

*Annual Review of Biophysics*

# Macromolecular Crowding Is More than Hard-Core Repulsions

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Annu. Rev. Biophys. 2022. 51:267–300

First published as a Review in Advance on  
March 3, 2022

The *Annual Review of Biophysics* is online at  
[biophys.annualreviews.org](http://biophys.annualreviews.org)

<https://doi.org/10.1146/annurev-biophys-091321-071829>

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## Keywords

depletion forces, excluded volume, macromolecular crowding, protein complex stability, protein stability, preferential interactions

## Abstract

Cells are crowded, but proteins are almost always studied in dilute aqueous buffer. We review the experimental evidence that crowding affects the equilibrium thermodynamics of protein stability and protein association and discuss the theories employed to explain these observations. In doing so, we highlight differences between synthetic polymers and biologically relevant crowders. Theories based on hard-core interactions predict only crowding-induced entropic stabilization. However, experiment-based efforts conducted under physiologically relevant conditions show that crowding can destabilize proteins and their complexes. Furthermore, quantification of the temperature dependence of crowding effects produced by both large and small cosolutes, including osmolytes, sugars, synthetic polymers, and proteins, reveals enthalpic effects that stabilize or destabilize proteins.

Crowding-induced destabilization and the enthalpic component point to the role of chemical interactions between and among the macromolecules, cosolutes, and water. We conclude with suggestions for future studies.

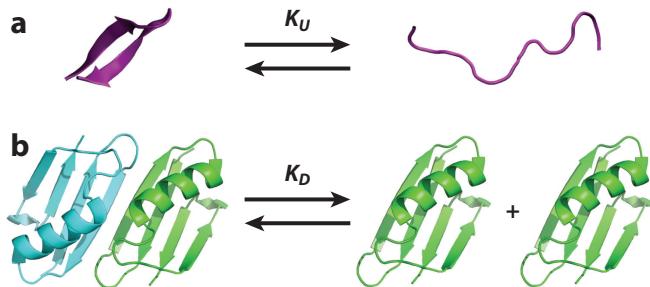
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## INTRODUCTION

### Rationale

The degree of crowding depends on the type of cell. Prokaryotes like *Escherichia coli* have cytoplasmic macromolecule concentrations of 200 to 450 g/L (203, 211, 239). The concentration of macromolecules in eukaryotic cells is approximately half the concentration in *E. coli*. The concentrations in the mitochondrion and the nucleus exceed the concentration in bacteria (233). The cytoplasms of amphibian and fish oocytes are less concentrated, with protein concentrations of 30 to 60 g/L (66, 119, 181, 209). For comparison, the protein concentration in an egg white is approximately 110 g/L (124). These data neglect the high concentrations of metabolites and other smaller solutes (14), which are tightly regulated. Some small solutes called osmolytes are used by cells to counteract environmental stresses, including osmotic stress, and can reach intracellular concentrations in the molar range (89, 228).

**Figure 1**

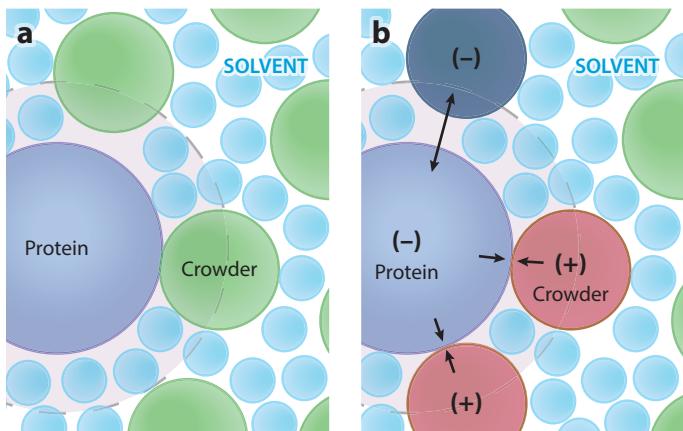
Schematics of crowding-modulated processes. (a) Unfolding of a model peptide (78). (b) Dissociation of the GB1 homodimer [2RMM (94)].

Globular proteins and simple protein complexes are the most important for this review. The processes of interest are mainly reversible protein folding and protein–protein association (**Figure 1**). These reactions are important because only the folded form of an enzyme is catalytically active, and for complexes, reactants and products can play different but equally essential roles. These macromolecules bathe in solutions comprising water and at least one cosolute. These cosolutes do the crowding and include small molecules (e.g., sugars and osmolytes), synthetic polymers, and other proteins.

Assuming a partial specific volume of 0.73 mL/g, proteins occupy 10% to 30% of the cytoplasmic volume in *E. coli* (239). Using an inexact elevator analogy (because elevators are much taller than a person), the situation corresponds to an unacceptable 12 to 18 people in a standard 3 m<sup>2</sup> car. Most *in vitro* studies on proteins are conducted at concentrations of less than 0.1 g/L protein, which would correspond to less than a single squirrel in the elevator.

The effects of crowding are determined by the interactions between the molecules in solution (**Figure 2a**). Simple crowding theories, which assume that all molecules are hard spheres, predict that the intracellular environment will stabilize proteins and their complexes due to hard-core repulsions, although shape can play a role (17). The idea is simple: The interaction free energy between the crowder and the protein depends on the distance between them. For uncharged atoms, the interaction is controlled by a Lennard-Jones-type potential; at short distances, the interaction is highly repulsive because of the difficulty in interpenetrating the electron shells of the species. The interaction is often slightly attractive at longer distances but eventually decays to zero. The repulsion experienced between molecules when they are close is one of the most important interactions in chemistry because it gives molecules their shapes. The interaction energy is rather like the behavior of billiard balls: The molecules do not notice each other until they try to overlap. The key idea is that this interaction energy is athermal. That is, there are no additional attractive or repulsive chemical, also called soft (161), interactions. The ensuing simple interaction free energy arises exclusively from entropic considerations because it only deals with the arrangement of molecules. As described below, soft interactions also play a role. They include charge–charge, dipole, nonpolar, and other interactions. Soft interactions are stabilizing or destabilizing depending on whether they are repulsive or attractive, respectively (171).

Until recently, most efforts to understand the structure and energetics of proteins focused on Lindström-Lang's four levels of structure (118): primary, i.e., the sequence of amino acids; secondary, i.e.,  $\alpha$ -helices,  $\beta$ -sheets, etc.; tertiary, i.e., the three-dimensional structure of globular proteins; and quaternary, in which subunits combine to form complexes. The importance of a fifth level, quinary structure, is becoming clear now that advances facilitate the study of biological



**Figure 2**

Hard and soft effects. (a) Illustration of a crowded solution of spherical particles possessing only hard repulsions. The shaded region depicts the volume that the protein excludes from crowders because of the hard interactions between the crowder and the protein. (b) Illustration of attractive and repulsive soft (i.e., chemical) interactions. Attractive interactions may include hydrogen bonding; weakly polar and hydrophobic interactions; and, as illustrated in this panel, interactions between complementary charges. Repulsive chemical interactions arise from the juxtaposition of like charges or from the sum of solvent– and cosolute–protein interactions. Illustration is not to scale.

macromolecules in living cells. The term quinary structure was coined several times (e.g., 32), and the loose use of the term has been criticized (159). Nevertheless, a reasonable working definition has been proposed: “Quinary structure comprises the transient interactions between macromolecules that provide organization and compartmentalization inside cells” (32, p. 403).

Understanding macromolecular crowding effects on proteins is important for both fundamental biology and human health. It is fundamentally important to know the properties of proteins where they function—in cells—including how crowding affects liquid–liquid phase separation (115). Understanding crowding effects on protein complexes is key because networks of protein interactions turn chemical signals into physiological responses that maintain homeostasis (35). Relevant to human health, two-thirds of missense mutations perturb protein–protein interactions (164), but there is little information on protein–protein interactions in cells (193, 197). In addition, there is an entire class of diseases related to protein aggregation (28), which is affected by solution conditions (200). For instance, aggregation of  $\alpha$ -synuclein, a protein implicated in Parkinson’s disease, is impeded in the crowded cellular interior, yet aggregation occurs readily in dilute solution (210).

Protein stability in high concentrations (hundreds of g/L) of small molecules (up to a few hundred Da) is important for at least two reasons. First, certain small molecules protect cells from stress (89, 228). Second, small molecules are used by the biotechnology industry to protect proteins (149), biologic drugs (139, 217), and vaccines. Recently, questions were raised about polyethylene glycol (PEG) in COVID-19 vaccines (50). Furthermore, protein enzymes are poised to revolutionize organic synthesis, but optimizing these reactions requires their stabilization in nonphysiological, and often nonaqueous, environments (67).

The effect of crowding by synthetic polymers is also important for the industrial and pharmaceutical applications mentioned above. In terms of biology, most efforts to understand macromolecular crowding employ synthetic polymers, but cells are crowded with proteins and nucleic acids. Further complicating efforts to understand protein biophysics in cells is the fact

that the nature of the cellular interior depends on many parameters, including the type of cell, the position of the protein of interest (also known as the test protein) within the cell, and even the position of the cell in the organism. For instance, as mentioned above, the protein concentration in bacteria can exceed 300 g/L, but the concentration in eukaryotic cells is approximately half that value (211), and changing the osmotic environment outside a cell changes the environment inside the cell (154, 155).

## History

Our goal is to review efforts to understand how cosolutes in general, and macromolecules in particular, affect protein and protein complex stability. The fundamental concepts were first elaborated by Gibbs (77), who related changes in surface tension (or alternatively, the free energy required to expose a surface or interface) to the accumulation or exclusion of a solute at that interface, typically expressed as the preferential interaction coefficient, which is discussed in more detail below.

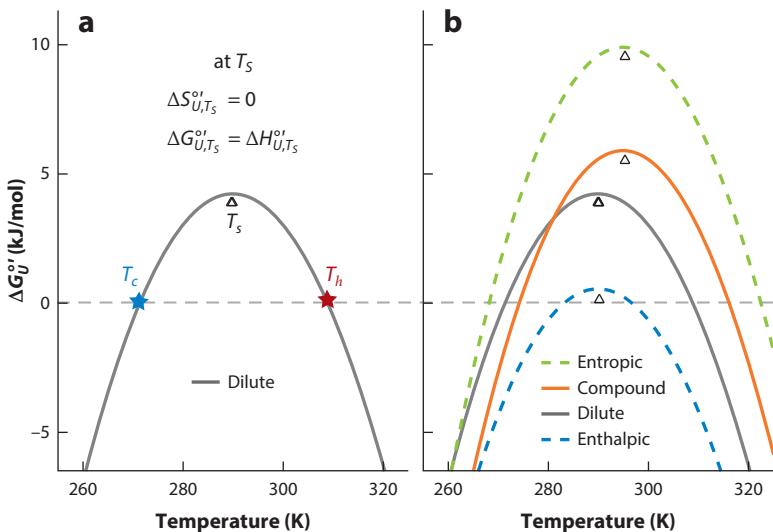
The first studies aimed at crowding and biology are probably Cohen's (34) 1942 paper on virus crystallization using the polymer heparin and the work of Ogston & Phelps (144) in 1961 using equilibrium dialysis to show that concentrated hyaluronic acid excludes serum albumin and  $\beta$ -lactoglobulin. Minton's seminal 1981 papers on the theory (131) and measurement (with Wilf; 134) mark the beginning of formal efforts to understand macromolecular crowding as it applies to proteins.

In the 1980s, it was commonly thought that “Equilibrium constants for such reactions can be increased by as much as two to three orders of magnitude, depending on the relative sizes and shapes of the reactants and products, and on those of the background macromolecules” (64, p. 115). Such an effect would amount to a maximum of approximately 4 kcal/mol in free energy at physiological temperatures, which is approximately seven times the available thermal energy,  $RT$ , where  $R$  is the gas constant, and  $T$  is the absolute temperature. Rarely, if ever, have increases this large in protein and protein complex stability been reported. An early challenge to the idea that only hard-core repulsions are important came from the Oas group, who demonstrated that the stability of monomeric  $\lambda$  repressor in cells is approximately the same as it is in buffer (76). Observations such as this led to the separation of the contributions of crowding into hard-core repulsions and chemical interactions (171).

As discussed above, hard repulsions arise because two molecules cannot occupy the same space at the same time. Hard repulsion-induced crowding is entropically stabilizing when the products occupy less of the space available to other proteins and crowding solutes than do the reactants. This situation is the norm for folding and for forming simple protein complexes. Attractive interactions, including hydrogen bonds and weakly polar, hydrophobic, and charge–charge interactions between cosolutes and test proteins, are destabilizing. Consider urea, which is destabilizing because it forms attractive interactions with the protein backbone, competing with the intraprotein interactions that stabilize the folded state (198). Repulsive charge–charge interactions increase stability because they add to the hard repulsions. Weaker interactions such as van der Waals forces between solution components can lead to apparent repulsion or attraction between cosolute and protein because of the tug of war among solvent, cosolute (or crowder), and protein.

## EQUILIBRIUM THERMODYNAMICS OF STABILITY

The term stability, as used for globular proteins and protein–protein complexes, refers to the standard-state free energy of unfolding ( $\Delta G_U^\circ$ ) or dissociation ( $\Delta G_D^\circ$ ). We assume that reactions are reversible and at equilibrium (**Figure 1**) unless stated otherwise. In the standard state, reactants and products have concentrations of 1 M. If protons are taken up or released, as is common in



**Figure 3**

Temperature dependence of protein stability. (a) Proteins in buffer with key temperatures labeled. (b) Effect of purely entropic stabilization, effect of attractive enthalpic interactions, and their compound effect. Parameters for panel *a* and the dilute curve in panel *b* are for superoxide dismutase in buffer from the Oliveberg laboratory (45). The enthalpic, entropic, and compound curves in panel *b* are based on Zhou's (236) superoxide dismutase data and ideas.

biology, then this standard state requires that experiments be performed at pH 0 (i.e., 1 M H<sup>+</sup>). To solve this problem, we define a biochemical standard state, specifically, pH 7, and denote this state by appending a superscript prime to changes in free energies, enthalpies, entropies, and heat capacities:  $\Delta G_{U/D}'$ ,  $\Delta H_{U/D}'$ ,  $\Delta S_{U/D}'$ , and  $\Delta C_p'$ , respectively.

A short description of the thermodynamics of protein stability is given below. For background, we suggest Anfinsen's (2) Nobel lecture. A feeling for the temperature dependence and the meaning of the concomitant thermodynamic parameters can be found in classic papers by Becktel & Schellman (12) and Dill and colleagues (22, 57). We limit our discussion to the simplest cases. For protein stability, we consider two-state unfolding involving only the folded (*F*) and unfolded (*U*) state, where  $\Delta G_U^o = -RT \ln(K_U)$  and  $K_U = [U]/[F]$ .

For protein complexes, we consider the dissociation of a dimer (*D*) to monomers (*M*) such that  $K_D = [M]^2/[D]$  and  $\Delta G_D' = -RT \ln(K_D)$ . For simple homodimers, the relationship between the fraction of dimer,  $f_D$ , and the equilibrium constant for dimerization,  $K_D$ , is

$$f_D = \frac{4P_T + K_D - \sqrt{K_D^2 + 8P_T K_D}}{4P_T}, \quad 1.$$

where  $P_T$  is the total protein concentration.

The temperature dependence of  $\Delta G_U^o$  is curved (Figure 3a) because the heat capacity of *U* is greater than that of *F*, i.e.,  $\Delta C_{p,U}^o > 0$ . The positive sign of  $\Delta C_{p,U}^o$  is mostly attributed to exposure of hydrophobic surface upon unfolding (152). The temperature dependence of the unfolding enthalpy ( $\Delta H_{U,T}'$ ) and entropy ( $\Delta S_{U,T}'$ ), assuming constant  $\Delta C_{p,U}^o$ , is described by Equations 2 and 3, where  $T_{\text{ref}}$  is a reference temperature:

$$\Delta H_{U,T}' = \Delta H_{U,T_{\text{ref}}}^o + \Delta C_{p,U}^o(T - T_{\text{ref}}), \quad 2.$$

$$\Delta S_{U,T}' = \Delta S_{U,T_{\text{ref}}}^o + \Delta C_{p,U}^o \ln(T/T_{\text{ref}}). \quad 3.$$

Using the Gibbs equation, we obtain

$$\Delta G_{U,T}^o = \Delta H_{U,T}^o - T \Delta S_{U,T}^o = \Delta H_{U,T_{\text{ref}}}^o - T \Delta S_{U,T_{\text{ref}}}^o + \Delta C_{p,U}^o [T - T_{\text{ref}} - T \ln(T/T_{\text{ref}})]. \quad 4.$$

Three temperatures are important (**Figure 3a**):  $T_c$ , the low temperature where  $\Delta G_U^o$  is zero;  $T_s$ , the temperature of maximum stability; and  $T_b$ , the high temperature where  $\Delta G_U^o$  is zero. The slope of the curve,  $(\partial \Delta G_{U,T}^o / \partial T)$ , at any temperature, is  $\Delta S_U^o$ . Therefore, at  $T_c$ , where the slope is zero, all the stability is due to  $\Delta H_{U,T}^o$ . When  $T_{\text{ref}}$  is defined as  $T_b$ ,  $\Delta S_{U,T_{\text{ref}}}^o$  can be eliminated from Equation 4 because  $\Delta S_{U,T_b}^o = \Delta H_{U,T_b}^o / T_b$ . We are then left with

$$\Delta G_U^o = \Delta H_{U,T_c}^o (1 - T/T_b) + \Delta C_{p,U}^o [T - T_b - T \ln(T/T_b)]. \quad 5.$$

The thermodynamics of protein stability does not depend on test protein concentration provided that the concentration is small, since  $K_U$  is unitless. The thermodynamics of dimer dissociation is more complicated because  $K_D$  has units of concentration (197), meaning that  $K_D$  depends on temperature and test protein concentration. Nevertheless, two differences between protein stability and the stability of a dimer made from two folded proteins are easily grasped. First, fewer bonds are broken to dissociate a dimer into folded monomers than to unfold a globular protein the size of the monomer. Second,  $\Delta C_{p,D}^o$  is less positive than  $\Delta C_{p,U}^o$  because less surface is exposed in dissociating a dimer made from two globular proteins than is exposed upon the unfolding of a globular protein.

As discussed above, the simplest mechanistic ideas about crowding effects due to steric or hard-core interactions predict stabilization of proteins and their complexes because the folded proteins occupy less space than unfolded proteins, and protein complexes occupy less space than their monomers. We denote crowding-induced changes in  $\Delta G_U^o$  and  $\Delta G_D^o$  as  $\Delta \Delta G_U^o$  and  $\Delta \Delta G_D^o$ , defined as  $\Delta G_{\text{crowded}}^o - \Delta G_{\text{dilute}}^o$ . We apply this directionality to changes in  $\Delta H_{U/D}^o$ ,  $\Delta S_{U/D}^o$ ,  $\Delta C_p^o$ , and  $T_s$ .

Zhou (236) presents a way to consider the crowding effects on the enthalpy and entropy of unfolding (**Figure 3b**). He chose  $T_s$  in dilute buffer as the reference temperature. The enthalpy of unfolding is then

$$\Delta H_{U,T}^o = \Delta H_{U,T_s}^o + \Delta C_{p,U}^o (T - T_s). \quad 6.$$

As described above, at  $T_s$ , we have  $\Delta S_U^o = 0$ . The entropy change is then

$$\Delta S_{U,T}^o = \Delta C_{p,U}^o \ln \frac{T}{T_s}. \quad 7.$$

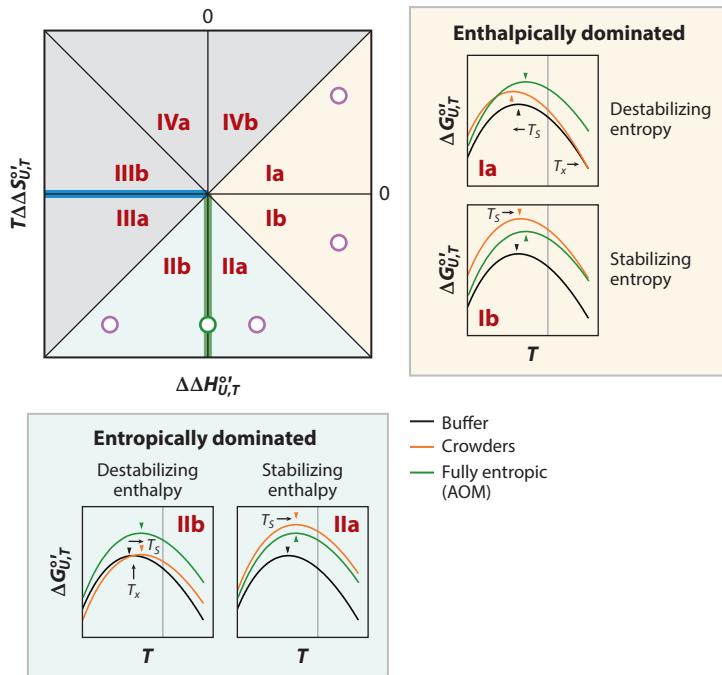
Zhou assumed that crowding does not change  $\Delta C_{p,U}^o$ , which is a reasonable first approach (but see 112). The effect of crowding on  $\Delta H_{U,T}^o$  and  $\Delta S_{U,T}^o$  is

$$\Delta \Delta H_U^o = \Delta \Delta H_{U,T_s}^o - \Delta C_{p,U}^o \Delta T_s, \quad 8.$$

$$\Delta \Delta S_U^o = -\Delta C_{p,U}^o \ln \left[ \left( 1 + \frac{\Delta T_s}{T_s} \right) \right], \quad 9.$$

where  $\Delta T_s$  is the change in the maximum of the stability curve. Zhou then considered three scenarios. The first corresponds to classic crowding theory: The stability increase is entirely entropic, which means  $\Delta \Delta H_U^o = 0$  such that

$$\Delta \Delta H_{U,T_s}^o = \Delta C_{p,U}^o \Delta T_s. \quad 10.$$



**Figure 4**

Thermodynamic footprints of crowder-induced stabilization. (*Top left*) A schematic entropy–enthalpy plot of crowder-induced effects. Stabilizing crowders ( $\Delta\Delta G_U'^o > 0$ ) are found below the diagonal, where  $T\Delta\Delta S_U' = \Delta\Delta H_U'$ , and can occupy four regions, each with different entropic and enthalpic contributions. Purple circles represent a crowder characteristic of each sector at a specific temperature,  $T^*$ . The satellite panels show schematics of the temperature dependence of  $\Delta G_U'^o$  representing crowders acting through different mechanisms. Gray vertical lines highlight the temperature  $T^*$  at which the entropy–enthalpy plot is constructed. For each curve, arrows highlight  $T_s$ , the temperature of maximum stability, and  $T_x$ , where the curves with and without crowders cross. The reference black curves are for protein in buffer. Green curves represent purely entropic (stabilizing) crowding, according to the Asakura–Oosawa model (AOM), corresponding to the vertical green line (separating regions IIb and IIa) with the green circle in the top left panel. Regions IIa and IIb are dominated by entropy (*cyan background*) and differ in whether the enthalpy is stabilizing (IIa) or destabilizing (IIb). In contrast, sectors Ia and Ib are both dominated by stabilizing enthalpy (*yellow background*) and differ by whether entropy is stabilizing (Ib) or destabilizing (Ia). The horizontal blue line in the top left panel represents a fully enthalpically destabilizing cosolute.

The consequence is a crowding-induced vertical up-shift, with  $\Delta T_s > 0$  (for  $\Delta C_{p,U}^o > 0$ ) and with  $\Delta\Delta G_U'^o$  increasing slightly with increasing temperature because  $\Delta S_U'$  is multiplied by  $T$  (**Figure 3b**).

Another way to present the same result is to consider changes in entropy and enthalpy due to the addition of crowders, as mapped in **Figure 4**. The diagonal  $T\Delta\Delta S_U' = \Delta\Delta H_U'$  separates stabilizing and destabilizing cosolutes. Destabilizing cosolutes occupy the shaded region above the diagonal, while stabilizers occupy the clear lower region. The first scenario—purely entropic stabilization—occupies the green line separating domains IIa and IIb.

The second of Zhou's scenarios is just as extreme: Crowding involves only an attractive protein–crowder interaction, with  $\Delta\Delta S_U'^o = 0$  and no change in  $T_s$ . The unfolded state exposes more attractive surface than does the folded state, which means  $\Delta\Delta H_U'$  is negative, and the stability curve

shifts downward with no change in shape compared to buffer (**Figure 3b**). This destabilization corresponds to the blue line separating domains IIIa and IIIb in **Figure 4**.

There are, however, more realistic scenarios. In the third case, the cosolute acts entropically (via steric interactions) but has, in addition, an attractive interaction with the protein. In this case,  $\Delta T_s$  is positive, and  $\Delta\Delta H_U^o'$  is negative. The result is that  $\Delta\Delta G_U^o'$  changes with temperature, which means that stability curves in dilute solution and in crowded solution intersect at a temperature  $T_X$ . For this scenario, crowding is stabilizing compared to buffer above  $T_X$  and destabilizing below. At  $T_X$ , we have  $\Delta\Delta G_U^o' = 0$  such that

$$T_X = \frac{\Delta\Delta H_U^o'}{\Delta\Delta S_U^o'} = \frac{-\Delta\Delta H_U^o'}{\Delta C_{p,U}^o \ln \left( 1 + \frac{\Delta T_s}{T_s} \right)} \approx \frac{-\Delta\Delta H_U^o'}{\Delta C_{p,U}^o \Delta T_s} T_s. \quad 11.$$

This situation corresponds to domain IIb in **Figure 4**. Minton and colleagues (95) discuss a similar phenomenon for protein complex stability under crowded conditions: entropic crowding with enthalpic penalties from a sticky solute.

Yet another scenario is common among stabilizing osmolytes. These cosolutes can have a hard interaction and an additional softer repulsion (at larger distances) that manifests as an added favorable enthalpic contribution to the stability or interaction free energy. This case corresponds to domain Ia in **Figure 4**. It follows from Equation 11 that, for these cosolutes, for which  $\Delta\Delta H_U^o' > 0$ , the value of  $\Delta T_s$  is negative. This stabilizing situation seems the opposite of steric crowding: Enthalpy is the driving force, and the entropic contribution is destabilizing, which apparently contradicts the essential idea of volume constraints as stabilizing. As discussed below, however, this mechanism just generalizes the crowding idea to the case where, because of soft interactions, exclusion is no longer athermal (166). Due to the near ubiquity of solution entropy–enthalpy compensation (120), scenarios Ia and IIb are more often observed than the others shown under the diagonal in **Figure 4**. Interestingly, smaller solutes typically inhabit region Ia, while large polymeric crowders often reside in region IIb (202).

In the next section, we discuss wet experiments, examine molecular-level explanations, and attempt to draw these topics together. It is not feasible to cite all of the work about the effects of crowding on biopolymers. We ignore crowding effects on nucleic acid–protein interactions and phase-separated states even though simulations and wet experiments prove their significant roles (115, 141, 147). Sometimes we make our point by citing the seminal example, a recent result, or a review. We hope that our effort complements and extends recent reviews (49, 151, 170, 188, 223, 237).

We end this section with a few words about the small magnitude but large importance of crowding-induced effects. As suggested by the curves in **Figure 3b**, cosolute-induced stability changes are small, yet changes even smaller than  $RT$  are important in biology. For example, globular proteins can go from 90% folded to 10% folded over a few °C (12), an increase in thermal energy of 0.01 kcal/mol. Human body temperature is 37°C, but a fever of 42°C is often fatal. *E. coli* has a maximum growth rate at 39°C, but stops growing and dies at 49°C (93). Temperature-sensitive missense mutants of yeast grow at 30°C but not at 37°C (218). Finally, in our favorite temperature change effect, increasing the incubation temperature of alligator eggs by 4°C changes the sex of hatchlings from 100% female to 100% male (71, 75).

## EXPERIMENTAL OBSERVATIONS

In this section, we begin with observations on disordered proteins (211) before discussing observations on globular proteins and protein complexes.

## Small Cosolutes

Small cosolutes include osmolytes (228) and the monomers of synthetic polymers. Proteins in solutions of these molecules follow the Stokes-Einstein and Stokes-Einstein-Debye relationships for translational and rotational diffusion, respectively (51, 60, 61). That is, their translational and rotational diffusion coefficients at a fixed temperature are inversely proportional to the macroscopic viscosity.

Molar solutions of trimethylamine oxide (TMAO) cause the collapse of the disordered protein carboxamidine ribonuclease, and urea prevents this collapse (153). High glucose concentrations collapse acid-denatured cytochrome *c* (48). For these proteins, collapse occurs because each protein possesses a unique and compact globular native state. However, glucose-induced collapse is weaker for the disordered protein,  $\alpha$ -synuclein (138). TMAO also tends to collapse disordered proteins, but again, the collapse is smaller than that observed for globular proteins (205).

Zwitterionic osmolytes are classic globular protein stabilizers (228), although it is important to bear in mind that changes in pH change zwitterions into singly charged ions, reversing the effect (189). TMAO also stabilizes proteins better than buffer in cell lysates (172) and than the absence of an osmolyte in living cells (195). Ethylene glycol, glucose, and sucrose, the monomers of PEG, dextran, and Ficoll, respectively, tend to stabilize foldable proteins and their complexes (162, 203). Polyols, such as mannitol and xylitol, also tend to be stabilizing (203). In contrast, N-ethylpyrrolidone is destabilizing (23), yet its polymer, polyvinylpyrrolidone (PVP), tends to be weakly stabilizing or neutral (109, 128).

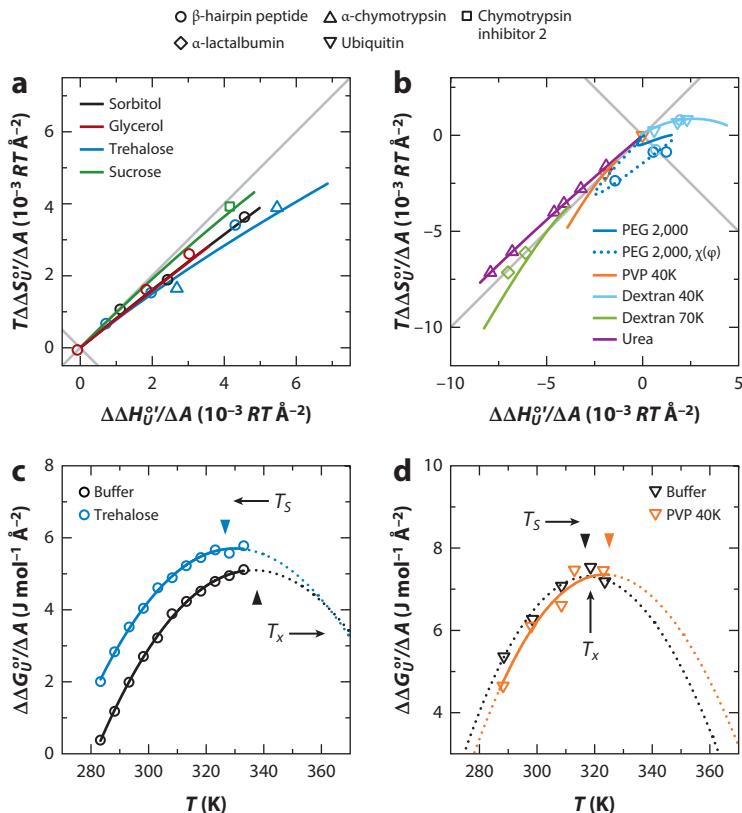
For stabilizing osmolytes, the thermodynamic mechanism is typically enthalpic (**Figures 4** and **5c**). Glycerol, sorbitol, other polyols, and trehalose enthalpically stabilize a folded  $\beta$ -hairpin (150, 203). Similar observations are reported for sucrose stabilization of chymotrypsin inhibitor 2 (16) and for stabilization of SH3 by TMAO and ethylene glycol (190). However, there are exceptions; for example, stabilization by the sugars glucose and sucrose is entropic for several proteins (111, 190), and Beg et al. (13) report that stabilization of apo  $\alpha$ -lactalbumin and lysozyme by six sugars is entirely entropic.

In summary, depending on the folding or association process, the enthalpic and entropic contributions from crowding inhabit different domains (**Figure 4**). Osmolytes often drive enthalpically dominated stabilization, while stabilizing synthetic polymer crowders are often entropically dominated. [As an aside, other small cosolutes destabilize proteins. A prominent and well-known example is urea (198), which preferentially interacts with protein surfaces, favoring the unfolded state because it exposes more surface than the folded state. As such, its behavior is characterized by a destabilizing enthalpic component that dominates the stabilizing entropy (domain IIIa in **Figure 4**; see also **Figure 5b**.)]

## Synthetic Polymers

PEG is by far the most commonly used synthetic polymer in crowding studies because of its high water solubility, high purity, low cost, commercial availability in close to a dozen molecular weights from 106 Da to 2,000 kDa, and well-studied physical properties (42, 96, 238). The glucose polymer dextran shares many of these characteristics and is commercially available in about half a dozen sizes from 6 to 150 kDa (1, 11). PVP (also known as Povidone) is also well characterized and available in at least five molecular weights from 10 kDa to 1,300 kDa (1, 109, 128). The cross-linked sucrose polymer Ficoll is available in molecular weights of 70 kDa and 400 kDa (73).

The quality of synthetic polymer solutions changes with concentration (161). At low concentration, polymers act like individual molecules, and the macroscopic viscosity, as measured, for example, with an Ostwald viscometer, increases monotonically with concentration. This is the



**Figure 5**

(*a,b*) Entropy enthalpy and (*c,d*) temperature dependence plots of crowder-induced stabilization show the thermodynamic footprint of crowder families. ○ indicates the β-hairpin peptide (150, 203), △ indicates α-chymotrypsin (106), □ indicates chymotrypsin inhibitor 2 (16), ◇ indicates α-lactalbumin (234), and ▽ indicates ubiquitin (181, 221). Panels *a* and *b* correspond to 298 K, except for the case of polyvinylpyrrolidone (PVP), which corresponds to 340 K. Thermodynamic quantities are normalized to the change in solvent-accessible surface area upon unfolding,  $\Delta A$  (168). Osmolytes often induce enthalpic stabilization mitigated by entropy (Ia in **Figure 4**). Macromolecular crowders mainly induce entropic stabilization mitigated by enthalpy (IIb in **Figure 4**). The osmolyte trehalose downshifts  $T_s$  (panel *c*). The polymer PVP upshifts  $T_s$  (panel *d*). Curves in panels *a* and *b* are fits to the mean field model (see 168). Panel *d* is adapted with permission from Reference 221.

so-called dilute region. Above a certain concentration, defined by polymer molecular weight, the chains form a mesh, and the viscosity increases steeply (1). The break between regimes is called the overlap concentration,  $c^*$  (161). Polymer solutions are referred to as semidilute above  $c^*$ . The existence of the semidilute regime makes synthetic polymer solutions fundamentally different than solutions of globular proteins because globular proteins can be treated more like spheres, which, rather than forming a mesh, jam at high concentrations. For this reason, it is unrealistic to treat a synthetic polymer molecule in a solution above  $c^*$  as a sphere. We return to this idea when discussing interpretations of crowding effects.

The diffusion of globular proteins in concentrated solutions of synthetic polymers deviates from Stokes-Einstein and Stokes-Einstein-Debye behaviors in that the viscosity they feel is less than the macroscopic viscosity (117). Furthermore, the deviation for rotational diffusion is greater

than that for translational diffusion, and the deviation increases with molecular weight. This behavior is also observed for disordered proteins (103, 219, 240). However, disordered proteins diffuse faster than folded proteins of similar size (219) in solutions crowded with globular proteins (219) and inside cells (103). This enhanced diffusional freedom of disordered proteins compared to their globular cousins may explain why disordered proteins are often selected by nature for use in signaling pathways, where speed is essential.

Crowding should not alter the structure of folded globular proteins because the interiors of these proteins are efficiently packed (157). Therefore, efforts focused on disordered states are important for understanding crowding effects. Data from single-molecule Förster resonance energy transfer (FRET) experiments show increasing collapse of a disordered protein with increasing PEG molecular weight at a fixed volume fraction, with the effect eventually saturating (191). Partial collapse without persistent structure formation is also observed for NUPR1 in solutions of Ficoll and dextran (18). Small-angle neutron scattering data show that protein crowders (BSA and lysozyme) induce two populations of disordered proteins, one compact and the other extended (8). We should remain cautious in our interpretation of macromolecular effects, however, because both sucrose and its cross-linked polymer Ficoll cause compaction of the disordered protein  $\alpha$ -synuclein (7).

An early study of PEG and globular protein stability from Lee & Lee (112) is also one of the most complete. These authors show that ethylene glycol and PEGs decrease the stability of several proteins. Destabilization increases with PEG molecular weight and is positively correlated with increasing test protein hydrophobicity.  $\Delta H_U^o$  at  $T_b$  increases with PEG molecular weight for most of the proteins.  $\Delta C_{p,U}^o$  for lysozyme and  $\beta$ -lactoglobulin changes from positive to negative with increasing PEG concentration, and the magnitude of  $\Delta C_{p,U}^o$  increases with increasing PEG molecular weight. PEG also destabilizes human  $\alpha$ -lactalbumin, whereas the more hydrophilic polymers, 70-kDa dextran and Ficoll, are stabilizing (182). Taken together, the results suggest that PEG's effects on globular protein stability are dominated by its hydrophobic nature and the hydrophobic nature of the unfolded state. However, as discussed above, the hydrophobicity of the protein may be key because PEG often collapses disordered proteins (191), which tend to be more hydrophilic (215).

Lee & Lee's (112) work included experiments showing that PEG affects  $\Delta H_U^o$  of lysozyme, but a study of lysozyme in dextran reports no change compared to buffer alone (174). Concentrated solutions of more hydrophilic polymers, such as dextran, Ficoll, and PVP, are generally stabilizing near room temperature and above, but the stabilization is often enthalpic (16, 45, 221). An elegant effort from the Ebbinghaus group shows that high concentrations of dextran stabilize ubiquitin enthalpically (181), but this interpretation has been challenged (13). Specifically, Minton and colleagues (127, 174) interpret the dextran-induced stabilization of lysozyme and the molten globule of cytochrome *c* as purely entropic. The same conclusion was reached by the Wittung-Stafshede laboratory in their study of dextran-induced stabilization of apoazurin (29), and the data in their figure 2 resemble those for pure entropic stabilization, as depicted in **Figure 3b**. Bear in mind that, in several of these efforts, a destabilizing cosolute, which might cause enthalpic compensation, was also present.

Uncharged non-PEG synthetic polymers and their monomers are generally stabilizing. PVP is an exception because its monomer is destabilizing (23). For stabilizing polymers, monomers are often just as potent as polymers on a g/L basis, and the source of the stabilization at and above room temperature is enthalpic, rather than entropic (16, 181). An extensive and systematic investigation has been reported on a  $\beta$ -hairpin peptide (203). In this instance, the PEGs, dextrans, and glucose are all stabilizing, but stabilization changes from entropic to enthalpic with increasing cosolute concentration.

There are few studies of charged polymers. Small zwitterions like sulfobetaine (216) and other zwitterionic osmolytes (228) typically stabilize proteins, but 100-kDa poly(sulfobetaine) at concentrations up to 250 g/L is destabilizing (100). The model monomer was not studied. An examination of the effect of a microgel comprising 10<sup>9</sup>-Da poly(*N*-isopropylacrylamide-coacrylic acid) on the stability and dynamics of chymotrypsin inhibitor 2 reveals only a small stabilizing effect and no effect on nanosecond internal dynamics compared to buffer, showing the size limit of crowding effects (129). Another recent effort shows that polyacetate destabilizes Cu/Zn superoxide dismutase (192). The destabilization probably arises from attractive interactions between the synthetic polymer and the unfolded state of the protein.

Polymer-based studies of protein complex stability have been reviewed (197). Generally, monomers and uncharged polymers have small stabilizing effects, but there are exceptions (226). The effect of PEG molecular weight on increases in stability is also mixed, and there are too few values of  $\Delta H_D''$  and  $\Delta S_D''$  to make definitive statements.

In summary, although sugar polymers may come the closest to pure entropic stabilization, observations of destabilization in concentrated solutions of PEG and charged synthetic polymers suggest the presence of attractive interactions between the polymer and the protein.

## Confinement

Confinement differs from crowding in that, in confinement, the test protein is trapped in a cavity just large enough to contain it and some hydrating water. A widely accepted model is that spherical confinement stabilizes globular proteins because there is no room to unfold. Although not always reversible, encapsulation in silica sol-gels increases  $T_h$  of several globular proteins compared to buffer (59), as does encapsulation of frataxin in a polyacrylamide gel (165). Thus, the results of both studies are consistent with this simple model. Confinement in reverse micelles with internal cationic or zwitterionic head groups, however, destabilizes model globular proteins (25, 180). Quantification of the temperature dependence of the stability suggests that destabilization arises from attractive interactions between the test protein and the inside of the micelles.

## Globular Proteins as Crowders In Vitro

The small molecules and synthetic polymers discussed above usually do not interfere with the optical spectroscopies used to assess stability, but how does one detect a test protein in a crowded sea of other proteins? In this case, nuclear magnetic resonance (NMR) is useful because one can enrich a protein with an NMR active nucleus (e.g., <sup>13</sup>C, <sup>15</sup>N) or label it with <sup>19</sup>F (19, 41). Fluorescent probes require much less protein but often require larger labels (83).

Globular proteins and synthetic polymers also differ in other respects. First, globular proteins are biologically relevant. After all, cells are crowded with proteins, not PEG, dextran, or Ficoll. Second, unlike synthetic polymers, globular proteins are compact and densely packed with atoms (157). Therefore, in some sense, globular protein crowders can be treated as spheres. Synthetic polymers, in contrast, form highly viscous semidilute solutions above  $c^*$  (161). Third, unlike most synthetic polymers used in crowding studies, proteins are charged. Fourth, it is difficult to control systematically the chemical interactions between protein crowders and a test protein. For instance, except for end effects, PEGs, no matter their size, have the same kind of chemical interactions with test proteins (102), but different proteins have different surfaces. Furthermore, there is no globular protein family in which increasing molecular weight leads to a smooth increase in size. In summary, proteins are physiologically relevant, but systematic studies of them are more challenging compared to studies using synthetic polymers as crowders.

Small-angle neutron scattering data on disordered proteins indicate that protein crowders (BSA and lysozyme) induce two populations, compact and elongated (8). The observation above, that the decrease in rotational diffusion is greater than the decrease in translational diffusion, is also observed for disordered and globular proteins in solutions crowded by globular proteins (194, 220).

High concentrations of globular proteins tend to destabilize test proteins (45, 130, 221), but there are exceptions (54). Destabilization is also seen in reconstituted cytosol (173); in chaperones (105); and, as discussed below, in living cells. The source of this decrease is the attractive interactions between the backbone of the test protein and the protein crowders (87). The stabilizing osmolyte betaine can overcome destabilization by reconstituted cytosol (172). Effects on protein folding kinetics in concentrated protein solutions have also been examined (79).

In a series of NMR experiments on the globular protein frataxin in high concentrations of hen egg white [mostly ovalbumin (124)], the Pastore and Temussi team (123) found that the components interact strongly with heat-unfolded frataxin but less strongly with the cold-unfolded protein. This observation is consistent with a role for the hydrophobic effect in attractive crowding-induced interactions because hydrophobicity increases with increasing temperature (177). Few efforts have assessed the temperature dependence of  $\Delta G_U^o$ , but both increases and decreases in  $\Delta H_U^o$  near  $T_s$  compared to buffer are observed (45, 190, 221).

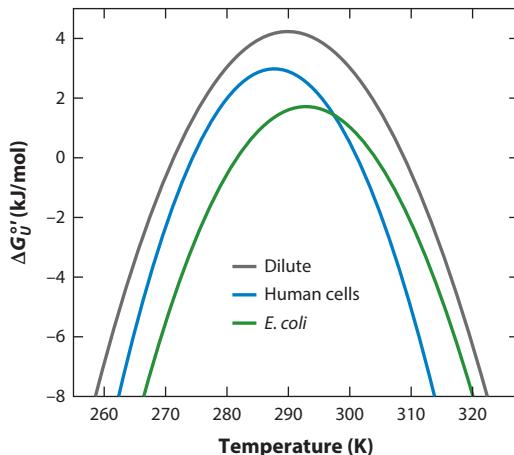
The effects of protein crowding on protein–protein interactions have been reviewed (197). As expected, chemical interactions play a role. As far as we are aware, there is only one study on the temperature dependence of protein complex stability under crowded conditions (194). This effort involves the interaction between an SH3 domain and a small unstructured peptide derived from the domain’s disordered binding partner. Given the small size of the peptide, which is therefore barely affected by hard-core repulsions, this effort probably reveals more about chemical interactions than about hard-core repulsions.

## In Cells

Studying protein and protein complex stability in living cells is both the ultimate goal of and the most challenging task in the study of crowding effects. Not only must the test protein be detected in the presence of every other molecule in the cell, but the conditions must also be such that the cells remain alive. Leakage of test proteins from dead or damaged cells is another problem that can result in artifacts (20, 43) unless important controls are performed (9).

A seminal publication (44) represents an early example of both in-cell NMR and protein disorder (196). This study’s data indicate that disorder can be maintained in cells. Later, the disordered protein flgM was shown to be more collapsed in *E. coli* cells than it is in buffer (52). Another early effort claimed that the periplasm of *E. coli* maintains recombinant  $\alpha$ -synuclein, a protein associated with Parkinson’s disease, in its disordered state (68, 126). Although the protein is disordered in cells (68, 210), this original interpretation (126) probably arose from a misunderstanding of amide–proton exchange rates (38, 46).

One of the first investigations of globular protein folding in cells used NMR (178) and a variant of protein L with seven lysine residues replaced by glutamic acids. In dilute buffer at room temperature, 84% of the molecules populate the denatured state, compared to 0.1% for the wild-type protein. It was expected that hard-core repulsions in the crowded interior of *E. coli* would fold the protein, yet the protein remained unfolded in cells. Furthermore, modest concentrations of salt fold the protein in dilute solution, but even increasing the salt concentration in cells does not result in folding. Thus, attractive chemical interactions overcome the stabilizing hard-core repulsive effect. These interactions have also been observed in cell lysates (40, 107, 108).



**Figure 6**

Temperature dependence of superoxide dismutase in buffer and cells based on parameters from the Oliveberg group (45). Comparison to **Figure 3b** shows that destabilization involves attractive enthalpic interactions and that these interactions are stronger in *Escherichia coli* cells, which are more crowded than human cells (211).

Protein stability in cells has been quantified using several tools, including NMR- and mass spectrometry-detected amide–proton exchange (76, 137), fluorescence techniques (58, 104), <sup>19</sup>F NMR (190), and heteronuclear NMR (45, 85). Many results support the idea that attractive interactions destabilize globular proteins compared to buffer alone (e.g., 24, 31, 45), and charge plays a role in this destabilization (31, 232), but stabilizing effects have also been observed (47, 55, 85). It seems that specific positions in the cell and the physiological state of the cell also affect stability (83).

Studying the same protein or protein complex in both *E. coli* and eukaryotic cells provides information about the relative importance of steric and chemical interactions. For instance, both the cytoplasm of a human ovary adenocarcinoma cell line and the cytoplasm of *E. coli* destabilize human superoxide dismutase compared to buffer alone, but destabilization is greater in the more crowded interior of *E. coli* (45) (**Figure 6**). There are few studies of the temperature dependence of stability in cells, but only decreases in  $\Delta H_U^o$  near  $T_s$  compared to buffer have been reported (45, 190).

Some of the first assessments of nuclear relaxation in cells showed that the environment affects transverse relaxation ( $T_2$ ) more than it does longitudinal relaxation ( $T_1$ ). Specifically, the increased viscosity compared to buffer assessed using transverse relaxation is larger than that measured via longitudinal relaxation (230–232). This counterintuitive result has been explained by fast exchange between the test protein and nearby cytoplasmic proteins (113).

Assessing protein complex stability in cells is even more challenging than measuring protein stability in cells. Most experiments use fluorescence detection. The Knop group made some of the first measurements of  $K_D$  using yeast (121). Unfortunately, the dilute solution values were not reported. Thus, the results cannot be used to assess the effect of the cellular environment. Sudhaharan et al. (199) studied the interactions between the RhoGTPase CDC42 and three of its effector proteins in Chinese hamster ovary cells. Comparison with *in vitro* data shows that the cellular environment decreases  $K_D$  by approximately a factor of two. The Wohland lab quantified complex formation between a CDC42 variant and an actin-binding scaffolding protein in zebrafish embryos (184). The  $K_D$  value of 100 nM in embryos is approximately fivefold larger than the value determined in buffer. Phillip et al. (148) identified a small decrease in rate constant for forming

the TEM1  $\beta$ -lactamase inhibitor protein complex in HeLa cells compared to buffer. These data suggest a role for charge, but  $K_D$  was not determined.

The Gruebele lab assessed complex formation between the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase in human U-2 OS cells (201). The stoichiometry changes from 1:1 in buffer to 2:1 in cells, and the  $K_D$  values were compared by taking the square root of the value determined in cells, resulting in a decrease in  $K_D$  from 20  $\mu\text{M}$  in buffer to 14  $\mu\text{M}$  in cells. Thus, quinary interactions stabilize this multienzyme complex.

The cellular interior destabilizes the homodimeric XIAP complex in HeLa cells compared to buffer (229). The crystal structure of the dimer shows that the protein is stabilized by a salt bridge. Destabilization probably arises because cells typically have a high ionic strength, which weakens the salt bridge.

Dimerization of the same protein has been examined in *E. coli* cells and *Xenopus laevis* oocytes (193). The complex is more stable in both cell types than it is in buffer. Charge–charge variants were also investigated: The more anionic is the homodimer, the stronger is the intermolecular repulsion between the dimer and the proteome in cells and the more stable is the complex. In summary, although there are few studies of this topic, the observation of both stabilizing and destabilizing effects points to a role for chemical interactions in cells.

## Summary of Observations

The sections above discuss two important points. First, chemical interactions play a key role in crowding. Second, stabilization or destabilization caused by small and large cosolutes often occurs via changes in enthalpy and entropy compared to the environment in buffer alone. The manifestation of these effects is evident in Zhou's (236) thermodynamic analysis, which is applicable to both stabilization by synthetic polymers and destabilization by protein crowders. The observations of crowding-induced destabilization and changes in enthalpy go against the simplest molecular interpretation of cosolute effects, which calls for purely entropically driven stabilization.

## MOLECULAR INTERPRETATIONS

To understand how interactions among several, sometimes many, components in solution alter the stability of solvated proteins and their complexes, we must explore the thermodynamics dictating the relationships among interactions, solution structure, and stability. In the following sections, we introduce the ideas used to explain the thermodynamics of crowding effects.

## Thermodynamics of Mixed Solvents

We consider the exposed interface of a protein or other large macromolecular solute particle,  $P$ , with a bathing solution comprising a binary mixture of water,  $W$ , and cosolute,  $C$ . Alternatively, it is common to use a notation corresponding to  $1 - W$ ,  $2 - P$ , and  $3 - C$  (176). Note that  $C$  can represent small molecules, synthetic polymers, or proteins that act as crowding agents.

The changes in free energy due to solutes are intimately tied to the excess or deficit of one solution component at the interface. The free energy change  $dG_p^o$  associated with immersing an interface in a binary mixture of cosolute and solvent is given by the Gibbs adsorption isotherm (145),

$$dG_p = -\Gamma_W d\mu_W, \quad 12.$$

where  $\Gamma_W$  is the preferential hydration (or solvation) coefficient, stated in terms of number of water molecules, and  $\mu_W$  is the molar free energy of the solvent, or simply its chemical potential. Gibbs developed this equation for large extended interfaces, but a similar relation holds for

macromolecular surfaces. This relation was realized by Wyman (225), is termed Wyman linkage, and is applied to proteins and other macromolecules. Equivalently, one can imagine preferential cosolvation defined by the expression  $dG_p^\circ = -\Gamma_C d\mu_C$ . These coefficients,  $\Gamma_W$  and  $\Gamma_C$ , represent the degree to which the molecules of one component are found next to the interface at an excess (positive values) or deficit (negative values) compared to their bulk concentrations.

Thus, we can relate the change in free energy associated with exposing the protein interface to the solvating mixture or, equivalently, the added exposure of solvent-accessible surface upon protein unfolding,  $\Delta\Delta G_U^\circ$ , or protein–protein dissociation,  $\Delta\Delta G_D^\circ$ , to the change in the preferential interaction coefficient,  $\Delta\Gamma_W$ . These quantities are related, at low protein concentrations, by

$$\Delta\Gamma_W = v_W^{-1} \frac{\partial \Delta\Delta G_{U/D}^\circ}{\partial \Pi}, \quad 13.$$

where  $\Pi$  is the osmotic pressure (in Pascals),  $v_W$  is the solvent's partial molar volume (in  $m^3\text{mol}^{-1}$ ), and free energy is in J. Equivalently, we can consider water's chemical potential,  $\mu_W$ , instead of osmotic pressure, since  $v_W d\Pi = -d\mu_W$ . This expression can also be stated in terms of the so-called *m*-value (81), used ubiquitously to characterize the effects of (usually denaturing) cosolutes on proteins, by realizing that  $m = \Pi\Delta\Gamma_W$  in the ideal, i.e., van't Hoff regime.

It is easy to associate stabilization,  $\Delta\Delta G_U^\circ$  (or  $\Delta\Delta G_D^\circ$ )  $> 0$ , with a positive change in preferential hydration, since this is equivalent to stating that more water molecules are adsorbed to the protein domain upon unfolding or dissociation, i.e., when protein surfaces are expanded and further exposed. Alternatively, one can speak of water molecules being released to solution upon folding or association, i.e., when exposed surfaces are buried. This stabilization can be achieved in two ways. First, the cosolutes can be preferentially excluded (equivalently, water can be preferentially included) at the protein–solvent interface, but exclusion is stronger for the unfolded state,  $\Gamma_{C,U} < \Gamma_{C,F} < 0$ . Second, the cosolutes can be preferentially included (equivalently, water can be preferentially excluded) at this interface but more strongly included in the folded state molecular interface than in the unfolded state,  $0 < \Gamma_{C,U} < \Gamma_{C,F}$ .

For stabilizing osmolytes, the former possibility—preferential exclusion of the cosolute—is much more common (37). Interestingly, however, Xie & Timasheff (227) report that, at room temperature, the osmolyte trehalose stabilizes ribonuclease A by preferential exclusion from the denatured state, but at higher temperatures, the mechanism changes to stabilization by preferential binding. Conversely, denaturing cosolutes, such as urea and guanidinium chloride, are preferentially included at protein surfaces, and thus  $0 < \Gamma_{C,F} < \Gamma_{C,U}$ ,  $\Delta\Gamma_C$  is positive, and the *m*-value is negative.

For heteropolymers like proteins, the extent of exclusion or inclusion is the sum of all contributions from all of the different chemical moieties in the protein chain. This idea is embodied in the transfer free-energy approach pioneered by Tanford and colleagues (5, 207). The idea that a complex protein interface can be considered a collection of well-defined molecular interfaces, each with its own preferential interaction with the solution, was implemented with some variations for proteins in solvent-plus-cosolute systems by the Record (56) and Bolen (6, 198) groups. By measuring transfer free energies per  $\text{\AA}^2$  of amino acid surface introduced into cosolute aqueous solutions, it is possible to calculate the cosolute contribution to protein stability, assuming additivity of the different components. The protein surface is regarded as a mosaic of chemical moieties. Thus, with knowledge of each piece of the solvent-accessible surface area and the experimentally derived transfer free energies from model compounds, it is possible to calculate the total free energy necessary to transfer a protein from water or buffer to cosolute solution. The free energy difference between two states (folded or unfolded protein or associated or dissociated complex) can then be calculated. This general approach has also been implemented

in simulations to assess cosolute effects on protein conformations (136) and the relative stability of globular (143) and intrinsically disordered proteins (90).

### Confluence of Molecular Interpretations

Although complete and formally correct, the above description does not elucidate the molecular mechanism. Guggenheim (84, p. 171) states, “[this is] as far as pure thermodynamics can take us,” or, as Lewis & Randall (116, p. 85) write, “Thermodynamics exhibits no curiosity, certain things are poured into its hopper, certain others emerge according to the laws of the machine.” These statements are a response to a particular and often-heard postseminar comment, namely, that crowding effects are explained by changes in the activity of water. Although crowding effects occur via changes in water activity, changes in water activity do not explain or reveal the mechanism.

Molecular mechanisms link the experimentally measured thermodynamic quantities to intermolecular interactions. Next, we review several approaches employed to resolve such mechanisms. Importantly, each mechanism must match the thermodynamic observables, namely the free energy, enthalpy (or internal energy), and entropy changes, as well as their dependence on solution composition. As outlined below, the different approaches to the thermodynamic mechanism, namely the crowding and the preferential interaction perspectives, are related via the Gibbs-Duhem equation (146), although interpretation of such data in terms of water binding remains controversial (37, 185, 187, 214).

### Dawn of Entropic Depletion Forces

Asakura & Oosawa (3, 4) set out to explain why “between two bodies immersed in a solution of macromolecules an interaction appears owing to characteristics of the medium,” and in doing so proposed a molecular mechanism, the Asakura-Oosawa model (AOM), for what has since been called the depletion interaction (114). They considered the interaction of two extended interfaces, representing the surfaces of large macromolecular solutes, bathed in a binary mixture of much smaller solvent and cosolute molecules. The solute–cosolute interaction is assumed to be completely repulsive due to hard-core interactions, which define an exclusion volume around the macromolecule into which the centers of mass of the cosolutes cannot penetrate.

If two macromolecules approach closer than a certain distance, their exclusion volumes overlap, decreasing the overall exclusion volume by  $\Delta V_{\text{ex}} < 0$ , which in turn increases the volume available to the cosolutes. The increase in available volume necessarily increases the translational (or mixing) entropy of the cosolutes, providing the driving force for association.

The change in free energy is conveniently described by  $\Delta \Delta G_{\text{AOM}} = \Pi \Delta V_{\text{ex}}$ . In the van ’t Hoff regime, where osmotic pressure is linear in cosolute molality, this expression is equivalent to the Gibbs adsorption isotherm, Equation 12, for concentration-independent  $\Delta \Gamma_W$ . The AOM is general and depends only on the effective macromolecule–cosolute interaction, which in turn is assumed to be completely steric and therefore temperature independent (i.e., athermal). Simply stated, the depletion force is entirely entropic. Beyond the van ’t Hoff regime, the virial expansion of the osmotic pressure can provide a more reliable concentration dependence of the free energy change. Theoretical considerations show that the stabilization afforded by the depletion force may be reduced due to crowder size polydispersity, even in the absence of enthalpic contributions (53).

### The Crowding Perspective

A conceptually similar approach to the AOM was introduced to explain the effects of cosolutes and crowding. The simplest implementation assumes that all species are hard spheres and then

defines the volume excluded by the macromolecule as a sphere with a radius equal to the sum of the cosolute and macromolecule radii (49). This idea is taught in first-year chemistry as a correction to the ideal gas law. The resulting exclusion of volume to the macromolecule favors compact states. This same idea was implemented by Cheung et al. (26) using an off-lattice model.

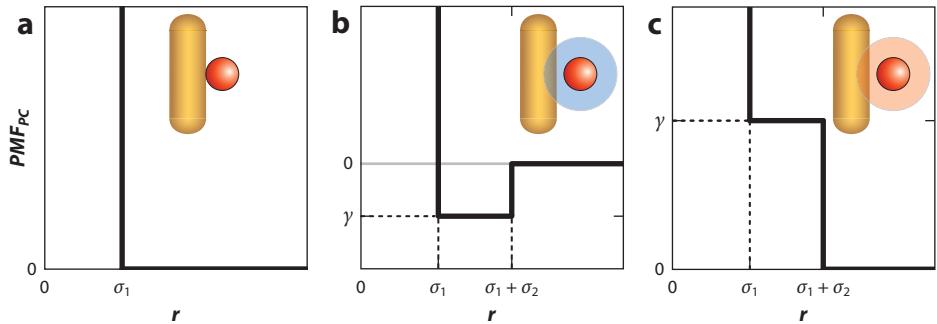
A potentially more accurate approach involves the use of scaled-particle (SP) theory (36, 110, 156, 235), which was developed for analyzing the thermodynamics of hard-sphere fluids. The concept was first applied to crowding by Minton and coworkers (132, 158, 237) and has since been used by others (17, 48, 80, 86, 175, 183, 208, 240). In this treatment, the dissociation or unfolding free energy is calculated by determining the reversible work required to form a cavity that can accommodate the different protein states (i.e., monomer versus dimer, unfolded versus folded). While the physical volume of the protein itself is assumed to be constant, the cavities that envelope it are not necessarily volume preserving because they reflect the change in volume excluded to solution. When the protein is much larger than the cosolute, the SP theory prediction reduces to that of the AOM, with the free energy scaling linearly with osmotic pressure. However, the more precise approach encoded in SP theory is more accurate when the relative sizes of protein and cosolute are comparable, such that molecular packing plays a crucial role. The expressions derived for different shapes and sizes of accommodating cavities are thus better for analyzing macromolecules of various shapes and sizes. In any case, since both the AOM and SP theory assume athermal, steric interactions, the mechanism remains entropic (202). SP theory makes an important prediction: At constant volume occupancy, small crowders have a larger effect than larger crowders because larger synthetic polymers leave more space than do smaller crowders (183).

There are several criticisms of SP theory. We introduce them below and direct the reader to more detailed information. In its favor, SP theory expresses the reversible work of creating a cavity in solution, which is a better approximation than that obtained from the AOM even when a virial expansion is applied (202). Yet SP theory has limitations. First, the theory, which involves fluids of hard particles, requires a virtual hard-core pressure (17), which is thousands of times the real pressure (17, 175, 183). Second, models based on hard spheres ignore the fact that the unfolded states of proteins and synthetic polymers at concentrations above the overlap concentration,  $c^*$ , are not hard spheres. However, Minton's (133) Gaussian cloud model of the unfolded state mitigates this problem. Another problem involves both good news and bad news. The good news is that SP theory allows an explicit estimate of the work required to make space for a protein in mixtures of hard spheres that are used as models of water–crowder solutions. The bad news is that there is no easy way to include interactions that are not hard core, so one is left with a hard-sphere representation of water and crowder alike. Thus, perhaps the most glaring shortcoming of SP theory is its neglect of crowder interaction free energies beyond hard-core excluded volume. This also means that SP theory cannot account for any temperature dependence. By contrast, the subject of the next section, Kirkwood-Buff (KB) solution theory, is fundamentally grounded in solution thermodynamics, but to be useful, it needs to be fed information about solution structure from other sources. Most notably, this information may be derived from computer simulations.

## Kirkwood-Buff Solution Theory

KB solution theory is a rigorous treatment of preferential interactions for mixtures involving protein, solvent, and cosolute (99) that links bulk macroscopic properties and the microscopic correlations between solution components. These correlations express local liquid structure and are usually stated in the form of pair correlation functions or radial distribution functions,

$$g_{ij}(r) = \frac{\rho_{ij}(r)}{\rho_{ij}^0}, \quad 14.$$



**Figure 7**

Protein surface–cosolute interaction in terms of a schematic potential of mean force,  $PMF_{PC}$ . (a) Athermal (i.e., steric) interactions are the basis of the Asakura–Oosawa model (AOM) and yield an entirely entropic effect. (b,c) A more realistic  $PMF_{PC}$  showing an additional temperature-dependent interaction at larger distances, as represented by Equation 17. The temperature-dependent, soft part of the potential can be (b) attractive or (c) repulsive. These soft interactions are represented by the blue and red haloes in the insets. Soft attractions mitigate steric interactions. In contrast, soft repulsion augments the strong steric repulsion experienced at smaller distances. This soft repulsion is characterized by the repulsive step height (which can be temperature dependent),  $\gamma = \gamma_H - T\gamma_S$ , with an enthalpic contribution  $\gamma_H$  and an entropic component  $T\gamma_S$ .

where  $\rho_{ij}(r)$  and  $\rho_{ij}^0$  are the local and bulk concentrations, respectively, of component  $j$  around component  $i$ . The theory then expresses the overall correlation between the two species by integrating over the entire volume,

$$G_{ij} = 4\pi \int [g_{ij}(r) - 1]r^2 dr, \quad 15.$$

where  $G_{ij}$  is termed the KB integral, whose units are [volume/molecule]. KB integrals can be calculated for each state of the protein—folded versus unfolded, dimer versus monomer. In the water–solvent–cosolute system, we can write the change in preferential hydration upon unfolding (or dissociation) using the relevant KB integral as (88, 158)

$$\Delta\Gamma_W = M_W [(G_{PW}^U - G_{PC}^U) - (G_{PW}^F - G_{PC}^F)], \quad 16.$$

where  $M_W$  is water molarity.

Generally, cosolute-induced stabilization depends on the interplay of water- and cosolute–protein preferential interactions. However, in many cases, Equation 16 can be further simplified to  $\Delta\Gamma_W \approx -M_W \Delta G_{PC}$  (167, 186). This simplification allows us to treat the solvent implicitly and thereby concentrate only on the solute–cosolute effective interaction. More formally, the potential of mean force, defined as  $PMF_{PC} = -RT \ln g_{PC}$ , implicitly incorporates all other solution degrees of freedom (i.e., solvent molecules) and gives the free energy as a function of protein–cosolute distance. Importantly, since the  $PMF_{PC}$  is a free energy, it has entropic and enthalpic components. Within the AOM, the  $PMF_{PC}$  is strictly hard core (**Figure 7a**) and is thus completely entropic. As we discuss next, however, there is often a considerable enthalpic contribution.

### Enthalpically Dominated Depletion Forces

To make connections to the AOM, we regard the quantity  $\Delta\Gamma_W/M_W$  from Equation 16 as an effective change in excluded volume,  $\Delta V_{\text{ex}}^{\text{eff}}$ . As stated above, the AOM assumes that the  $PMF_{PC}$  contains only hard, athermal interactions, but in experimental settings, additional chemical, soft, or quinary interactions play a major role (32), and these are generally temperature dependent. This realization necessarily implies that the emerging depletion force must have both entropic

and enthalpic components (166, 167). In fact, accumulating evidence, as reviewed above, indicates that most stabilization of proteins and their complexes induced by molecularly small solutes has a considerable enthalpic contribution (101, 150, 171, 202).

More specifically, stabilizing osmolytes that are necessarily preferentially excluded from proteins can induce enthalpically dominated stabilization, in which the entropic change is destabilizing (150, 171). Interestingly, even macromolecular crowders have an enthalpic contribution, often a destabilizing one, that partly counteracts the sterically induced stabilization (171, 221). To explain the discrepancies between the AOM prediction that stabilization is completely entropic and the experimental results, it is necessary to modify and extend the AOM-associated  $PMF_{PC}$  by including a soft component (**Figure 7**). For example, assume that the steric interaction is accompanied by another, longer-range, soft, attractive interaction between solute and cosolute (167) of the form shown in **Figure 7b**:

$$PMF_{PC}(r) = \begin{cases} \infty & r \leq \sigma_1 \\ \gamma & \sigma_1 < r \leq \sigma_1 + \sigma_2, \\ 0 & \sigma_1 + \sigma_2 < r \end{cases} \quad 17.$$

where we define a steric shell of width  $\sigma_1$  and a soft shell of width  $\sigma_2$  (in distance) and height  $\gamma$  (in energy). Generally, this soft part is temperature dependent and has both enthalpic and entropic contributions,  $\gamma = \gamma_H - T\gamma_S$ .

For enthalpically mitigated depletion forces (regime IIb in **Figure 4**), this longer-ranged interaction  $\gamma$  is negative and represents attraction, as in **Figure 7b**. This corresponds to the situation (**Figures 3** and **5d**) in which the driving force is still entropic but the stabilization is decreased by an unfavorable interaction energy (167).

In contrast, for enthalpy-dominated depletion forces (**Figure 4**), this longer-ranged interaction is repulsive (**Figure 7c**), serving to augment the hard-core stabilizing effect (166). Moreover, if this interaction also depends on temperature, such that the soft interaction weakens at higher temperatures, i.e.,  $\gamma_S > 0$ , then the resulting depletion force has a destabilizing (positive) entropic component and thus resides in domain Ia in **Figure 4** (167). For example, trehalose (**Figure 5a,c**) and TMAO are net repulsive, resulting in stabilization of the folded protein or the protein complex. In contrast, the denaturant urea is net attractive (122), which results in destabilization (**Figure 5b**).

Finally, when  $\gamma$  is sufficiently negative, an additional attractive force can arise between the solute particles due to bridging by cosolute (169). Bridging is also seen in systems not directly related to protein stabilization. For example, this kind of strong attraction is the origin of cononsolvency, where polymers collapse in mixtures of solvents that otherwise do not promote collapse and in colloidal systems that are compacted by PEG crowders, where the colloids form aggregates bridged by the sticky PEG molecules (163, 224).

## Mean-Field Theory of Closolute Effects

The effective interactions discussed above, along with contributions from cosolute associations, can be incorporated into a unified theory (168, 169) that allows an estimate of the free energy change associated with exposing protein surface upon unfolding or dissociation. The unified mean-field free energy has two contributions: first, cosolute–protein effective interactions, and second, the mixing free energy of cosolute in the local (protein) and bulk domains, described in the framework of Flory-Huggins solution theory (74, 92). Applying the model to wet experiment-based data by fitting the concentration dependence of  $\Delta\Delta G_{U/D}^o$  and the entropic and enthalpic contributions allows estimation of effective interaction coefficients in terms similar to those of

Equation 17. This approach gives averaged interaction coefficients over a protein surface that is a mosaic of moieties with different properties.

### Problem of Treating Spaghetti as a Sphere

Recall that SP theory predicts that, on a g/L basis, higher-molecular-weight crowders should have smaller effects (49, 183, 204), yet the opposite effect has been observed in efforts using synthetic polymers (240). Data from single-molecule FRET experiments show increasing collapse of disordered proteins with increasing PEG molecular weight at a fixed g/L concentration, with the effect eventually saturating (191), contradicting predictions from SP theory (49). This discrepancy is also observed for PEG-induced stabilization of a dimer formed from two disordered proteins (240), and hints of this behavior are seen in data on stabilization of lysozyme and  $\alpha$ -lactalbumin in 70- and 40-kDa dextran (182).

These discrepancies arise because, above the overlap concentration,  $c^*$ , synthetic polymers no longer act like individual molecules (i.e., spheres). Instead, they cross over each other, forming a confining mesh like the open space between strands of spaghetti. The Schuler lab (191, 240) tackled this problem by treating PEG via renormalization group theory in the AOM (142). Their application agrees with the data for both collapse and dimer formation (191, 240). In the mean-field theory (168), this shift in the properties of the synthetic polymer solution is baked into the mixing parameter of the Flory-Huggins parameter, the protein–cosolute interaction parameter, and the parameter defining the relevant length scale of polymer exclusion. In summary, at the concentrations and molecular weights usually employed to study crowding using synthetic polymers, the synthetic polymers cannot be treated as spheres. This conclusion also supports the arguments that synthetic polymers, although essential for the commercial protein and pharmaceutical industries, are not physiologically relevant because cells are crowded by globular proteins, not synthetic polymers.

### Simulations

Numerical simulations of crowding effects deserve their own review because the studies encompass everything from toy models to atomic-level molecular dynamics, from small-molecule osmolytes to the cytoplasm, and from peptides to giant protein assemblies. In this section, we provide a broad outline of some of these efforts and the resulting insights.

Early efforts concentrated on hard repulsions that can originate from confinement to a cavity or from crowders modeled as hard spheres. These simulations clarified that the stabilization of proteins can arise primarily from the entropic destabilization of the unfolded state of the protein originating from steric interactions. The free energy of the native state of the protein, by contrast, increases less than that of the unfolded state upon adding crowders. Interestingly, depletion-induced intramolecular attraction can promote unfolded structures that are compact and more structured than the unfolded state in the absence of crowders (26, 135, 206, 212).

All-atom molecular dynamics simulations shed molecular-level light on the properties of molecularly small cosolutes in aqueous solutions and their effects on protein structure. For example, the properties of TMAO have been simulated and analyzed in terms of its hydrophobic and dipolar characteristics (179). With molecular simulations, the preferential exclusion of TMAO from protein surfaces was demonstrated and linked to its underlying protein–cosolute interactions (21, 91, 160). The counteracting effects of TMAO and urea were studied as well, highlighting the subtle balance between urea and TMAO interactions with proteins. It is worth noting that such detailed all-atom simulations are helpful only when the underlying force fields (describing

the forces acting between and within molecules) are carefully calibrated. Force fields calibrated against experimentally derived KB integrals (Equation 15) have been particularly successful (222).

Wet experiments show that soft interactions between crowders and proteins can modulate the stabilizing effect exerted by confinement (25, 180). Simulations (98) show how attractive, soft interactions between crowder and protein can weaken stabilization or even destabilize proteins (IIb and IIIa in **Figure 4**, respectively). Moreover, crowder–protein repulsion that is not completely steric and has considerable temperature dependence can lead to enthalpic stabilization (Ia and IIb in **Figure 4**) (166). Notably, a temperature-dependent repulsion between cosolute and protein translates into an excluded volume that is also temperature dependent. Experiments show that electrostatic interactions can also modulate steric crowding effects (30, 31), and this phenomenon is observed in even simple models that consider charge (97).

How could crowder exclusion be temperature dependent? In solutions containing several solutes and solvent components, exclusion could be the result of a complex interplay among all constituents. Exclusion between the crowder and the protein could then result from the more favorable interaction of the crowder with the solvent compared to its interaction with the protein. Preferential interactions are thus a result of intermolecular forces among all components that can include a series of often attractive interactions such as van der Waals, hydrogen bond, weakly polar, hydrophobic, and charge–charge interactions. This complexity is particularly subtle when the cosolute or crowder is molecularly small, with a prominent example being osmolytes. Simulations containing atomic details suggest that, for small cosolutes such as TMAO and trehalose, enthalpic effects originate from the involvement of hydrogen bonds (15, 21, 78). Simulations further suggest that cosolutes change protein hydration, including the hydrogen bonding properties in the protein hydration shell, thus effectively creating temperature-dependent depletion (78). Simulations also point to changes in the solvation properties of water in the bulk solvating medium (15, 21), although these ideas remain controversial (10, 27). Such changes may manifest themselves in the observations of Ferreira et al. (72), who examine the effects of large and small cosolutes on solvatochromic dyes and correlate the changes with effects on protein stability.

Recent large-scale simulations exploit the full power of contemporary computational strength to model solutions that come close to the composition of realistic cellular cytoplasmic environments (62, 63, 69, 125). These efforts signal the need to resolve the discrepancies between experimentally observed stabilization or destabilization and predictions from simple crowding theories that consider only hard-core repulsions. These detailed models will also help decode how combinations of solutes impact proteins (69).

## CONCLUDING REMARKS

### Summary

Simple hard-core-based theories assume that crowding effects are purely entropic and stabilizing. Two facts distilled from the many experiment-based studies reviewed above indicate that this idea is insufficient. First, crowding can destabilize proteins. Destabilization means that hard-core stabilization is completely overcome by something else. Second, crowding effects, whether stabilizing or destabilizing, are often associated with a nonzero  $\Delta\Delta H_{U/D}^o$ , a situation not embodied in simple models based on hard-core repulsions alone. Chemical interactions among cosolute, protein, and water are missing.

The destabilization observed for many globular proteins in cells is brought about by attractive interactions with the protein backbone because protein unfolding exposes more of the attractive surface. The observations that proteins can be destabilized in both eukaryotic and *E. coli* cells (45, 213) show that attractive interactions are important, since stabilizing steric repulsions must

be larger in the more crowded *E. coli* cells. These destabilizing interactions, however, could help proteins fold, as suggested by the observation that the cytoplasm can disrupt non-native structure in the unfolded state (33).

Early evidence for the importance of weak attractive interactions came from Minton and colleagues' (95) study of protein complex formation. Other early evidence came from observations of changes in NMR spectra of cytochrome *c* in PEG (39). One could say that we should have seen this coming as early as 2001, based on comparing the predictions of large crowding effects (64, 65) with the observation from the Oas group that a variant of monomeric  $\lambda$  repressor appears to be no more stable in *E. coli* cells than it is in buffer (76). Recently, these interactions were quantified by changing protein surface residues and measuring the effect on diffusion in living cells (140). An essential point in favor of pushing our talented colleagues to understand these phenomena is that these weak, attractive, quinary interactions are what give structure to the insides of cells (32).

## Future Efforts

The most information could be gained with the least effort by using the data reviewed in the section titled Experimental Observations to test the ideas presented in the section titled Molecular Interpretations. Successes (and failures) will be limited, however, because most published experiment-based endeavors cover a limited number of crowders and a paucity of sizes and concentrations. Additional systematic efforts with synthetic polymers like those reported by Lee & Lee (112) and the Schuler lab (191, 240) are essential for testing the mechanisms and ultimately improving methods for storing and shipping industrial enzymes and biologic drugs (149). To obtain the most complete information, it is essential to test a range of synthetic polymers at concentrations and molecular weights that cross  $c^*$  so that both dilute and semidilute regimes are sampled. The prediction is that the data will be consistent with simpler theories below  $c^*$  and with renormalization group theory (142) in the AOM above  $c^*$ . Finally, let us not confuse synthetic polymer crowders with what goes on in cells; cells are packed with proteins and nucleic acids, not PEG and Ficoll. We now have the tools to study crowding in solutions of biologically relevant macromolecules, cell lysates, and living cells. Let us use them.

Interpreting experiment-based data in terms of the underlying entropic and enthalpic contributions would benefit from efforts that quantify the temperature dependence of transfer free energies. Experimental efforts would also gain from using a standard toolbox of commercially available crowder proteins with a variety of isoelectric points (e.g., BSA, lysozyme, RNase A, ovalbumin). Going further, can the roles played by van der Waals forces, protein charge, H-bonding, the hydrophobic effect, and other chemical interactions be understood by using site-directed variants of stable, highly expressed, and easily purified bullet-proof crowder proteins such as GB1 (82)? An even greater challenge is implementing the ability to vary the size of a protein crowder while holding its shape and surface chemistry constant. Finally, there have been few ventures into how the numerous small molecules in cells (14) modulate crowding effects.

The ultimate goal of biological research into crowding is to gain an understanding of the organization of the proteome inside living cells—the so-called quinary structure. A criticism surrounding the use of the term quinary is that current studies focus on defining the types of interactions that organize the cellular interior, but there have been no real tests to reveal these interactions in cells using proteins expressed at their normal levels (32, 159). Our expectations are buoyed by the fact that protein folding and stability can finally be tested in whole organisms (70). It now remains to extend the experimental and theoretical framework of macromolecular crowding discussed in this review to realistic biological cellular environments that are multicomponent, highly

dense, and nonequilibrium open systems. This endeavor will eventually reveal a fundamental and realistic understanding of protein function *in vivo*.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

## ACKNOWLEDGMENTS

Our research on crowding is supported by the National Science Foundation (grant MCB-1410854 to G.J.P.) and the United States-Israel Binational Science Foundation (grant BSF 2017063 to G.J.P. and D.H.). The Fritz Haber Research Center is supported by the Minerva Foundation. G.J.P. thanks Elizabeth Pielak, Bo Li, Huan-Xiang Zhou, Giuseppe Graziano, and Shikun “Rinco” Wang for helpful discussions and critical comments on the manuscript.

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