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A Difference between *In Vitro* and In-Cell Protein Dimer Formation

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ABSTRACT: The high concentration of macromolecules in cells affects the stability of proteins and protein complexes via hard repulsions and chemical interactions, yet few studies have focused on chemical interactions. We characterized the domain-swapped dimer of the B1 domain of protein G in buffer and *Escherichia coli* cells by using heteronuclear, multidimensional nuclear magnetic resonance spectroscopy. In buffer, the monomer is a partially folded molten globule, but that species is not observed in cells. Experiments using urea suggest that the monomer is unfolded in cells, but again, the molten-globule form of the monomer is absent. The data suggest that attractive chemical interactions in the cytoplasm unfold the molten globule. We conclude that the intracellular environment not only modulates the stability of protein complexes but also can change the species present, reinforcing the idea that chemical interactions are more important than hard repulsions in cells.

The *Escherichia coli* cytoplasm, where the macromolecular concentrations exceed 300 g/L, is crowded with proteins, nucleic acids, and small molecules.[1] In this complex environment, proteins experience interactions that are absent in dilute buffer, the condition under which most proteins are studied. Crowding alters the stability of proteins and protein complexes via hard repulsions and chemical interactions.[2] Hard repulsions arise because two macromolecules cannot occupy the same space at the same time.[3] Hard repulsions are entropic and tend to favor compact states. For instance, hard repulsions favor the more compact folded state of a globular protein over its less compact unfolded state, leading to stabilization.[4] Chemical interactions are enthalpic and modulate hard repulsions. Repulsive chemical interactions (i.e., those between like charges) reinforce hard repulsions and are therefore stabilizing. Attractive interactions (e.g., opposite charges and hydrogen bonds) weaken hard repulsions and are destabilizing.[4−7]

For decades, studies of macromolecular crowding focused on hard repulsions, ignoring the potential influence of chemical interactions between the test protein and its crowded environment.[2] This situation probably arose because most efforts employed relatively inert synthetic polymers as crowders,[4] but even these cosolutes are now known to exert chemical interactions.[8−10] A role for chemical interactions was also proposed in studies of globular proteins as crowding agents.[2] Furthermore, we know that the cytoplasm can modulate protein folding compared to that in buffer.[11] Here, we show that the cytoplasm can also change the species present in protein complex formation.

Studies of the stability of protein complexes in physiologically relevant environments demonstrate the importance of chemical interactions.[12] For instance, the stability of the 12.4 kDa B1 domain of the protein G (GB1) side-by-side dimer was quantified in *E. coli* and *Xenopus laevis* oocytes.[13] Both intracellular environments stabilize the dimer compared to a buffer. This increase probably arises from repulsive chemical interactions between cytosolic proteins and the monomer. Studies of variant GB1 dimers possessing changes in surface charge support this conclusion.[13] The data also show that the side-by-side dimer is more stable in oocytes than in *E. coli*,[13] despite the fact that the *E. coli* cytoplasm is twice as crowded,[1] in contradistinction to traditional theory, which predicts that an increased level of crowding increases stability. These observations indicate that chemical interactions can be more important than hard repulsions in cells. However, few in-cell efforts identify the species present.[11,12] Here, we show that the cellular interior can change the species present in a protein monomer−dimer reaction.

The L5V;F30V;Y33F;A34F variant of GB1 forms a domain-swapped dimer (DSD) in buffer that dissociates into molten-globule monomers with a K_D of 93 ± 10 μM at 298 K as shown by analysis of $^{15}N-^{1}H$ heteronuclear single-quantum coherence (HSQC) spectra.[14,15] We first tried to characterize this equilibrium in *E. coli* using fluorotryptophan labeling (Figure S1).[16] The dimer and monomer have unique ^{19}F chemical shifts and are in slow exchange on the NMR time scale.[13] We observed the dimer but not the monomer in cells (Figure S1). However, we switched to ^{15}N enrichment because a portion of the ^{19}F spectrum is obscured by metabolites (Figure S1).

Figure 1 shows HSQC spectra of the DSD in buffer and cells. At 1 mM GB1 in buffer (Figure 1A), both monomer and dimer cross-peaks are observed. Upon dilution to 30 μM (Figure 1B), the intensity of the monomer cross-peaks increases. These observations are consistent with Le Chatelier's principle because dilution increases the concentration of the monomer.[15,17]

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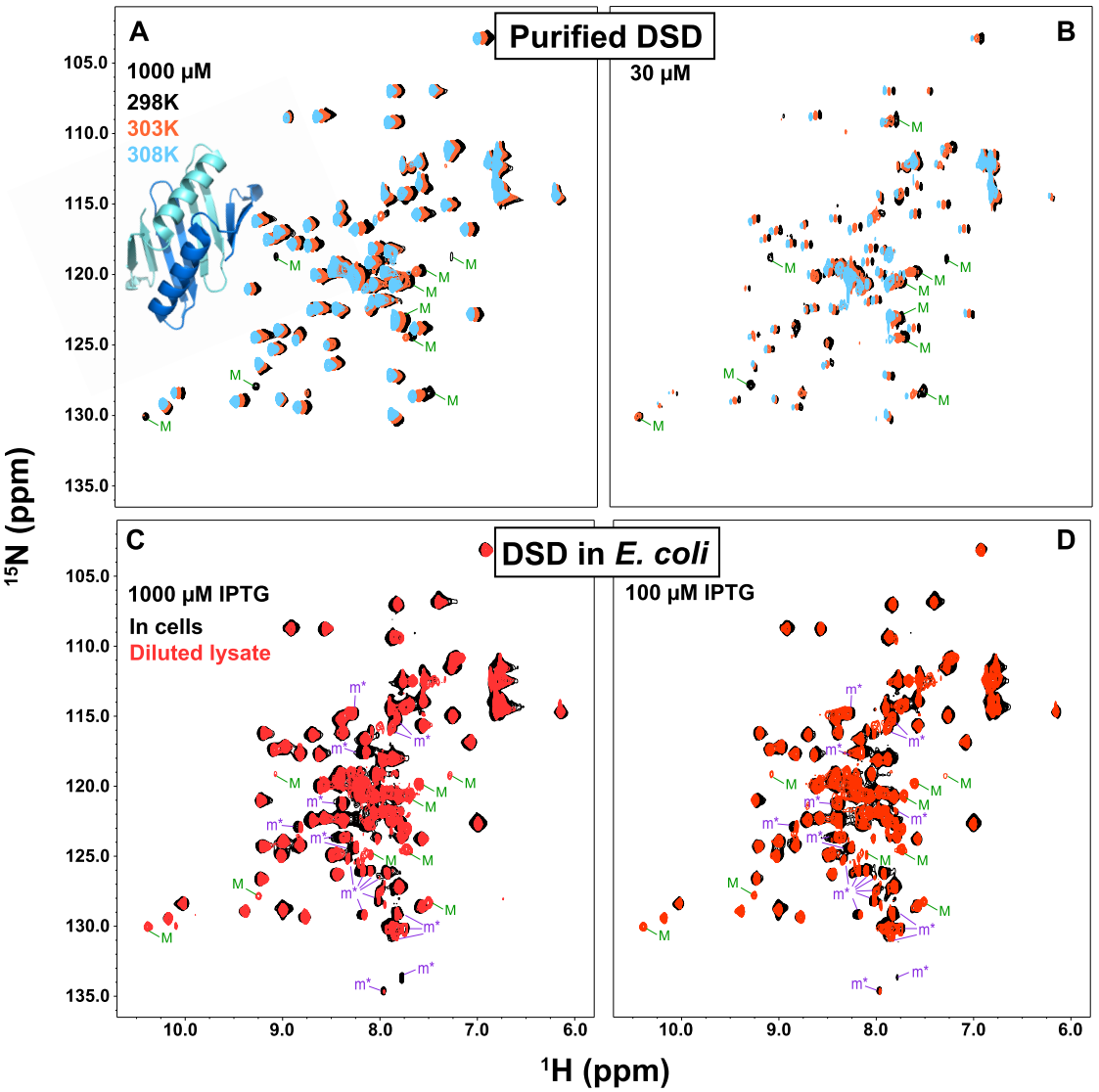

Figure 1. ^{15}N−^{1}H HSQC spectra of ^{15}N-enriched DSD in 20 mM phosphate buffer (pH 7.5) at 298 K (black), 303 K (orange), and 308 K (blue) at GB1 concentrations of (A) 1000 μM and (B) 30 μM. The concentrations in panels A and B were chosen because they are above and below the equilibrium dissociation constant (93 ± 10 μM at 298 K).[14,15] Overlaid spectra of DSD (298 K, pH 7.4) in cells (black) and diluted lysate (red) after induction with (C) 1000 μM and (D) 100 μM IPTG. M and m* denote monomer and metabolite cross-peaks, respectively. A ribbon diagram of the DSD (Protein Data Bank entry 1Q10) structure is shown in panel A.

To characterize the equilibrium in cells, the DSD-expressing plasmid was transformed into *E. coli* containing a deletion of lactose permease, which allows rheostatic control of expression by the inducer isopropyl β-D-thiogalactoside (IPTG).[17] The transformed cells were grown in ^{15}N-enriched minimal medium. As a control, a supernatant spectrum was acquired to assess protein leakage;[18] only metabolite signals are observed (Figure S2).[18] Dimer cross-peaks are observed in cells at 298 K. We do not observe monomer cross-peaks in cells induced with 1000 μM, 100 μM, or lower concentrations of IPTG (Figure 1C,D and Figure S3). Comparisons to other data[19] suggest that the intracellular concentration of GB1 upon induction with 100 μM IPTG is ∼1 mM. The monomer is readily detected *in vitro* at this concentration.[14] Induction with 25 μM IPTG gives a spectrum with only metabolite cross-peaks (Figure S3A). Induction with 31 μM IPTG results in weak dimer cross-peaks and new cross-peaks at the center of the spectrum but no monomer cross-peaks (Figure S3B). The narrow ^{1}H shift dispersion of these new signals is indicative of an unfolded protein.[20]

As a control, cells were lysed, the lysate was diluted, and another spectrum was acquired.[17] Both dimer and monomer cross-peaks are observed in lysate spectra from cells induced at high and low IPTG concentrations (Figure 1C,D). Some extra cross-peaks are observed in in-cell spectra that arise from ^{15}N-labeled metabolites, which we confirmed by performing an in-cell experiment without induction (Figure S2).[18] In summary, the absence of in-cell monomer signals contrasts with observations made in buffer where the protein behaves according to a simple dimer−monomer equilibrium.

The absence of monomer cross-peaks in cells could be caused by stabilization of the dimer or by the absence of the molten-globule monomer. To ascertain whether the monomer is absent in cells at 298 K or just unobservable, we acquired in-cell spectra at 308 K (Figure S4). A higher temperature should make the monomer easier to observe for two reasons. First, higher temperatures should increase the fraction of the

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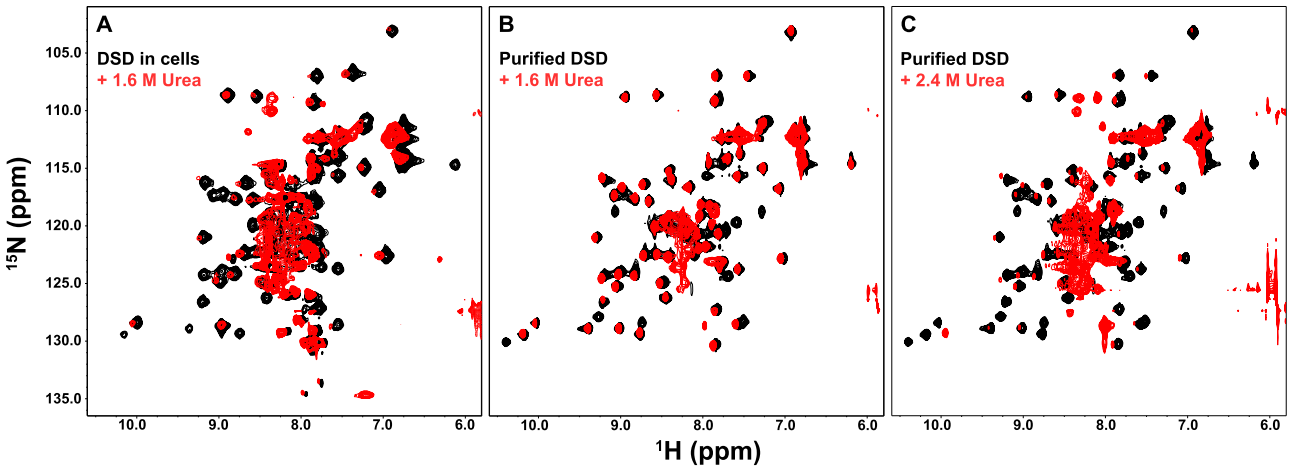

Figure 2. $^{15}N-^{1}H$ HSQC spectra of ^{15}N-enriched DSD in the presence (red) and absence (black) of urea in *E. coli* cells and buffer. (A) In-cell spectra (298 K, pH 7.4) in the absence and presence of 1.6 M urea. Overlaid spectra of 500 μM DSD in 20 mM phosphate buffer (298 K, pH 7.5) in (B) 1.6 M and (C) 2.4 M urea.

monomer because dissociation is endothermic. Second, increasing the temperature increases the rate of tumbling, resulting in sharper, easier-to-observe cross-peaks. However, like the 298 K data, no monomer cross-peaks are observed in cells at 308 K (Figure S4). Instead, new cross-peaks indicative of an unfolded protein[20] appear at the center of the spectrum.

As a comparison, we acquired spectra of the purified protein in buffer at 303 and 308 K. Surprisingly, monomer signals did not appear; instead, spectra of samples acquired at high and low GB1 concentrations showed the same trend: monomer cross-peaks disappear with an increase in temperature (Figure 1A,B). These *in vitro* results suggest that the monomer is unstable in cells, and as reported for other proteins,[21,22] the unfolded form sticks to other cytoplasmic proteins, which can decrease its mobility and increase the line width of cross-peaks making them less detectable.

Another way to assess the existence of the monomer in cells is by adding the denaturant urea,[23] which readily enters the *E. coli* cytoplasm[24] and destabilizes proteins in cells.[23] We used 0.6, 1.6, and 2.4 M urea, concentrations that do not affect viability.[24] Spectra acquired in the absence of urea and in 0.6 M urea are essentially identical (Figure S5). Figure 2A shows the in-cell spectrum in 1.6 M urea. Like the higher-temperature data discussed above (Figure S4) and the data acquired with induction with 31 μM IPTG (Figure S3), new cross-peaks indicative of unfolded protein[20] appear at the center and top of the spectrum. Importantly, we still did not observe molten-globule monomer cross-peaks.

To further test the effect of urea, we performed the experiment *in vitro*. In 1.6 M urea (Figure 2B), most monomer cross-peaks disappear, and a few new ones appear near the center of the spectrum. Inspection of spectra acquired in 2.4 M urea (Figure 2C) reveals reduced dimer cross-peaks with the concomitant appearance of signals from unfolded species, similar to our observation in cells.

To recap, we detect the monomer in buffer, but not in cells. In buffer, we observe the DSD, its molten-globule monomer, and, if we add urea, the unfolded state. In cells, we observe the DSD and, if we add urea or increase the temperature, the unfolded state, but not the monomer. We conclude that the cellular environment changes DSD formation from a dimer ⇌ molten-globule monomer ⇌ unfolded monomer equilibrium to a dimer ⇌ unfolded monomer equilibrium. This conclusion is consistent with the observation that the *E. coli* cytoplasm decreases the stability of wild-type GB1, which is exclusively monomeric.[5,25]

Although hard repulsions in cells should favor the dimer, our observation of only dimer in cells is more likely the result of attractive interactions that unfold the molten-globule monomer. In summary, attractive chemical interactions in cells can play a key role not only in protein stability, protein folding, and protein complex stability compared to buffer but also in determining the species involved in the formation of protein complexes. These conclusions illustrate the importance of accounting for chemical interactions when studying proteins in living cells.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.1c00780.

Materials and Methods, ^{19}F spectra of 5-fluorotryptophan-labeled DSD, $^{15}N-^{1}H$ supernatant spectrum and in-cell $^{15}N-^{1}H$ spectrum without IPTG induction, in-cell $^{15}N-^{1}H$ spectrum induced at low IPTG concentrations, in-cell $^{15}N-^{1}H$ spectrum at higher temperatures, in-cell $^{15}N-^{1}H$ spectrum at low urea concentrations, and supplementary references (PDF)

Accession Codes

L5V/F30V/Y33F/A34F variant of GB1: UniProtKB P06654.

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Author Contributions

I-T.C., C.J.S., S.L.S., and G.J.P. designed the experiments. I-T.C., C.J.S., and S.L.S. performed the experiments. I-T.C., C.J.S., S.L.S., and G.J.P. analyzed data. I-T.C. and G.J.P. wrote the manuscript. G.J.P. supervised the research and led project conceptualization.

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Notes

The authors declare no competing financial interest.

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