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Models of Macromolecular Crowding Effects & the Need for Quantitative Comparisons with Experiment

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Summary

In recent years significant effort has been devoted to exploring the potential effects of macromolecular crowding on protein folding and association phenomena. Theoretical calculations and molecular simulations have, in particular, been exploited to describe aspects of protein behavior in crowded and confined conditions and many aspects of the simulated behavior have reflected, at least at a qualitative level, the behavior observed in experiments. One major and immediate challenge for the theorists is to now produce models capable of making quantitatively accurate predictions of *in vitro* behavior. A second challenge is to derive models that explain results obtained from experiments performed *in vivo*, the results of which appear to call into question the assumed dominance of excluded volume effects in *vivo*.

Introduction

In accepting the editors' kind invitation to contribute to this issue I have been asked to review recent theoretical and computational studies that have advanced our understanding of macromolecular crowding, and to a lesser extent, confinement effects. To provide a little background to what follows I think I should start by acknowledging that, like many, my understanding of macromolecular crowding effects was first informed primarily by the work of Minton and Zimmerman [1,2] and that my initial knowledge of the termodynamic consequences of confinement was essentially defined by a single article written some years ago by Zhou and Dill [3]. When I was last closely involved in the field some years ago, explicit simulations of confinement effects on protein folding had just been reported by the Takada [4], Shea [5], and Thirumalai groups [6] and I had made my own contribution to the crowding field by showing that the effects of Ficoll70 on protein rebinding to GroEL could be quantitatively captured by molecular simulations [7].

In the intervening years, a considerable amount of work has been conducted in both the theoretical/computational and experimental arenas and this review will provide a brief summary of what I think I have learned from the most recent work. I will take as my starting point a review published by Zhou, Rivas and Minton in 2008 [8], and already cited, at the time of writing, 50 times, evidence of the current interest in the field. In keeping with journal policy, I will focus mostly on papers written in the last few years, although I will make reference to earlier works where necessary to make the review more self-sufficient. Since this article forms part of the "Theory & Simulation" issue of *Current Opinion in Structural Biology*, I will

primarily emphasize recent studies that have used theory or simulation approaches to explore crowding, confinement, or related issues, but since one of my hopes in writing this piece is to encourage the theoretical community to make more concerted efforts to connect with experiment I will also be highlighting some experimental studies that I think raise issues that we, as theorists, need to be able to explain. I make no claim to be comprehensive in my sampling of the literature and I hope that readers will forgive me if I have overlooked work that they feel is important. I also hope that my colleagues whose work is critiqued here will not object to a little gentle nose-tweaking: I ask that they remember that, in line with the title of this journal, what follows is only my opinion.

Macromolecular Crowding & Confinement Principles

Almost all discussions of macromolecular crowding effects start with some kind of statement stressing that the interior of the cell is a very congested place and that the high degree of crowding might cause significant differences between the behavior observed *in vitro* and that occurring *in vivo*. For an operational definition of the term 'macromolecular crowding effects' I will use that suggested by Zhou, Rivas and Minton [8]: such effects are those caused by "macromolecular cosolutes that are nominally inert with respect to the reaction of interest", with the crucial term 'inert' meaning that the only interaction between the crowder and the other macromolecular components of the system is an excluded-volume (i.e. steric) interaction. A similar operational definition of 'confinement effect' is any that results from the physical sequestration of a macromolecule from a dilute solution environment to the interior of a pore or slab (or any other geometry) whose walls engage in only excluded-volume (steric) interactions with the macromolecule of interest.

If we assume that crowding agents behave as defined above, it is relatively straightforward to use statistical mechanical theories to at least roughly predict their potential termodynamic consequences on processes such as protein folding and protein-protein association equilibria [1,2]. In fact, all that is required is some kind of structural model for the crowder (e.g. if it is modeled as a sphere we need only specify its radius), and a structural model for the initial and final states of the process of interest. In order to obtain qualitative effects, and perhaps obtain order-of-magnitude estimates of quantitative effects, these models need not be especially elaborate: modeling both the folded and unfolded states of proteins as spheres [9], for example, is often enough to get a good idea of the expected results. Statistical mechanics theories can also be used to predict the *kinetic* consequences of macromolecular crowding in an approximate way as long as some model for the transition state (ensemble) can be produced; more often, how ever, such effects are investigated via direct molecular simulation using techniques such as molecular dynamics, Langevin dynamics, or Brownian dynamics.

While it is easy to show that (idealized) crowding and confinement effects can in principle have significant effects on macromolecular behavior, there are at least three major issues that, I think, need to be kept in mind. First, there is the question of whether truly inert crowding agents exist that can be used in experiments to provide a direct read out of excluded volume effects *only*, or whether it is inevitable that all crowding agents will also cause additional effects that must be considered. Second, there is the perhaps related question of whether theoreticians can develop predictive models that can quantitatively describe the effects of the crowding agents used experimentally. Third, and arguably the most important issue, there is the question of whether an environment dominated by idealized macromolecular crowding agents such as Ficoll or Dextran is even a good mimic of a true intracellular environment such as the cytoplasm of *E. coli* (Figure 1). If it is not, and one is interested primarily in behavior *in vivo*, one might be tempted to view pursuit of macromolecular crowding effects as of only peripheral interest. Toward the end of this review I will briefly discuss a number of recent studies that touch on all three of these questions.

Calculations of crowding (and confinement) effects

In recent years, a number of groups have conducted theoretical and computational studies of the effects of macromolecular crowding on protein folding and protein-protein binding reactions. Several of these have reported combined computational and experimental studies, a strategy which, in principle, has significant potential to advance our understanding of such effects.

The Zhou group has been especially productive in recent years in the macromolecular crowding field. This group has employed experimental techniques to study the effects of Ficoll and Dextran on both protein stability [10•] and protein-protein binding affinity [11] and has used both scaled particle theory (SPT; [12]) and a variant of Widom's particle insertion method [13] to carry out corresponding sets of calculations [14,15•]. Building on the work of others [16], Zhou has also recently reported the use of SPT calculations to rationalize and predict potential crowding effects in membrane proteins [17]. The Zhou group's experimental work is a valuable contribution to the field since it sets quantitative goals that future models and calculations should attempt to reach. The calculation work on the other hand hints at the kinds of challenges likely to be faced in attempting to quantitatively reproduce the experimental numbers. For example, while good quantitative fitting of the observed effects of Dextran was reported by the group in three separate publications reported in the last year [10•,11,18], in all three cases a different (spherical) model for the Dextran was used in the calculations: in a study modeling crowding effects on the binding affinity of two subunits from DNA polymerase III, the radius of Dextran was assumed to have no dependence on its concentration [11]; in a study examining the effects of mixed crowding agents on the stability of the FKBP protein, the radius was assumed to be linearly dependent on concentration [10•]; and, in a collaboration with the Yang group exploring crowding effects on the forced-unfolding of tandem ubiquitin constructs, the radius was assumed to be quadratically dependent on concentration [18]. Since these three studies were all published in rapid succession, readers would be forgiven for being confused about what these studies tell us about how to model Dextran. Perhaps the key conclusion that we should draw is that, while quantitative agreement between calculation and experiment can be obtained, it can only be achieved currently by the use of fitting parameters, which them selves may need to take on different values depending on the system that the calculations are intended to describe. This in turn means that the models we have are on the right track, but cannot yet be considered *predictive*, which is what they need to be if we are to say that we truly understand the problem.

Statistical mechanics-based calculations have also been extensively used by Minton's group in attempts to model crowding effects in experimental system s. Guided by the radius of gyration distributions sampled in Goldenberg's Monte Carlo simulations of unfolded states [19], Minton has calculated the potential termodynamic effects of hard-sphere and hard-rod crowding agents on protein folding equilibria [20]. Similar kinds of calculations, albeit with significant technical differences, were carried out by Zhou (reviewed in [21]), with the latter predicting smaller effects of crowding on protein folding termodynamics than the former. In keeping with suggestions from both authors, how ever, one wonders if such calculations – which involve very simplified descriptions of both the protein (in its folded and unfolded states) and the crowding agent itself – might have reached the limit of their effectiveness, at least in so far as they are able to predict experiment at a quantitative level.

Perhaps one area where more simplified structural models may continue to be of use is as a means of quantitatively describing the colligative proper ties (e.g. the osmotic pressure) of protein solutions at very high concentrations [22]. In the 'effective hard particle' model advocated by Minton, proteins are to be modeled as spheres, with their effective radii reflecting not only their physical dimensions but also the nature of their intermolecular interactions:

proteins involved in predominantly repulsive interactions, for example, end up being ascribed larger radii than their structures would strictly dictate. Minton's group has recently shown that effective hard-sphere models parameterized against experimental data on one-component protein solutions can also quantitatively describe the behavior of two-component (i.e. mixed) solutions at high concentrations [23,24•]. Since, as noted by the authors, this nice result has so far only been demonstrated for mixtures of proteins that share similar pIs (and so are mutually electrostatically repulsive), it remains uncertain whether a similar kind of model can adequately describe the behaviors of systems where attractive interactions are significant. Even if they cannot, how ever, perhaps such models – since they are directly parameterized against experimental data – might be useful for predicting the effects that the modeled proteins may exert on, for example, the folding behavior of other proteins in cases where attractive interactions can be overlooked.

In recent years the Cheung group has conducted a number of interesting molecular simulation studies in collaboration with the experimental groups of Wittung-Stafshede [25•,26•,27] and, more recently, that of Waxham [28•]; the Cheung and Thirumalai groups pioneered the use of molecular simulation methods to directly simulate protein folding behavior in crowded conditions [29]. Directly simulating protein folding events is computationally expensive, but has the significant ad vantages that it (a) avoids potential concerns about the applicability of theories such as SPT to highly concentrated system s, (b) allows the kinetics of processes to be directly observed, without the need to apply termodynamic thinking to transition state ensembles, and (c) can lead to unanticipated predictions of conformational redistributions induced by the environment [26•,28•] that are not accessible by SPT or Widom-like insertion calculations (since, with the latter approach [14], for example, one must *a priori* know the structures of the conformations one wishes to explore). A challenge for the groups that do this kind of molecular simulation work, how ever, is to show that the behavior observed in their simulations is not just superficially consistent with that seen experimentally, but remains consistent when examined in greater detail. Ultimately, this will require that the experimental observations, such as % increases in helicity deduced from CD experiments [25•,26•], or donor-acceptor distances (and their variation with crowder concentration) derived from FRET experiments [28•], be more directly and accurately reproduced by the simulations than is currently the case.

A molecular simulation approach has also recently been used by the Thirumalai group to explore the effects of both crowding and confinement on protein folding behavior. In addition to the group's initial work in this area [29], they have attempted to model the Yang group's experiments [18] exploring crowding effects on the forced unfolding of ubiquitin constructs [30]. The group has also used simulations to explore the potential effects of crowding agents on the formation of amyloid fibrils [31] and to examine the effects of crowders on conformational distributions of a modeled homopolymer [32]; the latter work is less directly connected to experimental work than are the two former studies.

One new area in which molecular simulations have begun to be used is in explicitly modeling the kinetics of protein-protein association processes in crowded conditions. Two recent studies have focused on modeling the rates of association of spherical protein models [33,34•]. In one, the Yethiraj group showed, in line with theoretical expectations [8], that the rates of association of spheres can be either accelerated, or diminished, by crowding effects depending on the criteria used to define successful association. In the second, a similar kind of problem was considered but on a much more ambitious scale: the Ellison group reported modeling the diffusion-limited association of barnase and barstar in a coarse-grained model of the bacterial cytoplasm [34•]. In spirit, this model represents a (major) extension of one proposed in Bicout & Field's far-sighted study of many years ago [35], and, as far as I am aw are, is the first published working model of the cytoplasm that begins to look like the 'real thing'. The

disadvantage of the model is that it considers all macromolecules as spheres, and, while this is perhaps just about acceptable for modeling non-specific interactions, it may not be so for modeling highly specific interactions (such as barnase-barstar) since proteins have 'faces', and therefore care about which direction they are pointing. It will be interesting to revisit this issue with detailed models of the associating proteins.

In fact, a more structurally detailed simulation study looking at how a model protein-protein association event might be affected by macromolecular crowding has already been reported in the last year by Wieczorek & Zielenkiewicz [36•]. In this work, Brownian dynamics (BD) simulation techniques similar to those pioneered by Gabdoulline and Wade [37] were used to compute hundreds of trajectories of the proteins diffusing and associating in solutions of hard-sphere crowders. Again, it was shown that the rate of association could be significantly accelerated in crowded conditions depending on the model used. As the authors point out, a limitation of the study is that it does not consider electrostatic interactions between the proteins, which are certainly important in a number of diffusion-limited protein-protein associations (e.g. [38]), but presumably future studies will directly address this issue. Eventually, a goal for these kinds of simulations must be to establish a connection with the Schreiber group's groundbreaking studies of the association rates of protein-protein complexes in crowded solutions. Building on their own previous report [39], this group has recently published a comprehensive study [40••] exploring the effects of poly(ethylene glycol) (PEG) and Dextran, and also their monomeric equivalents (ethylene glycol and glucose), on the kinetics and termodynamics of a number of protein-protein complexes (including the venerable barnase-barstar system). For me, the data presented in this most recent report from the Schreiber group represent the kinds of 'gold standard' measurements that I think we as theoreticians need to be able to quantitatively reproduce.

In addition to theoretical and computational work that seeks to directly model the effects of macromolecular crowding, a number of similar works have attempted to model it indirectly, by restating it as a confinement problem, i.e. by assuming that when crowding is sufficiently high, proteins are effectively confined (e.g. [41–44]). Unfortunately, most of these studies make little or no connection with experiment and, when they do claim to make such comparisons, they are so indirect or selective that they are, in my view, effectively meaningless (e.g. [43,44]). This is a shame because there are certainly interesting experimental systems where confinement is clearly a potential issue and where there is still room for studies that attempt to quantitatively explain the observed behavior. One obvious biological example is the GroEL/ES chaperonin [45] from *E. coli*, which physically sequesters misfolded proteins in a cage-like environment (though one, it should be remembered, that actually contains holes through which water can pass). This system has already inspired a large number of computational studies [e.g. 4,5,7], and is likely to inspire still more owing to the interesting experimental effects reported when the internal volume of the GroEL cage is varied by lengthening (or deleting) the highly flexible C-terminal tails that form the base of the chamber [46,47]. At least two theoretical attempts have already been made to rationalize and explain the experimental results [48,49•]; in terms of explaining all of the observed behavior, how ever, there is still an enormous amount of work left to do.

A second biological example where confinement may be an issue concerns the behavior of nascent polypeptide chains as they traverse the ribosome's exit tunnel. Here again, at least three simulation studies have explored issues either directly or indirectly related to this question: the Thirumalai group considered the stabilization of an α-helix due to confinement within a cylindrical tunnel [50], I used coarse-grained molecular simulations to model the coupled synthesis and folding of proteins as they are translated [51], and the Pande group [52] used distributed computing to map out potential interaction sites of amino acid residues with the tunnel's wall (which was treated as completely inert in each of the two former studies). Again,

none of these studies can really be said to have addressed all of the potentially interesting issues and, in particular, there appears to be an opportunity to develop a much more direct connection with experiment in the form of the very clever studies conducted by the Deutsch group (e.g. [53,54•]).

A third, non-biological area where confinement is thought to play a role is in the behavior of proteins encapsulated within silica 'sol-gel' matrices. Although studies of encapsulated proteins had been conducted for some years previously, interest in this area, at least from the stand point of confinement effects, appears to have started mainly with the work of Eggers & Valentine [55]. Theoreticians have since focused almost exclusively on the fact that the melting temperatures of the proteins studied appear to be shifted upwards (which is an easy fact to explain at least qualitatively) but there are other important observations in this paper that have largely been ignored. At room temperature, for example, apomyoglobin was shown to have lost a great deal of its (helical) secondary structure when trapped within the gel; perhaps this observation is not so easily explained with excluded volume arguments. Interestingly, restoration of apomyoglobin's secondary structure within the gel could be achieved by adding 1M potassium phosphate, and investigation of other salts showed their stabilizing effects to be consistent with the Hofmeister series [55]. These, and other observations reported in the paper, strongly implicate the solvent, and its hydrogen bonding properties, as playing a significant role in determining behavior within the gel. More recently, Eggers' group has reported interesting work indicating that the inclusion of additional hydrophobic groups in the silica matrix can significantly stabilize proteins against thermal denaturation [56•,57]. Since these and other experimental works (e.g. [58,59]) continue to advance our understanding of protein folding in sol-gel system s, and since significant computational and theoretical attention has already been focused on modeling solvent-driven effects that control behavior of biomolecules in confined, hydrophobic environments (e.g. [60–62]), it appears that there should be opportunities for using simulations to understand sol-gel behavior at a level beyond that of pure excluded volume effects.

The status of the field

The clearest lesson that I think I have learned from preparing this review is that while obtaining some kind of qualitative agreement with experiment is relatively straightforward, it is much more difficult to predict, determine and understand crowding and confinement effects at a quantitative level. It must be, because in all the papers that I read I never once came across a table or figure that directly compared numbers from theory and experiment *except* in cases where the theoretical results had been aided by use of fitting parameters. In fact, during the course of researching this review I became somewhat dismayed by the readiness with which theoreticians were apt to claim in print that their calculations were "in agreement with experiment" when a close examination of the work would often reveal that the connection with experiment was very indirect. For a comparison between calculation and experiment to be truly meaningful it is important that they both be examining the same system (e.g. the same protein) and that they both be measuring exactly the same observable; there should be no differences that could act as sources of either discrepancies or fortuitous correspondence.

I raise the issue of performing quantitative comparisons with experiment because I think that our understanding of the basic concepts underpinning crowding and confinement effects is now sufficiently complete that we probably have little more to learn from qualitative comparisons. Pursuing quantitatively predictive models will certainly not be easy, and may even require us to discard some of our more cherished approximations. It is quite possible, for example, that we may find that our current structural models of crowding agents are insufficiently detailed to allow truly quantitative predictions of their effects to be made. Ficoll70, for example, is routinely modeled as a hard sphere, but use of a reasonable value for

its radius [63] leads to an unrealistic estimate that at 150mg/ml its volume occupancy is ~75% [25•]. This would suggest that Ficoll is, in fact, somewhat 'squishy' and this idea is strongly supported by experiments that have measured its diffusion through nanofabricated pores of known dimensions [64]. A quantitatively predictive model of Ficoll, therefore, may need to accurately mirror this behavior. A similar increase in sophistication may also be required in the modeling of Dextran, which in just the past few years has been modeled as a dumb-bell [27], a rigid rod [20], and a sphere [10•,11,18].

Since both Ficoll and Dextran appear to be sufficiently inert that their effects may be largely describable in terms only of excluded volume contributions, the additional structural detail required of newer-generation models for the two polymers may not be great: it may be that even comparatively coarse-grained models could prove sufficient. It should be borne in mind, how ever, that for true quantitative accuracy from calculations and simulations there might eventually be a need to include chemical details in the models. One recent experimental study that speaks to the possibility of subtle potential differences between the two crowders, and which may therefore only be resolvable with more chemically detailed models, comes from the Gai group [65• •]. This group compared the effects of Dextran70 and Ficoll70 on the termodynamics and refolding kinetics of three small folding motifs (two of 34 residues and one of 16 residues in length). The rationale for choosing small polypeptides to study was an interesting and, to me at least, compelling one: the authors argued that in studies of larger proteins the excluded volume effect is likely to be dominant "thus obscuring other subtle but important effects arising from the presence of crowding agents". One intriguing result found in the group's study was that Ficoll70 caused a 0.5 kcal/mol stabilization of the folding free energy of a 16-residue β-hairpin while Dextran70 had essentially zero effect. This, it may be noted, is in contrast to the results obtained by others on larger protein system s, where it is usually the case that Dextran exerts the bigger effect (e.g. [10•,27]) It would be nice to have an explanation for this difference.

It is now pretty clear that inertness is not a property shared by the third of the three 'usual suspects' considered when choosing a macromolecular crowding agent, PEG. In their 2008 review, Zhou, Rivas and Minton [8] presented a thoughtful argument that PEG should be avoided as a crowding agent precisely because of its potential for favorably interacting with the proteins it is intended to crowd; as a result, PEG is now fast becoming something of a pariah in the macromolecular crowding community. Certainly, if one's intention is to specifically dissect contributions from excluded-volume effects, then this sidelining of PEG makes perfect sense. But PEG is used in somany other scenarios (e.g. protein crystallization) that I think that there are good reasons for attempting to better understand its behavior anyway. Interesting new information on the nature of PEG-protein interactions has recently become available thanks to the work of the Crowley group who have performed NMR studies to explore the interaction between PEG and the small model protein cytochrome *c* [66•]. Notably, the largest chemical shift changes induced by the addition of PEG map to a region of the protein's surface implicated in interactions with other proteins. Since both atomically detailed [67,68] and coarse-grained [69] MD simulations have already been performed on PEG polymers in aqueous solution it may be interesting to see if the interactions observed in the NMR experiments can be recapitulated in direct simulations of PEG-protein interactions

If, as suggested above, obtaining quantitative and *predictive* models of crowding effects requires improvements in our modeling of crowding agents, then it may also require that greater consideration be given to solvation issues. A nice demonstration of potential solvation effects – at least in regard to confinement phenomena – has come from an explicit-solvent simulation study reported by the Pande group [70•]. In their work it was shown that, while confinement of a protein within a spherical cavity led to a clear relative stabilization of the folded state, imposing spatial confinement on the waters also – by the addition, in effect, of a hydrophobic

wall – led to a relative *destabilization* of the protein's folded state. The latter result cannot be explained by models that consider *only* excluded volume effects operating on the macromolecule. This does not necessarily mean, how ever, that an explicit treatment of solvent would be essential to capture the behavior: instead, it may be possible to adequately describe it by adding an attractive energetic interaction between the hydrophobic groups of the macromolecule and the confining wall, in much the same way that one was incorporated in the Shea group's early modeling of the GroEL/GroES cage [71]. Regardless of that issue, the study by Lucent *et al*. provides a nice illustration of how molecular simulations can be exploited and designed – in a way that experiments cannot – to explicitly address the role of a *single* variable (in this case, confinement of water) in determining observed behavior (in this case, folding of a model protein). Similar types of simulations, but with the hydrophobic wall replaced by one that mimics the surface of a sol-gel, might also be helpful in understanding the Hofmeister effects on protein stability noted earlier by Eggers and Valentine [55].

A final scenario where more elaborate modeling may ultimately be required to quantitatively predict confinement or crowding effects concerns the treatment of diffusion of macromolecules in highly crowded conditions. My group has recently shown that, in dilute solution conditions, correct modeling of the translational and rotational diffusion of proteins requires that hydrodynamic interactions (HI) be modeled between the flexible elements of the protein [72•]. It is unclear at this stage to what extent the use of a sophisticated HI treatment will alter the diffusion of proteins in highly crowded solutions relative to simulations that omit HI, although a very approximate modeling of such effects in crowded solutions of spheres has suggested that the effect might be significant [73]. If inclusion of HI is confirmed to make a big difference to diffusion in crowded conditions, then this will have obvious consequences for the ability of BD simulations such as those mentioned earlier to quantitatively reproduce diffusion-limited association data of the kind measured by the Schreiber group. If so, a recent advance made by Geyer & Winter is likely to be extremely important: in highly original work [74• •], this group has shown that an approximate factorization of the diffusion tensor (which is norm ally achieved by a computationally nightmarish Cholesky decomposition) can capture ~95% of the HI effect at a fraction of the computational cost. This advance, together with a subsequent extension of the thinking to a Langevin dynamics setting [75], may provide something of a revolution in the scale over which hydrodynamic effects are modeled in concentrated protein system s.

Do macromolecular crowding agents provide a 'cell-like' environment?

Implicit in essentially all of the studies that explore crowding effects is the notion that the studied conditions are much more representative of the cellular interior than would be, say, dilute solution conditions. But using crowding agents to mimic intracellular conditions is not the only way forward: increasingly, experimentalists have begun to take on the immense challenge of using biophysical methods to study protein behavior directly *in vivo* (see [76] for a review of recent developments). These studies are not without complications, and occasional difficulties of interpretation, but the results that have been obtained thus far raise, I think, sufficiently important issues that they should not be ignored by those in the crowding community whose primary goal is to mimic *in vivo* conditions. Particularly pertinent, I think, are the only two studies that have attempted to measure termodynamic stability of proteins *in vivo*. In the first, the Oas group showed that hydrogen/deuterium exchange experiments could be coupled with urea titrations of live *E. coli* cells to obtain quantitative measurements of the *in vivo* termodynamic stability of the protein $\lambda_{6\text{-}85}$, a 80-residue segment of the λ-repressor [77]. Taking into account a systematic offset in the free energies obtained from their methodology, their final result was that the protein's stability appeared to be essentially identical *in vitro* and *in vivo*. In the second study (summarized in [78• •]), the Gierasch group showed (again using urea titration) that a fluorescently-tagged version of the 137-residue

cellular retinoic acid binding protein, CRABP, had an apparent free energy of folding *in vivo* that was estimated to be ~1.4 kcal/mol *less* favorable than that measured *in vitro*.

The above results were considered in the review by Zhou, Rivas and Minton [8], and largely dismissed on the grounds that *in vivo* experiments are so difficult to interpret that a more fruitful avenue for future research would be to take a 'bottom-up' approach, focusing instead on understanding the effects of crowding agents in highly controlled solution conditions. I agree with many aspects of this view point, although I think it is worth noting that the Record group has shown that a significant degree of (osmotic) control can be exerted over intracellular conditions [79], and that this has enabled the Weisshaar laboratory to observe very interesting alterations to GFP's apparent diffusion coefficient in *E. coli* – not all of which appear to be explicable by excluded-volume considerations [80•]. And while our control over *in vitro* conditions is undoubtedly much greater, I also think that the results of the few 'top-down' *in vivo* studies that have so far been reported (those of the Oas and Gierasch groups) are so intriguing, and potentially important, that more studies along these lines are urgently needed. After all, I think that for the majority of crowding researchers it is the *in vivo* situation that ultimately counts, and it is perhaps quite telling that while I read a large number of papers in preparing this review I never once came across one that began "Ficoll and Dextran are incredibly important molecules and it is therefore vital that we understand their effects on protein folding and protein-protein associations".

So let us consider the result of Ignatova and the Gierasch group again and set aside for the moment whatever concerns a reader might have about the difficulties of obtaining, by extrapolation, a termodynamic stability *in vivo*. How are we to interpret it within the confines of conventional macromolecular crowding theory (i.e. under the assumption that only excluded volume effects operate)? I am almost certain that we cannot. Instead, I think that we need to consider other possibilities: for example, that there may be favorable interactions between the protein and other cytoplasmic macromolecules that differ in magnitude between the folded and unfolded states, or (less likely) that the cytoplasmic solvent is so disturbed that the hydrophobic effect is significantly diminished *in vivo*. Whatever might be the cause of the apparent destabilization observed by the Gierasch group [78• •], one major issue that it raises is the heretical notion that macromolecular crowding effects may not be dominant *in vivo*, but instead may be only one player whose importance can be overridden by other factors. A second major issue is that it brings into the question the idea that the use of inert macromolecular crowding agents in experiments performed *in vitro* will necessarily provide a good mimic of the intracellular environment. In fact, if one takes the argument to its logical limit, then one might conclude that a better *in vitro* mimic of protein termodynamics *in vivo* might be a solution containing mild concentrations of a denaturant (or even PEG). Please note that I am not advocating the use of guanidinium chloride as an intracellular mimic! I am just trying to point out that while adding macromolecular crowding agents (Figure 1) might make a solution *look* more like the complex, hectic intracellular environment [81], it is conceivable that it might make the *thermodynamics* of the solution less like that encountered *in vivo*. Since the above issues appear to strike at the heart of the accepted rationale for exploring crowding effects, it seems to me to be quite urgent that more attempts be made to measure the termodynamics of protein stability *in vivo*.

A call to arms

In closing, I would like to try to convince readers that it is time for us, as theoreticians, to stop declaring victory when a mere qualitative reproduction of crowding effects is obtained, and time for us to stop comparing experimental apples with simulated oranges (or bananas). Instead, I think that it is time for us to 'roll up our sleeves', 'to buckle down', and to invoke any other cliché that appears apposite, and take on the immense challenge of quantitatively *predicting*

the effects of macromolecular crowding agents – be they Ficoll, PEG, Dextran, proteins, or the Pielak group's current favorite: polyvinylpyrrolid one [82] – on the termodynamics and kinetics of folding or binding processes. With this goal in mind, perhaps it would be helpful to propose that a test set of 'gold standard' experimental data be compiled and used to provide a common yard stick against which to measure the performance of competing quantitative models. At the very least, this might force us as a community to set down future standards for what we think would constitute 'agreement with experiment'. From my reading of the recent literature, it seems that this is an important issue for us to resolve.

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of the proteins and their quite different secondary structures, it may well be that the agreement is fortuitous. It is perhaps also important to note that the title phrase “induces shape changes” is an interesting computational prediction, not a hard experimental fact. [PubMed: 18697933]

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at the end of the paper that the observed effects indicate one way in which chaperonins such as GroEL might work, but this seems a bit of a stretch, and the idea seems to have been dropped, or at least inverted, in more recent work [49•]. [PubMed: 17563390]

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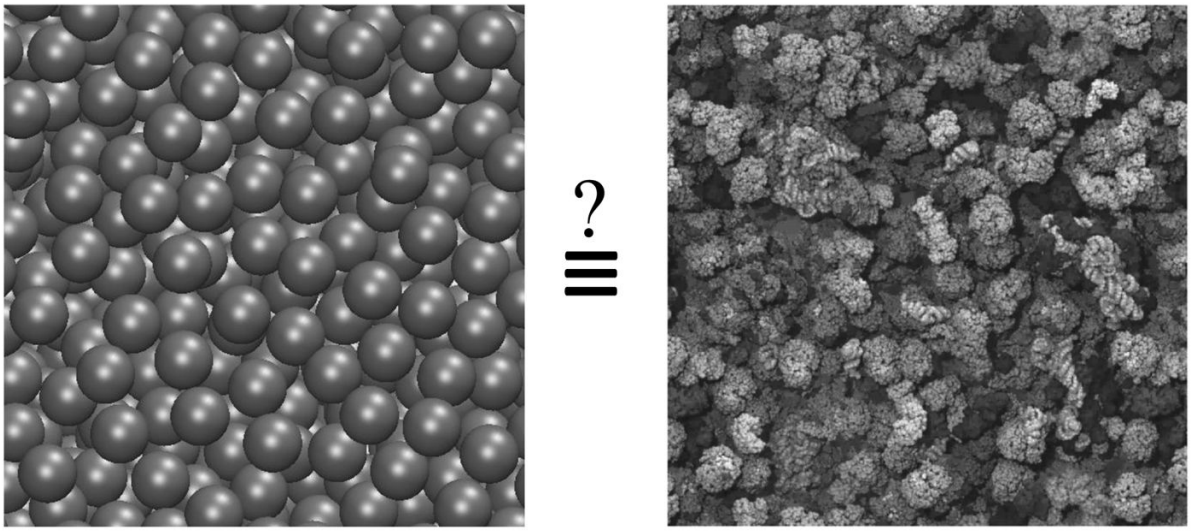

Figure 1. Do macromolecular crowding agents mimic intracellular environments ?
At left is shown a solution of an idealized, spherical crowding agent of the type typically used in calculations and molecular simulations (figure prepared with VMD [83]). At right is a representation of the type of system that such crowding agents are usually intended to mimic: an atomically detailed model of the cytoplasm of *Escherichia coli* (McGuffee & Elcock; unpublished). Are the differences between the two images more important than the similarities?