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Insights Into Crowding Effects on Protein Stability From a Coarse-Grained Model

Proteins aggregate and precipitate from high concentration solutions in a wide variety of problems of natural and technological interest. Consequently, there is a broad interest in developing new ways to model the thermodynamic and kinetic aspects of protein stability in these crowded cellular or solution environments. We use a coarse-grained modeling approach to study the effects of different crowding agents on the conformational equilibria of proteins and the thermodynamic phase behavior of their solutions. At low to moderate protein concentrations, we find that crowding species can either stabilize or destabilize the native state, depending on the strength of their attractive interaction with the proteins. At high protein concentrations, crowders tend to stabilize the native state due to excluded volume effects, irrespective of the strength of the crowder-protein attraction. Crowding agents reduce the tendency of protein solutions to undergo a liquid-liquid phase separation driven by strong protein-protein attractions. The aforementioned equilibrium trends represent, to our knowledge, the first simulation predictions for how the properties of crowding species impact the global thermodynamic stability of proteins and their solutions. [DOI: 10.1115/1.3127259]

1 Introduction

The unfolding, aggregation, and precipitation of proteins from high concentration solutions present formidable challenges in the manufacture, storage, and delivery of biopharmaceuticals. In vivo, these types of molecular processes have also been linked to a wide variety of disorders ranging from cataract formation to Alzheimer's, Parkinson's, Huntington's, and Creutzfeldt-Jakob diseases [1–5]. As a result, there is wide interest in characterizing the thermodynamic and kinetic parameters that influence the conformational stability and solubility of proteins in “crowded” cellular or solution environments.

A combination of experimental data and theoretical models has been used in recent years to investigate the mechanisms for protein unfolding and aggregation in a variety of different systems (see, e.g., Refs. [6–17]). These studies not only yielded important progress but also highlighted some of the inherent complications associated with understanding these phenomena. For example, protein aggregation is often net irreversible. Equilibrium fluctuations can play a key role in initiating the process, but kinetic transitions involving intermediate states that are difficult to isolate and characterize are also common. Unfortunately, and in part due to these issues, a comprehensive molecular theory for aggregation is lacking. Furthermore, exhaustive simulation of protein aggregation using atomistically detailed force fields is not yet a computationally feasible solution. One alternative approach, which we pursue here, is to explore what “coarse-grained” models of protein solutions predict about some of the equilibrium thermodynamic properties relevant for protein unfolding and aggregation.

Equilibrium conformational changes represent one type of molecular scale fluctuation that is important for protein stability (see, e.g., Refs. [3,6,8,18–20]) and can be qualitatively captured by coarse-grained models. When protein structure significantly fluctuates from the native fold, it often produces an increase in the

exposure of hydrophobic residues to the solvent, which in turn promotes protein self-association. In other words, changes to the system that preferentially destabilize the native state, such as modifications to solvent quality [8] or sequence mutations [7], can subsequently modify protein-protein interactions to make reversible or irreversible aggregation more favorable. The midpoint unfolding transition characterizes the set of states where, under equilibrium conditions, half of the protein molecules in the solution are unfolded. Understanding the location of this transition in the space of parameters available to the system (e.g., pH, temperature, protein concentration, etc.) is one of the key challenges in predicting protein stability.

A second equilibrium phenomenon of particular interest is liquid-liquid phase separation, where droplets of a concentrated protein phase form to take advantage of strong interactions between native or nonnative proteins. It has long been appreciated that polymerization reactions involving small organic molecules can induce phase separation [21–27], splitting a homogeneous fluid phase of monomers into polymer-rich and polymer-poor solutions. An analogous phase separation due to the oligomerization of proteins in solution has also been observed experimentally [28]. It has recently argued, based on a variety of experimental observations and theoretical predictions [29–38], that concentration fluctuations which occur under conditions where liquid-liquid phase separation is thermodynamically favored can play a key role in triggering protein precipitation in the form of crystals, fibers, amorphous aggregates, or gels. As a result, forecasting the location of liquid-liquid transitions on the phase diagrams of protein solutions is a basic step toward predicting when protein solutions are vulnerable to these basic routes of physical degradation.

In order for coarse-grained models of proteins to make useful predictions about the fluctuation phenomena discussed above, they must strike a balance. They need to be simple enough so that their global behavior can be studied computationally. However, they must also adequately reproduce three important physical aspects of the problem: the intrinsic free energy of folding of a protein in solvent, some basic structural features of the native and unfolded states, and the connection between protein structure and

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effective protein-protein interactions. We recently used these guidelines to develop a coarse-grained modeling strategy for studying the thermodynamics of concentrated protein solutions [39–41].

This approach, which builds on some of the collective insights provided by other studies (see, e.g., Refs. [42–53]), uses random heteropolymer collapse theory [54,55] as a means for calculating temperature- and species-dependent protein-protein interactions and the intrinsic thermodynamic stability of the native state. This information is then incorporated into transition-matrix Monte Carlo simulations [56–58], which can efficiently compute the equilibrium thermodynamic properties of this binary mixture of native and denatured proteins connected by the folding/unfolding “reaction” [39–41]. Although this type of coarse-grained strategy does not account for the details of secondary and tertiary protein structures, the basic sequence information included at the heteropolymer level allows the approach to successfully predict some of the qualitative experimental trends for how protein concentration affects the midpoint unfolding transition [39], how liquid-liquid phase separation can couple to denaturation of globular proteins [40], and how surface anisotropy (“patchiness”) of the native proteins relates to their unfolding and self-assembly behaviors in the solution [41]. Perhaps more importantly, this method allows one to qualitatively ascertain how effects of protein concentration (i.e., crowding) competes with protein-protein interactions to determine the thermodynamic behavior of the system.

In this article, we extend the use of this coarse-grained modeling strategy to probe a different basic question. How do the physical interactions of a cosolute crowding agent affect the conformational stability of proteins in solution and the tendency of these solutions to undergo liquid-liquid phase separation? Many previous molecular simulation studies we are aware of model the effects of macromolecular crowding by considering a single protein molecule confined to a pore [59–64]. Although an approximation, this type of model captures one main effect of crowding agents, namely, the reduction of conformational space available to a protein. While attempts at including crowding agent mobility in simulations were also made [53], these more detailed studies assume that the protein-crowder interaction is dominated by ex-

cluded volume effects. Moreover, even though simplified Go-like [65] or heteropolymer models [60] are used within the above mentioned simulations, typically only single protein chains remain computationally tractable. Using our coarse-grained modeling strategy, we are able to investigate solutions of interacting foldable proteins and mobile crowding agents.

We show here that our modeling strategy predicts that crowding agents either stabilize or destabilize native proteins in solution at low protein concentration, depending on how the protein and crowding species interact, while crowding agents always stabilize the native state at sufficiently high protein concentrations. Crowding agents also are predicted to reduce the tendency for liquid-liquid phase separation even when they exhibit weak favorable interactions with the proteins. Thus, their presence in solution might either reduce or increase the thermodynamic driving force for aggregation depending on the crowder-protein interaction and whether single-protein unfolding or concentration fluctuations associated with liquid-liquid phase separation represent the key step for the degradation process. As we will discuss, some of these predictions could be readily tested via experiments on model protein systems.

This paper is organized as follows. The coarse-grained protein model and simulation methods used in this study are described in Secs. 2 and 3, respectively. Results and discussion are provided in Sec. 4, and conclusions are given in Sec. 5.

2 Coarse-Grained Model

As mentioned in Sec. 1, the coarse-grained protein model used in this study is based on random heteropolymer collapse theory [54,55]. The theoretical treatment yields parameters for a reactive force field describing the free energetics of protein folding and the interactions between proteins in their native and denatured states. The full mathematical details of the coarse-grained modeling strategy are provided in Refs. [39–41], but we highlight some of the key assumptions and features of the approach below.

Limiting the focus to globular proteins of moderate size, it is assumed that the following effective “protein-protein” pair potential applies

$$u(r_{ij}) = \begin{cases} \infty & \text{if } r_{ij} \leq \sigma_{ij} \\ \frac{\epsilon_{ij}}{625} \left\{ \left[\left(\frac{r_{ij}}{\sigma_{ij}} \right)^2 - 1 \right]^{-6} - 50 \left[\left(\frac{r_{ij}}{\sigma_{ij}} \right)^2 - 1 \right]^{-3} \right\} & \text{if } \sigma_{ij} < r_{ij} < 2.5\sigma_{ij} \\ 0 & \text{if } r_{ij} \geq 2.5\sigma_{ij} \end{cases} \quad (1)$$

where r_{ij} is the distance between protein centers, ϵ_{ij} is the strength of the attractive interaction, σ_{ij} is the exclusion sphere radius for a given protein pair, and the subscripts i and j denote the states (native N or denatured D) of the two proteins of interest. Here, the solvent is implicit. The above coarse-grained interaction potential has been shown to capture many important aspects of the thermodynamics and phase behaviors of protein solutions [31,39–41,66]. The initial applications of this interaction potential to study protein solutions did not distinguish between folded and unfolded proteins [31,66], thus treating the protein solution as a single-component fluid of native molecules. This picture is adequate for studying protein solutions across thermodynamic conditions for which the native state is ultrastable. However, in order to gain insights into unfolding and physical degradation processes, it is necessary to account for native and denatured states of the proteins associated by the unimolecular folding reaction. As Cheung and Truskett [39] have discussed, heteropolymer collapse (HPC) theory provides one approximate means for accomplishing this.

Specifically, the parameters for the above interaction potential ϵ_{ij} and σ_{ij} as well as the infinite-dilution (i.e., intrinsic) free energy of folding ΔG_f^0 of a protein molecule can be determined within the coarse-grained model by using mean-field predictions of HPC theory [54,55]. Based on the observation that heteropolymers exhibit conformational folding behavior qualitatively similar to single-domain globular proteins [67], HPC theory assumes that a protein can be mapped to an equivalent heteropolymer composed of hydrophobic and polar segments. At a given temperature T , HPC requires as structural input the number of amino acid residues and the fraction Φ of those considered hydrophobic (e.g., based on a solubility criterion). An additional input is the free energy $\chi(T)$ associated with hydrating a hydrophobic polymer segment. This quantity characterizes the relative strength of the solvent-mediated attraction between hydrophobic residues relative to the other weaker types of inter-residue interactions. It is assumed that attractive protein interactions are driven solely by the tendency of hydrophobic surface segments to minimize their ex-

posure to solvent. Here, we adopt a parametrization of $\chi(T)$ introduced by Dill et al. [55] that qualitatively reproduces experimental trends for the partitioning of hydrophobic amino acids between an oily phase and liquid water at atmospheric pressure. Other studies also considered the effects of pressure [68] and pH [69,70] on the water-mediated interactions.

Specifically, HPC theory predicts R_D/R_N , the ratio of the radii of gyration of the denatured (D) state to the folded (N) one, as well as Θ , the fraction of solvent-exposed residues in the native state that are hydrophobic. Although hydrophobic residues are assumed to be uniformly distributed on the surfaces of native and denatured proteins in this work, the coarse-grained modeling strategy based on HPC theory was also extended to account for spatially nonuniform distributions (i.e., patches) of hydrophobic residues, which naturally lead to anisotropic protein-protein interactions [41]. For the unfolded state, the fraction of solvent-exposed residues that are hydrophobic is assumed to be equal to the fraction of hydrophobic residues in the sequence Φ . From a structural perspective, HPC theory correctly predicts that $R_D/R_N > 1$ and $\Phi > \Theta$. In terms of the interaction potential in Eq. (1), the relative exclusion radii are $\sigma_{DD}/\sigma_{NN}=R_D/R_N$ and $\sigma_{DN}/\sigma_{NN}=1/2[1+(R_D/R_N)]$. Based on this and simple mean-field calculations [39], the strongest attractive interactions of Eq. (1) are between denatured proteins, followed by denatured-native and native-native interactions, respectively, ($\epsilon_{DD} > \epsilon_{ND} > \epsilon_{NN}$). In addition to structural quantities, HPC theory also qualitatively predicts the experimental temperature-dependent trends for the infinite-dilution free energy of folding $\Delta G_f^0(T)$, which quantifies the intrinsic stability of a single chain in the absence of interactions with other proteins and solutes.

To investigate the effects of macromolecular crowding on conformational protein stability and protein solution thermodynamics, we studied two types of mobile crowding agents. The first type of model crowding agent consisted of simple hard spheres, which interact with native and unfolded proteins exclusively via excluded volume interactions characterized by length scales σ_{NN} and σ_{ND} , respectively. The second type of model crowding agent consisted of ultrastable (i.e., forbidden to unfold) native-state proteins with the same surface hydrophobicity and size as the foldable proteins in their native state. Because the only difference between the two types of crowders is that the latter possesses a short-ranged attractive interaction (see Eq. (1)), we will refer to them as hard-sphere and attractive crowders in what follows.

3 Simulation Methods

The above coarse-grained model can be treated as a reactive binary mixture, where protein folding (and unfolding) is a unimolecular chemical reaction with intrinsic free energy of reaction ΔG_f^0 . Of course, the total free energy of unfolding for the proteins also contains the effects of interactions with other proteins and crowders, and those effects can be determined via molecular simulation. Specifically, the thermodynamic properties of this model protein solution are computed using transition-matrix Monte Carlo simulation. While several implementations of transition-matrix Monte Carlo have been developed and used to study a broad range of phenomena [56–58,71–90], we chose, as in previous work with this protein model, to use a particular grand canonical implementation developed for multicomponent systems [40,41,58].

In the absence of crowders ($N_c=0$) and at fixed protein chemical potential μ and temperature T , an important statistical distribution of interest is the probability of observing N_i proteins (folded and unfolded) in a system of volume V . We denote this probability distribution as $\Pi(N_i; \mu, V, T)$ to emphasize that μ , V , and T are fixed. Notice that only a single chemical potential μ is specified because chemical equilibrium (protein folding and unfolding) requires that the chemical potentials of the native and denatured proteins (i.e., the reactant and product of a unimolecular

Table 1 Interaction parameters for coarse-grained protein model

Φ	σ_{DD}/σ_{NN}	σ_{ND}/σ_{NN}	ϵ_{NN}/k_B (K)	ϵ_{DD}/k_B (K)	ϵ_{ND}/k_B (K)	T_f (K)
0.500	1.259	1.129	411.476	1011.200	666.905	363.93
0.473	1.329	1.164	313.614	878.699	552.203	358.42
0.455	1.393	1.196	255.169	789.665	480.795	354.20
0.400	1.788	1.394	113.871	510.374	294.934	337.73

reaction) be identical. Although the computed distribution is unique to the chemical potential used in the simulation, the probability distribution can be determined straightforwardly at other chemical potential values using histogram reweighting [40,58,91]. Supplementing the distribution $\Pi(N_i; \mu, V, T)$ with easily collected canonical averages, such as the average number of denatured proteins $\langle N_D(N_i) \rangle$ and average total potential energy $\langle U(N_i) \rangle$ at a given value of N_i , the thermodynamic properties of the protein solution can be determined over a wide range of fluid densities using information from a single simulation. The statistical mechanical formalism for calculating properties such as pressure, composition, and energy from this information can be found in Ref. [58]. To study the influence of crowding agents or crowders on protein stability and protein solution thermodynamics, we simply calculate $\Pi(N_i; \mu, V, T, N_c)$, the probability of observing N_i proteins at chemical potential μ in the presence of N_c crowders. Computationally, this amounts to allowing the number of proteins in the system to fluctuate while fixing the number of crowders.

Grand canonical transition-matrix Monte Carlo simulations in this study consisted of the following types of trial moves: displacements, insertions/deletions, and identity changes. Trial displacements involved both proteins and crowders, while trial insertions/deletions and identity changes only involved the proteins. The overall breakdown of attempted trial moves was 25% displacements, 25% identity changes, and 50% insertions/deletions. Cubic simulation cells of length $9\sigma_{NN}$ were used. As in previous work [40,41], it was necessary to impose a constraint to prevent the system from freezing. This is important because the fluid-phase properties are of interest. Details of the imposed constraint can be found in Refs. [40,41]. Proteins with 154 residues and overall fractional sequence hydrophobicity $\Phi=0.400, 0.455, 0.473$, and 0.500 were studied, values consistent with medium size naturally occurring globular proteins [39,40]. Interaction parameters for each hydrophobicity value can be found in Table 1. For each protein (value of Φ), the distribution $\Pi(N_i; \mu, V, T, N_c)$ was calculated using crowder packing fractions between zero and 0.072 . To put the crowder packing fraction range into perspective, the maximum protein packing fraction that we were able to sample in the transition-matrix simulations was 0.35 . The temperature was set to the infinite-dilution midpoint folding temperature T_f of the protein being simulated, which is the temperature at which $\Delta G_f^0=0$. As noted above, two types of model crowding agents were studied, attractive and hard-sphere crowders. Using the information collected from the simulations, we calculated protein folding curves, that is the fraction f of proteins in solution in their native state as a function of dimensionless protein concentration $\rho\sigma_{NN}^3$, where ρ is the protein number density. To provide a calibration to experimental concentrations, a 100 mg/ml solution of ribonuclease A would have $\rho\sigma_{NN}^3 \approx 0.28$, assuming $\sigma_{NN}=4.0$ nm, and a molecular weight of 13.7 kDa. This is certainly considered a “high” concentration, consistent with the upper end of concentrations encountered in therapeutic protein solutions used for subcutaneous injections or of nonstructural proteins in cellular cytoplasm.

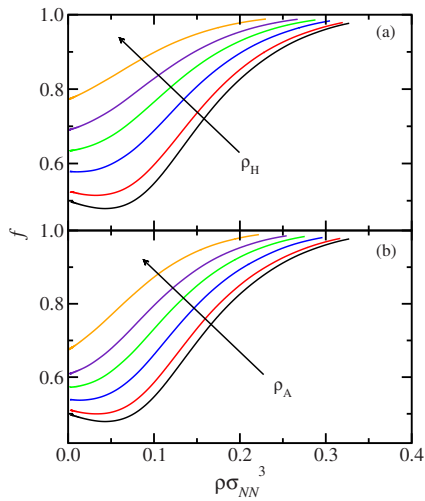


Fig. 1 Folding curves for the $\Phi=0.400$ protein in the presence of (a) hard-sphere crowders and (b) attractive crowders. The black curve represents the bare protein solution without any crowders at the infinite-dilution midpoint folding temperature. Dimensionless crowder concentrations $\rho_H\sigma_{NN}^3$ and $\rho_A\sigma_{NN}^3$ of 0.0137, 0.0412, 0.0686, 0.0960, and 0.137 were studied.

4 Results and Discussion

In Figs. 1(a) and 1(b), we show the folding curves for the lowest hydrophobicity protein ($\Phi=0.400$) in the presence of hard-sphere and attractive crowders, respectively. We emphasize that the quantities f and ρ refer to the folded fraction and number density of foldable proteins in solution. For point of reference, the “bare” protein folding curve in the absence of crowders ($N_c=0$) is also given. The nonmonotonic dependence of the bare protein folding curve on ρ is the manifestation of two competing physical effects [39]. The first effect is an energetic one that stems from attractive protein-protein interactions, or more precisely from the fact that $\epsilon_{DD} > \epsilon_{ND} > \epsilon_{NN}$. In short, assuming that there is enough space in solution to accommodate the transition to the more expanded denatured state, native proteins can increase their favorable attractive interactions with nearby proteins by unfolding. This effect dominates at low to moderate protein concentrations, which is why f initially decreases with protein concentration ρ . The second effect is the excluded volume trend typically associated with crowding. The native-state protein is smaller than the denatured protein ($\sigma_{DD}/\sigma_{NN} > 1$), and thus unfolding imposes a significant entropic penalty at high enough protein concentrations (f ultimately increases with ρ). These basic physics and their manifestation in experiments were discussed previously in Refs. [39–41].

Now we turn our attention to the effect of adding cosolute crowding molecules to the solution. Notice that the presence of crowders (hard-sphere or attractive) systematically enhances the stability of the native state of this low hydrophobicity protein, and that the degree of stabilization increases with crowder concentration. This indicates that excluded volume effects are dominating over the weak attractions that the attractive crowders can make with this protein in solution. The reason that the attractive interactions are particularly weak in this case is that low hydrophobicity proteins can make only a modest number of favorable hydrophobic residue contacts with attractive crowders. Of course, that is not to say that attractions have no effect here. One can see that the hard-sphere crowders are slightly more effective than the attractive crowders at stabilizing the native state. However, the attractive interactions between the attractive crowders and the proteins (which promote destabilization of the native fold) are simply too weak to compensate for the excluded volume interaction, and thus

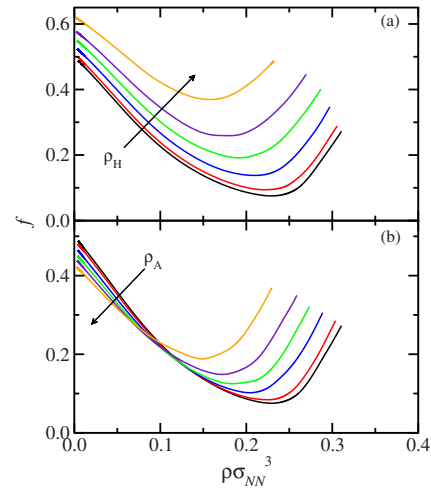


Fig. 2 Folding curves for the $\Phi=0.455$ protein in the presence of (a) hard-sphere crowders and (b) attractive crowders. The black curve represents the bare protein solution without any crowders at the infinite-dilution midpoint folding temperature. Dimensionless crowder concentrations $\rho_H\sigma_{NN}^3$ and $\rho_A\sigma_{NN}^3$ of 0.0137, 0.0412, 0.0686, 0.0960, and 0.137 were studied.

the presence of either attractive or hard-sphere crowders ultimately shifts the folding equilibrium toward the native state.

Folding curves for the $\Phi=0.455$ protein in the presence of crowding agents are shown in Fig. 2. In Fig. 2(a), we see that, as expected, the presence of hard-sphere crowders stabilizes the native state over the entire concentration range, and that protein stabilization increases with crowder concentration. In contrast, attractive crowders are *destabilizing* at low protein concentrations but stabilizing at high protein concentrations (see Fig. 2(b)). The magnitude of the effect (destabilization at low ρ and stabilization at high ρ) increases with crowder concentration, which is easy to understand. For low concentrations, there is ample free volume in the solution for native proteins to unfold into the more expanded denatured state. As a result, the addition of attractive crowders, which interact more favorably with the denatured state, shifts the equilibrium toward unfolding. In contrast, there is little volume available for unfolding in solution at high protein concentrations. That volume is decreased even further by the addition of crowders (attractive or hard-sphere in nature). As a result, crowders always promote the native state at sufficiently high protein concentrations. It is worth emphasizing that the physical driving forces in the $\Phi=0.400$ and the $\Phi=0.455$ protein solutions are precisely the same. The difference in their behaviors stems from the fact that higher hydrophobicity proteins can make more favorable hydrophobic contacts with attractive crowders, rendering the destabilizing effect of the attractive crowder interactions more important in that case.

Next, we examine the calculated folding curves for the $\Phi=0.473$ protein in Fig. 3. Again, the presence of hard-sphere crowders always favors the native state of the protein (Fig. 3(a)). Attractive crowders destabilize the protein at low concentrations due to strong, energetically favorable interactions, but then eventually stabilize the native state at high concentrations due to the excluded volume effect. However, this protein solution exhibits a feature in its bare folding curve ($N_c=0$) not found in the protein solutions discussed above: a thermodynamic liquid-liquid ($L-L$) phase transition in the absence of crowders. That is, without crowders, the solution phase separates into (a) a protein-rich fluid composed predominantly of denatured protein and (b) a comparatively protein-poor fluid with a slightly lower denatured protein composition. Phase separation in protein solutions induced by strong protein-protein interactions or protein oligomerization is known to

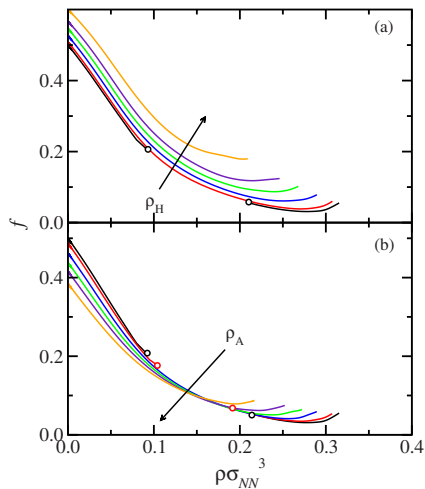


Fig. 3 Folding curves for the $\Phi=0.473$ hydrophobicity protein in the presence of (a) hard-sphere crowders and (b) attractive crowders. The black curve represents the bare protein solution without any crowders at the infinite-dilution midpoint folding temperature. Open circles of the same color denote coexisting fluid phases. Dimensionless crowder concentrations $\rho_H \sigma_{NN}^3$ and $\rho_A \sigma_{NN}^3$ of 0.0137, 0.0412, 0.0686, 0.0960, and 0.137 were studied.

occur experimentally. As discussed in Sec. 1, the concentration fluctuations associated with such a transition are thought to be an important driving force for protein aggregation and precipitation from solution [29–38]. In Fig. 3(a), we can see that the introduction of hard-sphere crowders prevents this phase separation from occurring at all. In the case of attractive crowders, the $L-L$ phase transition remains only for the smallest crowder concentration studied ($\rho_A \sigma_{NN}^3=0.0137$).

In Fig. 4, we can see that the highest hydrophobicity $\Phi=0.500$ protein solution has behavior that is qualitatively similar to the $\Phi=0.473$ protein solution. It also exhibits a $L-L$ phase transition in the absence of crowders. However, in this case, the

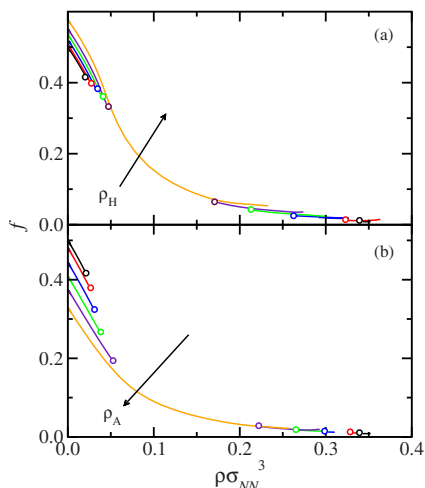


Fig. 4 Folding curves for the $\Phi=0.500$ hydrophobicity protein in the presence of (a) hard-sphere crowders and (b) attractive crowders. The black curve represents the bare protein solution without any crowders at the infinite-dilution midpoint folding temperature. Open circles of the same color denote coexisting fluid phases. Dimensionless crowder concentrations $\rho_H \sigma_{NN}^3$ and $\rho_A \sigma_{NN}^3$ of 0.0137, 0.0412, 0.0686, 0.0960, and 0.137 were studied.

width of the phase boundary is greater than that exhibited by the $\Phi=0.473$ protein solution (i.e., the properties of the coexisting fluid phases are more distinct). Nonetheless, the addition of hard-sphere or attractive crowders have the same effects as above, decreasing the width of this transition and even eliminating it for the highest crowder concentration studied. This type of elimination or shifting of a fluid-fluid phase transition due to crowding is analogous to what is observed for pure fluids under confinement. Presumably, it is related to the fact that the crowders (or surfaces of confined fluids) disrupt the strongly favorable energetic interactions that are responsible for the condensation of the high concentration (high density) fluid phase.

If crowding-induced elimination of phase separation generically occurs due to these simple physical principles, it should be possible to test experimentally. In particular, our results suggest that the addition of cosolute molecules might suppress the fluid-fluid phase transitions seen in oligomerizing protein solutions [28] or in polymerizing solutions of small organic molecules [21–27]. This might be an interesting question to explore further in future studies.

Finally, we emphasize that the results presented here only pertain to equilibrium behavior, namely, the effects that crowding agents have on protein unfolding curves and liquid-liquid phase separation. Since both processes appear intimately connected to protein aggregation and physical degradation processes, understanding and making predictions about them is a basic step toward ultimately understanding the factors that impact protein stability. However, quantitatively connecting such thermodynamic trends with kinetic models for making predictions about aggregation rates remains an outstanding challenge.

5 Conclusions

Using our recently developed coarse-grained protein modeling strategy, we investigated the effects of different model crowding agents on conformational protein stability and the thermodynamics of protein solutions. The results of this study can be summarized as follows. At low protein concentrations, depending on the strength of its interaction with the proteins, crowders can stabilize or destabilize the native state. Sufficiently strong crowder-protein attractions tend to unfold native proteins at low protein concentration (see $\Phi=0.455$, 0.473, and 0.500 protein solutions). At high protein concentrations, crowders tend to stabilize the native state simply due to excluded volume effects, irrespective of the strength of the crowder-protein attraction. Solutions of the more hydrophobic model proteins studied in this work ($\Phi=0.473$ and 0.500) phase separate into two distinct fluids at moderate protein concentrations in the absence of crowders. The width of the fluid phase boundary decreases with increasing crowder concentration and eventually vanishes. Based on these results, the presence of crowders in solution might either reduce or increase the thermodynamic driving force for loss of protein stability depending on the crowder-protein interaction and whether single-protein unfolding or concentration fluctuations associated with liquid-liquid phase separation represent the key step for the degradation process.

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