and reactant/product molecules and between crowder molecules.

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REFERENCES

- (1). Gnut D; Ebbinghaus S The macromolecular crowding effect - from in vitro into the cell. *Biol. Chem* 2016, 397, 37–44. [PubMed: 26351910]
- (2). Groen J; Foschepoth D; te Brinke E; Boersma AJ; Imamura H; Rivas G; Heus H; Huck WTS Associative interactions in crowded solutions of biopolymers counteract depletion effects. *J. Am. Chem. Soc* 2015, 137, 13041–13048. [PubMed: 26383885]
- (3). Jiao M; Li H-T; Chen J; Minton AP; Liang Y Attractive protein-polymer interactions markedly alter the effect of macromolecular crowding on protein association equilibria. *Biophys. J* 2010, 99, 914–923. [PubMed: 20682270]
- (4). Kim YC; Mittal J Crowding induced entropy-enthalpy compensation in protein association equilibria. *Phys. Rev. Lett* 2013, 110, 208102. [PubMed: 25167454]
- (5). Minton AP Quantitative assessment of the relative contributions of steric repulsion and chemical interactions to macromolecular crowding. *Biopolymers* 2013, 99, 239–244. [PubMed: 23348671]
- (6). Rosen J; Kim YC; Mittal J Modest protein-crowder attractive interactions can counteract enhancement of protein association by intermolecular excluded volume interactions. *J. Phys. Chem. B* 2011, 115, 2683–2689. [PubMed: 21361356]
- (7). Zhou H-X; Rivas G; Minton AP Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annu. Rev. Biophys* 2008, 37, 375–397. [PubMed: 18573087]
- (8). Rivas G; Minton AP Macromolecular crowding in vitro, in vivo, and in between. *Trends Biochem. Sci* 2016, 41, 970–980. [PubMed: 27669651]
- (9). Ando T; Yu I; Feig M; Sugita Y Thermodynamics of macromolecular association in heterogeneous crowding environments: theoretical and simulation studies with a simplified model. *J. Phys. Chem. B* 2016, 120, 11856–11865. [PubMed: 27797534]
- (10). Batra J; Xu K; Zhou H-X Nonadditive effects of mixed crowding on protein stability. *Proteins: Struct., Funct., Genet* 2009, 77, 133–138.
- (11). Du F; Zhou Z; Mo Z-Y; Shi J-Z; Chen J; Liang Y Mixed macromolecular crowding accelerates the refolding of rabbit muscle creatine kinase: implications for protein folding in physiological environments. *J. Mol. Biol* 2006, 364, 469–482. [PubMed: 17027032]
- (12). Kim YC; Best RB; Mittal J Macromolecular crowding effects on protein-protein binding affinity and specificity. *J. Chem. Phys* 2010, 133, 205101. [PubMed: 21133453]
- (13). Mittal J; Best RB Dependence of protein folding stability and dynamics on the density and composition of macromolecular crowders. *Biophys. J* 2010, 98, 315–320. [PubMed: 20338853]
- (14). Zhou B-R; Zhou Z; Hu Q-L; Chen J; Liang Y Mixed macromolecular crowding inhibits amyloid formation of hen egg lysozyme. *Biochim. Biophys. Acta, Proteins Proteomics* 2008, 1784, 472–480.
- (15). Zhou H-X Effect of mixed macromolecular crowding agents on protein folding. *Proteins: Struct., Funct., Genet* 2008, 72, 1109–1113. [PubMed: 18506780]
- (16). Minton AP Excluded volume as a determinant of macromolecular structure and reactivity. *Biopolymers* 1981, 20, 2093–2120.
- (17). Minton AP The effect of volume occupancy upon the thermodynamic activity of proteins: some biochemical consequences. *Mol. Cell. Biochem* 1983, 55, 119–140. [PubMed: 6633513]
- (18). Binder K Applications of Monte Carlo methods to statistical physics. *Rep. Prog. Phys* 1997, 60, 487–559.

- (19). Qin S; Zhou H-X An FFT-based method for modeling protein folding and binding under crowding: benchmarking on ellipsoidal and all-atom crowders. *J. Chem. Theory Comput* 2013, 9, 4633–4643.
- (20). Zhou H-X; Qin S Simulation and modeling of crowding effects on the thermodynamic and kinetic properties with atomic details. *Biophys. Rev* 2013, 5, 207–215. [PubMed: 23710260]
- (21). Hoppe T; Minton AP Incorporation of hard and soft protein-protein interactions into models for crowding effects in binary and ternary protein mixtures. Comparison of approximate analytical solutions with numerical simulation. *J. Phys. Chem. B* 2016, 120, 11866–11872. [PubMed: 27779417]
- (22). Fersht A *Structure and Mechanism in Protein Science*; W. H. Freeman: New York, 1999.
- (23). Atha DH; Ingham KC Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. *J. Biol. Chem* 1981, 256, 12108–12117. [PubMed: 7298647]
- (24). Laurent TC The Interaction between polysaccharides and other macromolecules. The solubility of proteins in the presence of dextran. *Biochem. J* 1963, 89, 253–257. [PubMed: 14084609]
- (25). Hall D; Minton AP Macromolecular crowding: qualitative and semiquantitative successes, quantitative challenges. *Biochim. Biophys. Acta, Proteins Proteomics* 2003, 1649, 127–139.
- (26). Beg I; Minton AP; Hassan MI; Islam A; Ahmad F Thermal stabilization of proteins by mono- and oligosaccharides: measurement and analysis in the context of an excluded volume model. *Biochemistry* 2015, 54, 3594–3603. [PubMed: 26000826]
- (27). Guzman I; Gelman H; Tai J; Gruebele M The extracellular protein VLS_E is destabilized inside cells. *J. Mol. Biol* 2014, 426, 11–20. [PubMed: 24013077]
- (28). Zimmerman SB; Minton AP Macromolecular crowding: biochemical, biophysical, and physiological consequences. *Annu. Rev. Biophys. Biomol. Struct* 1993, 22, 27–65. [PubMed: 7688609]
- (29). Wu D; Minton AP Quantitative characterization of nonspecific self- and hetero-interactions of proteins in nonideal solutions via static light scattering. *J. Phys. Chem. B* 2015, 119, 1891–1898. [PubMed: 25580677]
- (30). Kihara T Virial coefficients and models of molecules in gases. *Rev. Mod. Phys* 1953, 25, 831–843.
- (31). Kihara T Virial coefficients and models of molecules in gases. *B. Rev. Mod. Phys* 1955, 27, 412–423.
- (32). Harada R; Tochio N; Kigawa T; Sugita Y; Feig M Reduced native state stability in crowded cellular environment due to protein-protein interactions. *J. Am. Chem. Soc* 2013, 135, 3696–3701. [PubMed: 23402619]
- (33). Smith AE; Zhang Z; Pielak GJ; Li C NMR studies of protein folding and binding in cells and cell-like environments. *Curr. Opin. Struct. Biol* 2015, 30, 7–16. [PubMed: 25479354]
- (34). Huecas S; Andreu JM Polymerization of nucleotide-free, GDP- and GTP-bound cell division protein FtsZ: GDP makes the difference. *FEBS Lett.* 2004, 569, 43–48. [PubMed: 15225606]
- (35). Williams RC Jr. Concerted formation of the gel of hemoglobin S. *Proc. Natl. Acad. Sci. U. S. A* 1973, 70, 1506–1508. [PubMed: 4514319]
- (36). Munishkina LA; Ahmad A; Fink AL; Uversky VN Guiding protein aggregation with macromolecular crowding. *Biochemistry* 2008, 47, 8993–9006. [PubMed: 18665616]
- (37). Zimmerman SB Cooperative transitions of isolated *Escherichia coli* nucleoids: implications for the nucleoid as a cellular phase. *J. Struct. Biol* 2006, 153, 160–175. [PubMed: 16384714]
- (38). Brangwynne CP; Eckmann CR; Courson DS; Rybarska A; Høge C; Gharakhani J; Jülicher F; Hyman AA Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science* 2009, 324, 1729–1732. [PubMed: 19460965]
- (39). Feig M; Sugita Y Reaching new levels of realism in modeling biological macromolecules in cellular environments. *J. Mol. Graphics Modell* 2013, 45, 144–156.
- (40). McGuffee SR; Elcock AH Diffusion, crowding and protein stability in a dynamic molecular model of the bacterial cytoplasm. *PLoS Comput. Biol* 2010, 6, e1000694. [PubMed: 20221255]

- (41). Fodeke AA; Minton AP Quantitative characterization of temperature-independent and temperature-dependent protein-protein interactions in highly nonideal solutions. *J. Phys. Chem. B* 2011, 115, 11261–11268. [PubMed: 21846103]
- (42). Fodeke AA; Minton AP Quantitative characterization of polymer-polymer, protein-protein, and polymer-protein interaction via tracer sedimentation equilibrium. *J. Phys. Chem. B* 2010, 114, 10876–10880. [PubMed: 20677765]
- (43). Fernández C; Minton AP Effect of non-additive repulsive interactions upon the light scattering of concentrated protein-osmolyte mixtures. *J. Phys. Chem. B* 2011, 115, 1289–1293. [PubMed: 21175126]
- (44). Fernández C; Minton AP Static light scattering from concentrated protein solutions. II. Experimental test of theory for protein mixtures and weakly self-associating proteins. *Biophys. J* 2009, 96, 1992–1998. [PubMed: 19254559]
- (45). Minton AP Effective hard particle model for the osmotic pressure of highly concentrated binary protein solutions. *Biophys. J* 2008, 94, L57–L59. [PubMed: 18212007]

Figure 1.

Logarithm of the solubility of tracer species 3 relative to the solubility in dilute solution plotted as a function of the mass fraction of crowder species 1 in a mixture of equally sized crowders 1 and 2 jointly occupying volume fraction $\phi_{\text{tot}} = 0.2$. Curves were calculated with parameter values given in Table 1.

Figure 2.

Logarithm of the solubility of tracer species 3 relative to the solubility in dilute solution plotted as a function of the mass fraction of crowder species 1 in a mixture of unequally sized crowders 1 and 2 jointly occupying volume fraction $\phi_{\text{tot}} = 0.2$. Curves were calculated with parameter values given in Table 1.

Figure 3.

Equilibrium fraction of total tracer protein T in each of 5 compartments containing a crowder at volume fractions 0, 0.05, 0.1, 0.15, and 0.2, plotted for various ratios of tracer to crowder mass. Data were calculated using $\epsilon_{TC}/kT = -2$ (red), 0 (black), and 2 (blue).

Figure 4.

Logarithm of $\Gamma_{OC} = K_{OC}/K_{OC}^0$ plotted as a function of the mass fraction of crowder species 1 in a mixture of equally sized crowders 1 and 2 jointly occupying volume fraction $\phi_{tot} = 0.2$. Curves were calculated with $f_{exp} = 1.5$ and the parameter values given in Table 1.

Figure 5. Logarithm of Γ_{OC} plotted as a function of the mass fraction of crowder species 1 in a mixture of unequally sized crowders 1 and 2 jointly occupying volume fraction $\phi_{\text{tot}} = 0.2$. Curves were calculated with $f_{\text{exp}} = 1.5$ and the parameter values given in Table 1.

Figure 6.

Logarithm of $\Gamma_{1n} = K_{1n}(\phi)/K_{1n}^\circ$ plotted as a function of the mass fraction of crowder species 1 in a mixture of equally sized crowders 1 and 2 jointly occupying volume fraction $\phi_{\text{tot}} = 0.2$. Curves were calculated with $n = 2$ (panels A and B) and $n = 4$ (panels A' and B') and the parameter values given in Table 1.

Figure 7.

Logarithm of $\Gamma_{ln} = K_{ln}(\phi)/K_{ln}^\circ$ plotted as a function of the mass fraction of crowder species 1 in a mixture of unequally sized crowders 1 and 2 jointly occupying volume fraction $\phi_{\text{tot}} = 0.2$. Curves were calculated with $n = 2$ (panels A–C) and $n = 4$ (panels A'–C') and the parameter values given in Table 1.

Table 1.Parameter Values Employed in Calculations Plotted in Figures 1–2 and 4–7^a

figures	M_1/M_3	M_2/M_3	curve	e_{13}	e_{23}	e_{11}	e_{22}	e_{12}
1A, 4A, 6A and A'	1	1	black	0	0	0	0	0
			red			2	2	-2
			blue			-2	2	-2
1B, 4B, 6B and B'	1	1	black	2	-2	0	0	0
			red			2	2	-2
			blue			-2	2	-2
2A, 5A, 7A and A'	0.333	3	black	0	0	0	0	0
			red			2	0	0
			blue			-2	0	0
2B, 5B, 7B and B'	0.333	3	black	2	-2	0	2	-2
			red			2	2	-2
			blue			-2	2	-2
2C, 5C, 7C and C'	0.333	3	black	-2	2	0	-2	2
			red			2	-2	2
			blue			-2	-2	2

^aFor all calculations, $\phi_3 \rightarrow 0$, $\phi_{\text{tot}} = \phi_1 + \phi_2 = 0.2$, $r_3 = 1$ (relative units), and all $ij = 0.2$.