Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences*

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Key Words

excluded volume, configurational entropy, free energy, protein-protein interactions, protein folding, site-binding

Abstract

Expected and observed effects of volume exclusion on the free energy of rigid and flexible macromolecules in crowded and confined systems, and consequent effects of crowding and confinement on macromolecular reaction rates and equilibria are summarized. Findings from relevant theoretical/simulation and experimental literature published from 2004 onward are reviewed. Additional complexity arising from the heterogeneity of local environments in biological media, and the presence of nonspecific interactions between macromolecules over and above steric repulsion, are discussed. Theoretical and experimental approaches to the characterization of crowding- and confinement-induced effects in systems approaching the complexity of living organisms are suggested.

Contents

INTRODUCTION

Almost all proteins and other biological macromolecules in vivo exist, at least transiently, as components of structural and functional complexes (4). The number of published studies of macromolecular interactions has increased almost exponentially for the past 20 years and amounts to several hundred a year at present. However, almost all these studies are aimed at the characterization of attractive interactions that result in the formation of protein complexes or complexes of protein and other macromolecules (e.g., ribonucleoproteins). In contrast, repulsive interactions between macromolecules, by definition, do not lead to the formation of complexes and thus may not be observed directly. However, the presence and significance of repulsive interactions in fluid media containing a high total concentration of macromolecules and/or structural obstacles to the free motion of macromolecules may be observed indirectly through their effects on a variety of macromolecular reactions involving association and conformational isomerization (53, 56). Many of these effects may be predicted qualitatively (and sometimes quantitatively) using simple statistical-thermodynamic models and

observed experimentally by measurement of the dependence of thermodynamically based solution properties and reaction kinetics and equilibria on the concentration and composition of macromolecular cosolutes that are nominally inert with respect to the reaction of interest. The importance of excluded-volume interactions lies in their generality. Such interactions are universal and entirely nonspecific and have the potential to significantly modulate the kinetics and equilibria of a large number of macromolecular reactions taking place in physiological fluid media.

We classify an excluded-volume effect according to its origin: Macromolecular crowding refers to effects attributed to volume excluded by one soluble macromolecule to another, and macromolecular confinement refers to effects attributed to volume excluded by a fixed (or confining) boundary to a soluble macromolecule. A number of reviews of both crowding and confinement effects have appeared during the past five years (21, 29, 60, 62, 78, 96, 97, 100) and it is not our intention to recapitulate published material. Moreover, a separate review of the effect of crowding on macromolecular transport via diffusion appears elsewhere in the present volume (19). However, in the interest of completeness, in the following section we briefly summarize the various ways that crowding and confinement are expected to influence the equilibria and kinetics of macromolecular associations and isomerizations. Next, results of recently published (2004–2007) theoretical analyses, atomistic simulations, and experiments relating to effects of macromolecular crowding and confinement on a variety of macromolecular reactions are summarized. (References to work published prior to 2004 may be found in one or more of the reviews cited above.) The present review concludes with discussions of several topics related to the applicability of theoretical predictions or the results of experiments conducted in vitro to actual macromolecular processes taking place in living organisms.

A COMMON THERMODYNAMIC FRAMEWORK FOR ANALYSIS OF MACROMOLECULAR CROWDING AND CONFINEMENT

General features of macromolecular crowding and confinement are qualitatively exhibited by their effects on three prototypical macromolecular reactions:

Bimolecular Association

$$A + B \rightleftarrows AB$$
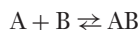
Association of a Soluble Macromolecular Ligand with a Specific Surface Binding Site

$$L + S \rightleftarrows LS$$
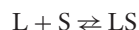
Two-State Folding of a Protein

$$U \rightleftarrows N$$

These three reactions are characterized, respectively, by three standard free energy changes and the corresponding equilibrium constants:

$$\Delta F_{AB} = -RT \ln K_{AB},$$
$$\Delta F_{LS} = -RT \ln K_{LS},$$

and

$$\Delta F_{UN} = -RT \ln K_{UN},$$

where R denotes the molar gas constant and T the absolute temperature. The effect of an environmental perturbation such as crowding or confinement on the equilibrium state of a particular chemical reaction may be analyzed by constructing a simple thermodynamic cycle as shown in **Figure 1a–c** and **Figure 2a–c**. The value of standard free energy changes and equilibrium constants for a particular reaction in bulk dilute solution is denoted by the superscript 0, e.g., ΔF_{AB}^0, K_{AB}^0. Then the free energy of hetero-association, site-binding, and unfolding in the bulk and perturbed environments (either crowded or confined) will be related by (62)

$$\Delta\Delta F_{AB} \equiv \Delta F_{AB} - \Delta F_{AB}^o$$
$$= \Delta F_{AB}^C - \Delta F_A^C - \Delta F_B^C, \quad 1.$$

$$\Delta\Delta F_{LS} \equiv \Delta F_{LS} - \Delta F_{LS}^o = \Delta F_{LS}^C - \Delta F_L^C, \quad 2.$$

Macromolecular crowding: effects of excluded volume on the energetics and transport properties of macromolecules within a solution containing a high total volume fraction of macromolecules

Macromolecular confinement: effects of excluded volume on the free energy and reactivity of a macromolecule situated in a cavity, bounded by impenetrable walls, having a smallest interior dimension only slightly larger than the largest dimension of the macromolecule
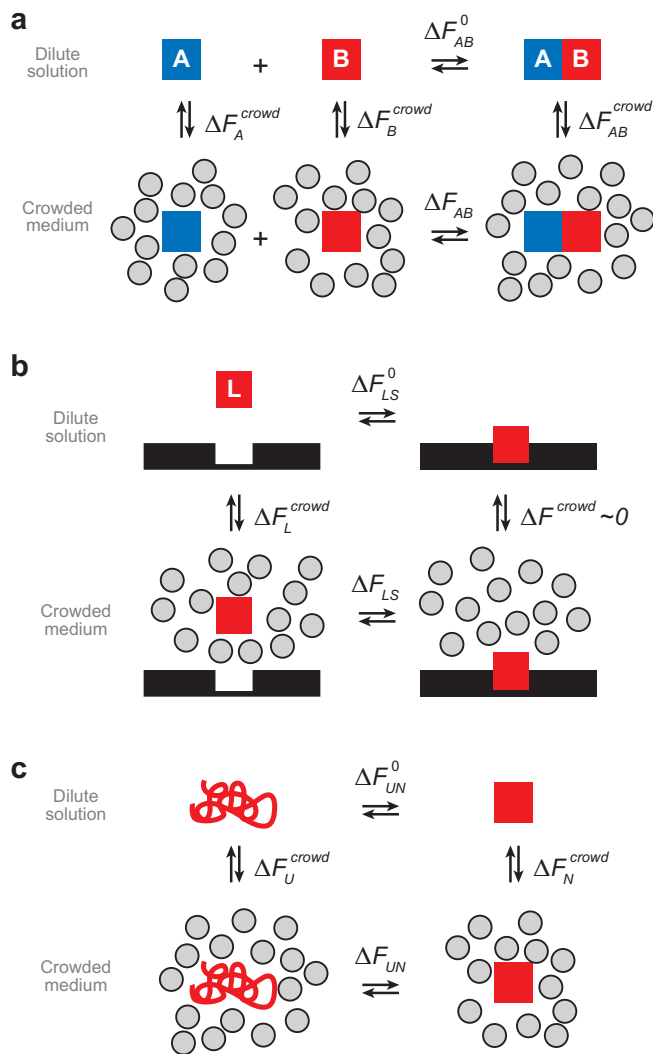
Figure 1

Thermodynamic cycles illustrating linkage between free energy of transfer of reactants and products from dilute solution to crowded medium and standard free energy of (*a*) association in solution, (*b*) site-binding, and (*c*) two-state folding of a protein.

and

$$\Delta\Delta F_{UN} \equiv \Delta F_{UN} - \Delta F_{UN}^{o}$$
$$= \Delta F_{N}^{C} - \Delta F_{U}^{C}, \qquad 3.$$

where ΔF_{X}^{C} denotes the standard free energy change associated with the transfer of X from bulk solution to the crowded or confined environment, as indicated in **Figure 1** and

Figure 2.[1,2] Free energy changes (on a per mole basis) are related to changes in the corresponding equilibrium constant by

$$K_{X} = K_{X}^{0} \exp(-\Delta\Delta F_{X}/RT), \qquad 4.$$

where X denotes *AB*, *LS*, or *UN*. The magnitude of the effect of crowding or confinement on a particular reaction equilibrium may thus be evaluated indirectly by comparing the free energies of transfer of reactants and products from bulk solution to the crowded or confined medium. Examples of such estimates are provided below.[3] In these examples, we focus on the excluded volume aspect of crowding and confinement, because this effect appears to be universal. Other nonspecific interactions of reactants with crowders and confining walls are discussed below.

CROWDING

Estimation of ΔF_{X}^{crowd}

The colligative properties of concentrated solutions of globular proteins, under conditions such that long-range interactions between protein molecules are damped out, are well described by models in which individual macromolecular species are represented

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[1]Equations 1–3 apply equally to changes in Helmholtz or Gibbs free energies. Simple models used to estimate the magnitude of ΔF_{X}^{C} generally assume constant volume and hence in the strict sense yield estimates of Helmholtz free energy changes. However, differences between Helmholtz and Gibbs free energy changes associated with reactions in the liquid state are not of qualitative significance.

[2]ΔF_{X}^{C} is formally equivalent to the difference between the equilibrium average free energy of interaction of X with the perturbing cosolutes or boundaries and the equilibrium average free energy of interaction of X with the constituents of bulk solvent that are replaced by cosolute or boundary. Thus ΔF_{X}^{C} implicitly takes into account any energetic consequence of desolvation that may accompany the transfer.

[3]The treatment presented here may be readily extended to a quasi-equilibrium analysis of the effect of crowding or confinement upon the kinetics of a transition-state limited association or isomerization reaction, in which case one must additionally estimate the free energy change associated with the transfer of the transition state from bulk to the crowded or confined medium (see for example the Appendix in Reference 59).

by hard convex particles that resemble the molecules in general size and shape (54, 63), justifying the use of hard-particle models for quantitative estimation of excluded-volume effects. The free energy of transfer of a macromolecule X from a dilute solution to a solution containing an arbitrary concentration of other macromolecules of the same or other species is then equivalent to the free energy of creating a cavity in the solution, free of any part of another molecule, that is large enough to accommodate the newly introduced macromolecule (47). The scaled particle theory (SPT) of hard-particle fluids, originally due to Reiss et al. (75), was devised specifically to calculate the free energy of cavity formation and is thus particularly appropriate for numerical estimation of the magnitude of crowding effects. Let us consider as an example a fluid containing a volume fraction ϕ of inert hard spherical particles with radius r_c. According to SPT (15, 77), the free energy or work of placing into this fluid a spherocylinder (sc), that is, a right circular cylinder capped on each end by a hemisphere, with a radius $r_{sc} = R_{sc}r_c$ and a cylindrical length of $2L_{sc} r_{sc}$, is given by

$$\Delta F_{sc}^{crowd}/RT =$$
$$-\ln(1-\phi) + A_1 Q + A_2 Q^2 + A_3 Q^3, \quad 5.$$

where

$$Q = \phi/(1-\phi)$$

$$A_1 = R_{sc}^3 + 3R_{sc}^2 + 3R_{sc} + 1.5L_{sc}(R_{sc}^2 + 2R_{sc} + 1)$$

$$A_2 = 1.5(2R_{sc}^3 + 3R_{sc}^2) + 4.5L_{sc}(R_{sc}^2 + R_{sc})$$

$$A_3 = 3R_{sc}^3 + 4.5 L_{sc}R_{sc}^2.$$

Note that the free energy of transfer of a spherical particle into this fluid is just the special case of Equation 5 with $L_{sc} = 0$.

Association Equilibria

Consider a bimolecular association reaction taking place in a solution of spherical crowding molecules. Let monomeric A and B be represented as identical hard spherical parti-
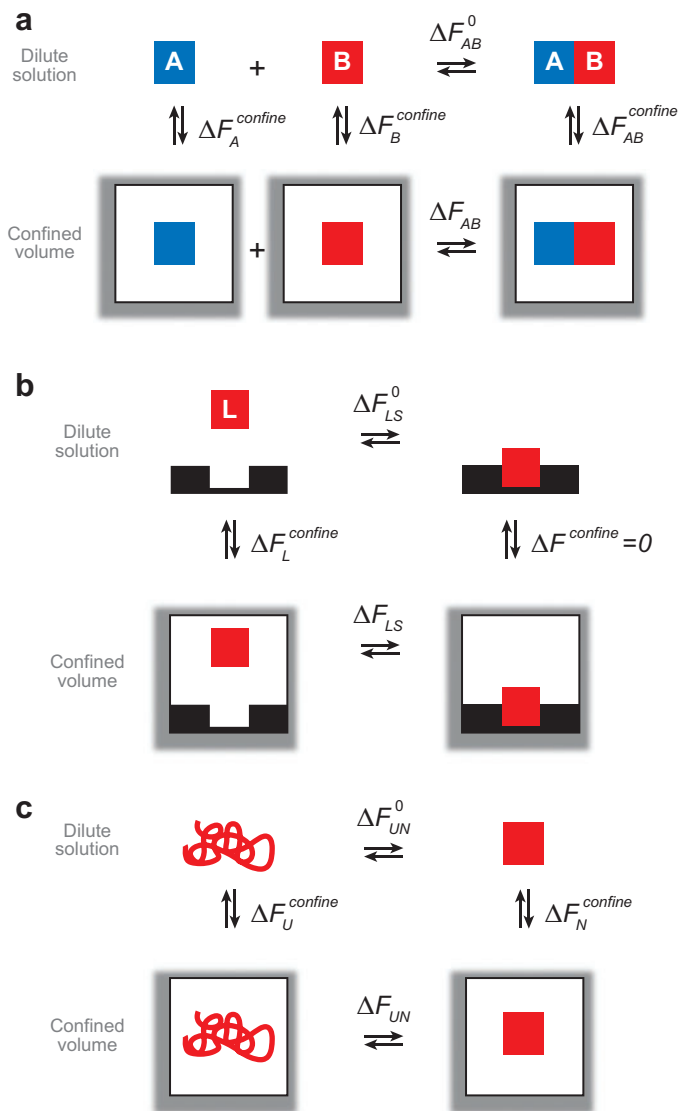
Figure 2

Thermodynamic cycles illustrating linkage between free energy of transfer of reactants and products from dilute solution to confined volume element and standard free energy of (*a*) association in solution, (*b*) site-binding, and (*c*) two-state folding of a protein.

cles with a radius equal to that of crowder (i.e., $R_{sc} = R_1 = 1$, $L_{sc} = L_1 = 0$). The dimer AB is represented as a spherocylindrical particle with a volume equal to twice that of monomer, but with $L_{sc} = L_2$ and $R_{sc} = R_2$ simultaneously adjustable to maintain constant volume, to illustrate the effect of different assumed shapes

Figure 3

Dependence of the equilibrium constant for formation of a spherocylindrical dimer from two spherical monomers ($R_1 = 1$) upon the volume fraction ϕ of hard-sphere crowders and dimer shape at constant dimer volume. (*a*) $L_2 = 0$, $R_2 = 1.26$ (sph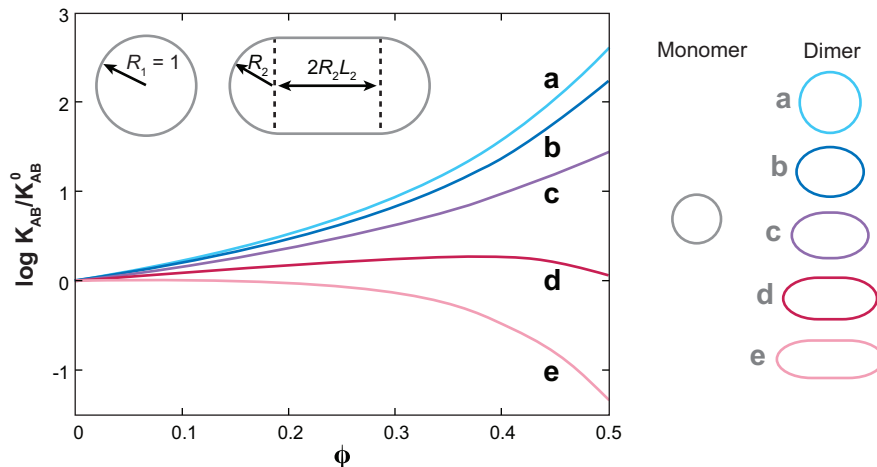erical dimer); (*b*) $L_2 = 2/3$, $R_2 = 1$; (*c*) $L_2 = 1$, $R_2 = 0.928$; (*d*) $L_2 = 1.5$, $R_2 = 0.851$; and (*e*) $L_2 = 2$, $R_2 = 0.794$.

for dimer. Results obtained using Equations 1, 4, and 5 are plotted in **Figure 3**. It is evident that crowding can substantially enhance the equilibrium tendency of A and B to dimerize when AB is compact ($L_2 \leq 1$), but crowding can also inhibit dimerization when the dimer is so aspherical that it excludes more volume to crowder than two monomers. Many proteins can also form higher-order oligomers. The effect of crowding on the equilibrium constant for concerted association of *n* monomers to 1 *n*-mer increases dramatically with the value of *n* (53).

Association Rates

The rate constant of association between two molecules can generally be written as (55, 102)

$$k_a = \frac{k_D k_{\text{react}}}{k_D + k_{\text{react}}}, \qquad 6.$$

where k_D is the rate constant under diffusion control and k_{react} is the rate constant under reaction or transition-state control. Crowding affects the rate constant in the two regimes differently. k_D is proportional to the relative diffusion constant of the two reactants. In-

creased crowding is expected to monotonically decrease the diffusion constant and thus acts to decrease k_D. At the same time crowding also induces an attractive interaction between the reactants, manifesting itself in the enhanced dimerization noted above, which acts to partially compensate for the decrease in k_D owing to the decrease in diffusion coefficient (98). However, the overall effect is such that generally k_D is expected to decrease with increased crowding (55).

In the reaction- or transition-state-controlled regime, the rate constant is dictated by the energy barrier arising from conformational changes necessary before forming the product. Because the transition state for association in solution is generally nearly as compact as the product complex, crowding is expected to effectively lower the energy barrier and thus increase the association rate constant by a factor that is close to the factor for increase of the equilibrium association constant. Correspondingly, a small effect of crowding on the dissociation rate constant is expected (59). Because fast associations are typically under diffusion control and slow associations are under reaction

control (5), crowding is generally expected to decelerate fast associations and accelerate slow associations (55).

Site-Binding Equilibria

To illustrate the effect of crowding on a site-binding reaction, let the free ligand be represented as a hard spherical particle ($L_{sc} = 0$) with radius R_{sc} times that of a hard spherical crowder. When the bound ligand is buried and thus inaccessible to crowders, crowding affects only the free energy of free ligand (i.e., $\Delta F_{LS}^{crowd} = 0$).[4] Results obtained using Equations 2, 4, and 5 are plotted in **Figure 4**, and it is evident that the enhancement of association by crowding can be much more significant for site-binding reactions than for bimolecular associations in solution. The same approximation may also be used to analyze the effect of crowding on protein solubility, which is modeled by assuming that crowding affects protein in the supernatant solution, but not in the condensed phase (46, 53).

Two-State Protein Folding Equilibria

The presence of crowder influences equilibria between conformational states of a macromolecule by favoring conformations that exclude less volume to crowder. In the case of protein folding, unfolded conformations are more expanded and thus crowding is expected to favor the native state. However, the use of the hard-particle model to estimate ΔF_U^{crowd} is unreliable, as the U state consists of a manifold of conformations instead of a single rigid conformation. Thus the extent to which crowding is predicted to stabilize the native state varies substantially between different models

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[4]The assumption that bound ligand excludes no volume to crowder provides an upper bound estimate of the effect of crowding, which may be less to the extent that bound ligand retains mobility and/or solvent exposure.
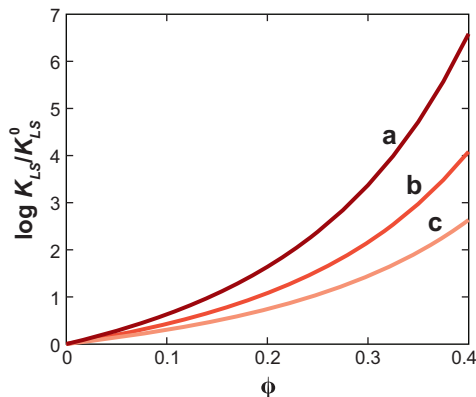

Figure 4

Dependence of the equilibrium constant for binding of a spherical macromolecular ligand to an immobile surface site upon the volume fraction ϕ of hard-sphere crowders, with $MW_{ligand}/MW_{crowder} = 2$ (curve *a*), 1 (curve *b*), and 0.5 (curve *c*).

for the unfolded state (see following section for examples).

Two-State Folding Rates

The rate constant for isomerization can be generally written as

$$k = k_0 \exp(-\Delta F^{\ddagger}/RT), \qquad 7.$$

where $\Delta F^{\ddagger}$ is the activation free energy (i.e., free energy difference between the transition state and the reactant state) and the prefactor k_0 depends on the dynamics of the isomerization process. Crowding can in principle affect both $\Delta F^{\ddagger}$ and k_0. For example, if the transition state is more compact than the reactant state, as in the case of a protein folding reaction, then crowding is expected to reduce $\Delta F^{\ddagger}$. If intrachain diffusion plays a significant role, crowding may act to reduce the rate of intrachain diffusion and hence the value of k_0 as well (see review of relevant literature below). When the transition state is more expanded than the reactant state, as in the case of protein unfolding, crowding is expected to decrease the rate constant.

CONFINEMENT

Estimation of $\Delta F_X^{confine}$

Consider a molecule X within a unit volume of bulk solution that can exist in a finite number of configurational states, each of which is specified by a set of positional coordinates denoted by r and a set of orientational coordinates denoted by θ. The free energy of transferring the molecule X from this volume to an equal volume of solution that may be bounded by hard walls in one, two, or three dimensions is given by the statistical-thermodynamic expression (25)

$$\Delta F_X^{\text{confine}} = -RT \ln \left(\frac{\iint_{allowed} d\theta dr}{\iint_{all} d\theta dr} \right), \quad 8.$$

where the multiple integral in the denominator is taken over all configurational states accessible in bulk solution, and the multiple integral in the numerator is taken over all allowed configurational states in the bounded volume, that is, all states in which no part of X intersects any hard-wall boundary.

While both confinement and crowding effects result from the reduction in possible configurations available to a macromolecule due to the presence of a high-volume fraction of other macromolecules or static barriers to movement, there is one major qualitative difference between the two phenomena. In contrast to the free energy cost of crowding, the free energy cost of confinement is not necessarily minimal for the molecular conformation that is globally the most compact in the sense of having the smallest radius of gyration. Rather, confinement favors conformations having a shape that is complementary to the shape of the confining volume. For example, although a spherical conformation may be favored in a quasispherical cavity, the preferred conformation in a cylindrical pore may be rod-like, and the preferred conformation in a planar pore (bounded by two parallel hard walls) may be plate-like. Thus numerical estimates of the magnitude of confinement effects are sensitive to the choice of models for the structure of both confining space and confined macromolecular species (56).

Association Equilibria

Model calculations of the effect of confinement in differently shaped pores on the association of two spherical monomers of identical size to form a dimer of twice the volume and varying shapes suggest that confinement has a small effect on bimolecular association and is expected to increase the equilibrium constant for bimolecular association by at most a factor of two or three. However, the effect of confinement on association constants for concerted formation of larger n-mers having a shape compatible with the shape of the confining volume increases strongly as the value of n increases (56).

Site-Binding Equilibria

If the bound ligand is completely immobile, then confinement affects only the free energy of the free ligand (i.e., $\Delta F_{LS}^{confine} = 0$). As a simple example, consider a spherical ligand with radius a confined in a spherical cavity with radius R_{cavity}. The change in the site-binding constant due to ligand confinement may be calculated from Equations 2, 4, and 8 to be

$$K_{LS}/K_{LS}^0 = \left(1 - \frac{a}{R_{cavity}} \right)^{-3}. \quad 9.$$

When $a = 0.5R_{cavity}$, the binding constant is increased by a factor of 8.

Two-State Protein Folding Equilibria

It is evident upon inspection of **Figure 2c** that the free energy cost of confining any partially or fully unfolded conformation of a protein is greater than the cost of confining the native state, and that confinement must stabilize the native state relative to any unfolded state. When summarizing the effects of confinement on protein folding, numerical estimates of the magnitude of the effect of confinement are sensitive to the nature of

approximations made in treating the effect of confinement on the (average) unfolded state. Zhou & Dill (101) presented a simple model in which the unfolded state is modeled as a random walk with a specified radius of gyration. They then estimated the value of $\Delta F_U^{\text{confine}}$ associated with the placement of U into a variety of confining geometries. Some of their results are plotted in **Figure 5**. According to this model, confinement is predicted to decrease the value ΔF_{UN} by as much as 20–30 RT in very small cavities, with a volume only slightly greater than that of the native state. This result makes intuitive sense. In such small cavities it is essentially impossible for proteins to unfold, as there is no space available for proteins to unfold into. By modeling the transition state for protein folding as a sphere with a somewhat larger radius than that of the folded state, a different but conceptually related model (30) has been used to estimate the change in the energy barrier for folding and to calculate the dependence of folding rate on the size of the confining cavity. A maximum acceleration in folding by tuning the cavity size is predicted.

In conclusion, enhancement of association and site-binding equilibria by confinement only occurs when the confined macromolecules are truly confined, i.e., they cannot equilibrate with bulk solution. When protein can equilibrate between a pore and bulk solution, the extent of association resulting in the formation of oligomers in the pore is less than in the bulk, and the extent of site-binding in the pore is equal to that in the bulk (56).

REVIEW OF THEORETICAL/ SIMULATION LITERATURE SINCE 2004

Most of the effects summarized in the preceding section are predicted on the basis of simple statistical-thermodynamic models. These models have the advantage that they are focused on specific aspects of crowding and confinement, that they are intuitive, and that their results can often be expressed in the
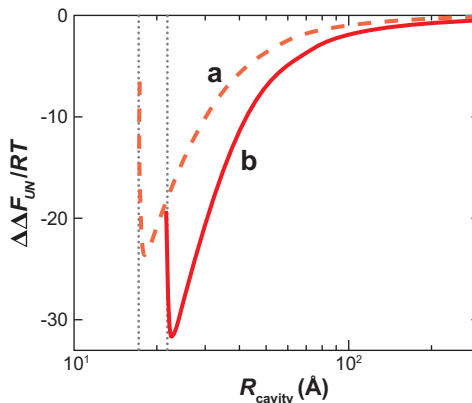

Figure 5

Effect of confinement in a spherical cavity on the free energy of two-state folding, calculated as a function of cavity radius according to the theory of Zhou & Dill (101), for proteins containing (*a*) $N = 100$ residues and (*b*) $N = 200$ residues. Vertical lines represent the radii of the hard spherical representation of the folded proteins, calculated according to r_N (Å) = $3.73 \, N^{1/3}$. The radius of gyration of the unfolded protein is calculated according to r_g (Å) = $3.27 \, N^{1/2}$.

form of reasonably simple analytical expressions. On the other hand, they do not allow for the exploration of complexities associated with crowding and confinement of real macromolecules by other real macromolecules. A complementary theoretical approach is by atomistic simulations. However, one needs to remain cognizant that the systems studied via simulation are still models instead of the real thing; not all idiosyncratic details of the model systems are of general value for understanding the real systems. For computational efficiency, in most of the atomistic simulation studies, amino acid residues were modeled by a pseudoatom representing C_α with or without a second pseudoatom representing the side chain, and solvent was not treated explicitly.

Effects of Crowding on Protein Folding

Zhou (98) considered the effect of crowding on the free energy of the unfolded state by treating the unfolded protein as a three-dimensional random walk in the presence of hard spherical obstacles. Calculation of the

probability that a random walk consisting of a certain number of steps will not encounter a crowding particle leads to a simple relation:

$$\Delta F_U^{\text{crowd}}/RT =$$
$$-\ln(1-\phi) + 3\phi y^2(1 + 2\pi^{-1/2}y^{-1}), \quad 10.$$

where y is the ratio of the radius of gyration of the unfolded chain in bulk solution to the radius of crowder. Equation 10 takes into account the possibility that an unfolded chain can in principle be accommodated within interstitial voids between spherical crowders that may be too small to accommodate a folded protein modeled as a hard particle. When the folded protein is modeled as a hard sphere, the treatment of Zhou (98) predicts that whereas at low-volume fraction of crowder, excluded-volume effects stabilize the folded state relative to the unfolded state, at very-high-volume fractions of crowder, excluded volume stabilizes the unfolded state relative to the folded state. ΔF_U^{crowd} for a self-avoiding chain can be lower than for an ideal chain with the same radius of gyration (100).

Minton (61) presented an effective two-state model for protein folding, with the unfolded state modeled as a compressible sphere. Because this model allows for intramolecular as well as intermolecular excluded-volume effects, more compact conformations of the unfolded chain are energetically more costly than in the random walk model, and as a result calculated values of ΔF_U^{crowd} are significantly more positive, and calculated values of $\Delta\Delta F_{UN}$ are significantly more negative than in the random walk model. This model also predicts the energetic consequences of neglecting intramolecular excluded volume and provides estimates of the extent to which the average radius of gyration of an unfolded polypeptide is reduced by the addition of hard-particle crowders.

Hu et al. (33) modeled the unfolded state as a chain of small tangent hard spheres. They predicted that smaller crowders stabilize the unfolded state relative to the native state, whereas larger crowders promote the stability of the folded form. This conclusion stands in contradiction to the results of prior theoretical treatments (61, 98), as well as experiment (72).

In a simulation study, Cheung et al. (12) found that, at a crowder volume fraction of 0.25, the folding stability of a WW domain was increased by 1.1 kcal mol^{-1}. Cheung et al. also found that the folding rate initially increases with crowder concentration but decreases at higher crowder concentrations. The decrease in folding rate was attributed to restriction by crowders of conformational fluctuations necessary for protein folding. A second factor may be an increase in the free energy of the transition state due to the decreased probability of finding voids that can accommodate the protein in an expanded transition state.

Miscellaneous Crowding Effects

Using simple space-filling models for both helix and coil, Snir & Kamien (83) predicted that crowding by hard spheres promotes the formation of helical conformations by random-coil polymers.

Hall and colleagues (27, 28) have modeled the folding of a tracer protein in the presence of crowders that can undergo folding/unfolding transitions or can self-associate. As the folded or self-associated crowders present less excluded volume, the model predicts that the stabilization of the native state by crowders will decrease as the crowders fold or self-associate.

Effects of Confinement on Isomerization and Protein Folding

By modeling the unfolded state as a polymer chain and the native and transition states as hard spheres, Hayer-Hartl & Minton (30) obtained simple analytical expressions describing the dependence of the rate of two-state folding within a spherical cavity on the radius of the cavity and the molecular weight of the confined protein. These expressions predicted that folding rates would

be maximized at an intermediate cavity size that increases with the molecular weight of the encapsulated protein. They quantitatively rationalized variations of the rate of refolding of several proteins within the central cavity of different mutants of the chaperonin GroEL/GroES that were specifically designed to change the volume of the cavity.

Maximization of folding rate at an intermediate cavity size has also been observed in atomistic simulations (13, 88, 93). These simulations also indicated that folding rate can be modulated by attractive interactions between the confined protein and the walls of the enclosing cavity. In addition, Cheung & Thirumalai (13) found that the folding yield can be increased by as much as 50% by repeated switching on and switching off of an attractive interaction between the cavity wall and the confined protein.

A simulation by Jewett et al. (39) suggests that the rate of folding in a confined environment can be increased via an alternative pathway in which a folding intermediate is transiently bound to the cavity wall. In another simulation study, Rathore et al. (73) suggested that although confinement-induced stabilization of the native state is dominated by entropy, stabilizing intramolecular interactions are not as optimal as in bulk solution. Net stabilization is hence sequence dependent and not as great as expected on the basis of entropic effects alone.

Ziv et al. (106) simulated the helix formation of peptides confined in an infinite cylinder and found that the helical state is stabilized relative to the coil state. They attempted to rationalize their results with a simple statistical-thermodynamic model, in which the coil state is modeled as a polymer chain and the helix is modeled as a stiffer polymer chain. The average helical content is predicted to be increased by confinement, but the increase is independent of peptide length, in contrast to the results of their simulation. Pande and coworkers (50, 85) simulated helix formation inside a cylindrical pore and folding of the villin headpiece inside a spherical cavity. Unlike previous simulations, solvent (water) was treated explicitly in these studies. Confinement in the spherical cavity favored helix formation when the solvent was allowed to equilibrate with the bulk, but, disfavored helix formation when solvent was trapped within the cavity. Zhou (99) has recently proposed that water trapped within the cylindrical pore has a higher thermodynamic activity than bulk water, and that hydrogen bonding between trapped water and the peptide backbone favors the coil/unfolded state.

Miscellaneous Confinement Effects

Elcock (20) simulated the cotranslational folding of three proteins. The proteins were fed off the peptidyltransferase active site with the ribosome large subunit, which was represented by atoms and pseudoatoms. No structure formation was observed while the nascent chain was in the ribosome exit tunnel, and the cotranslational folding of two single-domain proteins, CI2 and barnase, followed mechanisms identical to those in bulk water. However, for a two-domain protein, Semliki forest virus protein, cotranslational folding followed a mechanism different from that in bulk water. In the latter environment, the two domains first folded independently and then docked together. On the ribosome, the N-terminal domain folded first and the structure of the C-terminal domain then gradually accreted onto the preformed domain.

REVIEW OF THE EXPERIMENTAL LITERATURE SINCE 2004

Relevant experimental literature published during the past four years has been classified according to one of six categories: (*a*) effects of crowding on macromolecular association rates; (*b*) effects of crowding on macromolecular association equilibria; (*c*) effects of crowding on conformational isomerization; (*d*) effects of crowding on protein stability with respect to denaturation;

Table 1   Reported effects of macromolecular crowding on association rates

Addition of proteins, polysaccharides (dextran 138K, Ficoll 70K), and PEG (ranging in molar mass from 0.2K to 3.3K) increases the rate of amyloid fibrillation by α-synuclein (66). Approximately 10-fold acceleration in 150 g/L of PEG 3.3K and approximately threefold acceleration in 150 g/L Ficoll. Approximately five- to sixfold acceleration in 50 g/L lysozyme or BSA.

Addition of PEG 10K induces the fibrillation of β-synuclein in the presence of Zn^{2+} (94). Both PEG and Zn^{2+} are required for fiber formation.

Addition of Ficoll 70K accelerates HIV capsid protein self-assembly (16). The half-time for protein assembly is decreased approximately 10-fold in 100 g/L Ficoll.

Addition of PEG 3.3K induces the coassembly of the bacteriophage Φ29 monomeric capsid protein and dimeric scaffolding protein to form bona fide capsid particles (22). In the absence of the scaffolding protein, the capsid protein forms amorphous polymers in PEG.

Addition of Ficoll 70K and PEG (ranging in molar mass from 0.2K to 8K) reduces the second-order rate constant for diffusion-limited bimolecular association of beta-lactamase (TEM) and BLIP (43–45). At low crowder concentration, rate scales with rotational diffusion. At higher concentrations, rate decreases more strongly with increasing polymer concentration, possibly due to crowding-induced self-association of reactant species.

Abbreviations: BLIP, beta-lactamase inhibitor protein; BSA, bovine serum albumin; K, molar mass in kilograms; PEG, polyethylene glycol.

Table 2   Reported effects of macromolecular crowding on association equilibria

Addition of the unrelated protein RNase A promotes the dimerization of tracer apomyoglobin (107). Significant effects are observed at >50 g/L RNase. In contrast, an equivalent mass concentration of HSA did not promote apoMb dimer formation; this differential is much larger than expected from excluded-volume models.

Addition of dextran 10K enhances the formation of a decamer of bovine pancreatic trypsin inhibitor (84). Approximately 30-fold increase in decamer fractional abundance and ~5 × 10^5-fold increase in the association constant at ~200 g/L of dextran.

Addition of BSA or ovalbumin increases the binding affinity of replication protein RepA for specific DNA sequences (18). Approximately 10-fold increase of the equilibrium association constant for RepA-DNA complex formation in 150 g/L BSA.

Addition of dextran 70K inhibits the exchange of subunits between aggregates of α-crystallin (24). Effect can be attributed to crowding-induced lowering of concentration of monomeric subunit in equilibrium with aggregate.

High area occupancy of an amphiphilic peptide adsorbed onto a phospholipid membrane leads to conversion of peptide from an in-plane mode of adsorption to a transverse mode (3). Observed behavior is in qualitative accord with predictions of earlier theoretical model based upon excluded volume in two dimensions (58).

Abbreviations: BSA, bovine serum albumin; HSA, human serum albumin.

(*e*) effects of crowding on enzyme activity; and (*f*) effects of confinement on protein stability with respect to denaturation. Noteworthy findings in the six categories are listed in **Tables 1**–**6**.

EFFECT OF CROWDING ON THE COMPETITION BETWEEN PROTEIN FOLDING AND AGGREGATION

Many partially and fully unfolded proteins exhibit an increased propensity in vitro to form insoluble aggregates, leading to irreversible denaturation (1, 14, 37). Reference is made to two types of protein stability, namely, stability with respect to unfolding, called thermodynamic stability, and stability with respect to aggregation, called colloidal stability. These two types of stability are ordinarily interdependent and may be treated separately only under special conditions, such as in the limit of extreme dilution. The close relationship between the two types of stability is due to the similarity on an atomic scale between the noncovalent interactions

Table 3   Reported effects of macromolecular crowding on conformational isomerization

Addition of dextran reduces mean distance between residues 169 and 203 in partially unfolded adenylate kinase (36).
  Inter-residue distance, measured by time-resolved FRET, is reduced from 39 Å in 1 M GuHCl to 34 Å in 1 M GuHCl +
  270 g/L dextran. Inter-residue distance in native conformation (no GuHCl) is 29 Å.

**Addition of Ficoll 70K or PEG 20K shifts near-UV and far-UV CD spectra of RNase A in 2.4 M urea to that
  characterizing native RNase A** (90). Essentially complete transition of spectrum achieved at ∼35 wt-% added polymer.

**Addition of Ficoll 70K or PEG 20K significantly decreases the hydrodynamic radius of partially unfolded RNase A in
  2.4 M urea but does not affect the hydrodynamic radius of native protein** (90). Decrease of ∼25% in presence of
  35 wt-% polymer.

Addition of PEG 20K partially restores enzymatic activity of RNase A in 2.4 M urea (90). Enzyme activity increases from
  8% of native in the absence of polymer to 26% in the presence of 30 wt-% polymer.

**High concentrations of macromolecules in vitro and in the periplasm of *Escherichia coli* stabilize compact
  conformations of α-synuclein relative to expanded conformations** (51). Conformational transition from more compact at
  10°C to less compact at 35°C in dilute solution is suppressed in 300 g/L BSA or in periplasm of intact *E. coli* cells.

Pulling force required to unfold ubiquitin increases by 20% in the presence of 300 g/L dextran 40K (71). Observed
  effect is ∼10-fold greater than calculated via excluded-volume model.

**Addition of 30 wt-% Ficoll 70K or PEG 6K induces conversion of the unstructured C-terminal domain of histone
  H1 to a molten globule** (79).

Addition of concentrated Ficoll 70K enhances secondary structure of native apo- and holo-flavodoxin and VlsE (69, 87).

Abbreviations: BSA, bovine serum albumin; FRET, fluorescence resonance energy transfer; PEG, polyethylene glycol.

Table 4   Reported effects of macromolecular crowding on protein stability with respect to denaturation

Addition of Ficoll 70K increases the free energy of unfolding of FK506-binding protein (86). ΔG_{N-U} increases by
  approximately RT upon addition of 160 g/L dextran. Two-state transition verified. Magnitude consistent with prediction
  of excluded-volume model (98).

**Addition of dextran 30K stabilizes the molten globule conformation of apomyoglobin at pH 2 with respect to heat-
  and cold-induced unfolding** (52). T_{50} for cold denaturation reduced by ∼13°C and T_m for heat denaturation raised by ∼20°C
  upon addition of 270 g/L dextran. Results in qualitative agreement with prediction of excluded-volume theory.

Refolding rate of Rd-apocyt b_{562} increases by 30% at 30°C and by 80% at 20°C in the presence of 85 g/L PEG 20K
  (2). Unfolding rate little affected. Measurement via ^{15}N NMR spin-relaxation dispersion.

Addition of PEG 4K increases T_m for thermal denaturation of DNase I (81). **T_m increases by more than 15°C
  in 20 wt-% PEG.**

Addition of Ficoll 70K and dextran 70K increases T_m of apo- and holo-flavodoxin (69, 87). T_m increases by 14°C
  in low-salt buffer and 4°C in high-salt buffer upon addition of 300 g/L Ficoll 70K.

Abbreviations: NMR, nuclear magnetic resonance; PEG: polyethylene glycol.

that stabilize the native conformation of a
protein and those that stabilize intermolec-
ular noncovalent complexes. The difference
in free energy between a natively folded pro-
tein and a misfolded and/or aggregated pro-
tein may be small, and crowding can shift
the thermodynamic balance between the two
forms in either direction depending on the de-
tails of the folding and aggregation pathways
(21, 60).

A CAUTIONARY NOTE
ON THE USE OF
POLYETHYLENE GLYCOL
AS A CROWDING AGENT

The effect of macromolecular crowding on
a particular reaction is generally studied
experimentally by measuring changes in
reaction rates or equilibria in the presence
of different concentrations of putatively inert
macromolecular cosolutes. One of the most

Table 5 Reported effects of macromolecular crowding on enzyme activity

Enzymatic activity increases and then decreases with increasing concentration of protein crowding agents, but decreases monotonically with increasing concentration of polymeric crowding agents (17). Up to 10-fold increase in specific activity of urease in 30 wt-% hemoglobin.

Addition of PEG 6K increases enzyme activity of *Escherichia coli* AspP (65). 50 g/L PEG decreases K_m fourfold and increases V_{max} sixfold.

Addition of dextran 70K, Ficoll 70K, or PEG 6K increases enzyme activity of isochorismate synthase (40). At 25 wt-% of additive, K_m decreases two- to threefold.

Addition of dextrans (15K to 500K) or Ficoll 70K reduces rate of hydrolysis catalyzed by alkaline phosphatase (32). Larger dextrans have a larger effect per unit w/v concentration; 20 wt-% dextran 500K decreases rate approximately sevenfold while 20 wt-% dextran 15K decreases rate approximately twofold. Effect attributed largely to reduction in rate of enzyme-substrate encounter.

Specific activity of hexokinase reduced in high concentrations of BSA (67). At 250 g/L BSA, k_{cat} decreases ~33%, K_m decreases ~25%. Reaction rates measured calorimetrically.

Effects of small osmolytes and high concentrations of BSA on hexokinase activity are additive (68). Consistent with hypothesis that osmolytes (except urea) interact primarily with proteins via volume exclusion.

Addition of PEG 4K to 20K enhances activity of DNase I and S1 nuclease, does not significantly affect activity of exonuclease III, and decreases activity of exonuclease I (81). Polymer does not affect K_m, but increases V_{max} of DNase I ~20-fold in 20 wt-% PEG.

Abbreviations: BSA, bovine serum albumin; PEG, polyethylene glycol.

Table 6 Reported effects of macromolecular confinement on protein stability and conformation

Confinement in polyacrylamide gels increases T_m of several proteins (8). T_m increases with increasing polyacrylamide concentration in gel. 1.5°C to 5°C increase in 20% gels.

Confinement of glucose isomerase in functionalized mesoporous silica enhances retention of specific activity at high urea concentrations (48). In ~300 Å pores, enzyme activity in 8 M urea is equal to that in the absence of urea in bulk solution.

Confinement of GFP-mut2 in silica gel increases free energy of unfolding by GuHCl (9). Approximately $3RT$ increase at 37°C.

Structure of mutationally destabilized protein that is disordered in bulk solution reverts to native state when protein is encapsulated in reverse micelles (70).

Confinement of glucose isomerase in functionalized mesoporous silica and addition of 0.4 M urea increases specific activity (48) **by 50% without covalent cross-linking to silica and by 80% enhancement with covalent cross-linking.**

Confinement of several proteins in the cavity of wild-type and mutant forms of GroEL/GroES accelerates or decelerates rate of refolding in a size-dependent manner (89). Refolding is accelerated in larger cavities and decelerated in smaller cavities, in qualitative or semiquantitative agreement with predictions of confinement simulations and models (30, 41, 88).

popular cosolutes is the highly water-soluble synthetic polymer polyethylene glycol (PEG, or polyethylene oxide), which is actually a polyether with monomeric structure ($-CH_2-CH_2-O-$). PEG fractions with molecular weights in excess of a few thousand have a large and predominantly repulsive interaction with proteins and tend to induce macromolecular associations and compaction in qualitative accord with crowding theory (see, for example, References 38 and 74 and works cited in Tables 1–5). However,

a number of studies have shown that this interaction cannot be described quantitatively in terms of excluded volume alone, and several independent lines of evidence point to an attractive interaction between PEG and nonpolar or hydrophobic side chains on the protein surface (6, 7, 54, 91, 92). Thus the repulsive excluded-volume contribution to the PEG-protein interaction is partially compensated to an unknown extent by an attractive interaction, the strength of which can vary significantly between different proteins

of approximately equal size. A variety of other water-soluble polymers and proteins [e.g., dextrans, Ficoll, hemoglobin, defatted bovine serum albumin (BSA)] lack such an attractive interaction for other proteins, and their interactions with proteins can be described using pure excluded-volume models (46, 54, 76, 77, 80, 92). Because these readily available polysaccharides and proteins have the added advantage of resembling more closely the types of macromolecules encountered in a physiological medium, we recommend them as alternatives to PEG as crowding agents for use in quantitative studies.

BEYOND EXCLUDED VOLUME: EFFECTS OF OTHER TYPES OF NONSPECIFIC INTERACTIONS

Theoretical models for estimating the magnitude of crowding and confinement on macromolecular reactions generally assume that these effects are predominantly entropic in origin, i.e., deriving from the relative reduction in configurational entropy of reactants, transition state, and products due to crowding or confinement. Nevertheless, other nonspecific interactions such as electrostatic repulsion and attraction and hydrophobic attraction likely contribute significantly to overall energetics in highly crowded or confined media (54). The effects of such interactions on the colligative and association properties of highly concentrated solutions of a single protein may be satisfactorily accounted for by a simple semiempirical model in which the protein molecules are treated as effective hard spheres, the apparent size of which reflects short-ranged soft attractions or repulsions in addition to steric exclusion (29, 63, 64). However, the effective hard particle model cannot be expected to satisfactorily describe a medium containing high concentrations of multiple macromolecular species interacting via qualitatively different potentials, such as a solution containing two concentrated proteins that are oppositely charged at a particular pH (29, 54). At the present time it appears that

Monte Carlo and/or Brownian dynamics simulations provide the most promising approach to the theoretical study of crowding effects in such solutions, which should also be accompanied by additional experimental studies.

The results of experimental studies of the effect of high concentrations of a single species of inert macromolecule (crowder) on the associations of dilute test proteins have been interpreted in the context of effective hard-particle models that take into account nonspecific repulsive interactions deriving from steric exclusion and electrostatic repulsion as well as attractive interactions leading to association of the test molecules (76, 77). It is assumed in these models that crowder molecules interact with each other only via volume exclusion. One indication that the situation in physiological media may be more complex is provided by a recently published report (95), which showed that concentrated mixtures of protein and polysaccharide exhibit nonadditive effects on the refolding of lysozyme. Moreover, concentrated solution mixtures of dextran and PEG spontaneously separate into immiscible phases, between which proteins may partition in accordance with their relative affinity for each phase (49). The physical bases of these phenomena deserve closer study, as one would expect the local environment of most biological macromolecules in vivo to consist of more than one volume-excluding species.

We cannot yet adequately assess functional consequences of the nearly ubiquitous proximity of soluble proteins to the surfaces of biological structures (e.g., phospholipid membranes and protein fibers). However, some directions for future research have been suggested by relevant in vitro studies. A variety of proteins associate weakly with actin fibers, microtubules, DNA, and phospholipid membranes in a non-site-specific fashion (see Reference 11 and references therein). Proteins are localized at the surfaces of these structures by attractive electrostatic and/or hydrophobic interactions in addition to repulsive

volume-exclusion (hard-wall) interactions. The reduction in configurational entropy of the protein resulting from this dual mode of localization is greater than that achieved by hard-wall confinement alone and, in fact, magnifies significantly the consequences of confinement. Simple theoretical models (57) predict that adsorbed macromolecules, like hard-wall-confined macromolecules, have a stronger tendency to self- or hetero-associate than they do in bulk solution, and that the tendency to associate increases substantially with the strength of attraction between the soluble macromolecule and the surface. Adsorption may also increase the rate of macromolecular binding to specific surface sites (103). A number of macromolecular associations proceed more rapidly or to a greater extent on surfaces than in bulk solution (31, 42, 82, 104), suggesting that the consequences of localization via adsorption may be general and of potential importance in heterogeneous physiological media.

INTERPRETATION AND SIGNIFICANCE OF IN-CELL MEASUREMENTS OF PROTEIN STABILITY AND ASSOCIATION

Experimental techniques have been developed recently that provide information about either the stability and conformation or the association properties of specific labeled proteins within living cells (26). The use of some of these techniques to study diffusional transport of labeled macromolecules in cytoplasm and tissue is reviewed in Reference 19. In live-cell studies a labeled protein (or pair of labeled proteins) is introduced into a cell via expression of recombinant proteins or microinjection. Then, a signal that reflects conformation or association of labeled protein, or colocalization (hetero-association) of two labeled proteins, is monitored. In certain experiments, the intracellular environment is globally perturbed via addition of denaturant, temperature change, or application of hyper- or hypo-osmotic stress (23, 34, 35, 51). The monitored response of the labeled protein(s) within the cell to the applied perturbation is compared with that of the same protein(s) in dilute solution, and conclusions are drawn regarding the effect of the environment in vivo on the stability of the labeled protein(s).

Although each of these techniques does indeed provide information about aspects of the behavior of tracer proteins within a cell, one must be careful about the interpretation of the results of such experiments. The potential impact of labeling procedures on the spatial distribution of the tracer species, possible induction of artificial associations, and/or disruption of specific interactions of interest must be assessed. A number of additional questions must be answered: (*a*) How is the test protein distributed among the different local microenvironments within an intact cell? If it is found in multiple environments, how does one interpret the overall average signal? (*b*) If a tracer protein that is not native to a host organism such as *Escherichia coli* is highly overexpressed within that organism, how likely is it that the protein is situated within a microenvironment that closely resembles its native milieu? (*c*) How does the living cell respond to applied stress? Does this response alter the distribution of the test protein, the composition of the microenvironment(s) of the test protein, and the interactions between the test protein and its surroundings within each microenvironment? (*d*) Because a living cell is a homeostatic system, within which one cannot vary the composition of individual microenvironments in a systematic and quantifiable fashion, how can one determine the extent to which a particular tracer protein in a particular intracellular microenvironment is either confined or crowded?

NARROWING THE GAP BETWEEN IN VITRO AND IN VIVO

We suggest that the influence of crowding and confinement on macromolecular reactivity in vivo may be best explored by

means of a bottom-up approach, according to which the behavior of test proteins is studied quantitatively within a series of media in which features thought to be essential to a particular microenvironment are incorporated in a systematic fashion, from the most simple to the most complex (105). Such a bottom-up approach would ultimately lead to construction of a cytomimetic medium incorporating all the major elements thought to be present in the selected microenvironment. The ability to control and independently manipulate temperature, pH, salt, and osmolyte concentration; the types and concentrations of soluble bystander macromolecules; and the types and abundances of structural elements such as membranes and cytoskeletal filaments (if appropriate) in this model system provide a rigorous approach to the characterization of nonspecific interactions influencing the behavior of proteins and other macromolecules within a native-like environment. This is no simple task, but we believe that if our goal is to understand in quantitative terms the role of nonspecific interactions in biology—a role we believe is absolutely essential to the mechanism of life—we cannot avoid paying attention to the details of these inherently complex systems.[5]

[5]"It may not be a dream to imagine that, using reconstituted systems of increasing complexity, the coordinated motility of an artificial cell will eventually be mimicked.... However, in many instances the analysis of molecular events using classical biochemical and structural approaches remains the limiting factor for future progress" (10).

SUMMARY POINTS

1. Macromolecular crowding nonspecifically enhances reactions, leading to the reduction of total excluded volume. In general these reactions include the formation of macromolecular complexes in solution, binding of macromolecules to surface sites, formation of insoluble aggregates, and compaction or folding of proteins. The expected magnitude of the effect is strongly dependent on the relative sizes and shapes of concentrated crowding species and on dilute macromolecular reactants and products.

2. Macromolecular crowding is generally expected to increase the rate of slow, transition-state-limited association reactions and to decrease the rate of fast, diffusion-limited association reactions.

3. Simple statistical-thermodynamic theories based on coarse-grained structural models usually provide reliable predictions of qualitative effects and, in favorable circumstances, can provide reasonably good semiquantitative predictions of the magnitude of an expected effect.

4. Biological fluids are more complex than systems studied theoretically or experimentally in vitro because of increased heterogeneity and the probable presence of nonspecific attractive and repulsive intermolecular interactions in addition to volume exclusion. Theoretical and experimental explorations of model systems containing well-defined elements of added complexity are strongly encouraged.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

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Errata

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