Molecular crowding enhances native structure and stability of α/β protein flavodoxin

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To investigate the consequences of macromolecular crowding on the behavior of a globular protein, we performed a combined experimental and computational study on the 148-residue single-domain α/β protein, *Desulfovibrio desulfuricans* apoflavodoxin. *In vitro* thermal unfolding experiments, as well as assessment of native and denatured structures, were probed by using far-UV CD in the presence of various amounts of Ficoll 70, an inert spherical crowding agent. Ficoll 70 has a concentration-dependent effect on the thermal stability of apoflavodoxin (ΔT_m of 20°C at 400 mg/ml; pH 7). As judged by CD, addition of Ficoll 70 causes an increase in the amount of secondary structure in the native-state ensemble (pH 7, 20°C) but only minor effects on the denatured state. Theoretical calculations, based on an off-lattice model and hard-sphere particles, are in good agreement with the *in vitro* data. The simulations demonstrate that, in the presence of 25% volume occupancy of spheres, native flavodoxin is thermally stabilized, and the free energy landscape shifts to favor more compact structures in both native and denatured states. The difference contact map reveals that the native-state compaction originates in stronger interactions between the helices and the central β -sheet, as well as by less fraying in the terminal helices. This study demonstrates that macromolecular crowding has structural effects on the folded ensemble of polypeptides.

energy landscape theory | excluded volume effect | molecular simulations | protein folding

Protein folding *in vitro* has been extensively characterized in dilute conditions. However, the intracellular environment is highly crowded because of the presence of large amounts of soluble and insoluble macromolecules, including proteins, nucleic acids, ribosomes, and carbohydrates. This means that a significant fraction of the intracellular space is not available to other macromolecular species. It has been estimated that the concentration of macromolecules in the cytoplasm is in the range of 80–400 mg/ml (1, 2). All macromolecules in physiological fluids collectively occupy between 10% and 40% of the total fluid volume (3, 4). The term “macromolecular crowding” implies the nonspecific influence of steric repulsions on specific reactions that occur in highly volume-occupied media. Because of excluded volume effects, any reaction that amplifies the available volume will be stimulated by macromolecular crowding (5–8). It is proposed that crowding provides a stabilizing effect to the folded protein indirectly because of compaction of the denatured states. Crowding can be mimicked experimentally by adding high concentrations of inert synthetic or natural macromolecules, termed crowding agents, to the systems *in vitro*. Experimental and theoretical work has demonstrated large effects of crowding on the thermodynamics and kinetics of many biological processes, including protein binding, folding, and aggregation (1, 9–12).

Whereas theoretical simulations have focused on very small proteins or peptides (11), experimental crowding studies have mostly involved large complex proteins (i.e., multidomain and/or disulfide containing) and often extreme solvent conditions (such as acidic pH). For example, the effects of crowding agents on the

refolding of reduced denatured lysozyme (1, 9), oligomerization of GroEL subunits (13), self-assembly of the cell division protein FtsZ (14) and the capsid protein of HIV (15), and amyloid formation of the human apolipoprotein C-II (16) have been reported. A few studies have focused on the ability of crowding agents to induce conformational changes in unfolded states of proteins (17, 18). For example, it was shown that unfolded cytochrome *c* adopts a molten globule state in the presence of crowding agents at low pH (19). Notably, there are no detailed studies of the effects of macromolecular crowding on the behavior of small single-domain proteins that fold with simple mechanisms in dilute solutions.

To fill this gap, we have combined experimental and theoretical approaches to carefully assess the native and denatured structural ensembles, as well as the thermal stability, of a well behaved α/β model protein (i.e., apoflavodoxin) as a function of concentration of an inert spherical macromolecular crowding agent (i.e., Ficoll 70). Our *in vitro* and *in silico* results are in excellent agreement and demonstrate that crowding increases protein-thermal stability via structural enhancement in both the folded and denatured ensembles of molecules.

Results

Choice of Protein and Crowding Agent. The model system, *Desulfovibrio desulfuricans* flavodoxin (Fig. 1A), was selected because it is a small α/β protein (148 residues) with a common fold (i.e., the flavodoxin-like fold) in which a central β -sheet is surrounded by four helices (20–22). This protein has been extensively characterized previously in our laboratory in terms of its chemical and thermal unfolding behaviors in dilute solutions (23–27). It unfolds in an apparent two-state reaction upon chemical and thermal perturbations when monitored by far-UV CD. To be able to match with simulations, flavodoxin devoid of the FMN cofactor, i.e., apoflavodoxin, is used in all work herein. Apoflavodoxin has the same folded structure as with the cofactor; it exhibits a rather modest stability (≈ 15 kJ/mol, pH 7) at room temperature, indicating there is span for possible improvement because of crowding effects. The crowding agent selected for this work is Ficoll 70. This agent has many advantages; it is a polysaccharide (i.e., sucrose epichlorohydrin copolymer; average molecular mass of 74 kDa) that is inert, polar, and does not interact with proteins. It behaves like a semirigid sphere (radius of ≈ 55 Å) (28, 29); thus it is an attractive mimic of globular macromolecules that may be present in the biological setting where proteins normally fold. In addition, it can readily be

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Fig. 1. Protein system used in this study. (A) Model of *D. vulgaris* flavodoxin (2fx2). The sequence of *D. desulfuricans* flavodoxin, used in our *in vitro* experiments, is 46% identical to that of *D. vulgaris*. Green, β -sheets and loops; red, α -helices; blue, FMN cofactor (removed in our experiments). (B) Snapshot of apoflavodoxin and hard spheres of the size of Ficoll 70 (volume fraction = 25%) as used in the simulations.

represented in computer simulations as repulsive hard spheres of the appropriate size (Fig. 1B; for volume occupancy, ϕ_c , of 25%).

Ficoll 70 Increases Protein Thermal Stability *in Vitro*. Far-UV CD at 222 nm was used to probe thermal unfolding of apoflavodoxin as a function of increasing Ficoll 70 concentrations (0–400 mg/ml, pH 7). All reactions appear as single cooperative transitions and are 70–80% reversible; in addition, there is no scan-rate dependence in accord with equilibrated reactions. As demonstrated in Fig. 2A, the presence of Ficoll 70 has a dramatic effect on apoflavodoxin thermal stability. The more Ficoll 70 is present in the samples, the higher the thermal midpoint (T_m) for apoflavodoxin. In fact, T_m increases from 45°C to 65°C, going from 0 to 400 mg/ml Ficoll 70 in Hepes buffer, pH 7 (Table 1).

Apoflavodoxin is sensitive to buffer composition. For example, the protein is thermodynamically more stable in phosphate than in Hepes buffer (23). We found that by changing only the buffer conditions, the T_m value for apoflavodoxin can shift as much as 25°C (i.e., comparing phosphate plus salt vs. Hepes buffers at pH 7). This implies that population shifts within the native-state ensemble are possible and, moreover, that this ensemble contains a distribution of molecules with slightly different thermodynamic properties (30, 31). Nonetheless, the addition of Ficoll 70 always has a stabilizing effect on apoflavodoxin thermal stability, albeit the magnitude depends on the buffer choice (Table 1). The lower the protein stability without

Fig. 2. *In vitro* thermal stability as a function of crowding agent. (A) Thermal unfolding curves for apoflavodoxin probed by far-UV CD in the presence of various amounts of Ficoll 70. (B) T_m vs. Ficoll 70 concentrations for apoflavodoxin in three different buffer conditions (squares, 10 mM Hepes; circles, 20 mM phosphate; diamonds, 40 mM phosphate plus 250 mM NaCl, all at pH 7).

Table 1. T_m values (in kelvin) for apo-flavodoxin unfolding in different buffers (pH 7) combined with different amounts of Ficoll 70, as indicated

Buffer	Bulk	100 mg/ml	200 mg/ml	300 mg/ml	400 mg/ml
Hepes	317 ± 1	321 ± 1	325 ± 1	331 ± 1	337 ± 1
Phosphate	328 ± 1	330 ± 1	—	338 ± 1	341 ± 1
Phosphate + NaCl	342 ± 1	343 ± 1	—	346 ± 1	—
Simulation	365	372 (volume occupancy of 25%)			

The experimental midpoints were derived from CD-detected unfolding curves. All transitions were >80% reversible.

crowding agent in a particular buffer, the larger the stabilizing effect due to Ficoll 70 (Fig. 2B).

Structural Effects on Native State by Ficoll 70. In Fig. 3A, we show the far-UV CD spectra of native apoflavodoxin in the presence of increasing amounts of Ficoll 70 (0–400 mg/ml; pH 7.0, 20°C). Unexpectedly, we find that the negative far-UV CD signal grows larger, suggesting gain of secondary structure, as a function of added crowding agent. The negative signal at 222 nm is raised by $\approx 10\%$ in 200 mg/ml and by $\approx 15\%$ at 400 mg/ml Ficoll 70. Thermally denatured apoflavodoxin (at 95°C, pH 7) also displays changes in the CD signal as a function of Ficoll 70 concentration, but the spectral shape remains characteristic of that of unfolded polypeptides (Fig. 2B). For comparison, we also collected CD spectra of apoflavodoxin unfolded in 3 M guanidine hydrochloride (GuHCl) as a function of Ficoll 70 additions (at pH 7, 20°C). As seen in Fig. 3C, the addition of Ficoll (up to 250 mg/ml tested) to the chemically denatured protein has no major effect on secondary structure content.

Secondary structure estimations based on the far-UV CD spectra of folded flavodoxin (using the SOMCD neural network algorithm, <http://geneura.ugr.es/cgi-bin/somcd/index.cgi>) reveal that the helical content rises up to 20%, whereas the random coil contribution shrinks >10% when going from 0 to 400 mg/ml Ficoll 70 conditions (in buffer pH 7, 20°C). In the crystal structure of *Desulfovibrio vulgaris* flavodoxin (i.e., 2fx2, the closest structural homolog to *D. desulfuricans* flavodoxin in the Protein Data Bank), the protein has $\approx 37\%$ helix, $\approx 29\%$ β -sheet, and $\approx 34\%$ random coil; interestingly, this composition is very similar to that estimated for our apoflavodoxin in 400 mg/ml Ficoll 70. Thus, with Ficoll 70, the mean of the native-state ensemble distribution in solution shifts toward a structure that is similar to that in the crystals. We note that when the same Ficoll 70 titrations were repeated with folded and unfolded forms of holoflavodoxin (i.e., with FMN bound), similar trends as for the apoform were observed (data not shown). In addition, we found that another crowding agent, Dextran 70 (a glucose polymer that adopts a rod-shape structure), also induces additional structure in folded flavodoxin (data not shown).

Simulated Effects of Crowding Matches *in Vitro* Data. To assess the effects of macromolecular crowding on apoflavodoxin from a theoretical view, we computed thermodynamic properties and simulated the free-energy landscape for apoflavodoxin at different temperatures, with and without hard-sphere Ficoll 70 particles at a volume occupancy of $\phi_c = 25\%$ (scenario depicted in Fig. 1B). The folding transition temperature was calculated from the temperature dependence of the Q parameter to be 365 K without crowder. As in the *in vitro* experiments, apoflavodoxin unfolds in a single thermal transition *in silico* that is shifted to higher temperatures (i.e., T_m of 372 K) at $\phi_c = 25\%$ (data not shown). The stabilizing effect and the relative change in T_m value

Fig. 3. *In vitro* structural effects due to macromolecular crowding. Far-UV CD of (A) folded (pH 7, 20°C), (B) thermally unfolded (pH 7, 95°C), and (C) chemically unfolded (3 M GuHCl, pH 7, 20°C) flavodoxin in the presence of various amounts of Ficoll 70 (key for all in B).

due to the presence of crowders agree well between *in silico* and *in vitro* data (Table 1).

The trend of enhanced native-like structure in the presence of crowders is seen from 1D energy profiles, F as a function of Q , at a temperature close to the folding transition [supporting information (SI) Fig. 7]. The shifts in the minimum of the folded state, from $Q = 0.76$ (for bulk) to $Q = 0.80$ ($\phi_c = 25\%$) and $Q = 0.82$ ($\phi_c = 40\%$), indicate augmentation of native-like structures in the folded ensemble in the presence of crowders. This type of native-state change was not observed in the previous investigation of the WW domain (11). This difference may be due to the fact that flavodoxin is longer (148 residues) and contains more complex secondary and tertiary structures than the WW domain (34 residues).

In Fig. 4, we show the resulting 2D energy landscapes for apoflavodoxin as a function of radius of gyration, R_g , and fraction of native contacts, Q , at $T = 360$ K for bulk (Fig. 4A) and $\phi_c = 25\%$ (Fig. 4B) crowding conditions. Comparing Fig. 4A with B, it is clear that the shape of the energy landscape shifts toward the low R_g region in the presence of crowder. The ensemble structures of apoflavodoxin become more compact (i.e., R_g decreases) when crowders are added. Notably, this is apparent for both the unfolded and the native-state ensembles. For the native-state ensemble, the average R_g is $4.052 \pm 0.001\sigma$ ($\sigma = 3.8$ Å) for bulk conditions and $4.039 \pm 0.001\sigma$ for $\phi_c = 25\%$ conditions. Interestingly, despite the reduction in size of the native-state ensemble in the presence of crowders, the shape parameters remain the same in both cases (for bulk, $\Delta = 0.035 \pm 0.001$ and $S = -0.011 \pm 0.001$; for $\phi_c = 25\%$, $\Delta = 0.036 \pm 0.001$ and $S = -0.011 \pm 0.001$). This observation demonstrates that the effects on protein compaction due to crowding are isotropic.

To reveal the molecular origin of the crowding-induced protein compaction and increased structural content, we derived difference contact maps of the folded states of apoflavodoxin between the $\phi_c = 25\%$ and the bulk conditions (Fig. 5A). The same difference contact map was created between the two denatured states (Fig. 5B). Inspection of these maps reveal that the compaction of folded flavodoxin stems from improved interactions between the surrounding helices and the core β -sheet, as well as from less helix fraying in the terminal helices. Because helical contacts are stable in both bulk and crowded cases, these contacts do not show much of a color change in the difference map, although it is possible that the helices become more rigid in the presence of crowding agents. The extension of helices agrees well with the far-UV CD data that implied more α -helical content in folded apoflavodoxin at crowded conditions. In contrast, in the denatured state, crowding appears only to promote some native contacts between residue regions 70–90 (helix 2/loop region in folded protein) and 40–60 (loop/ β -strand region in folded protein) (Fig. 5B). From an overall perspective, structural fluctuations in the native state, as measured by the

rmsd for each residue, are diminished by a factor of 2 in the presence of crowders at $\phi_c = 25\%$ (SI Fig. 8).

Discussion

This is a combined experimental/computational study elucidating the effects of molecular crowding on thermal and structural properties of a small single-domain globular α/β protein (i.e., 148-residue apoflavodoxin). Earlier experimental work has focused on the effects of crowding on kinetic refolding of complex proteins and on protein nonnative states at extreme conditions, whereas theoretical approaches have instead involved simple lattice models or small peptide systems (1, 9–12).

An equilibrium statistical-thermodynamic model, developed primarily by A. P. Minton (32), predicts that macromolecular crowding should increase protein thermal stability (T_m) by a magnitude of ≈ 5 – 20°C at physiological solute conditions. However, there have been few experimental verifications of this prediction. The reason is likely that many proteins unfold irreversibly and aggregate in the presence of high concentrations of crowding agents. The (irreversible) thermal midpoint for G-actin was found to increase by 5°C in the presence of 100 mg/ml PEG (33); the presence of 300 mg/ml dextran had a 3°C favorable effect on lysozyme thermal stability at pH 2 (19), and the presence of 370 mg/ml dextran raised T_m by 3.5°C for cytochrome *c* (34). In contrast to these modest effects, but in agreement with the prediction, we discovered that Ficoll 70 dramatically enhances apoflavodoxin (reversible) thermal stability: stabilizing effects of up to 20°C were observed *in vitro*. Notably, the stabilizing effect on apoflavodoxin due to crowding is similar to that observed for α -lactalbumin when placed in pores of silica glass (35, 36).

The excluded-volume theory implies that macromolecular crowding acts on unfolded-state conformations. By making the

Fig. 4. *In silico* energy landscapes for apoflavodoxin with and without crowding. The 2D free-energy landscape as a function of R_g and Q at $T = 360$ K at volume fractions of crowder of zero (i.e., bulk) (A) and 25% (B). R_g is the radius of gyration in unit of σ ($\sigma = 3.8$ Å). Q is the fraction of native contacts. The color is scaled by $k_B T$.

Fig. 5. Mapping of *in silico* structural changes due to crowding against residue numbers. Difference contact map of the folded (A) and unfolded states (B) between 25% and 0% volume occupancy of crowders at $T = 360$ K. Secondary structure elements are indicated.

denatured state more compact and thereby less energetically favorable, the native state is indirectly stabilized (5–8). However, our experimental and computational observations of structural changes in both the native and denatured states of apoflavodoxin indicate that direct crowding effects on the native protein molecules are feasible (Fig. 6). We have observed both a compaction of the overall size of the native protein (i.e., effects on R_g) and a more native-like structure (i.e., more negative far-UV CD signal and Q value closer to 1) for apoflavodoxin in the presence of crowder. The structural effect appears larger in the folded than in the denatured ensemble although the compaction effect is more pronounced in the denatured ensemble.

We also analyzed the changes in the folded and unfolded states due to crowding from a thermodynamic point of view. For this, the simulation data were used to compute the energy (which roughly corresponds to enthalpy), E , and the entropy, S (37), for folded and unfolded states in buffer and in 40% of crowders at 350, 360, and 370 K (SI Fig. 9 A and B). From this analysis, it emerges that there are energetic and entropic effects due to crowding in both unfolded (i.e., $Q_U = 0.25$) and folded states (i.e., $Q_F = 0.8$) of apoflavodoxin: for the $\phi_c = 40\%$ condition,

$\Delta E_{FU} \approx -19$ kcal/mol, $\Delta S_{FU}/k_B \approx -24$ (S/k_B is unitless) (k_B , Boltzmann constant), and $\Delta G_{FU} \approx -1.72$ kcal/mol at 360 K. For apoflavodoxin in buffer (i.e., bulk), $\Delta E_{FU} \approx -47.4$ kcal/mol, $\Delta S_{FU}/k_B \approx -66$, and $\Delta G_{FU} \approx 0.12$ kcal/mol at 360 K (SI Fig. 10). Interestingly, the crowding effects on folded- and unfolded-state entropy values are favorable. This may be explained by the existence of a large number of substates within the folded and unfolded ensembles as a result of crowder–protein interactions at high volume fraction of crowders. In contrast, the effect on the enthalpy/energy change due to crowding appears unfavorable, which may be explained by crowding-induced compaction having a negative effect on internal bonds (compression of structural bonds; these are modeled as harmonic springs).

Crowded conditions may cause enrichment of a large number of slightly different protein structures that are all within the native ensemble of molecules but have the highest degree of order. This can be achieved by a shift (and broadening in terms of number of substates) of the average distribution of species within the native-state ensemble toward $Q = 1$. In agreement, the difference contact map reveals that the secondary and tertiary structures are not altered *per se*, but that they become better packed and exhibit less breathing/terminal fraying in the presence of crowders. Thus, crowding forces the native ensemble of the protein molecules in solution to be on average more like they are in the crystalline state. We propose that native-state structural effects caused by macromolecular crowding may be common *in vivo* for globular proteins that exhibit marginal stability. The volume fraction of macromolecules may be as high as 40% in living cells. For proteins with inherent plasticity in their native states, protein–crowder interactions may play a role in the modulation of local conformations at active sites. In addition, macromolecular-crowding effects may be of high significance for intrinsically unstructured proteins (for example, FlgM), which may be unfolded in dilute solutions *in vitro* but adopt folded structures in the crowded *in vivo* environment (38).

Materials and Methods

Protein Preparation. Flavodoxin from *D. desulfuricans* (American Type Culture Collection strain 29577) was expressed in *Escherichia coli* and purified as described (39, 40). In short, the apo-form of wild-type flavodoxin was isolated on a Q-Sepharose column and further purified by gel permeation on a Superdex-75 by using an FPLC system (Amersham–Amersham Pharmacia) (25).

In Vitro Measurements. Apoflavodoxin native- and unfolded-state far-UV CD spectra (1-mm cell, 200–300 nm) were monitored at 20°C and 95°C, respectively, on a Jasco-810 spectrometer. Thermal unfolding experiments were carried out by following CD at 222 nm as a function of temperature. All experiments were

Fig. 6. Crowding affects both folded and unfolded ensembles. Schematic free-energy profile for flavodoxin folding. The enhanced stability at $\phi_c \neq 0$ (dashed curve) is due to a combination of unfolded-state destabilization and stabilizing of the folded state.

performed in different buffers and in the presence and absence of various amounts of Ficoll 70 (Sigma). Three solvent conditions (10 mM Hepes, 20 mM phosphate, and 40 mM phosphate plus 250 mM NaCl, all at pH 7) were tested in separate experiments. Ficoll 70 was included in 100 mg/ml steps, from 0 to 400 mg/ml; higher concentrations of Ficoll 70 were not accessible due to limited solubility. Care was taken to let all buffer/Ficoll/protein mixtures equilibrate before measurements. Both unfolding (from 20°C to 95°C) and refolding (from 95°C to 20°C) data points were collected every degree with a scan rate of 2.5°C per minute scan rate. Scan rates were varied between 0.1°C per minute to 2.5°C per minute; there was no scan-rate dependence observed in the process. All unfolding transitions were >80% reversible; aggregation was never observed. Differential scanning calorimetry tests were performed on Ficoll 70 solutions to assure that Ficoll itself did not exhibit any phase transitions within the temperature range of flavodoxin unfolding. These experiments revealed no detectable transitions for Ficoll (not shown). Assuming a spherical shape (28, 29), 50 mg/ml Ficoll 70 corresponds to a volume occupancy, ϕ_c , of $\approx 25\%$; 100 mg/ml to $\phi_c \approx 50\%$; at Ficoll 70 concentrations >200 mg/ml; however, the spherical shape may be distorted (28, 29).

Model of Flavodoxin and Crowders. We used a coarse-grained C_α side-chain model (C_α -SCM) (11, 41, 42) to represent apoflavodoxin [Protein Data Bank (PDB) ID no. 2FX2; this structure is for *D. vulgaris* flavodoxin, which is the closest structural analog to *D. desulfuricans* flavodoxin for which there is a PDB structure). Each amino acid (excluding glycine) is represented by using two interaction sites, one corresponding to the C_α atom and the other the center of mass of the side chain. A modified Go-like (43) interaction is used for mimicking protein-like behavior in which the principle of minimal frustration is implemented to construct a funnel-like folding energy landscape (44–46). The structural Hamiltonian that includes bond-length potential, side-chain backbone connectivity potential, bond-angle potential, and dihedral potential follows Go-like behavior in which equilibrium values are justified from the native structure.

The interaction energy for nonbonded native side-chain interactions is:

$$E_{ij}^{\text{NB}} = \varepsilon_{ij} \left\{ \left(\frac{\sigma_{ij}}{r} \right)^{12} - 2 \left(\frac{\sigma_{ij}}{r} \right)^6 \right\}, \quad [1]$$

where a native contact between side chain i and side chain j , $i-j > 1$, is determined from the native structure by using the CSU program (47). Moreover, $\sigma_{ij} = 0.9(\sigma_i + \sigma_j)$. We take σ_i to be the distance between the center of mass of side-chain atoms and the C_α atom, which is the effective van der Waals radius of a side chain. For the native contact energies ε_{ij} , we use the Betancourt–Thirumalai (48) statistical potential. For backbone hydrogen-bond interactions, we consider an angular-dependent function to capture directional property of hydrogen bonds.

$$E_{ij}^{\text{HB}} = A(\rho)E_{ij}^{\text{NB}}, \quad [2]$$

where $A(\rho)$ is

$$A(\rho) = \frac{1}{1 + \left[(1 + \cos\rho)(1 - \cos\rho) \left(1 - \frac{\cos\rho}{\cos\rho_a} \right) \right]^2}. \quad [3]$$

In this equation, ρ is the angular alignment between two interacting strands of backbones and defined in ref. 41. ρ_a is the pseudo dihedral angle of a canonical helical turn, 0.466 (rad). A native pair of hydrogen bonding between C_α^i and C_α^j is determined by using the DSSP program (49). ε_{ij} for backbone hydrogen bonding between C_α^i and C_α^j is 2.5 kJ/mol. σ_{ij} is the hydrogen bond length, 4.6 Å. The crowding particle, Ficoll 70, is modeled as an inert sphere with a radius of 55.0 Å, which is 3.8 times the radius of gyration (R_g) of flavodoxin in the native state (14.6 Å). Interactions between Ficoll 70 spheres and flavodoxin are repulsive. We choose to study volume occupancy of crowders, ϕ_c , of 25% and 40%. In most simulations, $\phi_c = 25\%$ was used because of the limitation of computing resources.

Simulation Details and Calculations. Thermodynamics properties are simulated in a periodic cubic box in which each length (L) is 300σ , and σ is 3.8 Å, the average distance between two adjacent C_α s in the C_α -side-chain model. L is chosen to be at least twice as long as the extended flavodoxin polypeptide. In addition, pair correlation functions between the Ficoll 70 particles in the box are computed, and L is large enough to ensure that the finite size effects are avoided in the calculation. Simulation procedures follow those in the previous study by Cheung *et al.* (11). The Replica Exchange Method (REM) (50) is used to enhance the sampling efficiency in the molecular simulations (51). Thermodynamic properties such as the radius of gyration (R_g), shape parameters^{||} (Δ and S) (11), and the fraction of native contacts (Q) with respect to the crystal structure are computed to characterize the free-energy landscape of flavodoxin folding. The use of REM and Langevin equations of motion in the low friction limit (52) ensures that these thermodynamic properties, calculated with the weighted histogram analysis method (WHAM) (53) are converged.

^{||}For a sphere, Δ is 0; $S > 0$ (< 0) corresponds to prolate (oblate) ellipsoid.

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