Protein shape modulates crowding effects

Alex J. Guseman\textsuperscript{a}, Gerardo M. Perez Goncalves\textsuperscript{a}, Shannon L. Speer\textsuperscript{a}, Gregory B. Young\textsuperscript{b}, and Gary J. Pielak\textsuperscript{a,b,c,d,1}

\textsuperscript{a}Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; \textsuperscript{b}Department of Biochemistry & Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; \textsuperscript{c}Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; and \textsuperscript{d}Integrative Program for Biological and Genome Sciences, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Edited by Susan Marqusee, University of California, Berkeley, CA, and approved September 12, 2018 (received for review June 18, 2018)

Protein–protein interactions are usually studied in dilute buffered solutions with macromolecule concentrations of \(<10\) g/L. In cells, however, the macromolecule concentration can exceed \(300\) g/L, resulting in nonspecific interactions between macromolecules. These interactions can be divided into hard-core steric repulsions and “soft” chemical interactions. Here, we test a hypothesis from scaled particle theory; the influence of hard-core repulsions on a protein dimer depends on its shape. We tested the idea using a side-by-side dumbbell-shaped dimer and a domain-swapped ellipsoidal dimer. Both dimers are variants of the B1 domain of protein G and differ by only three residues. The results from the relatively inert synthetic polymer crowding molecules, Ficoll and PEG, support the hypothesis, indicating that the domain-swapped dimer is stabilized by hard-core repulsions while the side-by-side dimer shows little to no stabilization. We also show that protein cosolutes, which interact primarily through nonspecific chemical interactions, have the same small effect on both dimers. Our results suggest that the shape of the protein dimer determines the influence of hard-core repulsions, providing cells with a mechanism for regulating protein–protein interactions.

Macromolecular crowding \mid protein–protein interactions \mid scaled particle theory

Protein–protein interactions are essential for maintaining cellular homeostasis (1). Details of their equilibria under thermodynamically ideal conditions have provided a trove of information. Ideality in this sense refers to dilute solutions, where each monomer contacts only solvent or another monomer, conditions far removed from those in cells where protein–protein interactions evolved. In the cytoplasm, and other cellular compartments and biological fluids, macromolecules can occupy up to \(30\%\) of the volume, and their concentrations often exceed \(300\) g/L (2).

Protein molecules take part in more-complex interactions under nonideal conditions. The surrounding macromolecules influence proteins in two ways, neither of which is significant in dilute solution. Hard-core repulsions arise from high volume occupancy, because two molecules cannot occupy the same space at the same time. This volume exclusion favors the most compact state of a protein (3). Chemical interactions comprise transient contacts between protein surfaces arising from the diverse chemical landscapes of proteins (4). When repulsive (i.e., like charges), they favor the state that maximizes the distance between charges, adding to the hard-core repulsions and stabilizing the native state. Attractive chemical interactions (e.g., opposite charges, hydrogen bonds) are destabilizing. We are beginning to understand how hard-core repulsions and chemical interactions affect protein stability (5), but there are few studies about crowding effects on protein–protein interactions (6–9).

Given the existential roles of both protein–protein interactions and crowding in biology (10), we are undertaking efforts to determine the effect of crowding on the simplest of protein complexes, homodimers. Our first endeavor, which involved a side-by-side homodimer, highlighted chemical interactions and showed a small contribution from hard-core repulsions (11, 12). These results were predicted by Berg (12), who used scaled particle theory to suggest that side-by-side dimers would be only mildly influenced by hard-core repulsions. Berg also predicted that more-compact dimers are more likely to be stabilized by hard-core repulsions. Here, we test this idea by changing the shape of a dimer in a controlled fashion.

Scaled particle theory is based on statistical mechanics. For situations like those investigated here, the theory considers solution nonideality as arising from the presence of cosolutes (13–15). As often applied, the solvent and cosolutes are considered hard spheres, which leads to three consequences: Molecules only interact when they touch, the interaction energy is purely repulsive, and the energy has a strong distance dependence. The inability of billiard balls to interpenetrate is a macroscopic example. Water and cosolute concentration are expressed as volume occupancy, \(\Phi\), the unitless parameter indicating the fraction of the volume they occupy. The reversible work (i.e., free energy) to produce a monomer- and dimer-sized hole in water and in a cosolute-containing solution is calculated, and a thermodynamic cycle (16) is used to estimate the change in the free energy of dissociation (\(K_{D-M}\)) in cosolute solution minus the value in water, i.e., \(\Delta \Delta G_{D-M}^{\text{c}}\), where a positive value indicates stabilization.

The theory has several shortcomings, including its spherical assumptions, neglect of chemical interactions, including solvation, and its extreme sensitivity to sphere size and density (17, 18). Nevertheless, it has been successfully applied to assess the effects of crowding-induced hard-core interactions on protein stability and protein–protein interactions (18–22). For instance, its application to protein stability led to the idea that crowding

**Significance**

Macromolecular crowding influences protein–protein interactions via hard-core repulsions and chemical interactions. Scaled particle theory predicts that the effect of hard-core repulsions depends on the shape of the protein complex, and simple ideas about chemical interactions predict a dominant role for the chemical qualities of the protein surface. The theory predicts that a collapsed, ellipsoidal, dimer will be stabilized by hard-core repulsions, whereas a less collapsed, side-by-side, dimer will not. We applied scaled particle theory to two dimers with nearly identical surfaces but different shapes. Our results support the predictions; crowders that interact primarily through hard-core repulsions stabilize the ellipsoidal domain-swapped dimer more than the side-by-side dimer, whereas crowders that interact via chemical interactions have the same effect on both dimers.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Published under the PNAS license.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1810054115/-/DCSupplemental.

Published online October 9, 2018.
effects comprise both hard-core repulsions and attractive or repulsive chemical interactions (16, 23).

We use the implementation of scaled particle theory (13) described by Berg (12) to assess crowding effects. The monomers are assumed to be spheres. The dimers have twice the volume of the monomer, but their shapes, quantified as the eccentricity, $L$, is varied (Fig. 1). At the compact extreme, $L = 0$, the dimer is a sphere. At the other extreme, $L = 1$, the dimer comprises touching monomers (kissing spheres). Berg’s application shows that shape matters. Treating water as a sphere of radius 1.4 Å, crowding favors more-compact (smaller $L$) dimers and stabilization. Shaded areas indicate the estimated eccentricities of the GB1 dimers. The 4.1-Å species represents sucrose, and the 35-Å species represents BSA.

Scaled Particle Theory Predictions. Positive values of $\Delta G^{\circ}_{D-M}$ indicate an increase in dimer stability compared with dilute solution. Scaled particle theory calculations (Table 1) were performed as described by Berg (12) using the dimerization reaction with a variable cavity shape (Berg’s equations, SI Appendix, Eqs. S1–S8). Cosolutes were assumed to be spheres. Small molecule radii were estimated using molecular volume data from the ChemAxon chemicalize database (https://chemicalize.com). Solvation was ignored. Protein radii were estimated from their surface areas and volumes obtained using Volume Area Dihedral Angle Reporter (VADAR) and their structures [Protein Data Bank (PDB) ID code 3V03 for BSA and PDB ID code 1DPX for lysozyme] (34).

To apply Berg’s (12) ideas, we first estimated the dimer eccentricities. Distances between the centrally located 5′-proton on trp43 (35) and the protein surface were measured using a Python-based molecular-conversion utility (PyMOL) (36) giving a monomer radius of 11.8 Å, and an eccentricity of 0.71 for the side-by-side dimer and 0.56 for the domain-swapped dimer (Fig. 1). We also assessed eccentricity by fitting the solvent-accessible surface areas and volumes to geometric shapes, which
yielded an eccentricity of 0.77 for the side-by-side dimer and 0.53 for the domain-swapped dimer. The ranges are shaded in the figure. A complete list of input parameters is compiled in SI Appendix, Table S1.

**Observations Using $^{19}$F NMR.** To test the predictions from scaled particle theory, we measured $\Delta G^o_{D\rightarrow M}$ using the approach described in Materials and Methods and 5-fluorotryptophan-labeled GB1 in buffer (Fig. 4) and in a variety of small and large cosolutes at pH 7.5 and 298 K (Table 1). Comparison of $^{15}$N–$^1$H heteronuclear single quantum coherence (HSQC) data for the domain-swapped dimer (SI Appendix, Fig. S4) and the side-by-side dimer (37) show that fluorine labeling does not significantly affect structure of the proteins.

**Discussion**

**Scaled Particle Theory.** The predictions for the cosolutes tested (Table 1) agree with Berg’s (12) prediction that the hard-core component of crowding has a larger stabilizing effect on the more compact domain-swapped dimer (38). Our calculations ignore the effect of solvation on size and the fact that the cosolutes, especially urea, are poorly modeled as spheres. These limitations do not, however, change the fact that the hard-core component has a larger effect on the domain-swapped dimer. We did not apply the theory to the synthetic polymers, because these macromolecules cannot be accurately modeled as spheres, because, at the high concentrations used here (39–41), the individual polymer molecules overlap to form a mesh (42). The reason is exemplified by the concentration dependence of viscosity. At low concentrations (the so-called dilute regime), there is an approximately linear relationship between viscosity and concentration, as is observed for small molecules. At higher concentrations, however, synthetic polymers form a mesh (the semidilute regime), and there is a much steeper dependence. The intersection between these regimes is called the overlap concentration, $c^*$. Perhaps the best rationale for not treating synthetic polymers as spheres is that polymers form these highly viscous solutions, whereas spheres jam at a volume occupancy of ~0.6 (43).

**Control Small-Molecule Cosolutes.** We divided the cosolutes into three classes: small-molecule denaturants and stabilizers, synthetic polymers and their monomers, and proteins (Fig. 5). The denaturant urea at 100 g/L is predicted by scaled particle theory to stabilize the domain-swapped and side-by-side dimers by 0.61 kcal/mol and 0.31 kcal/mol, respectively. We observe destabilizations of 1.31 ± 0.05 kcal/mol and 0.25 ± 0.04 kcal/mol for the domain-swapped and side-by-side dimers, respectively. The predicted and observed results are contradictory in both sign and magnitude. The sign discrepancy highlights a key shortcoming of scaled particle theory: It ignores chemical interactions between the cosolute and the test protein, in this instance the attractive interaction between urea and the protein backbone (44). The magnitude discrepancy means the domain-swapped dimer should be more stabilized (less destabilized) than the side-by-side dimer. This discrepancy probably arises because the monomers are fundamentally different. For the side-by-side dimer, the monomer is a stable folded protein (26), but, for the domain-swapped dimer, the monomer is a molten globule with an ensemble average of the GB1 fold, but lacking stabilizing hydrogen bonds and salt bridges (SI Appendix, Fig. S1) (25, 27). Urea likely unfolds the globule, resulting in a destabilizing chemical interaction larger than the predicted stabilization from scaled particle theory.

Trimethylamine-N-oxide (TMAO) at 39 g/L is predicted to stabilize the domain-swapped and side-by-side dimers by 0.25 kcal/mol and 0.13 kcal/mol, respectively. We observe stabilizations of 0.16 ± 0.06 and 0.50 ± 0.06 kcal/mol, respectively. The less than expected stabilization of the domain-swapped dimer may arise because the monomer is only partially structured (SI Appendix, Fig. S1) (25–27), and undergoes domain swapping to form the most thermodynamically favorable state. TMAO, a known protein stabilizer, compacts proteins (45–47). Such compaction likely favors a monomer conformation similar to that of wild-type GB1, which would reduce the tendency to swap domains and dimerize. Molecular dynamics simulations (48) suggest dimerization occurs via a slightly expanded monomer. TMAO would limit the expansion because it stabilizes compact states.

**Synthetic Polymer Cosolutes.** These macromolecules are traditionally used as cosolutes to mimic hard-core repulsions under crowded conditions. We tested Ficoll-70, a 70-kDa branched sucrose polymer, at 300 g/L and 8-kDa polyethylene (PEG) at 200 g/L (Fig. 5A).

We first studied their monomers, sucrose and ethylene glycol. Sucrose stabilizes the domain-swapped dimer by 0.26 ± 0.06 kcal/mol; TMAO would limit the expansion because it stabilizes compact states.

### Table 1. $\Delta G^o_{D\rightarrow M}$ Values at 298 K predicted by scaled particle theory (SPT) and calculated from NMR data (pH 7.5) for the domain-swapped ($L = 0.5$) and side-by-side dimer ($L = 0.7$)

<table>
<thead>
<tr>
<th>Cosolute</th>
<th>g/L</th>
<th>SPT $^a$</th>
<th>NMR $^b$</th>
<th>SPT $^a$</th>
<th>NMR $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Domain-swapped</td>
<td>Side-by-side</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>300</td>
<td>1.38</td>
<td>0.26 ± 0.06</td>
<td>0.67</td>
<td>0.03 ± 0.04</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>200</td>
<td>1.50</td>
<td>−0.30 ± 0.06</td>
<td>0.76</td>
<td>−0.29 ± 0.05</td>
</tr>
<tr>
<td>TMAO</td>
<td>38</td>
<td>0.25</td>
<td>0.16 ± 0.06</td>
<td>0.13</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>Urea</td>
<td>100</td>
<td>0.61</td>
<td>−1.31 ± 0.05</td>
<td>0.31</td>
<td>−0.25 ± 0.04</td>
</tr>
<tr>
<td>BSA</td>
<td>100</td>
<td>0.14</td>
<td>0.51 ± 0.05</td>
<td>0.10</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>50</td>
<td>0.09</td>
<td>−0.12 ± 0.05</td>
<td>0.05</td>
<td>−0.18 ± 0.05</td>
</tr>
<tr>
<td>Ficoll-70</td>
<td>300</td>
<td>N/A $^c$</td>
<td>0.71 ± 0.06</td>
<td>N/A $^c$</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>8-kDa PEG</td>
<td>200</td>
<td>N/A $^c$</td>
<td>0.39 ± 0.06</td>
<td>N/A $^c$</td>
<td>−0.22 ± 0.03</td>
</tr>
</tbody>
</table>

$^a$Positive values indicate increased dimer stability.

$^b$As described by Berg (12) using parameters from SI Appendix, Table S1.

$^c$From Guseman and Pielak (37).

$^d$Ficoll and PEG cannot be simulated with scaled particle theory at these concentrations.

---

Fig. 3. The van der Waals volumes, SASAs, measured free energies of dissociation, electrostatic surface potentials from PyMOL (36), and geometric representations of the dimers.
other protein mass-per-volume concentration. Ficoll stabilizes the side-by-side more stabilizing than their respective monomers at the same stabilizing macromolecular effect. That is, both polymers are destabilizes this dimer by

\[ \Delta G_{D \rightarrow M}^{0} \text{ by } -0.29 \pm 0.05 \text{ kcal/mol} \] (37). The increased stabilization of the more compact domain-swapped dimer by sucrose is also consistent with theory (12, 22). The destabilization of the dimers by ethylene glycol is likely due to attractive interactions with the test protein; ethylene glycol interacts favorably with protein surfaces (49–52).

Having assessed the monomers, we tested for, and found, a stabilizing macromolecular effect. That is, both polymers are more stabilizing than their respective monomers at the same mass-per-volume concentration. Ficoll stabilizes the side-by-side dimer by 0.15 ± 0.04 kcal/mol compared with buffer, and PEG destabilizes this dimer by -0.22 ± 0.03 kcal/mol (37), whereas both synthetic polymers stabilize the domain-swapped dimer by 0.71 ± 0.06 kcal/mol for Ficoll, and 0.39 ± 0.06 kcal/mol for PEG. This result is consistent with observations on other protein–protein interactions (37, 53), but differs from observations on protein folding (54). As discussed above, we cannot make direct comparisons with scaled particle theory, but the Ficoll and PEG results are consistent with Berg’s proposal that the influence of hard-core repulsions on dimer formation depends on the shape of the dimer complex. Specifically, the more compact domain-swapped dimer is stabilized more than the less compact kissing-sphere-shaped side-by-side dimer.

**Protein Cosolutes.** Unlike synthetic polymers, globular proteins are roughly spherical (55, 56), allowing the application of scaled particle theory. To understand how chemical interactions affect the dimers, we used globular proteins with opposite net charges at pH 7.5, BSA (66 kDa, pI 5) and lysozyme (14 kDa, pI 9), as cosolutes (Fig. 5B).

Theory predicts that BSA at 100 g/L stabilizes the domain-swapped dimer by 0.14 kcal/mol and the side-by-side dimer by 0.10 kcal/mol. We observe larger stabilizations, 0.51 ± 0.05 kcal/mol and 0.48 ± 0.06 kcal/mol, respectively. At pH 7.5, BSA is predicted to have a charge of −19, while both GB1 dimers are predicted to have a charge of −4. The more-than-predicted stabilization can be explained by electrostatic repulsions between BSA and acidic patches on the surface of each dimer (29).

For lysozyme (50 g/L), scaled particle theory predicts stabilizations of 0.09 kcal/mol and 0.05 kcal/mol for the domain-swapped and side-by-side dimers, respectively, but we observe destabilizations of 0.12 ± 0.05 kcal/mol and 0.18 ± 0.05 kcal/mol, respectively. These results are also explained by charge–charge interactions, because lysozyme has a positive charge of +7 at pH 7.5, while the dimers have a charge of −4. In summary, these results show, for both dimers, that chemical interactions can modulate, and even overcome, the effects of hard-core repulsions.

The effect of protein cosolutes on \( \Delta G_{D \rightarrow M}^{0} \) is the same for each dimer. This agreement between values supports our hypothesis that similar protein surfaces result in similar chemical interactions. The GB1 dimer system effectively decouples the difference in hard-core repulsions by changing the shape of the protein complex while producing indistinguishable differences in chemical interactions, making it the ideal system to test scaled particle theory predictions.

**Conclusions**

We tested Berg’s (12) idea that shape can control the effects of crowding on protein complex stability, by using two nearly identical proteins that form dimers with different shapes but similar surfaces. Scaled particle theory predicts that the more compact dimer is generally more stabilized by crowding. This observation suggests that shape dependence may have been used by biology to control which proteins interact. That the compact domain-swapped dimer is more influenced by hard-core repulsions from the synthetic polymers than is the side-by-side dimer might be important for two reasons. First, it highlights which architecture would be favored by the crowded cellular interior, providing a means of stabilizing complexes important for metabolism, signaling, and maintaining biological homeostasis. Second, cells could use more self-contained proteins that would form dumbbell-shaped dimers to prevent weak protein–protein interactions from being stabilized by the crowded interior. We observe these differences in concentrated solutions of the relatively inert synthetic polymers, PEG and Ficoll. However, the predicted differences can be entirely counteracted by electrostatic interactions, as shown by the stabilizing effect of BSA and the destabilizing effect of lysozyme, and attractive interactions, as shown by ethylene glycol. It is important to point out, however, that, unlike the GB1-based system, most natural domain-swapped dimers exhibit extremely slow off-rates. We conclude that, to gain biologically useful knowledge about protein–protein interactions, we must
Materials and Methods

they were selected, the so called physiological state.

19F NMR. Experiments were performed at pH 7.5 and 298 K on a Bruker Avance III HD spectrometer operating at a 19F Larmor frequency of 470 MHz equipped with a cryogenic QCI probe and a tunable H/F channel. Spectra comprising 31,047 points were acquired with a 2-s delay, an acquisition time of 1.4 s, an exponential line-broadening of 31,047 points were acquired with a 2-s delay, an acquisition time of 1.4 s, an exponential line-broadening was applied to each free-induction decay before Fourier transformation. The 5-fluorotryptophan 43 in the domain-swapped dimer produces two resonances in slow exchange (37). The downfield resonance at −124.2 ppm corresponds to the monomer. The upfield resonance at −125.6 ppm corresponds to the dimer (Fig. 4) (shifts for the side-by-side dimer are published) (37). Integration of each resonance was supported by National Cancer Institute (Grant P30 CA016086).

The GB1 variants were expressed and purified as described by ref. 37. A detailed description is found in SI Appendix, Supplemental Materials and Methods.

Cosolutes. Solutions were prepared to the desired concentration in 20 mM sodium phosphate buffer. The pH was adjusted to 7.5 using concentrated HCl or NaOH. Lyophilized lysozyme and BSA were purchased from Sigma-Aldrich. Their concentrations were monitored using extinction coefficients at 280 nm or NaOH. Lyophilized lysozyme and BSA were purchased from Sigma-Aldrich.


The GB1 variants were expressed and purified as described by ref. 37. A detailed description is found in SI Appendix, Supplemental Materials and Methods.

Cosolutes. Solutions were prepared to the desired concentration in 20 mM sodium phosphate buffer. The pH was adjusted to 7.5 using concentrated HCl or NaOH. Lyophilized lysozyme and BSA were purchased from Sigma-Aldrich. Their concentrations were monitored using extinction coefficients at 280 nm or NaOH. Lyophilized lysozyme and BSA were purchased from Sigma-Aldrich.

Data Analysis. NMR spectra were analyzed using ToppSpin3.5Pro. A 10-Hz exponential line-broadening was applied to each free-induction decay before Fourier transformation. The 5-fluorotryptophan 43 in the domain-swapped dimer produces two resonances in slow exchange (37). The downfield resonance at −124.2 ppm corresponds to the monomer. The upfield resonance at −125.6 ppm corresponds to the dimer (Fig. 4) (shifts for the side-by-side dimer are published) (37). Integration of each resonance was supported by National Cancer Institute (Grant P30 CA016086).

The GB1 variants were expressed and purified as described by ref. 37. A detailed description is found in SI Appendix, Supplemental Materials and Methods.

Cosolutes. Solutions were prepared to the desired concentration in 20 mM sodium phosphate buffer. The pH was adjusted to 7.5 using concentrated HCl or NaOH. Lyophilized lysozyme and BSA were purchased from Sigma-Aldrich. Their concentrations were monitored using extinction coefficients at 280 nm or NaOH. Lyophilized lysozyme and BSA were purchased from Sigma-Aldrich.
