Correction

BIOCHEMISTRY

The authors wish to note, “After our paper was accepted, we determined that the pH in cells was closer to 5.8 than 7.6. Experiments were repeated at the correct pH (1). The result changes the conclusion about the GB1 stability in cells. Specifically, the result indicates that the protein is less stable in cells than it is in buffer. The results for BSA at pH 7.6 are unchanged.” The corrected Fig. 6 and its legend appear below.

Fig. 6. $\Delta G^\circ$ values for WT GB1 residues in 100 g/L BSA, buffer, and cells. Error bars represent the SD of the mean from three trials.


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Residue level quantification of protein stability in living cells

William B. Monteith* and Gary J. Pielak* b, c, 1

*Department of Chemistry, bDepartment of Biochemistry and Biophysics, and cLineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

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The intracellular milieu differs from the dilute conditions in which most biophysical and biochemical studies are performed. This difference has led both experimentalists and theoreticians to tackle the challenging task of understanding how the intracellular environment affects the properties of biopolymers. Despite a growing number of in-cell studies, there is a lack of quantitative, residue-level information about equilibrium thermodynamic protein stability under nonperturbing conditions. We report the use of NMR-detected hydrogen–deuterium exchange of quenched cell lysates to measure individual opening free energies of the 56-aa B1 domain of protein G (GB1) in living Escherichia coli cells without adding destabilizing cosolutes or heat. Comparisons to dilute solution data (pH 7.6 and 37 °C) show that opening free energies increase by as much as 1.14 ± 0.05 kcal/mol in cells. Importantly, we also show that homogeneous protein crowders destabilize GB1, highlighting the challenge of recreating the cellular interior. We discuss our findings in terms of hard-core excluded volume effects, charge–charge GB1-crowder interactions, and other factors. The quenched lysate method identifies the residues most important for folding GB1 in cells, and should prove useful for quantifying the stability of other globular proteins in cells to gain a more complete understanding of the effects of the intracellular environment on protein chemistry.

Proteins function in a heterogeneous and crowded intracellular environment. Macromolecules comprise 20–30% of the volume of an Escherichia coli cell and reach concentrations of 300–400 g/L (1, 2). Theory predicts that the properties of proteins and nucleic acids can be significantly altered in cells compared with buffer alone (3, 4). Nevertheless, most biochemical and biophysical studies are conducted under dilute (<10 g/L macromolecules) conditions. Here, we augment the small but growing list of reports probing the equilibrium thermodynamic stability of proteins in living cells (5–9), and provide, to our knowledge, the first measurement of residue-level stability under nonperturbing conditions.

Until recently, the effects of macromolecular crowding on protein stability were thought to be caused solely by hard-core, steric repulsions arising from the impenetrability of matter (4, 10, 11). The expectation was that crowding enhances stability by favoring the compact native state over the ensemble of denatured states. Increased attention to transient, nonspecific protein–protein interactions (12–15) has led both experimentalists (16–19) and theoreticians (20–22) to recognize the effects of chemical interactions between crowder and test protein when assessing the net effect of macromolecular crowding. These weak, nonspecific interactions can reinforce or oppose the effect of hard-core repulsions, resulting in increased or decreased stability depending on the chemical nature of the test protein and crowder (23–26).

We chose the B1 domain of streptococcal protein G (GB1) (27) as our test protein because its structure, stability and folding kinetics have been extensively studied in dilute solution (28–38).

Its small size (56 aa; 6.2 kDa) and high thermal stability make GB1 well suited for studies by NMR spectroscopy. Quantifying the equilibrium thermodynamic stability of proteins relies on determining the relative populations of native and denatured states. Because the denatured state ensemble of a stable protein is sparsely populated under native conditions, stability is usually probed by adding heat or a cosolute to promote unfolding so that the concentration ratio of the two states can be determined (39). However, stability can be measured without these perturbations by exploiting the phenomenon of backbone amide H/D exchange (40) detected by NMR spectroscopy (41). The observed rate of amide proton (N–H) exchange, kobs, is related to equilibrium stability by considering a protein in which each N–H exists in an open (exposed, exchange-competent) state, or a closed (protected, exchange-incompetent) state (40, 42):

\[
\frac{k_{cl}}{k_{op}} \approx \frac{\text{open}(N-H)}{\text{closed}(N-D)} \approx \frac{k_{int}}{k_{int}}
\]

Each position opens and closes with rate constants, k_{op} and k_{cl} (where K_{eq} = k_{op}/k_{cl}), and exchange from the open state occurs with intrinsic rate constant, k_{int}. Values for k_{int} are based on exchange data from unstructured peptides (43, 44). If the test protein is stable (i.e., k_{cl} >> k_{op}), the observed rate becomes:

\[k_{obs} = k_{eq}k_{int}/(k_{cl} + k_{int})
\]

Exchange occurs within two limits (42). At the EX1 limit, closing is rate determining, and k_{obs} = k_{eq}. This limit is usually observed for less stable proteins and at basic pH (45). Most

Significance

Proteins function in a sea of macromolecules within cells, but are traditionally studied under ideal conditions in vitro. The more details we amass from experiments performed in cells, the closer we will get to understanding fundamental aspects of protein chemistry in the cellular environment. In addition to furthering our essential knowledge of biochemistry, advancements in the field of macromolecular crowding will drive efforts to stabilize protein-based therapeutics. Here, we show that protein stability can be measured at the residue level in living cells without adding destabilizing cosolutes or heat.

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1To whom correspondence should be addressed. Email: gary_pielak@unc.edu.

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globular proteins undergo EX2 kinetics, where exchange from the open state is rate limiting (i.e., k\text{cl} >> k\text{obs}), and k\text{obs} values can be converted to equilibrium opening free energies, $\Delta G_{\text{op}}$ (46):

$$k_{\text{obs}} = \frac{k_{\text{op}}}{k_{\text{cl}}} = K_{\text{op}} k_{\text{int}}$$

where $RT$ is the molar gas constant multiplied by the absolute temperature.

The backbone amides most strongly involved in H-bonded regions of secondary structure exchange only from the fully unfolded state, yielding a maximum value of $\Delta G_{\text{op}}$ (47–49). For these residues $\Delta G_{\text{op}}$ approximates the free energy of denaturation, $\Delta G_{\text{den}}$, providing information on global stability. Lower amplitude fluctuations of the native state can give rise to partially unfolded forms (50), resulting in residues with $\Delta G_{\text{op}}$ values less than those of the global unfolders.

In summary, NMR-detected H/D exchange can measure equilibrium thermodynamic stability of a protein at the level of individual amino acid residues under nonperturbing conditions. Inomata et al. (51) used this technique to measure k\text{obs} values in human cells for four residues in ubiquitin, but experiments confirming the exchange mechanism were not reported and opening free energies were not quantified. Our results fill this void and provide quantitative residue-level protein stability measurements in living cells under nonperturbing conditions.

**Results**

**In Cells.** We attempted to measure GB1 stability directly in cells by pairing H/D exchange with in-cell NMR (52) and conventional serial $^{15}$N-$^2$H heteronuclear single quantum coherence (HSQC) analysis (53), because GB1 is one of the few proteins that gives reasonable spectra in E. coli (13, 14, 54, 55). The signal-to-noise ratios of the spectra, however, were insufficient for quantification. We overcame this problem by modifying the approach of Ghaemmaghami and Oas (7) for measuring stability in discrete, quenched cell lysates (Fig. 1, Fig. S1, and SI Materials and Methods). Briefly, the cells are transferred and washed into D$_2$O, where they remain viable (SI Materials and Methods). An aliquot of cell slurry is removed at defined times, the cells lysed, exchange quenched, and the lysate analyzed by NMR. The dead time is \sim 1 h. Representative HSQC spectra of the initial and final lysates of WT GB1 illustrate the decrease in N–H cross-

The decay of the T18 cross-peak illustrates the upper limit for measuring exchange. Quantification of k\text{obs} for T18 required a lower contour level and fitting to fewer times than the 17 more slowly exchanging residues. At contour levels lower than those shown in Fig. 2, resonances from six additional residues (K10, A20, A24, T25, Q32, and N35) are detectable in the spectrum from the initial time point, but decay is too rapid to obtain k\text{obs}. Rates for these residues are listed as >k\text{obs, T18}.

Cross-peaks from 24 backbone amides do not appear to exchange. We conclude that these residues are quench labeled, that is, they are least protected from solvent and, therefore, are labeled with protons immediately before quenching, when the proton concentration increases 10-fold. This conclusion arises from two considerations. First, the side chain amides, which are solvent exposed, behave similarly. Second, as discussed below, quench labeling is not observed in the serially acquired (i.e., no quench step) dilute solution data. Exchange rates for quench-labeled residues are also listed as >k\text{obs, T18}, even though their rates are probably even larger than those for the six residues described above. We attempted to assign the remaining residues, but the lysate was not stable enough for acquisition of 3D NMR data.

In summary, 48 of the 56 residues provide information on exchange. For the 17 slowly exchanging residues, k\text{obs} values were converted to free energies of opening, $\Delta G_{\text{op}}$, by using Eq. 4 (Fig. S2 and Table S2). The value for T18 was not included because its rate in cells was obtained from limited data. Elevated rates of intrinsic exchange (10–100 s$^{-1}$) under our conditions (pH$_{\text{corr}}$ 7.6, 37 °C) prevented quantification of 30 residues. Based on our results for T18, we conclude that the k\text{obs} values for these 30 residues are $>7 \times 10^{-4}$ s$^{-1}$ in cells and $>3 \times 10^{-4}$ s$^{-1}$ in buffer (see In Dilute Solution).

**In Dilute Solution.** To compare the in-cell rates to those acquired in dilute solution (pH$_{\text{corr}}$ 7.6, 37 °C), we mimicked the discrete sampling method (Fig. 1) using purified GB1 instead of the GB1-containing lysate (SI Materials and Methods). The exchange behavior is similar under both conditions; i.e., the same residues exchange slowly, the same residues exchange too rapidly to quantify, and the same quench labeling is observed. However, quantifying the exchange of T18 was possible because the dead time is <5 min, compared with \sim 1 h for the in-cell studies (Fig. 3). Similar to the in-cell data, T18 is the fastest exchanging quantifiable residue. The k\text{obs} values are tabulated in Table S1, and the
values were used to calculate the changes in stability of the variant in cells compared with buffer (ΔΔGcell,enu = ΔΔGcell,enu WT - ΔΔGcell,enu I6L). Second, we calculated the change caused by the mutation (ΔΔΔGden,mut = ΔΔΔGden,mut WT - ΔΔΔGden,mut I6L) in cells. Third, we calculated the effect of the mutation in dilute solution (Fig. 7 and Fig. S3). We use these data to assess the thermodynamic cycle in the Discussion.

Calorimetry. We used differential scanning calorimetry to quantify the free energy of denaturation, ΔΔΔGden (SI Materials and Methods). Due to the high thermal stability of the WT protein (Tm = 79.0 °C at pHcorr 7.6), it is difficult to obtain adequate posttransitional baselines for robust fitting while maintaining reversibility (58). To solve this problem, we used the calorimetric enthalpy, ΔHcal′, of the destabilized I6L variant and the Tm values of the two proteins to calculate ΔΔΔGden,mut with the equation, ΔΔΔGden,mut = ΔHcal′ (Tm,WT - Tm,I6L)/Tm,WT (59). The value of ΔΔΔGden,mut (−0.68 ± 0.06 kcal/mol) is consistent with the average ΔΔΔGcell,mut (−0.8 ± 0.1 kcal/mol) from dilute solution H/D exchange experiments.

Discussion

We obtained, in triplicate, backbone amide exchange rates in cells and buffer for 18 residues distributed throughout GB1: Y3, K4, L5, I6 and L7 in β1; T18 in β2; A26, E27, K28, V29, K31, Y33 and A34 in the α-helix; T44 and D46 in β3; and T51, F52 and T53 in β4. The set includes 14 (in bold) of the 16 residues suggested by the EX2 mechanism (26). We repeated this strategy with the destabilized GB1 variant, I6L (57). Comparisons of ΔΔΔGcell,enu values were possible for 12 residues (Tables S5–S7). We made three comparisons. First, we calculated the change in residue-level stability of the variant in cells compared with buffer (ΔΔΔGcell,enu WT - ΔΔΔGcell,enu I6L). Second, we calculated the change caused by the mutation (ΔΔΔGden,mut = ΔΔΔGden,mut WT - ΔΔΔGden,mut I6L) in cells. Third, we calculated the effect of the mutation in dilute solution (Fig. 7 and Fig. S3). We use these data to assess the thermodynamic cycle in the Discussion.

In Vitro Crowding. To assess the effect of individual protein crowders (24, 56) on GB1 stability, we acquired exchange data in 100 g/L solutions of either BSA or lysozyme (SI Materials and Methods). Of the 17 common GB1 residues quantified in cells and in buffer, 13 yielded measurable rates in BSA; the others exchanged too quickly (Table S4). The corresponding opening free energies, ΔGcell,enu were compared with those obtained in buffer and in cells (Fig. 6). BSA destabilizes GB1 compared with dilute solution, whereas the protein is stabilized in cells. In lysozyme, exchange rates are so large that the backbone N–H signals have completely decayed by the first acquisition (~20 min). We conclude that lysozyme destabilizes GB1 by ~1 kcal/mol compared with buffer alone.

I6L Variant. Recently, we used a thermodynamic cycle comprising kobs measurements of residues in chymotrypsin inhibitor 2 and a variant, in buffer and in reconstituted cytosol, to confirm the EX2 mechanism (26). We repeated this strategy with the destabilized GB1 variant, I6L (57). Comparisons of ΔΔΔGcell,enu values were possible for 12 residues (Tables S5–S7). We made three comparisons. First, we calculated the change in residue-level stability of the variant in cells compared with buffer (ΔΔΔGcell,enu WT - ΔΔΔGcell,enu I6L). Second, we calculated the change caused by the mutation (ΔΔΔGden,mut = ΔΔΔGden,mut WT - ΔΔΔGden,mut I6L) in cells.
they arise from globally exchanging residues, then we expect constant values of $\Delta G_{\text{op}}$ and $\Delta G_{\text{op,cell}}$ across the primary structure (Fig. 4 and Fig. S2). Although there is deviation, the range is <1 kcal/mol. For this reason we remain cautious about overinterpreting these deviations because data from 20 proteins (60) show that global unfolders yield $\Delta G_{\text{op}}$ values within 1 kcal/mol of $\Delta G_{\text{den}}$ obtained from thermal or cosolute denaturation. In addition, if some residues exchange by local unfolding and others by global unfolding we might expect a correlation between $\Delta G_{\text{op}}$ and $\Delta G_{\text{op,cell}}$, but this is not the case. In summary, we believe these residues exchange through global unfolding or high-energy fluctuations that are energetically indistinguishable from global unfolding under physiological conditions. With this caveat in mind, we suggest two additional sources of deviation. First, although most evidence points to equilibrium two-state folding of GB1 (36–38), there is evidence of complex kinetic pathways, so we cannot rule out the possibility that intermediates may be populated at equilibrium in cells or in buffer. Second, despite our knowledge that $k_{\text{int}}$ values do not change under crowded conditions (61), deviations could arise because intrinsic rates are derived from model peptides, not the specific primary structure of GB1.

**Exchange in Buffer.** As stated in the Introduction, to convert $k_{\text{obs}}$ values to $\Delta G_{\text{op}}$ values, the test protein must be stable (i.e., $k_{\text{cl}} \gg k_{\text{op}}$) and $k_{\text{int}}$ must be rate determining (the EX2 limit). GB1 is highly stable in dilute solution (28, 29), and intrinsic exchange rates in buffer are known (43, 44). Proof that intrinsic exchange is rate determining for GB1 in buffer comes from two sources. First, stopped-flow measurements provide a lower limit of $\sim 10^5$ s$^{-1}$ for $k_{\text{cl}}$ (30), whereas $k_{\text{int}}$ values are $<10^2$ s$^{-1}$. In addition, H/D measurements at two $p$H values can be used to assess the exchange mechanism, because intrinsic exchange is base catalyzed above $p$H 4 (62). Specifically, if $k_{\text{int}}$ is rate determining, changing the $p$H by one unit should change $k_{\text{obs}}$ by a factor of 10. Consistent with this idea, a plot of log $k_{\text{obs}}$ versus log $k_{\text{int}}$ for GB1 residues in buffer at $p$H 7.6 and 6.7 (Fig. S4) has a slope of 0.9 ± 0.1 and an intercept ($\sim 1.1 \pm 0.3$) equal to the difference in $p$H. Lastly, because our exchange experiments yield data for residues involved in global unfolding, the $\Delta G_{\text{op}}$ values should approximate $\Delta G_{\text{den}}$ from calorimetry. This approximation holds for both WT GB1 and the I6L variant (SI Materials and Methods).

**GB1 Structure in Cells.** Although the folding kinetics (5, 6, 8, 51, 63) and equilibrium thermodynamic stability (5–9) of globular proteins can be influenced by crowding, their tertiary structures should remain unchanged (51, 54, 64) because the packing densities of globular proteins approximate those for ideal packing of hard spheres (65). As discussed above, the ability to overlay the in-cell spectrum with that from dilute solution is consistent with this expectation.

Furthermore, the exchange data show similar patterns along the primary structure in both cells and buffer, supporting the conclusion that the tertiary structure is unchanged. More specifically, the pattern of solvent-accessible surface area (SASA) along the sequence has an approximate inverse relationship with $\Delta G_{\text{op}}$ (Fig. 4). The average SASA for the 17 residues with quantifiable exchange rates (excluding T18), the seven residues

![Fig. 5. GB1 (1PGB) is stabilized in cells. Residues are colored by the magnitude of $\Delta G_{\text{op,cell}}$. Gray residues are unassigned.](image)

**Fig. 5.** GB1 (1PGB) is stabilized in cells. Residues are colored by the magnitude of $\Delta G_{\text{op,cell}}$. Gray residues are unassigned.

**Exchange in Cells.** The fact that the $^{15}$N/$^1$H HSQC spectrum of GB1 can be overlaid with the spectrum in buffer (13) indicates the protein is stable in cells. Further, $k_{\text{int}}$ values do not change significantly in reconstituted cytosol compared with buffer (61). Unfortunately, we cannot test the requirement that $k_{\text{int}}$ is rate determining by changing the $p$H because we cannot accurately manipulate the intracellular $p$H. For this reason we turned to the thermodynamic cycle (26), mentioned in Results (Fig. S3). Briefly, if $k_{\text{int}}$ is rate determining in cells, the change in $\Delta G_{\text{op}}$ caused by a mutation ($\Delta G_{\text{op,mut}}$) should be the same in buffer and cells (Fig. 7). Of the 12 comparable residues, 3 (K4, T51, and T53) of the $\Delta G_{\text{op,mut}}$ values agree within 1 SD of the mean, and another 3 (Y3, A26, and A34) within 2 SDs. The remaining 6 residues (K28, V29, K31, T44, D46, and F52) differ by more than 2 SDs. We were puzzled that not all of the residues satisfied the condition $\Delta G_{\text{op,mut,cell}} \approx \Delta G_{\text{op,mut,buff}}$, because, as discussed above, all 12 residues are exposed only on global unfolding and possess similar protection factors. We hypothesize that the exceptions arise because the thermodynamic cycle neglects the possibility that mutations introduce interactions (with respect to WT) between GB1 and the cytoplasm that are absent in buffer. We are currently testing this hypothesis.

To provide further, albeit indirect, evidence that we are measuring free energies, we estimated the effect the intracellular environment would need to impose on GB1 to move exchange to the EX1 limit ($k_{\text{obs}} \approx k_{\text{op}}$). Using a $k_{\text{cl}}$ of $10^5$ s$^{-1}$ (30) and the average value of $\sim RT \ln(k_{\text{obs}}/k_{\text{op}})$ for the 17 residues quantified in cells, the cytoplasm would have to decrease $k_{\text{cl}}$ $10^5$–$10^6$-fold and decrease $k_{\text{cl}}$ by an order of magnitude compared with dilute solution to force exchange into the regime where $k_{\text{cl}}$ is rate determining. Such drastic effects are unlikely and have never been observed in cells (5, 6, 8, 63). In summary, the data are consistent with the assumption that we are measuring free energies of opening in cells.

![Fig. 6. $\Delta G_{\text{op}}$ values for WT GB1 residues in 100 g/L BSA, buffer, and cells. Error bars represent the SD of the mean from three trials.](image)
that decay too quickly, and the 24 quench-labeled residues are 0.6 ± 0.3 Å², 1.3 ± 0.9 Å², and 2 ± 1 Å², respectively, where the uncertainties are the sample SDs. Hence, the quench-labeled and rapidly exchanging residues are more likely to have greater solvent exposure. These exposed backbone amide nitrogen atoms are found in loops, the outer strands of the four-stranded sheet (β2 and β3), and the ends of the helix. The observations about SASA are consistent with dilute solution studies of GB1 structure (28, 32), dynamics (31), and H/D exchange (33). We conclude that the cellular interior does not change the structure of GB1 compared with buffer.

**Cellular Environment and GB1 Stability.** The cytoplasm of *E. coli* stabilizes GB1 residues by 0.43 ± 0.06 to 1.14 ± 0.05 kcal/mol compared with buffer at the same pH and temperature (Figs. 4 and 5). Recent advances in both the experimental (5–9, 13, 14, 16–19, 24, 26, 56, 63) and theoretical (20–22, 25) aspects of macromolecular crowding allow this stabilization to be rationalized in terms of the properties of GB1 and the *E. coli* cytoplasm.

The net effect of macromolecular crowding arises from the relative effects of hard-core repulsions, which are always stabilizing, and longer range interactions, which may be stabilizing or destabilizing (19, 23). GB1 has a pI of 4.8, similar to that of the majority of *E. coli* proteins (66), and a net charge of −4 at pH 7.6. These properties are expected to result in a large number of charge–charge repulsions in cells. Indeed, it has been suggested that these repulsions are what allow GB1 to tumble freely in the cell and yield high quality in-cell 15N–1H HSQC spectra (13, 14, 54, 55, 67, 68). These repulsive interactions enhance the volume excluded by hard interactions in cells, thus favoring the compact intracellular environment.

Contrary to the stabilization of GB1 in cells, individual protein crowders destabilize the protein compared with buffer alone (Fig. 6). GB1 is destabilized such an extent in 100 g/L lysozyme that quantification was not possible. This destabilization can be understood by the prevalence of weak, attractive interactions between positively charged lysozyme (pI = 11.3) and anionic GB1. The attractive interactions are destabilizing because the unfolded state possesses more reactive surface than the folded state, lowering the free energy of the denatured state ensemble relative to the native state.

**Effect of Protein Crowders in Vitro.** Contrary to the stabilization of GB1 in cells, individual protein crowders destabilize the protein compared with buffer alone (Fig. 6). GB1 is destabilized to an extent of 100 g/L lysozyme that quantification was not possible. This destabilization can be understood by the prevalence of weak, attractive interactions between positively charged lysozyme (pI = 11.3) and anionic GB1. The attractive interactions are destabilizing because the unfolded state possesses more reactive surface than the folded state, lowering the free energy of the denatured state ensemble relative to the native state.

A similar explanation for destabilization by BSA is less straightforward. Based on our rationale for in-cell stabilization, we expected stabilization of GB1 in 100 g/L BSA (pI = 4.7) compared with buffer alone because both GB1 and BSA have anionic surfaces. However, this destabilization is in agreement with our observations (24, 26, 56, 70) for chymotrypsin inhibitor 2 (CI2, pI = 6.0), supporting the hypothesis that nonspecific, attractive backbone interactions can overcome charge–charge effects and hard-core repulsions. Moreover, the fact that cells are not crowded with only one protein complicates such simplistic comparisons. Nevertheless, the effect of BSA and other protein crowders can be rationalized via Zhou’s realization that despite the presence of stabilizing, repulsive soft interactions between a test protein and a crowder, there exists a temperature above which crowding will be stabilizing (71). Given our data, we expect a cross-over above 37 °C for the BSA–GB1 pair, which is reasonable because the cross-over for CI2–BSA is 37 °C (71).

**Contributions to Protein Stability in Cells.** Recent work has shown that the effects of macromolecular crowding on globular protein stability depend on the nature of the crowder (21, 25, 26, 70). Synthetic polymers tend to act as inert spheres and are stabilizing. Physiologically relevant crowders (e.g., proteins, cytoplasm) modulate the hard-core effect through longer range interactions: Attractive forces between the crowder and test protein favor destabilization, and repulsive interactions enhance stability. However, this idea may be too simple, as indicated by our observation on the effect of BSA and the temperature dependence of crowding discussed by Zhou (71). Another complication is the role the cell has in modulating stability via compartmentalization, as highlighted by Gruebele and coworkers (5, 9). Although the present study enriches our knowledge of the forces stabilizing proteins under native conditions, more studies are necessary to bring about a comprehensive understanding of the effects of cellular crowding on protein stability.

**Materials and Methods.** Plasmid information and protocols for protein growth, purification, H/D exchange and calorimetry experiments can be found in SI Materials and Methods, along with tables of exchange rates, opening free energies, and supporting figures. Unless otherwise stated, pH readings are uncorrected for the deuterium isotope effect (72). Intrinsic rate constants from the online Server Program for Hydrogen Exchange Rate Estimation, SPHERE (44), were calculated for exchange at 37 °C and pH 7.2. Experiments were performed in triplicate. Uncertainties are the SD of the mean.

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