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Macromolecular Crowding Fails To Fold a Globular Protein in Cells

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Supporting Information

ABSTRACT: Proteins perform their functions in cells where macromolecular solutes reach concentrations of >300 g/L and occupy >30% of the volume. The volume excluded by these macromolecules stabilizes globular proteins because the native state occupies less space than the denatured state. Theory predicts that crowding can increase the ratio of folded to unfolded protein by a factor of 100, amounting to 3 kcal/mol of stabilization at room temperature. We tested the idea that volume exclusion dominates the crowding effect in cells using a variant of protein L, a 7 kDa globular protein with seven lysine residues replaced by glutamic acids; 84% of the variant molecules populate the denatured state in dilute buffer at room temperature, compared with 0.1% for the wild-type protein. We then used in-cell NMR spectroscopy to show that the cytoplasm of *Escherichia coli* does not overcome even this modest (∼1 kcal/mol) free-energy deficit. The data are consistent with the idea that nonspecific interactions between cytoplasmic components can overcome the excluded-volume effect. Evidence for these interactions is provided by the observations that adding simple salts folds the variant in dilute solution but increasing the salt concentration inside *E. coli* does not fold the protein. Our data are consistent with the results of other studies of protein stability in cells and suggest that stabilizing excluded-volume effects, which must be present under crowded conditions, can be ameliorated by nonspecific interactions between cytoplasmic components.

The effects of high macromolecule concentrations on equilibrium properties arise from two phenomena. The first, excluded volume, is the result of the impenetrable nature of atoms. The volume excluded by the crowding molecules is unavailable to the test protein. The native folded state of a globular protein takes up less space than the denatured state. Application of Le Chatelier's principle leads to the conclusion that volume exclusion favors the native state because it occupies less space.[1]

The other phenomenon involves specific and nonspecific intermolecular chemical interactions. If the crowding molecule interacts with only the native state, the effect is stabilizing. If the crowder has an affinity for protein in general, the effect is destabilizing. These opposing effects are reminiscent of ligand binding and urea denaturation. Binding pulls the equilibrium between the native and denatured states toward the native state because the crowder binds this state. Urea pulls the equilibrium toward the denatured state because that state exposes more surface area. Although urea also introduces an excluded-volume effect,[2] this contribution is smaller than that from the chemical interactions. Thus, one cannot know a priori how crowding will affect globular protein stability.

Until recently, the stabilization afforded by volume exclusion was thought to dominate both in vitro and in cells, although there were hints of compensation.[3−5] We have shown that nonspecific, noncovalent intermolecular interactions and excluded-volume effects compete to affect diffusion.[6] Here we tested this idea in terms of protein stability in cells.

We chose as our test protein the immunoglobulin G binding domain of protein L (ProtL) from the mesophile *Streptococcus magnus*. This well-studied[7,8] 7 kDa protein has the properties expected for a protein from a mesophilic organism[9] and exhibits reversible unfolding at 25 °C in dilute solution via a two-state reaction with a stability of 4.3 kcal/mol.[9] Changing seven of its lysines to glutamic acids lowers the stability, causing the majority of the protein molecules to be in the denatured state in dilute buffer. The destabilization arises from the variant's decreased solvent-accessible surface area, not the increase in negative charge.[9] The variant does fold reversibly, however, upon addition of Na^+ salts[9] or, as described below, K^+ salts. If excluded-volume effects dominate crowding, then the fraction of the variant molecules in the native state should increase in cells relative to dilute solution. We tested this idea.

ProtL locations were determined by osmotically shocking *Escherichia coli*.[10] SDS-PAGE results showed an increase in ProtL expression over 4 h of induction (Figure S1 in the Supporting Information). The variant was located almost entirely in the cytoplasm through 4 h. The wild-type protein was primarily located in the cytoplasm from 0 to 1 h. Later, it was present in both compartments. At all times, the level of the wild-type protein was greater than that of the variant. These observations are consistent with the idea that increasing expression causes cytoplasmic proteins to migrate to the periplasm.[11] For studies of the wild-type protein, we limited the expression time to ensure that the protein was in the cytoplasm.

First, we monitored wild-type ProtL using the $^1H-^{15}N$ heteronuclear single-quantum coherence (HSQC) experiment. The protein was folded, as indicated by the large dispersion of ^1H chemical shifts (Figure 1a). The folding was also assessed using ^{19}F NMR spectroscopy. ProtL contains three tyrosines, so 3-fluorotyrosine (3-FY) substitution[12] should yield three resonances. This prediction was borne out (Figure 2a). The resonances were well-separated, as expected for a folded protein.

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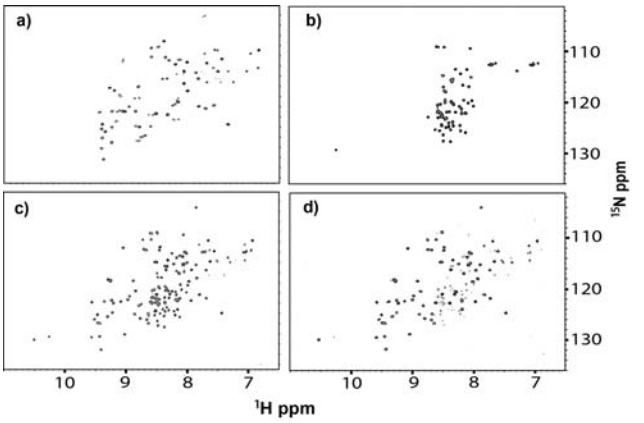

Figure 1. HSQC spectra (20 mM phosphate buffer, pH 6.0, 25 °C) of the ^{15}N-enriched (a) wild-type protein and (b−d) Kx7E variant in (b) 0, (c) 0.3, and (d) 0.8 M NaCl.

The purified ^{15}N-enriched Kx7E variant was dissolved in 20 mM phosphate buffer solutions containing 0−1 M NaCl. The limited ^{1}H chemical shift dispersion (Figure 1b) indicated that the variant was unfolded in buffer.[9,13−15] When the NaCl concentration was increased to 0.3 M, the dispersion increased to that of a folded protein, although cross-peaks from the unfolded form were visible (Figure 1c). In 0.8 M NaCl, almost all of the variant was folded (Figure 1d), and the cross-peak pattern was similar to that of wild-type ProtL.

The average intensities of the unfolded and folded Kx7E cross-peaks at each NaCl concentration (Table S1) were used to produce a titration curve (Figure S2). The transition was 50% complete at 0.3 M NaCl and reached a maximum at 0.8 M NaCl. The data were fit to a two-state model.[16] The unfolding free energy at 0 M NaCl was −1.0 kcal/mol (root-mean-square deviation of 2%); this represents the free energy required to fold half the variant molecules in the absence of NaCl.

We also monitored the folding of the variant using ^{19}F NMR spectroscopy. The purified ^{19}F-labeled protein was dissolved in 20 mM phosphate buffer solutions containing 0−1 M NaCl. The spectrum in the absence of added NaCl (Figure 2b) showed the three expected resonances in a narrow chemical shift range, indicative of an unfolded protein. As the NaCl concentration was increased, two resonances moved downfield, and the intensities of the resonances from the unfolded protein decreased (Figure 2c−g). At 1 M NaCl, most of the protein was folded, although resonances from the unfolded protein remained at low intensities. The chemical shifts of the variant in 1 M NaCl were consistent with those of the wild-type protein (Figure 2a). The increasing fraction of folded protein with increasing NaCl concentration is consistent with the HSQC data, although not enough data points were acquired for quantification.

We used in-cell NMR spectroscopy to assess wild-type ProtL in *E. coli*. The HSQC spectrum of a cell slurry containing the ^{15}N-enriched wild-type protein is shown in Figure 3a. The spectrum was the same as that of cells without an expression vector (Figure S4). That is, the spectrum of the protein was unobservable; the cross-peaks were from ^{15}N-enriched metabolites.[17] These observations are consistent with results showing that ^{1}H−^{15}N HSQC spectra from many globular proteins are not detectable in *E. coli*.[18−21] After lysis, the wild-type protein spectrum was detected (Figure 3b). Furthermore, the spectrum was unchanged in the presence of 1 M NaCl (Figure 3c), as expected for a

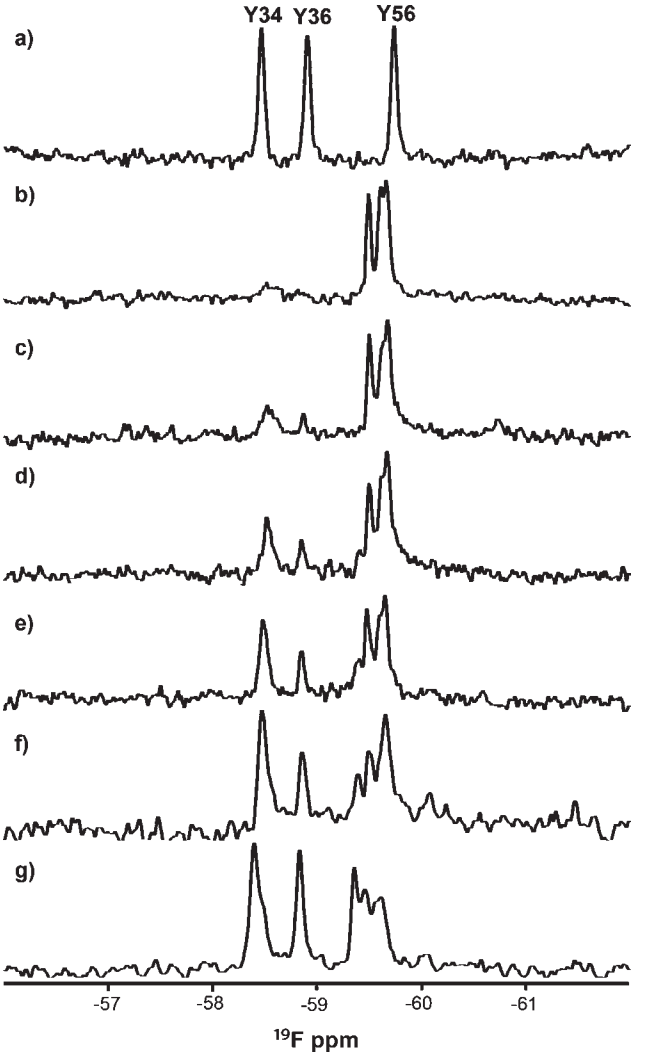

Figure 2. ^{19}F spectra (20 mM phosphate buffer, pH 6.0, 37 °C) of the ^{19}F-labeled (a) wild-type protein and (b−g) Kx7E variant in (b) 0, (c) 0.1, (d) 0.2, (e) 0.3, (f) 0.5, and (g) 1 M NaCl. Assignments were made by using mutagenesis (Figure S3).

mesophilic protein. The spectrum of the cell supernatant showed no protein cross-peaks (Figure S5), confirming that the protein was in the cells. To detect the protein in cells, we turned to ^{19}F NMR spectroscopy.[21]

The in-cell experiments were repeated with the ^{19}F-labeled wild-type protein. Three resonances from the protein were detected in the ^{19}F spectrum of the cell slurry (Figure 4a). The fourth resonance, from unincorporated 3-FY, was sharper and overlapped the most upfield protein resonance. Although broad, the resonances were well-separated and similar to those from the in vitro spectrum. The supernatant spectrum also showed a sharp resonance from unincorporated 3-FY (Figure 4b) and three additional small, sharp resonances. The positions of these resonances matched those of wild-type ProtL in dilute solution, indicating that a small amount of protein (<10%) leaked from the cells. These data confirmed that wild-type ProtL is folded in *E. coli* and showed that it can be detected by ^{19}F NMR spectroscopy.

Disordered proteins are detectable in cells by ^{1}H−^{15}N HSQC NMR spectroscopy because of their internal motion.[18−20] The HSQC spectrum of the cell slurry exhibited a narrow

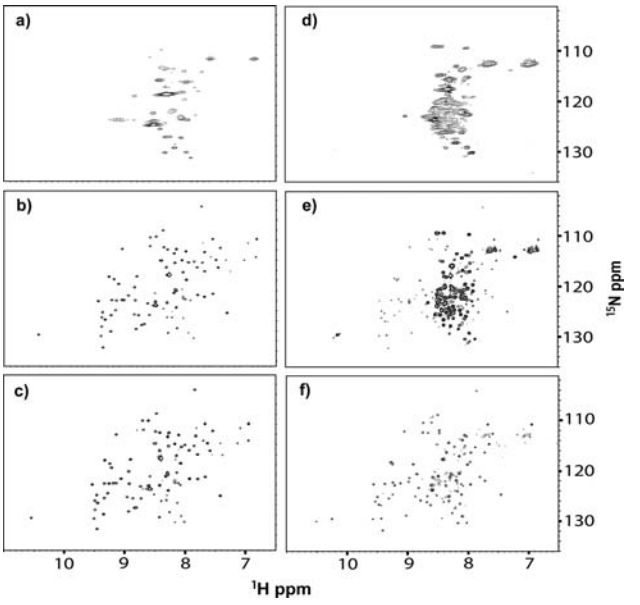

Figure 3. HSQC spectra (25 °C) of the ^{15}N-enriched wild-type protein and the Kx7E variant in *E. coli*. (a) Cell slurry expressing the wild-type protein. (b) Lysate of cells from (a). (c) Lysate of cells shown in (a) upon addition of NaCl to a final concentration of 1 M. (d) Cell slurry expressing the Kx7E variant. (e) Lysate of cells from (d). (f) Lysate of cells shown in (d) upon addition of NaCl to a final concentration of 1 M.

chemical shift dispersion in the ^{1}H dimension (Figure 3d), characteristic of an unfolded protein. The ability to detect the HSQC spectrum of the variant in cells showed that the protein is soluble in the cytoplasm. The spectrum of the cell supernatant showed no protein cross-peaks (Figure S5), indicating that the spectrum in Figure 3d arises from the variant inside cells. The protein remained unfolded upon cell lysis (Figure 3e) but folded when the NaCl concentration (Figure 3f) or the KCl concentration (Figure S6) was increased. These results demonstrated that although the variant is foldable, the *E. coli* cytoplasm is unable to fold it.

The ^{15}N results were confirmed with ^{19}F NMR spectroscopy. The spectrum of the Kx7E cell slurry displayed an envelope of broad resonances in a narrow chemical shift range and a sharp peak from unincorporated 3-FY (Figure 4c). The position of the envelope was inconsistent with the resonances from the folded variant (Figures 2a and 4a) but consistent with that of the unfolded variant (Figure 2b). The absence of the downfield resonance from the folded variant was especially evident. No resonances from the variant were detected in the supernatant, indicating that the protein did not leak (Figure 4d).

It has been known for over 40 years that the *E. coli* intracellular K^+ concentration can be manipulated by adjusting the osmolality of the medium.[22] An attempt was made to fold the variant in *E. coli* by increasing the intracellular concentration of K^+ by growth in a hyperosmotic (1.05 Osm) medium.[3,23−25] However, the ^{19}F spectrum of the Kx7E cell slurry in hyperosmotic minimal medium (Figure 4e) showed no resonances from the folded protein. The upfield shift of the resonance envelope of the unfolded protein was probably caused by the increase in K^+ concentration.

Many in vitro studies of crowding have focused on volume exclusion. In these experiments, the environment has contained only one or a few different types of crowders. Furthermore, it has usually been assumed that the crowders have little interaction with the test protein unless their van der Waals surfaces attempt to overlap, at

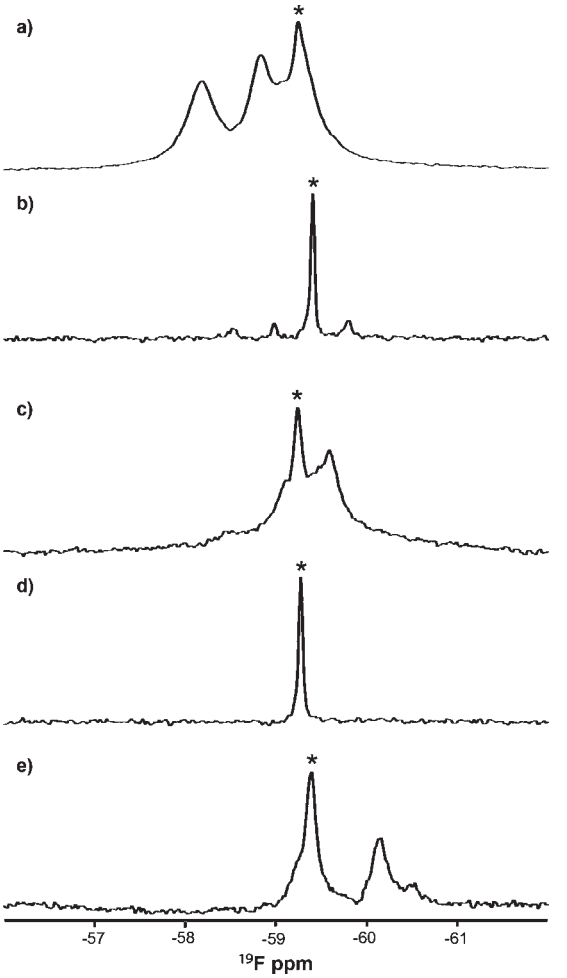

Figure 4. ^{19}F spectra (37 °C) of the ^{19}F-labeled wild-type protein and the Kx7E variant in *E. coli*. (a) Cell slurry expressing the wild-type protein. (b) Supernatant from the sample used in (a). (c) Cell slurry expressing the Kx7E variant. (d) Supernatant from the sample used in (c). (e) Cell slurry expressing the Kx7E variant grown and induced in hyperosmotic media. Asterisks denote the resonance from free 3-FY.

which point the repulsive forces increase exponentially. Recent work, however, has demonstrated the importance of nonspecific intermolecular interactions.[5,6,26,27] Thus, one cannot ignore chemical interactions.

We examined the unstable Kx7E variant of protein L. The variant folds in the presence of high concentrations of salt,[9] prompting the question of whether the crowded *E. coli* cytosol would have a similar effect.

We acquired HSQC spectra of the variant in vitro by adding increasing concentrations of NaCl. The spectra allowed the construction of a folding titration curve. The unfolding free energy of Kx7E in the absence of NaCl was −1.0 kcal/mol. This NMR-derived value compares favorably to those derived by Tadeo et al.[9] from guanidinium chloride and urea titration experiments with CD detection (−0.4 to −0.5 kcal/mol). Therefore, the *E. coli* cytosol would have to deliver between 0.4 and 1.0 kcal/mol of stabilization to fold 50% of the variant molecules.

The in-cell NMR data show that wild-type ProtL is folded but the variant remains unfolded. We conclude that the volume exclusion provided by the highly crowded intracellular environment is insufficient to overcome the unfavorable free energy of folding.

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We also tested whether increasing the K^+ concentration in cells could fold the variant. The cytoplasmic K^+ concentration in *E. coli* is ~0.2 M[23,25] for cells grown in minimal media (0.1 Osm). Nearly all of the cytoplasmic K^+ in such *E. coli*, however, is associated with polyanions such as DNA and RNA.[24] Thus, not enough salt is available to fold the variant in *E. coli*.

We increased the cytoplasmic K^+ concentration by increasing the osmolality of the medium from 0.16 to 1.05 Osm. Under these conditions, the cytoplasmic K^+ concentration reaches 0.5−0.9 M.[23,25] Importantly, at least half of this upshift is due to an increase in free cytoplasmic K^+.[23] Therefore, the concentration of free cytoplasmic K^+ under hyperosmotic conditions was at least 0.3 M, which leads to easily detectable levels of the folded protein in vitro. Even under these conditions, we did not detect the folded form in cells. Furthermore, it is unlikely that a significant amount of the unfolded form was associated with chaperones because the observation of cross-peaks in Figure 3d was inconsistent with the molecular weight of a Kx7E-chaperone or proteasome complex.[28]

Our results suggest that any stabilizing excluded-volume effect, which must exist because of the high concentration of macromolecules in the cytoplasm, is more than offset by a destabilizing effect. The destabilization probably arises from nonspecific protein−protein interactions. Wang et al.[6] have shown that rotational diffusion of a globular protein is slowed beyond what would be expected from viscosity alone in crowded protein solutions and *E. coli* lysates and that the difference is due to nonspecific intermolecular interactions with the test protein. These nonspecific interactions in cells can lead to irreversible denaturation,[29] explaining why the Kx7E variant is unfolded in cells. Such nonspecific protein−protein interactions may also explain why the variant does not fold completely in vitro even at high salt concentrations.

Although volume exclusion would be expected to stabilize the native state of globular proteins,[30] the *E. coli* cytosol contains biologically active molecules. These molecules chemically interact with the protein under study, and these interactions can be stabilizing or destabilizing. The few available in-cell studies of globular protein stability indicate no change or a slight destabilization in *E. coli* relative to dilute solution.[3,4] Although dilute solution data may be physiologically relevant for stable globular proteins, our data show that nonspecific intermolecular interactions can overcome volume-exclusion-induced compaction, causing unstable globular proteins to remain unfolded. The situation is different for intrinsically disordered proteins, where the crowded environment in cells can result in compaction.[31,32] The dissimilar behavior is probably caused by the different amino acid compositions of these two protein classes.[33]

In view of the fact that KCl folds the variant in vitro, it is striking that increasing the intracellular K^+ concentration fails to have the same effect. This discrepancy indicates that nonspecific intermolecular interactions may prevent refolding of globular proteins even when solution conditions favor it.

■ ASSOCIATED CONTENT

Supporting Information. Materials and methods, Table S1, and Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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