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Macromolecular crowding increases structural content of folded proteins

Michael Perham[a,1], Loren Stagg[b,1], Pernilla Wittung-Stafshede[a,b,c,*]

[a] Department of Chemistry, Rice University, 6100 Main Street, Houston, TX 77251, United States
[b] Department of Biochemistry and Cell Biology, Rice University, 6100 Main Street, Houston, TX 77251, United States
[c] Keck Center for Structural Computational Biology, Rice University, 6100 Main Street, Houston, TX 77251, United States

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Abstract **Here we show that increased amount of secondary structure is acquired in the folded states of two structurally-different proteins (α-helical VlsE and α/β flavodoxin) in the presence of macromolecular crowding agents. The structural content of flavodoxin and VlsE is enhanced by 33% and 70%, respectively, in 400 mg/ml Ficoll 70 (pH 7, 20 °C) and correlates with higher protein-thermal stability. In the same Ficoll range, there are only small effects on the unfolded-state structures of the proteins. This is the first *in vitro* assessment of crowding effects on the *native-state* structures at physiological conditions. Our findings imply that for proteins with low intrinsic stability, the functional structures *in vivo* may differ from those observed in dilute buffers.**

Keywords: Macromolecular crowding; Excluded volume effect; Circular dichroism; Secondary structure

1. Introduction

The intracellular environment is crowded due to the presence of high concentrations of macromolecules including proteins, nucleic acids, ribosomes, and carbohydrates [1]. 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 [2,3] corresponding to a volume occupancy of up to 40%. Due to excluded volume effects, any reaction that amplifies the available volume will be stimulated by macromolecular crowding [4–7]. It is proposed that the major result of macromolecular crowding, or space confinement, is a stabilizing effect on the folded protein that originates in lowered entropy of the unfolded state because of polypeptide compaction. Experimental and theoretical work has demonstrated large effects of crowding on the thermodynamics and kinetics of many biological processes *in vitro*, including protein binding, folding, and aggregation [2,8–11].

Crowded conditions can be created experimentally by adding inert synthetic or natural macromolecules, termed crowding agents, to the systems *in vitro*. Experimental studies of crowding effects on folding have mostly involved large, complex proteins and often at extreme solvent conditions (such as acidic pH) [2,8,12,13]. A few studies have focused on the ability of crowding agents to induce conformational changes in unfolded states of proteins. For example, unfolded cytochrome *c* was found to adopt a molten globule state in crowding agents at low pH, [14] and two intrinsically unstructured proteins (FlgM and a variant of RNase T1) were discovered to fold in crowded conditions [15,16]. No *in vitro* studies have focused on the effects of macromolecular crowding agents on the folded structures of small, single-domain proteins at physiological conditions.

To address this issue, here we investigate the native- and unfolded-state structures of two model proteins (*Borrelia burgdorferi* VlsE and *Desulfovibrio desulfuricans* flavodoxin) as a function of macromolecular crowding *in vitro* (*i.e.*, using synthetic crowding agents). The two proteins were strategically selected to include contrasting folds and sizes: VlsE (341 residues) has 50% α-helices and the rest is mostly unstructured loops [17,18], whereas flavodoxin (148 residues) has a mixed α/β topology with a flavin mononucleotide (FMN) cofactor (Fig. 1) [19]. Both proteins have been characterized previously in our laboratory in terms of chemical and thermal unfolding behaviors in dilute solutions [17,20,21]; both proteins have rather low thermal (T_m of ~50 °C; pH 7) and thermodynamic (ΔG_U of 15–20 kJ/mol; pH 7, 20 °C) stabilities. Ficoll 70 (*i.e.*, a highly branched copolymer of sucrose and epichlorohydrin building blocks) and dextran 70 (*i.e.*, a flexible long-chain poly(D-glucose) with sparse and short branches) are polysaccharides that are inert, polar and do not interact with proteins. Ficoll behaves like a semi-rigid sphere (radius ~55 Å) whereas dextran is best modeled as a rod-like particle [22,23]. Both polymers are attractive mimics of macromolecules that may be present in the biological setting where proteins normally fold.

2. Materials and methods

Proteins were prepared as described [17,21]. Ficoll 70, dextran 70, polyethylene glycol (PEG1,450), GuHCl and urea (Sigma) were of highest purity; special care was taken to mix all solutions due to the high viscosity of some samples. Equilibration times (at 20 °C) were checked before measurements; 2–16 h were sometimes needed. Aggregation was not detected in these mixtures as assessed by manual inspection and by light scattering at 300 nm. Far-UV CD (Jasco J-810 instrument) was measured in a 1-mm cell (200–300 nm); 10 mM HEPES or phosphate buffer, pH 7, with/without GuHCl/urea as indicated. In most experiments, 20 μM flavodoxin or 10 μM VlsE was

*Corresponding author. Address: Department of Chemistry, Rice University, 6100 Main Street, Houston, TX 77251, United States.
E-mail address: pernilla@rice.edu (P. Wittung-Stafshede).

[1]These authors contributed equally to this work.

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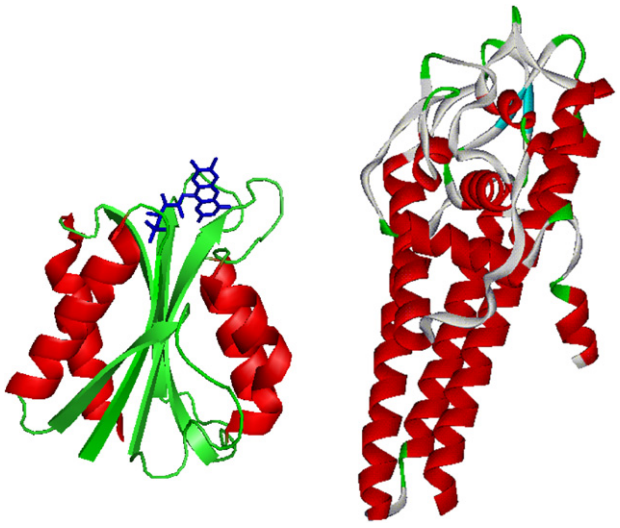

Fig. 1. Models of *Desulfovibrio vulgaris* flavodoxin (a. *D. desulfuricans* homolog, left) and *Borrelia burgdorferi* VlsE (right) based on the crystal structures 2fx2 and 1L8W. The FMN cofactor in flavodoxin is shown in stick; red corresponds to α-helices, green represents β-strands in flavodoxin and turns in VlsE.

used. Thermal unfolding experiments were probed by CD at 220 nm (20–95 °C; scan rate 1 deg/min) for samples of protein mixed with different fixed concentrations of Ficoll 70 or dextran 70 (in the range 0–400 mg/ml). Varying the scan rate between 0.5 and 2.5 deg/min did not change the profiles. The thermal unfolding reactions were not fully reversible: for both proteins, only 50–70% (depending on buffer conditions and amount of crowding agent) of the starting CD signal was recovered upon cooling. Thermal irreversibility has been noted earlier for flavodoxin [21].

3. Results

In Fig. 2A, we show the far-UV circular dichroism (CD) spectra of native flavodoxin in the presence of increasing amounts of Ficoll 70 (0–400 mg/ml; pH 7.0, 20 °C). It is clear that the negative far-UV CD signal grows larger, suggesting gain of secondary structure as a function of crowding. The negative signal at 220 nm increases by 24% in 200 mg/ml and by 33% at 400 mg/ml Ficoll 70. By contrast, in both 3 and 4 M GuHCl, denaturant conditions known to cause complete flavodoxin unfolding [20,21], there is no effect by the same Ficoll 70 additions (Fig. 2B). Thermally unfolded flavodoxin (at 95 °C, pH 7) displays minor changes in CD signal as a function of Ficoll 70 concentration albeit the spectral shape remains characteristic of that of unfolded polypeptides (Fig. 2C). We note that there was no contribution to the CD signal from the Ficoll 70 itself and no protein aggregation occurred in any of the solutions.

Similar effects on the folded (Table 1) and unfolded (not shown) states were obtained when dextran 70 was incubated with flavodoxin. Moreover, when the same Ficoll and dextran experiments were repeated with folded and unfolded forms of apoflavodoxin (*i.e.*, no FMN), again, the same trends were observed (not shown). Secondary structure estimations (using SOMCD neural network algorithm), based on the CD spectra

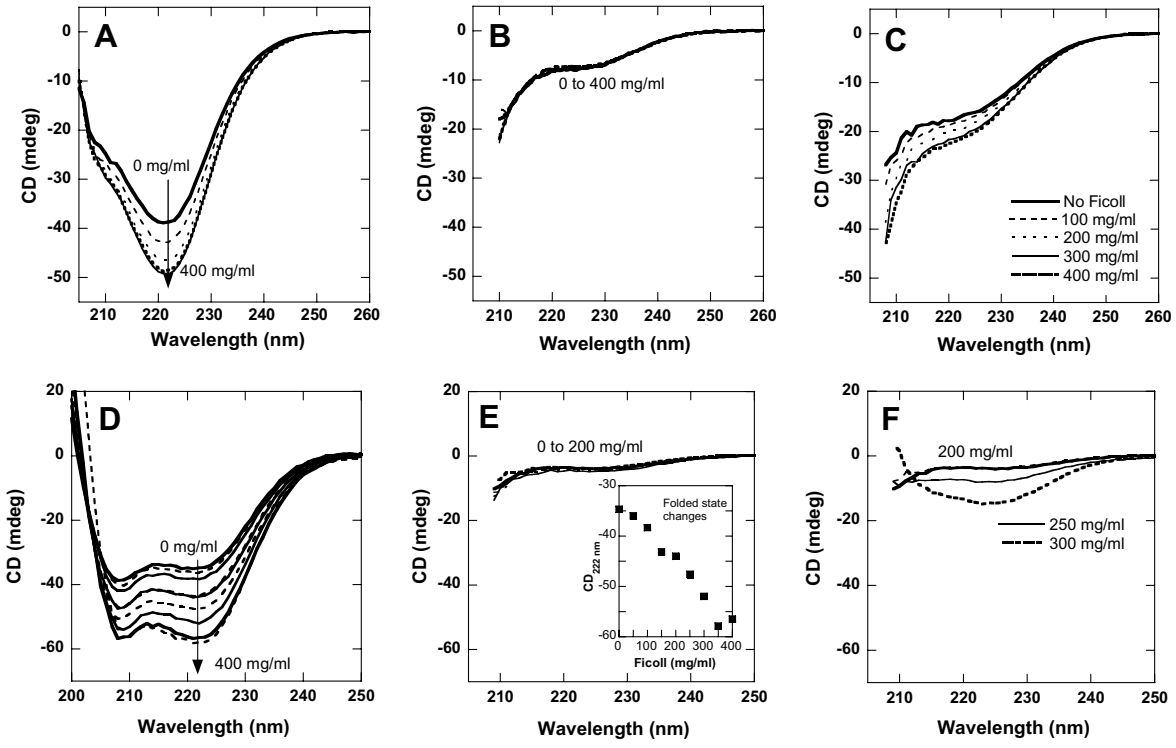

Fig. 2. Far-UV CD of (A) folded (pH 7, 20 °C), (B) chemically-unfolded (3 M GuHCl, 20 °C) and, (C) thermally-unfolded (95 °C) flavodoxin in the presence of various amounts of Ficoll 70 (0–400 mg/ml). Far-UV CD of (D) folded (pH 7, 20 °C) VlsE in the presence of various amounts of Ficoll 70 (0–400 mg/ml, 50 mg/ml increments) and, (E and F) chemically-unfolded (2.5 M urea, 20 °C) VlsE in the presence of various amounts of Ficoll 70 as indicated. Inset in E shows CD at 222 nm versus Ficoll 70 concentration for folded VlsE (*i.e.*, based on the spectra in D).

Table 1
Effects of different macromolecular crowding agents on the native-state structures of flavodoxin and VlsE (buffer, pH 7, 20 °C) as measured by far-UV CD

Protein	Agent	% change
Flavodoxin	Ficoll 70	24 ± 4
	Dextran 70	10 ± 3
	PEG 1,450	5 ± 2
	Glycerol	0 ± 3
VlsE	Ficoll 70	30 ± 3
	Dextran 70	33 ± 3
	PEG 1,450	20 ± 4
	Glycerol	0 ± 2

The percentages represent increases in negative CD signal at 220 nm (*i.e.*, 100% *CD(crowder)/CD(buffer) – 100%) when 200 mg/ml of crowding agent was added. The small osmolyte, glycerol, (known to stabilize proteins) was also tested as a control. Note that for Ficoll 70, the changes are found to be higher in 400 mg/ml (see text and Fig. 2A, D).

for folded flavodoxin at different conditions, revealed that the helical content increased while the random coil content decreased, with no change in β-sheet content, when going from 0 to 400 mg/ml Ficoll 70 (in buffer pH 7, 20 °C).

To test the generality of the observation, we performed the same experiments with another, structurally-different protein: the α-helical VlsE. Based on the increased negative far-UV CD signal, there is a dramatic build up of helical content in the native state of VlsE upon additions of Ficoll 70 (Fig. 2D). The negative CD signal at 220 nm increases approximately linearly with Ficoll concentration up to 400 mg/ml (Inset, Fig. 2E). Structural estimates using the SOMCD algorithm disclosed that the helix content rises from 52% to 80% when going from 0 to 400 mg/ml Ficoll 70 (in buffer pH 7, 20 °C). Again, at denaturant conditions causing complete VlsE unfolding (2.5 M urea, pH 7, 20 °C) [17], there is no effect on the CD spectra for Ficoll 70 additions up to 200 mg/ml (Fig. 2E). However, at 250 and 300 mg/ml Ficoll 70, a distinct non-native form of VlsE appears (Fig. 2F) exhibiting a negative far-UV CD peak centered at 220–225 nm, in agreement with β-sheet/turn content [24]. Interestingly, this species resembles a non-native state of VlsE detected in high concentrations of alcohols [25]. We previously proposed that this conformation may be biologically significant as alcohols mimic dielectric properties of the environment near membranes. Like in the case of flavodoxin, dextran 70 had similar effects on the native-state conformation of VlsE as Ficoll 70 (Table 1).

Since excluded-volume theory predicts that protein stability is enhanced by crowding, we tested if the structural effects observed on the native states correlated with increased thermal stability. In Fig. 3 we show thermal profiles, as monitored by far-UV CD, for flavodoxin as a function of increasing amounts of Ficoll 70 (A) and dextran 70 (B). As expected, both macromolecular crowding agents have stabilizing effects on flavodoxin. The thermal midpoint, T_m, increases gradually from 48 °C with no crowder (pH 7, HEPES buffer) to 64 °C in 400 mg/ml of Ficoll 70, and to 70 °C in 400 mg/ml of dextran 70 (pH 7). Analogously VlsE was found to gradually shift its thermal midpoint from 49 to 55 °C when the Ficoll 70 concentration was increased stepwise from 0 to 400 mg/ml (Fig. 3C). We note that the thermal transitions were only partially reversible (probed by the return of the native-like CD signal).

4. Discussion

This is the first assessment of protein native-state structural effects by macromolecular crowding in solution at physiological pH. Surprisingly, we discovered that for two unrelated single-domain proteins (Fig. 1), their folded states become more ordered, in terms of secondary-structure content, in the presence of crowding agents (*i.e.*, spherical Ficoll 70 and rod-shaped dextran 70) (Fig. 2; Table 1). These effects on the native-state conformations correlate with increased thermal stability in both proteins (Fig. 3). Our findings imply that the crowded cellular environment can have conformational effects on the *folded* states of proteins. Since the same results are obtained for two unrelated proteins with different folds, we propose that this may be a general phenomenon for small, single-domain proteins *in vivo*. In support of this idea, an increased structural content in the native state of a third, unrelated protein, *Pseudomonas aeruginosa* azurin was also detected (not shown). It is possible that observed differences between NMR-solution and crystallographic structures of proteins [26,27] are due to macromolecular crowding effects (due to the close packing of identical proteins) in the crystals. Clearly, there are parallels between high-volume occupancy

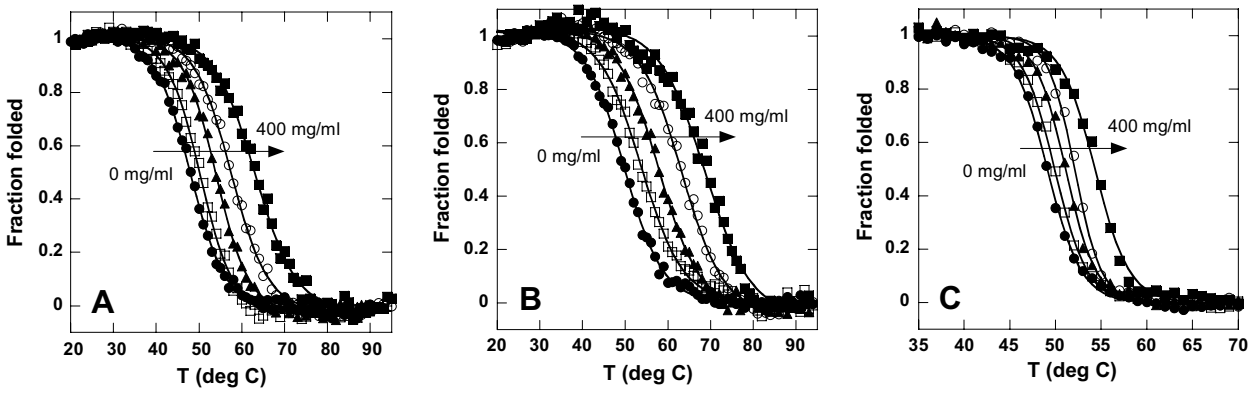

Fig. 3. Thermal profiles for flavodoxin (CD at 220 nm) as a function of Ficoll 70 (A) and dextran 70 (B) amounts (pH 7, 10 mM HEPES). (C) Thermal profiles for VlsE as a function of Ficoll 70 amounts (pH 7, 20 mM phosphate). Filled circles, no crowder; open squares, 100 mg/ml; filled triangles, 200 mg/ml; open circles, 400 mg/ml; filled squares, 400 mg/ml of crowder. Solid lines are two-state fits to the data points. In accord with a two-state mechanism for VlsE, CD-detected transitions match fluorescence-detected curves at each condition (fluorescence data shown).

of macromolecular-crowding agents in solution and close packing of proteins in crystalline states.

Since Ficoll 70 and dextran 70 are both sugar-based polymers, we also tested the effect of a chemically-different crowding agent, *i.e.*, polyethylene glycol (PEG 1,450). PEG is best modeled as a spherical particle. We found that the native-state structures of flavodoxin and VlsE were similarly perturbed in the presence of PEG, as in the presence of Ficoll and dextran (Table 1). However, the extent of the change (as measured by CD) was less with PEG. This may be due to the smaller size of the PEG variant we used as compared to the sugar-based polymers (MW of 1450 for PEG versus 70,000 for Ficoll and dextran). The excluded-volume theory implies that macromolecular crowding affects the unfolded states of proteins, thereby indirectly making the folded states more stable [4–7]. Although this theory states that changes in protein stability are due mostly to unfavorable effects on the unfolded-state ensemble, direct effects on the folded state is not excluded [28,29]. Thus, our findings are not in direct disparity with excluded-volume theory but they clearly emphasize that structural perturbations on the native state should be given deeper consideration in future experiments and theoretical models.

Analogously to our results presented here, upon encapsulation in a silica gel with hydrophobic modifications, apo-myoglobin acquired a higher helical content than that found for the folded state in solution [30]. In earlier studies of unfolded apo-myoglobin in different sol–gel systems, it was proposed that its ability to fold was influenced by the properties of confined water molecules in the silica pores [31,32]. The effect of water on hydrophobic and ionic interactions in small molecules confined in water droplets [33] as well as on protein-conformational changes in confined spaces [34,35] have recently been addressed. We speculate that interactions with confined water molecules, in combination with other factors, play a role in the formation of the non-native VlsE species observed in high amounts of Ficoll at denaturing conditions (Fig. 2F). We note that VlsE was found as a dimer in the crystal structure and at some solution conditions [18,36]; therefore, we probed the possibility of VlsE dimers at our crowded conditions *in vitro*. Based on cross-linking experiments, we conclude that a fraction (20–30%) of VlsE dimers may exist at high Ficoll 70 amounts (*i.e.*, 300–400 mg/ml) whereas only monomers (>95%) are detected in buffer and at low Ficoll amounts (*i.e.*, 100–200 mg/ml). Since the dimer and monomer forms of VlsE have about the same amount of α-helices [18,36], dimer formation does not explain the increased structural content in the presence of crowding agents.

We propose that crowded conditions cause enrichment of a subset of protein structures, within the folded ensemble of species, which are most ordered. Extension of α-helices may be the dominant consequence of macromolecular crowding as the random coil-to-helix process can be viewed as a simple one-dimensional phase transition [37]. This agrees with our observations of non-cooperative, gradual changes in protein structure as a function of Ficoll 70 concentration (*e.g.* inset, Fig. 2E). As β-sheets involve long-range interactions, their formation will require cooperativity and spatial precision. Notably, all proteins studied here, and previous ones found to fold in crowded conditions [15,16,30], contain some helical elements. In addition, for both VlsE and flavodoxin, the structural effects found in Ficoll/dextran correspond to increases in α-helix, and not β-sheet, content as based on CD spectra predictions.

In an attempt to correlate the native-state structural effects to thermodynamics, the thermal-unfolding data in Fig. 3 was analyzed. Since thermal unfolding of flavodoxin is non-two-state [21], two-state analysis of the curves is not valid. Instead, we measured the calorimetric enthalpy change for flavodoxin unfolding in buffer (using differential scanning calorimetry; $\Delta H_{cal} = 275 \pm 20$ kJ/mol) and, together with the ΔT_m values from the CD measurements at various crowded conditions, change in protein stability (ΔG_U) was derived as described in Eq. (20) in [38]. For the largest ΔT_m (*i.e.*, in 400 mg/ml dextran 70), the estimated increase in ΔG_U is ~16 kJ/mol. In contrast, VlsE unfolding is two-state and the enthalpy change was obtained using van't Hoff equation [38] and the thermal profile in buffer ($\Delta H_{vH} = 460 \pm 15$ kJ/mol). Using this value and the ΔT_m for VlsE in 400 mg/ml Ficoll 70, we estimated an increase in ΔG_U of ~9 kJ/mol. Thus, both proteins appear to be substantially stabilized in the presence of crowding agents. We note that further thermodynamic analysis is not reliable since the reactions in high Ficoll concentrations are not reversible and, for VlsE, the protein adopts a non-native state.

In conclusion, our findings propose that 'native' states of proteins (at least for small proteins with marginal stability) *in vivo* and *in vitro* may differ: there appears to be 'room for improvement', *e.g.* tightening of loops and helix extensions, depending on the surrounding solute environment. Future studies using other detection methods (*e.g.* NMR), to pinpoint the location of the structural changes in the sequence, as well as correlations with protein activity will be of high interest.

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