Cell Volume Controls Protein Stability and Compactness of the Unfolded State

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S Supporting Information

ABSTRACT: Macromolecular crowding is widely accepted as one of the factors that can alter protein stability, structure, and function inside cells. Less often considered is that crowding can be dynamic: as cell volume changes, either as a result of external duress or in the course of the cell cycle, water moves in or out through membrane channels, and crowding changes in tune. Both theory and *in vitro* experiments predict that protein stability will be altered as a result of crowding changes. However, it is unclear how much the structural ensemble is altered as crowding changes in the cell. To test this, we look at the response of a FRET-labeled kinase to osmotically induced volume changes in live cells. We examine both the folded and



unfolded states of the kinase by changing the temperature of the media surrounding the cell. Our data reveals that crowding compacts the structure of its unfolded ensemble but stabilizes the folded protein. We propose that the structure of proteins lacking a rigid, well-defined tertiary structure could be highly sensitive to both increases and decreases in cell volume. Our findings present a possible mechanism for disordered proteins to act as sensors and actuators of cell cycle or external stress events that coincide with a change in macromolecular crowding.

INTRODUCTION

The interior of the cell, where most proteins perform their function, is heterogeneous and dynamic. Proteins have evolved to perform optimally under the native crowded conditions that exist inside the cell.¹⁻⁴ However, the idea of a constant, "native" cellular environment is at odds with the transient conditions often found in live cells. Even in multicellular organisms, cells change volume in the "rounding" process leading to mitosis,^{5,6} during metastasis⁷ and blebbing,⁸ and as a result of shear forces⁹ or while squeezing through tight junctions.^{10,11} These morphological changes carry with them changes to the composition of abundant small solutes as well as larger biomolecules.^{8,12-14} Such a change in the cellular environment can have dramatic effects in certain cases. For example, pioneering work by Eaton and co-workers^{11,15} highlights how even small volume changes of erythrocytes can expedite the highly cooperative aggregation of mutated hemoglobin in sickle cells.

More recently, researchers have examined how cellular volume changes alter protein function in less cooperative processes. The fact that cellular volume is a highly tunable parameter, controllable by changes to external osmotic pressure, makes it convenient to use as a perturbation method to probe protein function inside the cell.^{12,16–19} For example, cellular volume changes were shown to reduce protein stability²⁰ or alter complex concentrations formed by relatively weak protein—protein interactions in live cells.¹² In another example, volume oscillations were used to reveal the osmotic-

stress mitigation mechanism in yeast cells¹⁶ or the role of the cell wall in *E. coli* cell growth.¹⁷ This body of work complements numerous other studies comparing between protein stability in dilute solutions and protein stability in the crowded cellular interior.^{21–25} Overall, the emerging picture highlights the remarkably heterogeneous effect of the cellular milieu on protein stability.

Groundbreaking work by Minton and co-workers has shown that a crowded solution can increase protein stability by favoring a more compact state that takes up less space in solution.^{2,26} The increased stability can be understood in terms of a shift toward higher melting temperature (T_m) —the temperature at which the population of a two-state protein is equally divided between its folded and unfolded states. This T_m shift increases the fraction of proteins in the folded state at a given temperature without changing the structure of the two states.^{27,28} Since its inception, macromolecular crowding theory has been refined, Today it is understood that macromolecules can have both repulsive interactions with protein surfaces (mainly entropic) that often stabilize the native state of proteins or sticking interactions (at least partly enthalpic) that could either stabilize or destabilize pro-

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teins,^{29–31} depending on the protein's shape and electrostatic/ hydrophobic interactions of its surface with the surrounding solution.³² These enthalpic or "soft" interactions can lead to unexpected consequences in the presence of macromolecular crowders.^{33–36}

As one such example, there is accumulating evidence that the unfolded state is especially susceptible to structural changes that occur from crowded environments. Recent work shows how changes to crowded conditions induced by the addition of inert polymers such as Ficoll or dextran have a significantly larger effect on unfolded proteins than on folded ones.^{37–39} Experiments by Pielak and co-workers have even highlighted this sensitivity in *E. coli* cells.⁴⁰ The importance of the unfolded state should not be underestimated: at any given moment, a non-negligible percentage of well-folded proteins is predicted to be unfolded, even when the cell is in optimal conditions.^{41,42} If we account for intrinsically disordered proteins, which amount to roughly 30% of the human proteome,⁴³ a significant percentage of the proteome may be responsive to volume changes. Since such volume changes occur naturally, we wanted to test the sensitivity of a model protein under folding and unfolding conditions to crowding changes.

We use live cell microscopy to directly test the sensitivity of the unfolded state to crowding changes. We change in-cell crowding by controlling the media's osmotic pressure and follow the fluorescence of a FRET-labeled protein transiently expressed in cells to understand the effect of these changes on its structure. We use fPGK, a modified, active phosphoglycerate kinase that is conjugated with AcGFP1 and mCherry at the N- and C-terminus, respectively. The FRET signal from fPGK has been shown to be a good proxy for its structure.^{44–46} For this work, we use fPGK_{LT}, a low-melting temperature mutant of fPGK (sequence in Supporting Information, Table S2) that unfolds in the cell at $\sim 35 \pm 1$ °C—a temperature where the cell is still viable. fPGK_{LT}, like other fPGK variants, exists in a pseudo-two-state equilibrium inside cells (as indicated by a single transition between two end states detected by our experiment; strictly speaking, PGK is a multistate folder).⁴⁴ We tune the population distribution between these two states in situ by controlling the temperature of the cell's surrounding media. We then test the response of $fPGK_{IT}$ to cellular volume change by altering the osmotic pressure around the cells and measuring the average cellular FRET signal using epifluorescent microscopy. A reciprocal change in green and red fluorescence was previously shown to be indicative of a change in positioning between the two FRET labels—caused by a structural change in the protein.^{44,47}

Our experiments show that the folded state of fPGK_{LT} is not very susceptible to cellular volume changes: when fPGK_{LT} populations are primarily folded, increasing or decreasing the cell's water volume by up to 30% has little or no effect on protein compactness, as judged by FRET. This makes sense because many enzymes, especially ones involved in crucial metabolic cycles, evolved to function well even when the cell itself is under duress.⁴⁸ As the surrounding media's temperature increases, an unfolded fPGK_{LT} population emerges. Our experiments show that the FRET signal from this unfolded population is responsive to volume changes: decreasing cellular volume can compress the unfolded fPGK_{LT}, in a manner similar to that observed for other proteins due to macromolecular crowding.^{49,50} Upon volume increase, the structure of fPGK_{LT} further expands, indicating that the original unfolded population was not completely extended in the

cell.^{51,52} Our data shows that both increased and decreased cellular crowding induces conformational changes in the structure of the unfolded state.

The work shown here highlights the sensitivity of proteins to changes that can occur naturally or as a result of environmental stress in live cells and indicates that more research should be done regarding how proteins act and interact under conditions that fluctuate around the optimum. Specifically, the unfolded state of proteins, or proteins that are primarily disordered, should be probed to see if function is altered or turned on/off when crowding in the cell changes.

METHODS

Plasmid Design. The plasmid for the $fPGK_{LT}$ fusion construct was designed with an in vitro melting temperature of \sim 35 °C, so that the protein could be unfolded at temperatures where mammalian cells are viable. This mutant was designed on the basis of an enzymatically active, less destabilized triple mutant (Y122W/W308F/W333F) of yeast PGK, fPGK_{HT}, with a melting temperature of ~42 $^{\circ}$ C.⁴⁵ To further destabilize the protein, we made the enzymatically active point mutations (F333W, P204H)⁵³ and loop insertion (89_90insSGGGGAG).⁵⁴ The protein was labeled at the N-terminus with AcGFP1 and the C-terminus with mCherry, with a two amino acid linker between the protein and the label. To assist with in vitro purification, a 6xHis-tag and thrombin cleavage site were added to the N-terminus of the AcGFP1. This gene was cloned between BamHI and NotI in the pDream 2.1 expression vector (GenScript Biotech), which has a CMV promoter for expression in mammalian cells.

Materials. U-2 OS cells from ATCC were grown in DMEM supplemented with 10% FBS, 5% penicillin streptomycin, and 5% sodium pyruvate in 75 cm² cell culture flasks. Imaging was done in FluoroBrite media (Gibco) supplemented with NaCl or diluted with Milli-Q water. 40 mm round #1.5 coverslips (Bioptechs) were cleaned by holding them to an open flame for 1 s, sonicating once in 1 M KOH for 10 min, twice in water for 10 min, and storing in 70% ethanol.

Transfection. Cells were transfected using 10 μ L of Lipofectamine 2000 and 4 μ g of the appropriate plasmid once the culture reached 70–80% confluence. After incubation in DMEM without penicillin streptomycin for 5–6 h, cells were trypsinized and plated on precleaned 40 mm round #1.5 coverslips (Bioptechs) in 60 mm round dishes (FisherBrand) for 14 h.

Flow Cell Microscopy. All flow cell experiments were done using an FCS2 chamber (Bioptechs). This is a closed system with perfusion tubes for near laminar flow and temperature control, that can maintain constant temperature. The coverslip with adhered, transfected cells was washed twice with 2 mL FluoroBrite media before being placed on a microaqueduct slide with a 100 μ m thick 14 mm \times 22 mm rectangular silicon gasket. The chamber was assembled, locked, and connected to the flow system, making sure that there was no bubble in between the coverslip and microaqueduct slide. The FCS2 chamber was used to control temperature inside the flowcell by using a temperature controller. A second probe for the temperature, a J-type thermocouple, was inserted directly into the outlet tube of the FCS2 chamber. Temperatures measured from the inlet tube and the outlet tube have a difference of less than 0.5 °C (Figure S1).

Media reservoirs were placed in a water bath with temperature control to create a stable baseline for media



Figure 1. Volume modulation and protein response. (A) 3D reconstruction of confocal images of cell surfaces before (top) and after (bottom) -0.1 Osm (left) or +0.5 Osm (right) change. (B) Change in cell free volume V_f (eq 4) as a function of osmolarity change. Gray points are individual cells, red points represent the average. Lines are standard error (red) and standard deviation (blue) of the data. (C) In-cell D/A of fPGK_{LT} and fPGK_{HT} measured by volume modulation (solid lines) or T-jump (dashed lines) as a function of temperature. In-cell temperature was obtained by temperature calibration as described in Figure S6. The majority of fPGK_{LT} was folded at 18 and 24 °C (left structure), the unfolded population increased at 33 °C, and nearly the entire protein was unfolded at 39 °C (right structure). (D) Schematic of the volume-modulation setup, as detailed in the Methods section.

temperature. All media lines were isolated with cotton and aluminum foil to reduce heat loss. Once the temperature in the flowcell equilibrated at the appropriate temperatures for 90 s, imaging was initiated. Initially isosmotic (0.3 Osm) media was flowed over the cells at a rate of 3 mL/min for 10 s. We then switched from the isosmotic reservoir to a non-isosmotic one by using a distributor (IDEX) and flowed this media for an additional 120 s at the same rate, before switching back to the isosmotic media. The flow rate was controlled using a nitrogen flow controlled by a piezo valve (Elveflow OB1) and coupled to a flow meter to adjust pressure in order to maintain a constant flow and prevent focus drift. A flow-related focus drift occurred when switching between reservoirs. A temperaturerelated focus drift occurred after switching, since it took a few seconds for the FCS2 chamber to reach equilibrium (Movie S1 A and B). The entire setup was timed and controlled using a home-built LabVIEW program (National Instruments).

Fluorescence imaging was done by exciting the donor or the acceptor at 470 or 590 nm, respectively, using an LED (ThorLabs M470-L3, M590-L3). For FRET, following donor excitation, emitted light was split into green and red channels by a dichroic (Chroma) and directed onto adjacent spots on a Phantom V12.1 CMOS camera. Imaging was performed at 24 frames per second with 800 × 600 resolution using a 63× NA 0.85 N-achroplan air-immersion objective (Zeiss). Our imaging shows that U-2 OS cells can survive in extreme hypoosmotic (~0.1 Osm) or hyperosmotic (~0.8 Osm) media and can recover rapidly once the osmotic stress disappears (Figure S2, jp8b08216_si_002.avi, jp8b08216_si_003.avi, and jp8b08216_si_004.avi).

Osmolarity Calibration. To determine the time dependence of osmolarity change during flow cell microscopy, we rely on separate calibration experiments. Calibration experiments use one reservoir containing water and the other reservoir containing water with 0.2 μ M fluorescein. Imaging was performed in the same way as flow cell microscopy (see above). The rate of media switching is quantified by the changes in the fluorescein fluorescence signal, as shown in Figure S3, and used to assess the switching between isosmotic and non-isosmotic media. To determine the calibration uncertainty, independent measurements were performed on different slides and different imaging positions on a single slide and showed good reproducibility.

FRel Experiments. 25 × 75 mm², 1.0 mm thick slide (VWR) coverslips with adhered, transfected U-2 OS cells were attached to slides using a 120 μ M thick spacer sticker (Grace biolabs). The FReI microscope setup was described in previous works.^{19,45,46} To obtain sharp stair-stepping temperatures (temperature jump, T-jump), a 2200 nm IR diode laser was focused on the imaged cells, which was heated rapidly by modulating the laser amplitude. Imaging was performed at 60 frames per second with 800 × 600 resolution using a 63× NA 0.85 N-achroplan air-immersion objective (Zeiss).

Data Analysis. MATLAB (MathWorks) was used to analyze all data. To quantify changes in protein structure resulting from osmotic modulation, we measured the fluorescence of both green and red channels. We examine the change in fluorescence before and after volume change, $\Delta F = F_{\text{stress}} - F_{\text{iso}}$, where the subscripts stress and iso denote the osmotically stressed or isosmotic signal, respectively. The fluorescence during imaging can be affected by bleaching, cell volume V change, temperature change, and cross-talk between the two channels (eq 1)

$$\Delta F = \Delta F(V) + \Delta F(B) + \Delta F(T) + \Delta F(\text{cross-talk})$$
(1)



Figure 2. fPGK_{LT} in single U-2 OS cells responds to cell volume modulation. (A) Micrographs of U-2 OS cells before (top), during (middle), and after recovery (bottom) from volume changes. Relative volume changes ΔV_f are written at the bottom right-hand side. Volume changes were calculated from media osmolarity (Figure 1B, Figure S3). The temperature and volume changes of all cells are specified at the top of each column. Scale bars are 10 μ m. (B) Relative free cell volume change as a function of time. Switching occurs at 10 and 130 s. (C) Temperature measured by thermocouple at the outlet of the FCS2 chamber. The dips in temperature seen at 39 °C and at ~40 and ~150 s are a result of flow switching. We correct for this artifact as described in Figure S5 and Table S1. (D) Whole-cell averaged green and red fluorescence intensity normalized to their initial values. (E) FRET signal normalized to initial values. In panels B–E shaded areas correspond to regions averaged to obtain changes from crowding modulations.

The three terms on the right-hand side correspond to the change in fluorescence due to volume change $\Delta F(V)$, bleaching $\Delta F(B)$, and temperature change $\Delta F(T)$. The bleaching term $\Delta F(B)$ was corrected by subtracting a linear function fitted to the plateau after temperature equilibration. The temperature change correction is described in Figure S5. The fluorescence changes as a result of volume change are of the main interest here. From this signal, the protein structural change can be assessed by D/A or the relative FRET change χ (eq 2 and eq 3). D/A was calculated by

$$D/A = \frac{F_{\text{green}}}{F_{\text{red}}}$$
(2)

where F_{red} and F_{green} are the intensities of the acceptor (mCherry) and the donor (AcGFP). Relative FRET change was calculated by

$$\chi = \frac{S_{\text{stress}} - S_{\text{iso}}}{S_{\text{iso}}} \tag{3}$$

where S_{stress} and S_{iso} are observed signals after and before osmotic challenge.

We assume that the water content of the cell, which is not taken up by large molecules such as other proteins, is initially 70% of the total volume.^{12,55,56} We then estimate the change in free cellular volume upon osmotic duress, since the macromolecular content of the cell remains largely constant during short osmotic challenges.^{4,18} We calculate the free volume (i.e., that not occupied by macromolecules) in the cell, $V_{\rm ff}$ following an osmotic challenge through eq 4

$$V_{\rm f} = 1 - \frac{1 - \frac{V}{V_0}}{0.7} \tag{4}$$

where V_0 and V are the volume before and after osmotic challenge, respectively.¹² We calculate the change in relative cell volume by $\Delta V_{\rm f} = (V_{\rm f,\ stress} - V_{\rm f,\ iso})$.

RESULTS

Osmotic Pressure Correlates with Cell Volume Change. In order to perturb the cell's volume, we use DMEM media supplemented with purified Milli-Q water (for hypoosmotic conditions) or with added NaCl (for hyperosmotic conditions) (Figure 1A). We have previously quantified the relationship between cell volume change and media osmotic pressure by using fast 3D confocal microscopy to measure cell volume¹² (Figure 1B). We use this relationship to report directly on the change of free volume (the volume



Figure 3. Green and red fluorescence changes due to volume modulation below, around, and above the melting temperature after temperature correction. Fluorescence is normalized to isosmotic period averaged between 4 and 10 s after we start imaging. Temperature is specified at the top of each column, and volume change is specified at the top of each panel. Solid lines are means of between 7 and 21 experiments from at least four separate transfections. Shaded areas are SD of repeats. More details and raw data are shown in Figure S4.

not occupied by macromolecules) in the cell, rather than the external osmotic pressure. We do this by estimating the initial free volume (e.g., the volume occupied by water in the cell) of \sim 70% and attribute any changes in total volume changes to this free volume (eq 4). This is rationalized by the fact that water moves in and out of the cell orders of magnitude faster than even small solutes.⁵⁷

Controlling the Partitioning of fPGKLT between the Folded and Unfolded States. We use temperature to tune how the cellular fPGK_{LT} population is distributed between the folded and unfolded states. The in-cell melting temperature $(T_{\rm m})$ of fPGK_{LT} was measured using FReI (Methods). Similar to what was reported previously for a higher $T_{\rm m}$ PGK mutant (fPGK_{HT}, $T_{\rm m}$ = 42 ± 1 °C, Figure 1C), fPGK_{LT} melting shows a characteristic sigmoid shape, with the midpoint at 35 ± 1 °C (Figure 1C). Below this point, the baseline represents a population that is largely folded. Above this point, the baseline represents a population that is largely unfolded. We picked temperatures below (18 and 24 °C), around (33 °C), and above (39 °C) the melting temperature to change the ratio between folded and unfolded fPGK_{LT} populations. We use a microscope setup equipped with a flowcell and temperature control (Figure 1D) to modulate crowded conditions in the cell through changes to media osmotic pressure. The readout temperature was calibrated by measuring the $T_{\rm m}$ of fPGK_{LT} and fPGK_{HT}, as described in Figure S6.

Representative experiments at low and high temperature as cell volume decreases are shown together with control experiments where the volume is not changed in Figure 2. Throughout the experiment, the surface area of the cell remains constant (Figure 2A), meaning the volume loss occurs primarily in cell-height loss, as previously reported.^{12,19} In experiments where the media remained isosmotic after the switch, no change was observed in green or red fluorescence (Figure 2B–E, first and third columns). A signal change is

observed only when hypo/hyperosmotic media was flowed over the cell, decreasing its volume (Figure 2B–E, second and fourth columns). In all cases except high temperature and volume change,¹⁹ the return to basal volume causes complete recovery of the fluorescence signal to their original levels, indicative of a highly reversible structural change induced by volume changes.

Free Cell Volume Changes Perturb Unfolded But Not Folded fPGK_{LT} Structure. To understand how volume changes affect the different structural ensembles of fPGK_{LT}, we performed volume modulation experiments below, around, and above the $T_{\rm m}$ and followed the change in green and red fluorescence that occurred as a result (Figure 3). We first noted that, when the cells are at room temperature (18 °C, Figure 3, left column) and the majority of $fPGK_{LT}$ is in the folded population (as indicated by the in-cell melting curve in Figure 1C), little change occurs in both red and green fluorescence as the cell volume is reduced. Slightly larger, but still small, is the change in green and red fluorescence when the cell volume increases. Increasing the temperature to 24 °C shows a slight increase in the amplitude at the largest and smallest volume conditions, but an overall small change is shown.

As the temperature increases further to 33 °C, the amplitude upon volume perturbation of both green and red fluorescence grows significantly. This coincides with the onset of unfolding, as shown in Figure 1C. At this temperature, the protein population is estimated to be $20 \pm 10\%$ unfolded. The change in green and red fluorescence becomes even larger at 39 °C, when nearly the entire protein population is unfolded. Notably, the green fluorescence changes are smaller than the red. This may be explained by (1) the fact that we are considering a relative change and that the absorption and fluorescence quantum yield of AcGFP1 are significantly greater than those of mCherry⁵⁸ and (2) the fact that not all proteins contain

both tags: Since the donor is at the N-terminal of the protein, incomplete expression or subsequent cleavage would create a donor-only construct. A donor-only fPGK (or just monomeric GFP) population is not expected to change its fluorescence signal even when structural changes occur in its protein conjugate, since there is no acceptor for FRET; as such, this population acts to reduce the overall change in fluorescence signal seen in Figure 3.

One key observation is the sensitivity of the unfolded ensemble to crowding decrease. Little attention has been paid to the fact that volume increase through water influx, a process that occurs routinely in the cell cycle, 5,6,59 carries with it a reduction in crowded conditions. We find that the folded state remains fairly insensitive to the decrease in crowded conditions, as noted by the small fluorescence changes at 18 and 24 °C. This is not surprising: the folded state is held together tightly by thousands of noncovalent interactions, and we do not expect large conformational changes resulting from decreased crowding. The unfolded state, however, has been shown to display more flexibility *in vitro* as well.^{40,50,60}

DISCUSSION

The signal changes shown in Figure 3 can be explained by two scenarios. In one scenario, which we term the " $T_{\rm m}$ shift" scenario, the protein coexists between a folded and an unfolded ensemble with lower and higher D/A ratios, respectively. As crowding changes, protein population is redistributed, and a change in fluorescence is observed. Such redistribution essentially amounts to a shift in melting temperature $T_{\rm m}$ and does not involve structural change in the ensembles. Indeed, we recently showed that, in the presence of increasing concentration of the polymeric crowder Ficoll, the $T_{\rm m}$ of fPGK increases.²⁵ This is shown in Figure 4A, top. The other scenario, which we term "change in unfolded compactness", attributes the change in fluorescence to compaction or expansion of the unfolded state. In this scenario the $T_{\rm m}$ may remain constant as shown for the latter in Figure 4B, top.

To differentiate between the " $T_{\rm m}$ shift" and the "change in unfolded compactness" scenarios, we calculate the fluorescence change for both in Figure 4. We use characteristic values of green and red fluorescence for the folded and unfolded states (as obtained from Figure 1C) as input. The estimated change in fluorescence as a result of a $T_{\rm m}$ (stability) shift due to a crowding increase (volume decrease) is shown in Figure 4A (middle), and that due to a crowding decrease (volume increase), in Figure 4A (bottom). A prominent feature of the crowding-induced change in fluorescence, ΔF , is a peak that rises near the $T_{\rm m}$. We show analogous simulations due to changes in unfolded state compactness in Figure 4B. For this scenario, ΔF continues to increase with temperature until it plateaus. In both cases, crowding changes at low temperatures have no effect on the change in fluorescence. Not shown is a scenario where native state compactness changes, which would result in fluorescence changes at low temperature that are clearly not observed in our experiments.

For the case of crowding increase (middle panels in Figure 4A,B), we cannot rule out either scenario without additional data taken at even higher temperatures which are difficult to perform in live cells. For the case of crowding decrease (bottom panels in Figure 4A,B), however, we do not observe a peak in the green nor red fluorescence changes under our incell conditions. The experimental data (symbols in Figure 4)



Figure 4. $T_{\rm m}$ shift shift vs change in unfolded compactness. (A) $T_{\rm m}$ shift or (B) change in unfolded compactness a result of crowding are shown for a parameter-free model (lines) and for data from Figure 3 (large symbols are averages of all individual cells, shown as smaller, semitransparent symbols). Error bars are SD of the data. Our model assumes a sigmoidal dependence of folded population on temperature, with a basal midpoint of 35 °C, and a decay constant of 2 °C. The green and red fluorescence is then calculated in crowding increase (middle panel) and decrease (bottom panel). The ΔF values correspond to the change in fluorescence based on the folded ensemble. These are calculated using basal values for folded (1500 and 500 AU for green and red, respectively) and unfolded conformations (1700 and 200 AU for green and red, respectively). For panel A, T_m shift is ± 2 °C, as estimated from experiments in Ficoll.²⁵ For part B, there was no $T_{\rm m}$ shift. Instead, the unfolded state green and red fluorescence was varied by $\pm 15\%$, as indicated from experiments at high temperatures.

instead show a plateau at high temperature and no effect at low temperature. Thus, the "change in unfolded compactness" best matches crowding decrease experiments, whereas the shift in stability or native state compactness must make smaller contributions. We also note the possibility of other scenarios, such as a destabilization of the unfolded state under increased crowding recently observed by Cohen and Pielak using NMR.⁴⁰ Due to the low resolution provided by our FRET experiments, our data does not definitively support any one scenario.

Thus, our model does not rule out small contributions from stability or native state compactness that occur simultaneously: a small shift of $T_{\rm m}$ (<2 °C) would result in a peak contribution to the fluorescence curve that is overwhelmed by the plateau due to unfolded state compactness. Alternatively, a large $T_{\rm m}$ shift toward increased stability would result in a monotonic increase in fluorescence change over the temperature range we used. Changes in the native or unfolded baselines of the melting curve may also carry with them a range of behaviors that we did not consider. Finally, we do not account for possible changes in the cooperativity of the unfolding transition, which would alter the slopes of the curves seen in the top panels of Figure 4A and B. All of these factors can, individually or together, improve the fit of the experimental data to the simulated curves. Nonetheless, our model can rule out certain possibilities. Upon decreased crowding, the peak in

 ΔF occurs at lower temperatures than the original $T_{\rm m}$ if the effect is due to protein stability—this is certainly not observed, and thus for decreased crowding, there is no significant shift of protein stability in the cells (Figure 4A, bottom). In addition, we can rule out significant structural changes of the folded state when crowding changes. In the case of structural change in the folded state, changes to green and red fluorescence would be noticeable at low temperature as well. Such changes are not observed, as shown in Figure 3.

Our interpretation of the data shows that, while additional crowding can compact the unfolded protein's structure, decreased crowding has a similar but opposite effect, causing the unfolded protein's structure to further expand. This implies that the unfolded protein is slightly collapsed even when the population is unfolded. This collapsed state responds to changes in crowding as predicted by entropic crowding forces, and demonstrated in vitro for both intrinsically disordered proteins⁵⁰ and expanded polymers.⁶¹ An alternative explanation is that the unfolded protein contains some residual structure even at temperatures well past $T_{\rm m}$. This residual structure can be compacted by an increase of cellular crowding, as expected for an extended conformation.^{62,63} While these two cases cannot be differentiated by our experiments, our data implies that, for PGK, the interactions holding these structures together are weak and driven by crowding, and not by "sticking" of the unfolded state to the cytoplasmic matrix.

CONCLUDING REMARKS

This work highlights the dynamic nature of the unfolded state inside cells. Our experiments suggest that the unfolded state of proteins may be sensitive to changes in the cellular conditions that result from either routine cell cycle events or environmental duress. Such modulation of expanded protein structure would be particularly relevant for certain intrinsically disordered proteins that are known to contain residual structure.^{49,64} We suggest that intrinsically disordered proteins could be possible sensors and actuators of cellular phenotype upon change in volume and/or crowding. Changes in cell volume are relevant even in the case of multicellular organisms, which generally maintain a constant osmotic pressure environment, due to natural processes that cause cell volume changes such as entry into mitosis or shape changes during cell motility.

In most cases, proteins are perturbed in vitro by changing temperature or pressure, adding denaturants or stabilizers, or titrating a drug or a binding partner. The in vitro solution conditions in these cases can always be well-controlled, and because of this, the etiology of the observed effect can usually be traced to the perturbation performed.³¹ Here, we use perturbations that are routinely done in vitro but perform them in cells. The response of the fPGK protein probe to these perturbations integrates the physical-chemical effects that would occur in an aqueous solution, with the additional responses of the cell that are, to a large degree, unknown. To minimize at least the adaptive response (e.g., transcription upregulation upon stress) of the cell to osmotic or temperature stress, our experiments are kept short, on the order of 2-3min. In this time span, regulatory transcription is kept to a minimum, and the macromolecular components of the cell do not change dramatically in their organization.

Of course, we cannot rule out that secondary effects due to changes in the cytoplasmic matrix beyond volume, such as changing concentrations of nonlabeled species such as ions and other metabolites with our model protein, can affect the measurements.¹⁹ Indeed, recent work by our group and others points to the importance of abundant small solutes including ions and ATP in determining protein structure and interactions.^{19,24,65} Despite these currently unknown factors, this study presents ample evidence that cell volume changes could be an important contributor to protein structure and function, and especially the function of intrinsically disordered proteins.^{52,66,67}

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.8b08216.

Temperature measurement, cell volume change images, osmolarity calibration, temperature correction and fluorescence changes before temperature correction, incell temperature calibration, and protein sequences (PDF)

Movie showing focus drift at 18 °C during imaging (AVI)

Movie showing focus drift at 39 $^\circ C$ during imaging (AVI)

Movie showing endosomal movements during imaging (AVI)

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The authors declare no competing financial interest.

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