

Macromolecular Crowding Remodels the Energy Landscape of a Protein by Favoring a More Compact Unfolded State

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Abstract: The interior of cells is highly crowded with macromolecules, which impacts all physiological processes. To explore how macromolecular crowding may influence cellular protein folding, we interrogated the folding landscape of a model β -rich protein, cellular retinoic acid-binding protein I (CRABP I), in the presence of an inert crowding agent (Ficoll 70). Urea titrations revealed a crowding-induced change in the water-accessible polar amide surface of its denatured state, based on an observed ca. 15% decrease in the change in unfolding free energy with respect to urea concentration (the m -value), and the effect of crowding on the equilibrium stability of CRABP I was less than our experimental error (i.e., ≤ 1.2 kcal/mol). Consequently, we directly probed the effect of crowding on the denatured state of CRABP I by measuring side-chain accessibility using iodide quenching of tryptophan fluorescence and chemical modification of cysteines. We observed that the urea-denatured state is more compact under crowded conditions, and the observed extent of reduction of the m -value by crowding agent is fully consistent with the extent of reduction of the accessibility of the Trp and Cys probes, suggesting a random and nonspecific compaction of the unfolded state. The thermodynamic consequences of crowding-induced compaction are discussed. In addition, over a wide range of Ficoll concentration, crowding significantly retarded the unfolding kinetics of CRABP I without influencing the urea dependence of the unfolding rate, arguing for no appreciable change in the nature of the transition state. Our results demonstrate how macromolecular crowding may influence protein folding by effects on both the unfolded state ensemble and unfolding kinetics.

Introduction

The intracellular environment is highly crowded with macromolecules; total macromolecular concentrations range from 50 to 400 g/L.^{1–3} Due to excluded volume effects, cellular crowding increases the chemical potential of macromolecules, and hence the thermodynamic driving force for them to react.⁴ Macromolecular crowding also affects the nature of diffusion within the cell. Hence, ligand binding events, protein–protein interactions, enzymatic activities, protein folding, and essentially all other biochemical processes are expected to be modulated relative to their propensities in dilute solution by the high concentrations of macromolecules in cells. We have been studying how protein folding energy landscapes are altered by the cellular environment. Our results point to striking differences in the nature of a urea denaturation process inside *Escherichia coli* cells from that in dilute solution.^{5,6} Among the many factors that differ between typical *in vitro* environments and those encountered *in vivo*, macromolecular crowding would seem to

be a potentially major one that may influence the folding/unfolding equilibrium *in vivo*.

The impact of macromolecular crowding on protein folding has been explored both theoretically and experimentally.^{7–10} Yet despite numerous studies, fundamental gaps remain in our understanding of the effects of crowding. Theoretical predictions of crowding effects on protein stability vary significantly depending on how the unfolded state is modeled.^{11–13} It is crucial that we gather direct experimental data on the impact of macromolecular crowding on the nature of the unfolded state. There are many experimental studies examining how crowding affects protein folding energetics,^{14–22} and the results mostly point to a modest stability effect. Experimental studies on the effects of crowding on kinetics of folding or unfolding do not form a clear picture as yet, with reports of retarded unfolding,^{21,23} no effect on unfolding kinetics,^{14,24} acceleration of refolding,^{22,24–26} or retardation of refolding.^{21,26} Studies characterizing crowding effects on ensemble distributions of the unfolded state are sparse and inconclusive.^{27,28} Crowding has been observed to favor

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aggregation and misfolding,^{25,29,30} potentially complicating experimental investigation of folding under crowded conditions.

In this study, we have used an inert macromolecular crowding agent (Ficoll 70) to probe the impact of crowding on the folding landscape of a model β -sheet-rich protein, cellular retinoic acid-binding protein (CRABP I), a monomeric 18-kDa protein with a well-studied folding mechanism in dilute solution.^{31–35} Three native tryptophans (Trp 7, Trp 87, and Trp 109) all contribute to the intrinsic fluorescence of CRABP I and provide probes that are sensitive to local structure and environment. Our initial finding based on equilibrium urea denaturation experiments was that crowding caused a decrease in the dependence of unfolding free energy on the concentration of urea (the m -value). The observed m -value change is most likely a result of compaction of the denatured state in the presence of a crowding agent. Thus, we directly investigated how macromolecular crowding modulates the properties of the fully populated denatured state by measuring tryptophan accessibility using iodide quenching of Trp fluorescence and cysteine susceptibility to chemical modification. We obtained compelling experimental evidence that crowding leads to unfolded state compaction. Crowding significantly increased the urea concentration for half-maximal unfolding (C_m), although the effect on protein stability at zero urea concentration (ΔG°) was not significant, given the experimental uncertainty (i.e., <1.2 kcal/mol). In addition, our results from investigating urea-induced unfolding over a wide range of Ficoll concentrations also provide a clear picture of how crowding affects the unfolding rate of CRABP I. We observe significant crowding-induced retardation without significant change in urea dependence of unfolding rate, implying no appreciable change in the nature of the transition state. In this

work, we propose a correction for the concentration of small molecules to account for volume exclusion by the crowding agent. Application of this correction enabled a better dissection of the effect of crowding on the m -value and how unfolding kinetics changed with crowding agent concentration.

Materials and Methods

Chemicals. Ultrapure urea (>99% pure, fw = 60.06) was obtained from MP Biomedicals, LLC (Solon, OH). Ficoll 70 (fw = 74 kDa) was from Fluka Biochemika (Buchs, Sweden), and *O*-(2-maleimidoethyl)-*O'*-methyl-polyethylene glycol 5000 (Mal-PEG, fw = 5 kDa, $\geq 90\%$ purity (NMR)) was obtained from Fluka (Buchs, Switzerland). KI and KCl (certified ACS) were from Fisher Scientific Co. (Fair Lawn, NJ). Na₂S₂O₃ (99%) was from Aldrich (Milwaukee, WI). *N*-Acetyl-L-tryptophanamide (NATA) was from Sigma-Aldrich (St. Louis, MO). All other chemicals are reagent or molecular biology grade.

Protein. For expression and purification, we followed the published protocol³¹ with only minor changes. Instead of sonication, a microfluidizer with 16 k-atm of pressure was used to rupture the cells. Purified protein was lyophilized and stored at -20°C . CD spectroscopy and fluorescence emission scans were detected for each batch of purified protein. Proteins were expressed with a His-tag for purification, which was not removed; previous studies showed no influence of the His-tag on stability or folding/unfolding kinetics.³² Two CRABP I mutants were utilized in this study: (i) P85A, in which a stabilizing R131Q mutation has also been introduced, was used in all studies, and (ii) WT*, with only the R131Q mutation, was used only in unfolding kinetics studies. CD and fluorescence emission spectra show that there are no structural differences between the CRABP I variants.^{33,36}

Equilibrium Urea Denaturation. Urea denaturation was performed to determine m -value, ΔG° , and C_m . Urea samples for denaturation titrations were prepared by mixing urea stock (in Tris buffer: 10 mM Tris·HCl, pH 8.0, with temperature dependence taken into account) with Tris buffer in a series of specified ratios, with both having the same concentration of Ficoll 70. Protein was then added to each sample from a single stock in Tris buffer/Ficoll 70, to a final concentration of 0.8–6.4 μM , with thorough and gentle mixing. Urea, Ficoll solution, and protein stock were always made fresh for each measurement, and each had 2 mM tris(2-carboxyethyl)phosphine (TCEP) present as reductant. Urea denaturation samples were equilibrated in a closed incubator (to avoid water condensation) at 20 or 37 $^\circ\text{C}$ for a time determined to be sufficient to reach equilibrium at a given temperature (at least 8.5 and 5.5 h for 20 and 37 $^\circ\text{C}$, respectively), and then monitored by fluorescence emission at 350 ± 3 or 4 nm with excitation of 280 ± 2 nm using a PTI QM-1 fluorimeter with Peltier temperature control (Photon Technology International, New Brunswick, NJ) with emission scan (300–380 nm) also collected. A limited number of titrations were also monitored by far-UV CD signal (210–260 nm) to ensure that consistent results were obtained and thus to check the two-state behavior of the system. Background for urea or Ficoll solutions was always subtracted as blank. All samples were centrifuged before measurements.

Urea denaturation curves were analyzed using an equilibrium two-state model and assuming validity of linear extrapolation of unfolding free energy with respect to urea concentration.^{37,38} Global fitting was performed on multiple data sets (usually 3–4, and 6 for 37 $^\circ\text{C}$ /150 g/L Ficoll) to obtain parameter values and standard errors. Besides conventional fitting of the ΔG° and m -value together, we also fitted the C_m and m -value together to estimate the error of C_m ; otherwise there was no difference for the two kinds of fitting. Origin 8 software was used for regression analysis with the default “asymptotic symmetry” method used for standard error estimation.

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Iodide Quenching of Tryptophan Fluorescence. Dynamic quenching of tryptophan fluorescence by iodide was used to probe Trp accessibility.^{39,40} Quenching measurements were performed for a series of samples of increasing [KI] at 37 °C, basically as previously described.³⁹ To ensure constant ionic strength as the iodide concentration was increased, the total KI plus KCl concentration was held constant at 300 mM (330 mM also checked with no differences observed). Fresh KI and KCl stocks, in a solution of 8.0 M urea, 0 or 150 g/L Ficoll 70, with Tris buffer plus 2 mM TCEP, were added to protein or NATA stock dissolved in the same solution and incubated for 4 h. Final protein concentrations were 3–5 μ M, in the same range as used in urea denaturation experiments. A small amount of $S_2O_3^{2-}$ (1 mM) was added to minimize I_3^- formation. Fluorescence emission scans (300–380 nm) were collected using the PTI QM-1 fluorimeter with excitation at 295 ± 3 nm. Background signal from the same solutions without protein or NATA was subtracted.

Cysteine PEGylation. PEGylation, i.e., reaction with polyethylene glycol bis-maleimide (Mal-PEG), has been used to map topological accessibility of Cys residues in native proteins⁴¹ and was used in this study to probe Cys accessibility in the unfolded state. P85A CRABP I samples were unfolded in 8.1 M urea/Tris buffer with 0 or 150 g/L Ficoll 70 and 2 mM TCEP for over 4.5 h at 20 °C. For PEGylation, 2 μ L of Mal-PEG stock (50-fold excess over the total Cys concentration, given that each protein has three cysteines) was added to 200 μ L of unfolded protein solution (with protein concentration of 5 μ M), and the sample was thoroughly mixed. After 30 s of PEGylation reaction, 1 μ L of Cys-HCl was added at 40-fold excess of [Mal-PEG] to quench the reaction. After 2 min, the reaction solution was combined with 5X gel loading buffer and boiled for 7 min before running 10% SDS-PAGE. PEGylation time was tested in the presence or absence of Ficoll to ensure complete reaction.

Protein Unfolding Kinetics. Native protein stock was added to urea solutions (both in Tris buffer/Ficoll 70 with 2 mM TCEP) to a final protein concentration of 3–5 μ M to initiate unfolding at 20 or 37 °C. The unfolding traces were monitored by fluorescence emission at 350 ± 3 –4 nm with excitation of 280 ± 2 nm using the PTI QM-1 fluorimeter. Quartz cuvettes (750 μ L) equipped with a magnetic stirring bar were used to mitigate possible photobleaching effects due to slow diffusion in crowded solutions. An emission scan from 300 to 380 nm was collected at the end of each kinetic trace to confirm that the protein was completely unfolded. Single exponentials were found to provide good fits to the kinetic traces.

Results

Ficoll 70 Is an Inert, Nonperturbing Crowding Agent.

Consistent with a previous report that Ficoll 70 (74 kDa) is an inert crowding agent,¹⁰ we found no significant soft interaction of Ficoll with urea by vapor pressure osmometry (see Supporting Information and Figure S1), and we found the partial specific volume of Ficoll 70 to be independent of urea concentration (see Supporting Information). We saw no perturbation to the structure of CRABP I, native or unfolded, upon addition of crowding agent (see Supporting Information and Figure S2), in contrast to recent reports.^{19,27,42} Our characterization of unfolded-state properties was mostly performed at 150 g/L Ficoll, while our kinetics study spanned a wider range of Ficoll concentrations. The Ficoll concentrations studied clearly revealed trends from addition of

crowding agent and fell into the ranges of macromolecular crowding reported for various cellular environments.^{1–3,43}

Urea Concentration Correction. The effect on small molecule concentration of the substantial excluded volume in solutions containing high concentrations of macromolecular species has been widely neglected, with a few exceptions (for example, see refs 44–46). Comparing urea denaturation and urea-induced kinetic studies in the presence and absence of an inert crowding agent, we noticed that crowding agent is not the only factor that is varying between the two systems and that urea activity coefficient also varies at the same apparent urea concentration. An inert crowding agent increases the activity coefficient of any species by decreasing its available volume through pure steric repulsion ($\gamma_i = 1/f_{\text{available},i}$, where $f_{\text{available},i}$ is the fractional volume available to species i).^{8,47} Therefore, to be able to attribute observed thermodynamic/kinetic effects to crowding agent alone, we corrected the effect of the excluded volume of crowding agent on the urea activity coefficient. We propose a simple way to take into account this effect by subtracting the solvent-excluded volume of crowding agent (calculated using its partial specific volume) from the total solution volume to approximate the volume available to small molecules considering their negligible size. The activity coefficient, $1/f_{\text{available}}$, determined in this way is then used to correct the apparent urea concentration measured by refractive index⁴⁸ (we verified that this measurement is not affected by Ficoll). Unless specified, urea concentration in this paper denotes the corrected concentration ($[\text{urea}]_{\text{eff}}$).

Urea Denaturation of P85A CRABP I in Ficoll Follows a Reversible Two-State Transition. To be able to attribute any observed effects of macromolecular crowding to altered properties or shifts in population of the two end-states, folded or denatured, we needed to ensure that our conditions were consistent with a two-state model, i.e., that there were no complications due to protein aggregation or misfolding, both of which are likely to be enhanced under crowding conditions.^{25,29,30} CRABP I is a well-behaved protein that reversibly refolds in dilute solution without detectable equilibrium intermediates, although it visits kinetic intermediates upon refolding from urea.^{31–34,36} Under the conditions investigated (0, 75, and 150 g/L Ficoll, 0.8–6.4 μ M protein), two-state equilibrium behavior was confirmed by observation of a single transition in urea denaturation curves, monitored by intrinsic fluorescence at 350 nm and by CD at either 218 (dominated by backbone contributions) or 232.5 nm (dominated by aromatic packing) (Figure S3). Denaturation data from all three measurements are well fit by a two-state transition with, importantly, the same C_m and m -value. Folding reversibility under crowded conditions was demonstrated by the agreement of fluorescence emission spectra of protein unfolded and refolded to the same urea concentration (in the transition region) (Figure S3), confirming the absence of aggregation, which is consistent with the observation of no protein concentration dependence of urea denaturation. The single transition and reversibility justify the applicability of an equilibrium two-state model to analyze urea denaturation curves.

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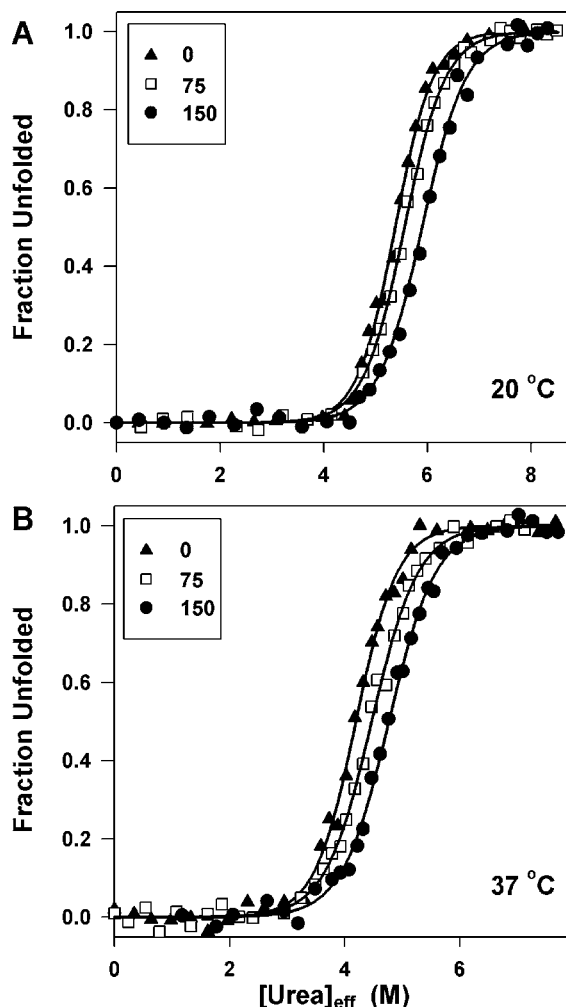


Figure 1. Representative urea denaturation curves of CRABP I in the presence of different amounts of crowding agent at 20 (A) and 37 °C (B). Fraction unfolded was determined by monitoring fluorescence at 350 nm. The symbols are the same for both panels: 0 (▲), 75 (□), and 150 (●) g/L Ficoll. The lines through the data are nonlinear fits to an equilibrium two-state model.

Ficoll Increases C_m and Decreases the m -Value. Urea denaturation of P85A CRABP I has been performed in 0, 75, 150 g/L Ficoll at either 20 or 37 °C (Figure 1). The data were analyzed in an unbiased way, and the resulting parameter values plus standard errors of m -value, C_m , and ΔG° from global fitting (with m -value and C_m or ΔG° shared, and parameters for native and unfolded state base lines floated for each data set) are reported in Table 1. Parameter values obtained from fitting individual curves at each condition yield mean values no different from the globally fitted parameter values and have significantly smaller standard deviations than the uncertainties obtained from global fitting (most are $\leq 50\%$ the latter), indicating high reproducibility. A few conclusions can be drawn from these data: First, as revealed by an increased C_m , crowding makes protein more resistant to urea denaturation, but the effect of crowding on protein stability (ΔG°) is less than the experimental uncertainties of our fits (≤ 1.2 kcal/mol). Second, we find that crowding reduces the m -value to a small but statistically significant extent (by 13–15% between 0 and 150 g/L Ficoll). Although an apparent decrease in m -value can arise from accumulation of intermediate in the transition zone, we find the evidence for two-state equilibrium unfolding of CRABP I in crowded solution (see above) to be strong. Finally, protein

stability decreases significantly as temperature increases from 20 to 37 °C whether in the presence of Ficoll or not, but the effect of Ficoll is comparable at either temperature. Correcting urea concentration for excluded volume enabled the trends in both m -values and unfolding kinetics to be observed. Figure S3A, as an example, compares a denaturation curve in the presence of crowding agent as a function of uncorrected [urea] with one plotted versus $[\text{urea}]_{\text{eff}}$ and shows that plotting against $[\text{urea}]_{\text{eff}}$ leads to a higher C_m and a smaller m -value with negligible difference in ΔG° .

Macromolecular Crowding Leads to a More Compact Ensemble for the Unfolded Protein. The m -value for chemical denaturation has been correlated with the change in solvent-accessible surface area (ΔASA) between the folded and unfolded states.⁴⁹ Subsequent studies have pointed out that the m -value is actually probing the change in solvent-exposed polar amide surface upon unfolding.^{50,51} With regard to a local bulk domain model interpretation of the m -value,⁵² we conclude that the proportionality between the m -value and the ΔASA remains the same in the presence of crowding agents, so long as the [denaturant] is corrected for the excluded volume of crowding agents (see Supporting Information). Thus, the reduced m -value observed in the presence of crowding agent suggests that crowding either caused the unfolded state to become more compact or caused the native state to become more expanded. The former is much more likely, based on our having observed no structural change in crowded conditions and on theoretical grounds^{7,53} and other reports.⁵⁴

Thus, we directly characterized the unfolded state for its compaction in two ways: First, we investigated how crowding affects accessibility of tryptophan residues by assessing their susceptibility to iodide quenching. In contrast to some previous work,^{27,28} we were characterizing a fully populated unfolded state with or without crowding agent. Parallel measurements of iodide quenching of unfolded protein in 8.0 M urea in 0 or 150 g/L Ficoll were carried out at 37 °C, with NATA as a control for fully accessible tryptophan. The Stern–Volmer plots (Figure S4) of both protein and NATA are linear, consistent with a dynamic quenching mechanism over the concentrations of iodide used.^{39,40,55} To determine the accessibility of Trp's, we used a modified Stern–Volmer law,⁵⁵ according to which, if there are n accessible fluorophores with the same value of Stern–Volmer constant (K_Q) and the rest are inaccessible, the following relation exists between the fluorescence in the absence (F_0) or presence of quencher (F), the molar quencher concentration (X), and the fractional fluorescence arising from accessible fluorophores (f_a):

$$F_0/(F_0 - F) = 1/(f_a K_Q X) + 1/f_a \quad (1)$$

Based on eq 1, a plot of $F_0/(F_0 - F)$ vs $1/X$ yields a straight line with intercept $1/f_a$. If each exposed fluorophore has a different K_Q or if there is static quenching, the plot of $F_0/(F_0 -$

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Table 1. Effect of Macromolecular Crowding on Folding Energetics of P85A CRABP I^a

Ficoll 70 (g/L)	20 °C			37 °C		
	<i>m</i> -value (cal/(mol·M))	<i>C</i> _m (M)	Δ <i>G</i> ^o (cal/mol)	<i>m</i> -value (cal/(mol·M))	<i>C</i> _m (M)	Δ <i>G</i> ^o (cal/mol)
0	1568 ± 109	5.36 ± 0.02	8401 ± 580	1698 ± 127	4.27 ± 0.02	7252 ± 537
75	1448 ± 143	5.61 ± 0.03	8123 ± 791	1551 ± 92	4.48 ± 0.02	6951 ± 412
150	1326 ± 118	5.99 ± 0.04	7942 ± 681	1477 ± 90	4.81 ± 0.02	7100 ± 431

^a The parameter values plus the standard errors at each condition are the result of a global fitting of independent measurements (three or four data sets in general, and six for 37 °C/150 g/L Ficoll).

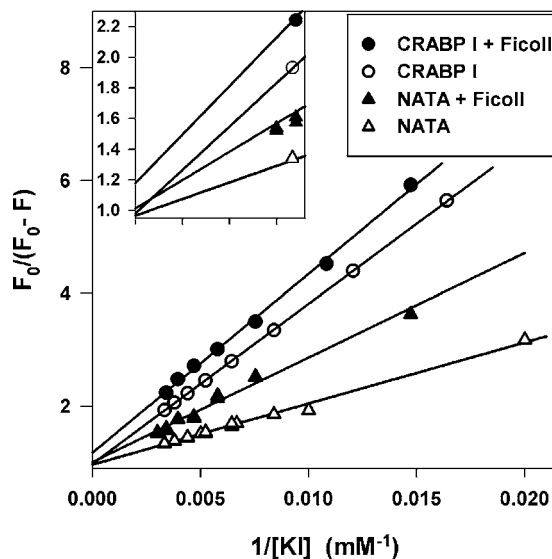


Figure 2. Representative modified Stern–Volmer plots for unfolded CRABP I at 37 °C in 150 (●) and 0 (○) g/L Ficoll and NATA (in 8.0 M urea) in 150 (▲) and 0 (△) g/L Ficoll. Lines are fits to eq 1. The inset is the plot of the region near the intercept.

F) vs *1/X* will show curvature. The intercept, $1/f_a$, for NATA is 1 in the presence or absence of Ficoll, as anticipated for a fully accessible Trp. Our analysis of iodide quenching data for the unfolded protein gives a linear plot (Figure 2), suggesting that the three Trp residues are indistinguishable in terms of iodide quenching. The intercept value of 1.00 ± 0.02 (the standard deviation among three independent measurements) for unfolded CRABP I in dilute solution reveals full accessibility of the three fluorophores to iodide in the absence of macromolecular crowding. The intercept value of 1.16 ± 0.02 for unfolded protein in 150 g/L Ficoll indicates a reduction of 14% ($f_a = 86\%$) in Trp accessibility to iodide quencher and, therefore, a more compact unfolded state in the presence of crowding agent.

The second method we used to test the possible compaction of the unfolded state in the presence of macromolecular crowding was assessing cysteine side-chain accessibility by modification with PEG-maleimide, which reacts with exposed cysteine sulfhydryls and adds approximately 5 kDa to the molecular weight of the modified protein per PEG group transferred. At a urea concentration of 8.1 M and 20 °C, 100% of the CRABP population is unfolded in the presence or absence of 150 g/L Ficoll according to our equilibrium urea denaturation data. CRABP has three Cys residues, all of which are sequestered in the native state and, as shown in Figure 3, readily PEGylated in the unfolded state in the absence of crowding agent. Upon addition of 150 g/L Ficoll, the band for un-PEGylated protein is significantly more intense and bands for PEGylated protein (whether adding one, two, or three PEG groups) are weaker. In other words, the Cys residues are significantly less accessible to PEGylation in the presence of

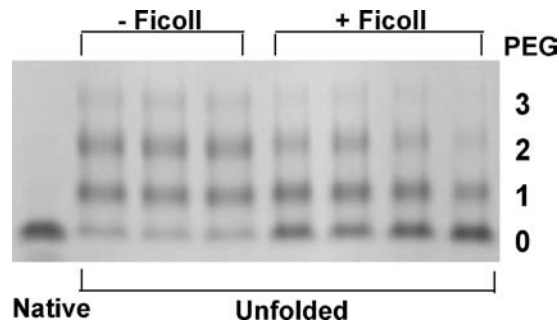


Figure 3. Extent of PEGylation of unfolded CRABP I in 0 or 150 g/L Ficoll at 20 °C. Native protein was used as a control since all three cysteines are inaccessible in the native state in 0 or 150 g/L Ficoll. The number of PEGylated residues is indicated at right.

macromolecular crowding agent, again providing direct support for a more compact unfolded state. Quantification of the gel bands from several independent reactions in dilute (11) and crowded (14) solutions gives an average $18 \pm 3\%$ decrease in PEGylation due to macromolecular crowding. The different extents of PEGylation are not due to crowding effects on PEGylation kinetics (see Supporting Information and Figure S5).

Macromolecular Crowding Substantially Retards CRABP Unfolding. Unfolding kinetics were monitored at urea concentrations where the protein would be fully unfolded at equilibrium (see Figure S6 for a representative raw unfolding trace). We examined unfolding kinetics for CRABP I (WT*) in 0, 150, 200, 300, and 350 g/L Ficoll at 37 °C (Figure 4), and to ensure that observed kinetics were not affected by a slow cis–trans isomerization around the Leu84–Pro85 peptide bond previously found to contribute a minor phase to folding kinetics,³⁶ we also measured kinetics for P85A CRABP I in 0, 75, 150 g/L Ficoll at 20 and 37 °C and saw no appreciable differences in the trends (Figure 5). We report here a distinct retarding effect on protein unfolding rate by crowding agent, and we are able to assess this kinetic crowding effect as a function of crowding agent concentration. For both variants of CRABP I, the urea dependence of unfolding rate (from which the m_u -value is derived from the slope of $\ln(\text{unfolding rate})$ as a function of [urea]) does not change significantly with crowding agent concentration, arguing that macromolecular crowding does not appreciably alter the nature of the transition-state ensemble. Again, we observed a marked impact of urea concentration correction on unfolding kinetics under crowding conditions. When plotted as a function of apparent [urea], the unfolding kinetics show no systematic trend as Ficoll concentration increases. Some rates are observed to be accelerated, contrary to predictions based on excluded volume effects, at and below 200 g/L Ficoll. However, when the unfolding rate is plotted as a function of $[\text{urea}]_{\text{eff}}$, a clear

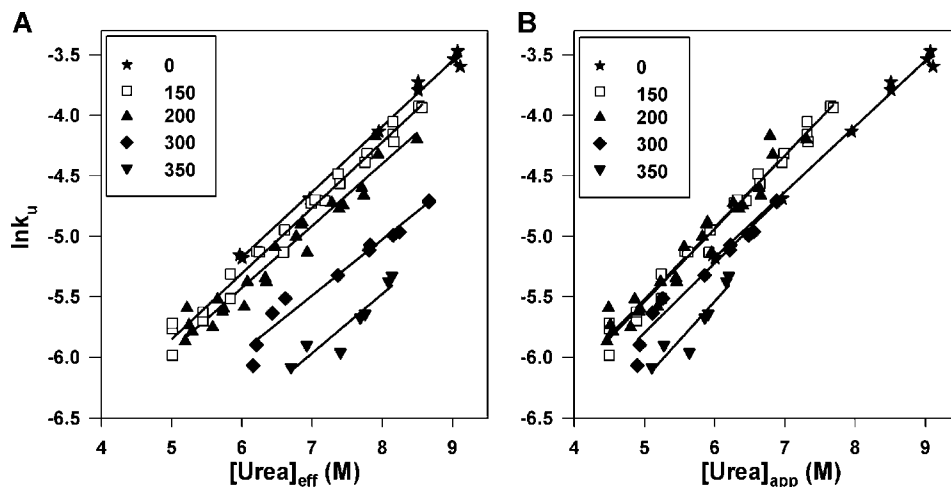


Figure 4. Unfolding rate of WT* CRABP I at 37 °C plotted as a function of corrected urea concentration, $[\text{urea}]_{\text{eff}}$ (A), and of apparent urea concentration, $[\text{urea}]_{\text{app}}$ (B), in 0 (★), 150 (□), 200 (▲), 300 (◆), and 350 (▼) g/L Ficoll. Straight-line fits of the data are shown.

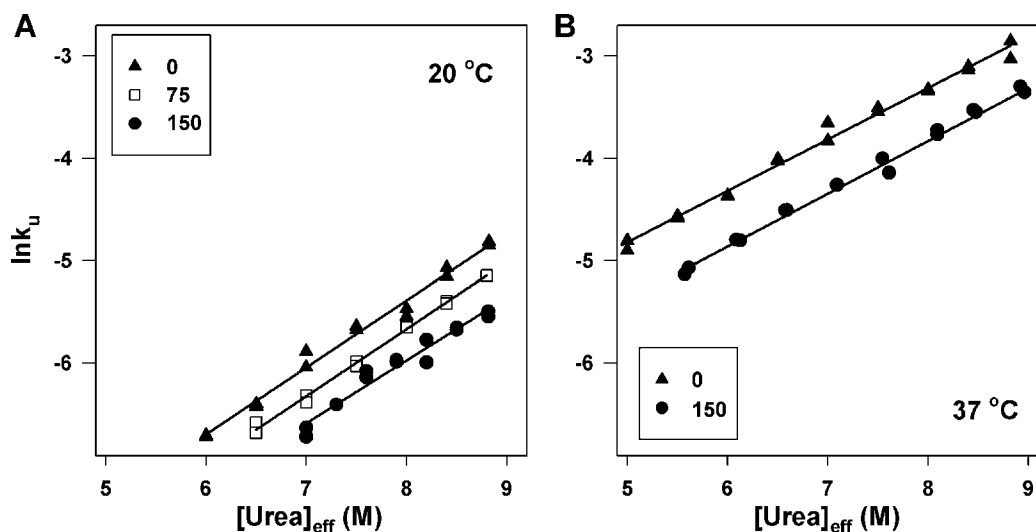


Figure 5. Unfolding rate of P85A CRABP I as a function of $[\text{urea}]_{\text{eff}}$ at 20 (A) and 37 °C (B) in 0 (▲), 75 (□), and 150 (●) g/L Ficoll. Straight-line fits of the data are shown.

trend emerges, with the rate becoming smaller with increased concentration of crowding agent (Figure 4).

Discussion

In this study, we interrogated the folding landscape of the β -rich monomeric protein, CRABP I, in the presence of an inert macromolecular crowding agent and found that it refolds reversibly by a two-state equilibrium process and suffers no competing aggregation under the conditions used. Therefore, observed effects of macromolecular crowding can be attributed to the altered populations or properties of the two end states (native and denatured). We found that the change in CRABP I stability from crowding was less than the error limits of our experiments (viz., 1.2 kcal/mol) for all Ficoll concentrations tested at either 25 or 37 °C. We observed a significant upward shift in the half-maximal urea concentration for unfolding (C_m) upon addition of crowding agent and conclude that it arose primarily from a crowding-induced decrease in the urea dependence of the equilibrium constant for denaturation (the m -value), arguing for a change in the nature of the unfolded ensemble in the presence of crowding agent. Our direct characterization of a fully populated denatured state, as assured

by our equilibrium urea denaturation data, showed that the crowding agent causes a significant ($\sim 15\%$) compaction.

Our data point to a nonspecific and random compaction due to crowding, because the extent of compaction deduced from the reduction in solvent-exposed polar amide surface (13–15%) (based on the m -value decrease), the decrease in Trp accessibility (14%) (from iodide quenching), and the lowered Cys accessibility (18%) (from PEGylation) due to 150 g/L Ficoll is similar. This may account for the lack of change detected in CD or fluorescence spectra. To understand the meaning of this change in solvent-accessible surface, we estimated the magnitude of the reduction in polar amide surface area under crowding condition. Using the proportionality of m -value/ $\Delta\text{ASA}_{\text{polar amide}}$ ⁵¹ and neglecting its temperature effect, the observed m -value decrease from 0 to 150 g/L Ficoll of 242 or 221 cal/(mol·M) (at 20 and 37 °C, respectively) corresponds to 291 or 267 Å² of polar amide surface, about the size of 11 or 12 backbone groups.⁵¹ The observation of a more compact unfolded state in the presence of a crowding agent is consistent with predictions by Minton.¹² We suspect that comparable previous experiments have not revealed this crowding effect on the unfolded state either because they were confounded by the coexistence of

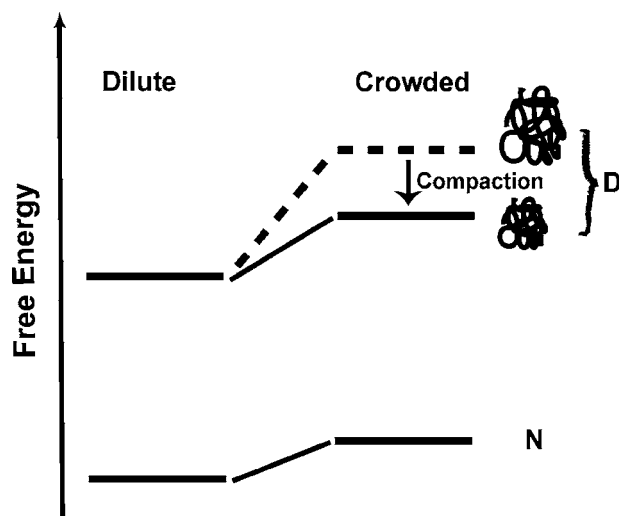


Figure 6. Crowding favors a more compact denatured state (D), which leads to a smaller difference in free energy between native (N) and D state in crowded solution than one would expect if a more expanded unfolded state (indicated as dashed line) were present in the crowded solution.

intermediate and unfolded states, or by the formation of aggregates under crowded conditions,²⁷ or because they were not designed to provide results quantitative enough to measure this small but significant effect.²⁸ Compaction of an unfolded state ensemble, reflected by a change in the m -value of two-state equilibrium unfolding, has been previously reported; in this case, the compaction occurred in response to a pH change.^{56,57}

Compaction of the unfolded state in the presence of macromolecular crowding agents has an important general thermodynamic consequence, as illustrated by Figure 6. Due to their large excluded volume effect, macromolecular crowding agents increase the free energy of all macromolecular solutes; the larger the molecule, the larger the effect.^{4,58} The differential increase in the free energy of native and unfolded states determines how crowding affects the stability of the native relative to the unfolded state. A more compact unfolded state will suffer a smaller free energy increase due to the excluded volume effect than a more expanded unfolded state. Thus, our data showing compaction of the unfolded state argue for a smaller free energy difference between the unfolded and native states in the presence of Ficoll than one would expect if a more expanded unfolded state were present in the crowded solution. In this argument, we are neglecting the energy effect due to chain entropy reduction in a more compact unfolded state, which we believe will be small, but unfavorable, given the relatively modest extent of chain compaction.

We observed a distinct retarding effect of crowding agents on the unfolding rate of CRABP I. The retardation cannot be explained simply by elevation of the activation energy due to the differential increase in the free energy of the transition state relative to the native state. For example, the decrease in unfolding rate of P85A by 150 g/L Ficoll at 20 and 37 °C corresponds to an activation energy increase of 330 cal/mol. Using scaled particle theory, modeling all macromolecules as

hard spheres,⁵⁸ and using a radius of 2 nm for the native protein (estimated from the crystal structure⁵⁹) and 5.5 nm for Ficoll,⁴⁶ we found that, in order to increase activation energy by ~ 330 cal/mol, the transition state must be expanded in radius by 2.5-fold, in which case the transition state would be nearly as expanded as a fully unfolded state.⁶⁰ This interpretation is inconsistent with the small ratio of m_u to m (the Tanford β value^{61,62}) for P85A CRABP I (0.24 at 20 °C or 0.18 for 37 °C), which argues for a quite native-like transition state. Therefore, there must be other factors affecting unfolding rate. One possibility is a viscosity effect that acts on the pre-exponential factor.⁶³ Protein unfolding is likely to be accompanied by a large local viscosity change due to collision with the macromolecular crowding agent during polypeptide chain rearrangements involved in the unfolding process. The lack of change in the m_u -value in the presence of crowding agent implies that crowding has little effect on the compaction of the transition-state ensemble, consistent with the observation of a native-like transition state. The lack of change in m_u -value and decrease in m -value imply a reduced urea concentration dependence of the overall refolding rate in Ficoll solution.

We observed that crowding agent leads to an increase in C_m , which together with the decreased m -value indicates that the protein becomes more resistant to urea denaturation in crowded solution. We are limited by experimental error in our efforts to determine the effect of crowding on stability and can conclude only that the crowding-induced stability change is <1 – 1.2 kcal/mol. Indeed, the macromolecular crowding effect on the stability of CRABP I is not expected to be large, given the small size of CRABP I relative to Ficoll 70 (ratio of molecular weights approximately 1:4)¹² and the compaction of the unfolded state (see above). Scaled particle theory provides an estimate of the upper bound for the stability effect of crowding, based on modeling all macromolecules in all states as hard spheres.⁵⁸ Using a radius of 2 nm for the native protein, 5 nm for the unfolded protein (assuming a 2.5-fold increase with respect to native⁶⁰), and 5.5 nm for Ficoll,⁴⁶ we estimate a maximal increase in stability of 326 (345) cal/mol at 20 (37) °C from addition of 150 g/L Ficoll. Consistent with this work, other studies involving proteins that are small relative to the crowding agent have also reported only modest stabilizing effects at similar concentration of crowding agent.^{16,18} When the protein and crowding agent are comparable in size, the effect of crowding on protein stability (ΔG°) is expected to be significantly larger and the stabilizing effect to increase greatly as crowding agent concentration increases.¹²

The observation that crowding makes the unfolded state more compact represents one way that a protein's folding landscape in the crowded intracellular environment is markedly different from that sampled in dilute solution in the test tube. Interestingly, the retarding effect on unfolding kinetics by crowding agent suggests that macromolecular crowding alone can make a protein kinetically more stable. Moreover, our data say that crowding reduces the response of a protein to destabilizing conditions such as chemical denaturation. However, revisiting our urea denaturation experiments using FIAsH-labeled CRABP

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in *E. coli* cells, where the urea melting isotherm was characterized by a significantly lower C_m and a higher m -value than the dilute solution isotherm,⁶ we conclude that factors apart from macromolecular crowding, such as chaperone binding, heterogeneity of the cellular environment, weak protein–protein interactions, physiological responses to urea addition, different salt concentrations, and so on, must cause the markedly different urea melt. In addition, we saw more rapid unfolding kinetics and equilibration times *in vivo* than *in vitro*, which is the opposite of our observations in the presence of inert macromolecular crowding agents. We are currently pursuing the other factors that contribute to the complexity of the *in vivo* folding process and how they may remodel the energy landscape.

Conclusion

We provide here compelling experimental evidence that macromolecular crowding makes the unfolded state of a protein more compact. As discussed, such compaction has potential influence on protein folding energetics and reduces the response of protein to chemical denaturation. We also provide strong evidence that crowding retards protein unfolding but without changing the urea dependence of unfolding rate, implying little change in the nature of unfolding transition state. Finally, these

observations do not account for in-cell urea denaturation studies we have carried out, emphasizing the complexity and multitude of factors that influence folding in the cell.

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Supporting Information Available: Discussion of VPO determination of soft interaction of urea with Ficoll 70; CD and fluorescence data showing that Ficoll 70 does not perturb the structure of CRABP I; justification that the proportionality between m -value and the change of water-accessible surface area (Δ ASA) upon protein unfolding is the same in the presence or absence of crowding agent when using corrected urea molar concentration; control experimental data showing that reduced PEGylation in crowded solution is not due to a crowding effect on PEGylation kinetics; determination of partial specific volume of Ficoll 70; and Figures S1–S6. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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