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Electrostatic Contributions to Protein Quinary Structure

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Supporting Information

ABSTRACT: There are four well-known levels of protein structure: primary (amino acid sequence), secondary (helices, sheets and turns), tertiary (three-dimensional structure) and quaternary (specific protein−protein interactions). The fifth level remains largely undefined because characterization of quinary structure, the transient but essential macromolecular interactions that organize the crowded cellular interior, requires the measurement of equilibrium thermodynamic parameters in living cells. We have overcome this challenge by quantifying the pH-dependence of quinary interactions in living *Escherichia coli* cells using the B1 domain of protein G (GB1, 6.2 kDa). To accomplish this goal, we buffered the cellular interior and used NMR-detected amide proton exchange to quantify the free energy of unfolding in cells. At neutral pH, the unfolding free energy in cells is comparable to that in buffered solution. As the pH decreases, the increased number of attractive interactions between *E. coli* proteins and GB1 destabilizes the protein in cells relative to buffer alone. The data show that electrostatic interactions contribute to quinary structure.

Protein "quinary structure" was independently described three times: by Vaĭnshteĭn in 1973, by Edelstein in 1980 and by McConkey in 1982.[1−3] The latter contribution motivated the work described here. McConkey observed that evolutionarily distant protein homologues have similar overall charges; this is completely unexpected if their exteriors need only be hydrophilic, and suggests that the cellular interior is highly organized. In 1983, Srere consolidated numerous findings that Krebs' cycle enzymes can be isolated together and coined the term metabolon to describe the organizing effect of quinary interactions.[4] The transient nature of quinary interactions allows cells to alter their metabolism in response to the environment and is essential for survival.[5] However, there is no quantitative information regarding the strength of the interactions that comprise quinary structure because they can only be studied in living cells. Here, we quantify the potential contribution of charge−charge interactions.

Efforts to understand protein behavior under physiologically relevant conditions began with studies using high concentrations of uncharged synthetic polymers.[6,7] These results were interpreted mostly in terms of hard-core repulsions, which occur because two atoms cannot occupy the same space at the same time.[8] As expected, these polymer solutions often stabilize proteins.[9] Synthetic polymers, however, do not adequately mimic the cellular interior, because biologically relevant crowding molecules also interact chemically with proteins.[10−13] These so-called soft interactions, which define quinary structure, modulate the effect of hard-core repulsions and can stabilize or destabilize proteins, depending on whether interactions between the crowder and the test protein are repulsive or attractive, respectively.[14]

The B1 domain of streptococcal protein G (GB1, 6.2 kDa, pI 4.6)[15,16] is the quintessential test protein to probe the potential of quinary structure in *Escherichia coli* because GB1 has been extensively characterized, it is a two-state folder and it is not native to *E. coli*, which minimizes the likelihood of specific interactions.[17−20] In addition, GB1 can be studied in cells using NMR because GB1 and the majority of proteins in *E. coli* are polyanions at physiological pH,[21] and the consequent net charge−charge repulsions facilitate the acquisition of high quality in-cell HSQC spectra.[22]

There are no histidines in wild-type GB1. By installing a histidine at position 10 (K10H GB1) to measure the intracellular pH, we developed a buffer to control the intracellular pH of *E. coli*, and showed that the cytosolic pH affects the quality of in-cell $^{15}N-^{1}H$ HSQC spectra.[23] Specifically, as the pH is decreased, the accumulation of positive charge on the surrounding *E. coli* proteins increases the attractive interactions with polyanionic GB1, slowing GB1 tumbling and broadening its crosspeaks into the background. These results qualitatively demonstrated that the intracellular pH modulates quinary structure. Here, we used NMR-detected amide proton exchange to quantify the pH-dependence of K10H GB1 stability in buffer and in cells.

Protein stability, $\Delta G^{\circ'}_{U}$, is the Gibb's free energy of the unfolded (U) state minus that of the folded (F) state, such that

$$\Delta G^{\circ'}_{U} = -RT\ln(K_U) = -RT\ln\left(\frac{[U]}{[F]}\right)$$

where R is the universal gas constant and T is the absolute temperature.

Amide proton exchange is a powerful tool for quantifying protein stability in *vitro*[24,25] and in living cells.[26−28] For many proteins, the free energy required to open the protein and expose a particular amide proton to solvent, $\Delta G^{\circ'}_{op}$, can be determined by dividing the observed rate of exchange, k_{obs}, by the intrinsic rate of exchange, k_{int}, such that

$$\Delta G^{\circ'}_{op} = -RT\ln\left(\frac{k_{obs}}{k_{int}}\right)$$

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One might expect all amide protons that are exposed only upon complete unfolding to give the same $\Delta G^{\circ\prime}_{op}$. However, this is not the case because of the inherent uncertainty in k_{int} values, which are derived from model peptides, not the particular protein being studied. Nevertheless, the method is valid because exchange data from 20 proteins show that global unfolding residues yield $\Delta G^{\circ\prime}_{op}$ values within 1 kcal/mol of $\Delta G^{\circ\prime}_{U}$ from thermal or cosolute denaturation.[29] Most importantly for this study, the method is valid for GB1 because stability measurements from amide proton exchange data have been confirmed by differential scanning calorimetry.[27,28]

We define the strength of quinary interactions, $\Delta\Delta G^{\circ\prime}_{quin}$, as the stability of GB1 in cells minus its stability in buffer at the same pH and temperature (37 °C).

$$\Delta\Delta G^{\circ\prime}_{quin} = \Delta G^{\circ\prime}_{op,cells} - \Delta G^{\circ\prime}_{op,buff}$$

Negative $\Delta\Delta G^{\circ\prime}_{quin}$ values reflect protein destabilization in cells and suggest an increase in attractive quinary interactions.

We showed previously that replacing a negatively charged residue with more positively charged amino acids destabilizes GB1 in cells in a charge-dependent manner.[28] Other groups have shown that protein surface charge plays a role in quinary interactions.[22,30,31] To investigate further the role of electrostatic interactions in quinary structure without perturbing the protein sequence, we quantified GB1 stability at pH 7.4, 6.0 and 5.0.

At pH 7.4, K10H GB1 and the majority of *E. coli* proteins (pI < 7)[21] are polyanions. K10H GB1 remains polyanionic between pH 7.4 and 5.0, whereas many *E. coli* proteins change from polyanions to polycations as the pH is decreased below their pI. *E. coli* are known to survive these slightly acidic conditions.[32,33] We hypothesized (Figure 1) that the accumulation of positive

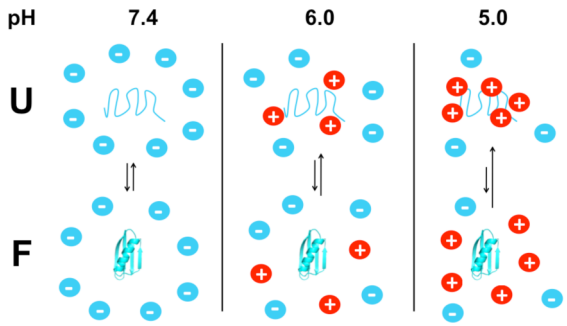

Figure 1. Interactions between *E. coli* proteins (red and blue circles) and the unfolded (U) and folded (F) states of GB1 (PDB ID 3PGB) as a function of pH. As the pH is decreased, *E. coli* proteins become more positively charged, and GB1 is destabilized. The relative lengths of the arrows are not to scale; $\Delta G^{\circ\prime}_{U}$ is >0 at all three pH values (Figures S1–S3).

charge on *E. coli* proteins as the cytosolic pH is lowered would preferentially increase attractive charge–charge interactions with the unfolded ensemble of GB1, because the unfolded protein has more accessible surface, and thus decrease GB1 stability in cells compared to buffer.

The pET-11a plasmid harboring the K10H variant,[34] the isolation and purification of GB1[15,23] and the protocol for NMR-detected amide proton exchange in cells and in buffer have been described.[27] The construct also carries the T2Q variant to prevent N-terminal deamidation.[34] For dilute solution experiments, 2.2 mg of lyophilized protein was resuspended in 500 μL of 75 mM HEPES/75 mM bis-tris propane/75 mM citrate, 99.9% D_2O at the desired pH, and $^{15}N-^{1}H$ HSQC spectra were acquired serially.

The quenched lysate method is an established approach for quantifying protein stability in cells.[26,27] Briefly, expression of the K10H variant was induced with 1 mM (final concentration) of isopropyl-β-D-thiogalactopyranoside when *E. coli* cells reached an optical density of 0.6 at 600 nm. After 2 h, chloramphenicol was added to stop protein expression and the cells were harvested by centrifugation. A timer was initiated after resuspension in the D_2O-containing buffer. Individual aliquots were removed at discrete time points and hydrogen exchange was quenched while cells were simultaneously lysed by vortexing with glass beads.[27] After centrifugation, the supernatant was transferred to an NMR tube and a spectrum acquired. At pH 7.4 and pH 6.0, aliquots were removed within 2 h after exchange was initiated. At pH 5.0, aliquots were removed ∼1, 2, 3, 8 and 15 h after initiating exchange.

Exchange rates were converted to $\Delta G^{\circ\prime}_{op}$ values using intrinsic rates of exchange from SPHERE (37 °C, alanine oligopeptide basis).[35,36] Figures S1–S3 showing $\Delta G^{\circ\prime}_{op}$ values as a function of residue number at each pH value and Tables S1–S3 listing k_{obs} and $\Delta G^{\circ\prime}_{op}$ values are provided in the Supporting Information (SI). The stability measurements were confirmed by differential scanning calorimetry using a Microcal VP-DSC calorimeter (Table S4). For calorimetry, the temperature was increased from 20 to 95 °C at a rate of 60 °C/h for three scans to yield $\Delta H^{\circ\prime}_{cal}$ and T_m. Analysis was performed as described by Becktel and Schellman[37] to obtain $\Delta G^{\circ\prime}_{D}$.

The number of quantifiable residues is limited by crosspeak overlap and large k_{obs} values. We were able to quantify $\Delta G^{\circ\prime}_{op}$ for the 12 residues that have k_{obs} values of $<9.7 \times 10^{-4}$ s^{-1}. Quantifiable residues are well distributed in the secondary structure: β1 (Y3, K4), α1 (A26, K28, V29, K31, A34), β3 (T44, D46) and β4 (T51, F52, T53). These 12 residues are known to exchange upon complete GB1 unfolding; therefore, the mean $\Delta G^{\circ\prime}_{op}$ approximates $\Delta G^{\circ\prime}_{U}$.[19,27] Backbone amide protons in β2 exchange too quickly to quantify.[19] At pH 7.4, we can quantify $\Delta G^{\circ\prime}_{op}$ for K4, A26, T51 and T53 in buffer, but the crosspeaks decay too quickly for quantification in cells. Hydrogen exchange is base-catalyzed,[25] and we attribute the limited data and the larger uncertainties at the highest pH to faster exchange.

At pH 7.4, the average $\Delta G^{\circ\prime}_{op}$ in buffer is 6.80 ± 0.07 kcal/mol (Figure S1), where the uncertainty is the standard deviation of the mean. This value is approximately equal to that in cells, 6.65 ± 0.09 kcal/mol (Figure S1). The $\Delta\Delta G^{\circ\prime}_{quin}$ values (Figure 2) are modestly positive or negative. Their average value, 0.1 ± 0.1 kcal/mol (the uncertainty is the standard deviation of the mean), is insignificant, indicating that the net interaction between GB1 and the cellular milieu is negligible at this pH. Lowering the pH to 6.0 decreases the stability of GB1 in buffer and cells (Figure S2), but the destabilization is more dramatic in cells; the average $\Delta\Delta G^{\circ\prime}_{quin}$ is −0.80 ± 0.05 kcal/mol. At pH 5.0, the destabilization is even more dramatic in cells (Figure S3), and the average $\Delta\Delta G^{\circ\prime}_{quin}$ is −1.12 ± 0.06 kcal/mol. These data indicate a significant role for electrostatic interactions in quinary structure (Figure 3).

Studies of globular proteins have long emphasized the requirement for a well-packed and hydrophobic interior[38] with nearly complete formation of internal hydrogen bonds.[39] Despite the fact that these studies were conducted in simple buffered solutions, they provided physiologically relevant

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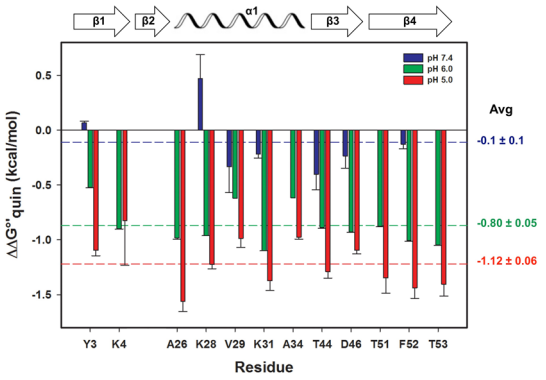

Figure 2. $\Delta\Delta G^{\circ\prime}_{quin}$ values for K10H GB1 at pH 7.4 (blue), pH 6.0 (green) and pH 5.0 (red) at 37 °C. Error bars represent the uncertainties propagated from triplicate (dilute solution) and duplicate (cells) experiments. Dashed lines represent the average at each pH. The uncertainties are the standard deviation of the mean.

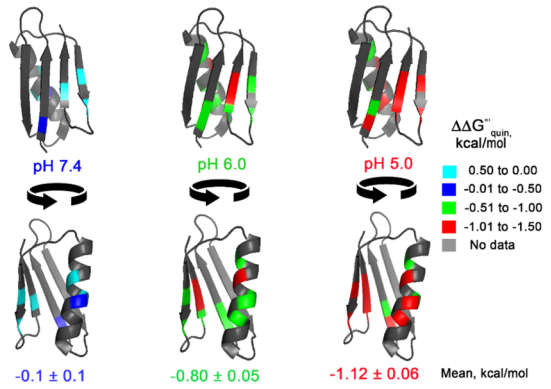

Figure 3. Structure of GB1 (PDB ID 3PGB) with residues colored by the magnitude of quinary interactions at each pH value.

information about protein interiors because interior atoms experience the same environment in cells and in buffer: they are surrounded by atoms belonging to that same protein.

Despite the ideas of McConkey,[3] interest in protein surfaces remained stagnant: defining them as hydrophilic was sufficient. Our data indicate that exteriors are as important as interiors for understanding protein chemistry in cells, but this physiologically relevant information was hidden because studies were performed in simple buffered solutions. The key idea is that exterior atoms are exposed to mostly water in simple buffered solutions, but in cells, these atoms are exposed to the complex, crowded and dynamic cytoplasm.[40,41] There is now an emerging realization that exteriors play an important role in cells by forming the transient interactions that comprise protein quinary structure.[22,31,42,43]

Quinary interactions, by definition, are absent in dilute solutions, but they play an important role in the dense cellular environment. Traditional crowding theory predicts that the cellular environment should stabilize proteins strictly due to the effects of hard-core repulsions. Although such steric effects must be present, they are modulated by transient chemical interactions that either reinforce or oppose them.[10,14,44,45] Several studies[10,28,46] have shown that the cellular interior destabilizes proteins, but the nature of quinary interactions remains mostly unchartered territory.

As shown here, at pH 7.4, the stabilizing effect of hard-core repulsions is balanced by attractive quinary interactions to the extent that GB1 stability is approximately equal in cells and in buffer. In other words, near neutral pH, attractive interactions between proteins are tempered because both GB1 and the majority of *E. coli* proteins are negatively charged.[21] In buffer, GB1 stability decreases by 1.3 kcal/mol when the pH is lowered from 7.4 to 5.0. The pH-induced destabilization, however, is much more dramatic in cells: GB1 is destabilized by almost 2.5 kcal/mol when the pH in cells is decreased from 7.4 to 5.0. The extent to which pH modulates quinary structure is remarkable (Figure 3), causing a 30% decrease in stability.

Additional sources of the stability changes in cells compared to buffer must also be considered. For instance, the pH changes in cells could unfold other proteins or change the unfolded state ensemble of GB1, exposing additional polar and hydrophobic groups. Such interactions,[28] and changes in metabolite ionization, may also contribute to our observations. Changes in chaperone activity are not likely to contribute because as enzymes, chaperones do not affect equilibria, which are the basis for the measurements described here.

Ultimately, the changes in interactions that alter GB1 stability in cells compared to buffer arise from manipulating the intracellular pH. In buffer, protein stability is governed by hydrophobic interactions and specific intramolecular interactions.[39,47] Despite their predicted importance,[1−3] the role of protein surfaces, with few exceptions,[48,49] has been overlooked, and the contribution of intermolecular interactions in cells has been ignored. Our results provide quantitative evidence that charge−charge interactions are important factors in quinary structure. In summary, surfaces cannot be ignored when proteins are studied in their native environment because there is more to protein stability than a well-packed, hydrophobic core.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b07323.

Figures for the free energy of opening at each pH value and tables containing the results from NMR-detected amide proton exchange experiments (PDF)

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Notes

The authors declare no competing financial interest.

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