

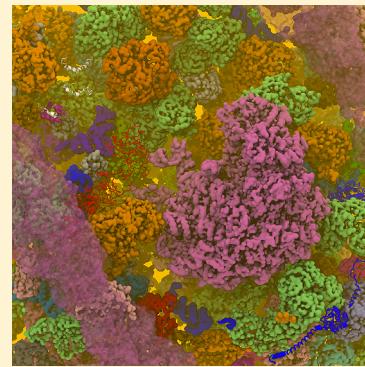
Protein Folding Dynamics in the Cell

Irisbel Guzman[†] and Martin Gruebele*,[‡]

[†]Department of Biochemistry, University of Illinois, Urbana, Illinois 61801, United States

[‡]Department of Chemistry, Department of Physics, Center for the Physics of Living Cells, and Center for Biophysics and Quantitative Biology, University of Illinois, Urbana, Illinois 61801, United States

ABSTRACT: Protein folding is a remarkably fast unimolecular reaction, spanning microseconds to hours at room temperature. Thus, free energy differences and activation barriers on the free energy landscape of proteins are rather small. This opens up the possibility of living cells modulating their protein's landscapes, providing cells another way to control the function of their proteomes after transcriptional control, translational control, and post-translational modification. In this Feature Article, we discuss advances in physicochemical studies of protein stability and folding inside living cells. We focus in particular on our studies using fast relaxation imaging (FREI). Although the effect of the cell on protein free energy landscapes is only a few kT , the strong cooperativity of many folding and binding processes allows small modulation of the energy and entropy to produce a large population modulation. Lastly, we discuss some biomolecular processes that are particularly likely to be affected by in-cell modulation of the proteome, and thus of interest for quantitative physical chemistry studies.



1. INTRODUCTION

Protein folding is an important and now fairly well understood physicochemical process required for cell function. *In vitro* and computational studies have been the major driver of protein folding science. Much has been learned with the advent of site-specific techniques like hydrogen exchange or Phi- and Psi-value analysis,^{1–3} powerful NMR methods,^{4,5} single molecule experiments,^{6,7} and the development of fast relaxation techniques for direct comparison with long folding simulations.^{8–10} A quantitative statistical mechanical model has emerged to explain the experimental and computational observations from these studies.^{11,12} We know that unfolded states of proteins already contain significant residual structure and are biased for folding.^{13,14} When there is no competition with aggregation, the protein energy landscape funnels small proteins toward the native state in a stochastic yet efficient quest^{15,16} for the lowest energy state encoded by the sequence.¹⁷ This process suffers some frustration due to the function for which proteins have evolved¹⁸ but generally with relatively few traps or off-path conformations sampled during folding.^{19,20}

A funneled energy landscape means that enthalpy and entropy of the protein and its solvent compensate, resulting in small free energy barriers and a very rapid folding reaction. Even “slow folders” that take minutes to react are much faster than typical small organic molecule reactions at room temperature. Folding is robust, with several alternative low free energy paths available even to small fold topologies.^{21–23} Since Boltzmann weighting of populations is exponentially sensitive on the $k_B T$ energy scale ($\sim 2.5 \text{ kJ/mol}$ at physiological temperatures),⁸ a predominant path usually exists for a single solvent condition and amino acid sequence.²⁴

Together with its proteins, the cell has evolved into an enormously complex environment for metabolism, information transfer, and proof reading needed to support homeostasis (Figure 1).²⁵ The cell depends on the function of a very diverse set of proteins to sustain these processes. Cells spend a considerable free energy cost transcribing and translating their genetic information to produce a functional proteome.²⁶ Protein function therefore needs to be highly regulated to obtain the optimal outcome. This regulation does not end with control of expression but continues with post-translational modification,^{27–29} translocation or insertion of proteins into different cellular compartments or membranes,^{30,31} specific checkpoints during the cell cycle,³² and programmed degradation.³³ In addition, quinary structures (weak transient macromolecular interactions on the order of $k_B T$) control protein function in ways too subtle for pull-down assays or similar strong-binding assays to detect.^{34–36} These weak interactions in the cell are an important frontier for protein regulation and folding science.

All but the most stable ordinary globular proteins unfold and refold again many times after translation,³⁷ providing stochastic checkpoints for degradation. Other proteins are unfolded in isolation *in vitro*,³⁸ yet may gain structure in the cell, or fold upon binding.³⁹ In addition, proteins in the cellular matrix are subject to macromolecular crowding,^{40,41} electrostatic interactions,^{42,43} and solvent–protein interactions (e.g., hydrophobicity) that vary among compartments⁴⁴ and in time.⁴⁵ Many of these inter- and intramolecular interactions have been

Received: February 22, 2014

Revised: May 8, 2014

Published: May 30, 2014

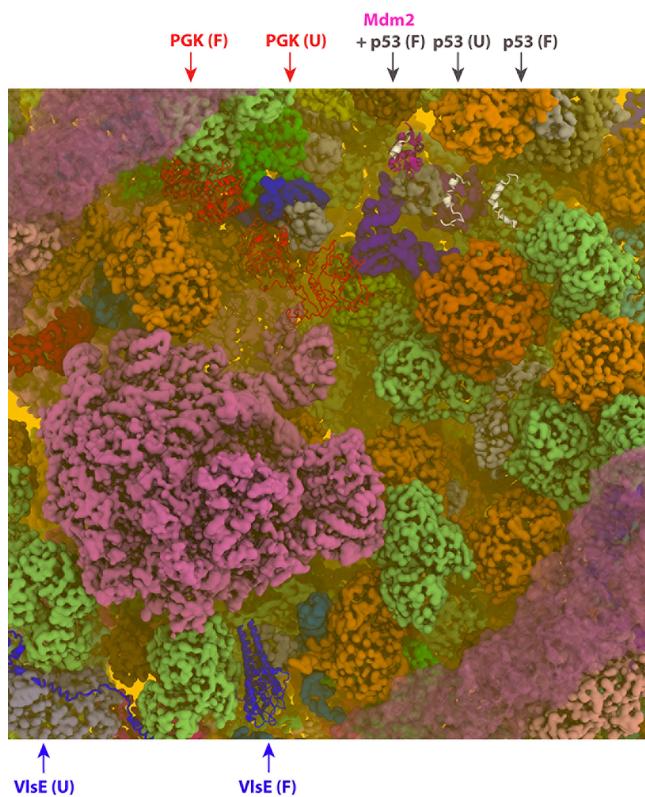


Figure 1. Crowded cytosol illustrating several folding and binding processes. Red ribbon structures: The folding (F)–unfolding (U) equilibrium of phosphoglycerate kinase depends on macromolecular crowding. White ribbon structures: p53 has a disordered N terminus in aqueous solution (U), which may be shifted toward folding by crowding and nonspecific interactions in the cytoplasm (F) or binding to its inhibitor Nmd2 (purple). Blue ribbon structures: the extracellular protein VlsE folding equilibrium is shifted in the opposite direction from phosphoglycerate kinase in the cytoplasm; favorable contacts formed by the unfolded state (U) may overcome crowding effects that favor the folded state (F). (The figure was prepared by Prof. Taras Pogorelov with data from Prof. Adrian Elcock.)

studied *in vitro*,^{46–49} but their relative contributions could vary within the cell.

Within this complex environment, proteins face selective pressure not just for function but also for avoidance of undesirable interactions that could lead to false signaling or aggregation. As chaperoning mechanisms appeared, some proteins and assemblies were free to evolve to large size, even though their folding or assembly yields are very small *in vitro* without assistance.⁵⁰ Furthermore, a significant fraction of proteins with well-known function inside cells cannot be studied *in vitro* because they aggregate or lack their in-cell structure in aqueous buffer.⁵¹ For example, a comparative study showed that the information from model-protein studies only represents 8.4% of *E. coli* soluble proteins. This difference is even larger for membrane proteins.⁵¹

Some of the effects of the cell on its proteins may be incidental; they are the stochastic byproducts of a complex environment. Others have evolved. Cells are clearly not well-stirred chemical reactors, so the cell probably evolved to control protein folding and function in space and time. Therefore, it is of interest to ask how much the cellular environment modulates *in vitro* folding mechanisms, and whether such modulation could be critical for time- and space-selective function. The big

question for the future is, to what extent has the cell evolved to avoid undesirable protein interactions and misfolding, while promoting desirable control over its proteome's function in space and time?

A number of model proteins have now been studied in synthetic environments mimicking some aspects of the cell,^{52,53} and recently, these studies were extended to the intracellular environment to deconvolute “intrinsic” from “cellular” effects on folding and function.^{39,44,45,54–57} The physicochemical information obtained from *in-cell* protein folding studies is the first step toward a comprehensive understanding of how cytoplasmic physical gradients and compartmentalization work at the molecular level. Therefore, these studies are not only important for the protein-folding field, but they are also relevant for cell physiology, specifically, how cells maintain homeostasis with minimal driving forces and expenditure of free energy. With these important points in mind, we review in this Feature Article recent experimental and computational progress toward understanding of protein folding inside cells. We emphasize our own crowding and fast relaxation imaging (FREI) studies inside living cells,^{45,55–60} and finish with open inquiries and questions for the *in-cell* protein folding field.

2. MOVING TOWARD A CELL-LIKE ENVIRONMENT

Macromolecular Crowding. Macromolecular Crowding is one of the most important features of the *in-cell* environment. Cells are highly crowded,^{40,61} and depending on the organelle or subcellular location, 5–40% of the cell total volume is occupied by macromolecules.⁴⁰ The mass fraction can be even higher with a protein density of about 1.4 g/cm³.⁶² Crowding specifically refers to a steric effect (excluded volume) modeled by a short-range repulsive interaction.⁶³ Longer range interactions (e.g., electrostatic repulsion or enthalpically favored sticking) may also occur but are not referred to as “crowding”.⁴²

Simple excluded volume models predict that protein states of greater entropy (such as unfolded chains or dissociated complexes) are disfavored by the reduced volume. According to Hammond’s principle,⁶⁴ this implies faster folding and association due to a lower reaction barrier when a protein is in a crowded environment.

Many *in vitro* experiments using sugar polymers or protein crowders have confirmed this picture.^{43,52,60,65} The cellular retinoic acid-binding protein I (CRABP1) was crowded by Ficoll-70, a spherical, nonperturbing, and inert macromolecular crowder. The CRABP1 urea-denatured state is more compact in Ficoll-70 buffer than in aqueous buffer.⁶⁶ Also, our studies of phosphoglycerate kinase (PGK) reveal higher enzymatic activity in 200 mg/mL Ficoll; the increase in activity is attributed to a more compact native state with proximal active sites.⁶⁰ Additionally, crowding studies of λ_{6-85} protein using the thermophilic protein SubL as a crowder revealed stabilization of λ_{6-85} and enhanced downhill folding.⁶⁵

The situation is not always this simple even *in vitro*. For example, a pH-dependent mutant of protein Cl2 was slightly destabilized when BSA and lysozyme were used as crowders.⁴³ A unified model has been developed to explain the switch from stabilization to destabilization, which occurs at a different crossover temperature for different crowders.⁶⁷ As we shall see, the cell can use competing effects of crowding and electrostatics to fine-tune which direction stability of a protein is shifted in.

Macromolecular Confinement. Macromolecular Confinement occurs when a small compartment or pore reduces the conformational space of a protein, as opposed to mobile

macromolecules. Confinement occurs in many places in the cell, such as the ribosomal exit channel,⁶⁸ translocon pores in membranes,⁶⁹ and the inside of chaperones such as GroEL.⁷⁰ Confinement can generate repulsive or attractive interactions,⁶¹ and requires rather small compartments of 10–100 Å to be significant.⁷¹

In vitro studies have shown how a confined environment modulates the energy landscape of protein folding by reducing the conformational entropy of the unfolded state. For example, NMR spectroscopy reveals stabilization of apomyoglobin and ubiquitin in zeolite pores.⁷² Also, the melting temperature of ribonuclease A increases by about 30 °C when it is confined in 25 Å pores of silica glass.⁷³ Additionally, an unfolded mutant of protein α_3 W was encapsulated in sodium bis(2-ethylhexyl) sulfosuccinate reverse micelles, where it folded into a helix bundle.⁷⁴

Longer Range Interactions. In addition to steric effects, longer range electrostatic interactions as well as stickiness (hydrophobicity when water-mediated) play a role in the cell. These have been studied *in vitro* by many techniques, including small-angle X-ray scattering (SAXS).^{73,75} For example, we developed a method to fit interaction potentials to concentration-dependent SAXS data without assuming analytical potentials.⁴⁹ The potential of mean force in Figure 2 highlights

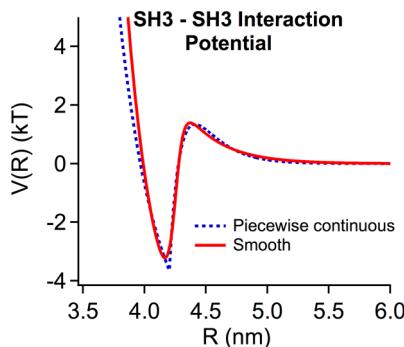


Figure 2. Interaction potential between pairs of SH3 protein molecules is determined by fitting a piecewise potential of mean force and a continuous potential of mean force to concentration-dependent small-angle X-ray scattering data as a function of protein concentration.⁴⁹ This particular potential reveals both long-range repulsive and attractive interaction before the short-range repulsive wall (crowding) is reached.

that both repulsive interactions (e.g., long-range electrostatics of charges on the protein surface) and attractive interactions (e.g., medium-range exclusion of solvent from the space between proteins increases solvent entropy) can occur. These interactions will complicate simple crowding and confinement in the cell, with its large variety of differently shaped and charged macromolecules and complex mix of ionic species. Long range interactions also occur between proteins and water molecules: on a ns time scale, NMR experiments show a strongly perturbed solvent layer 1–2 water molecules deep.⁷⁶ Terahertz spectroscopy reveals perturbations of the water network on a sub-ps to ps time scale up to 2 nm.⁷⁷ Although the magnitude of the perturbation per molecule drops at greater distance for the protein, the number of affected solvent molecules in a shell increases as r^2 with distance r from the protein.

3. METHODS TO STUDY PROTEIN FOLDING DYNAMICS IN THE CELL

SUPREX. The stabilities of unpurified proteins from rates of H/D exchange (SUPREX) were the first quantitative data obtained from protein folding studies inside *E. coli*. This method is based on the fact that protons in proteins exchange with the protons from the solvent (H/D exchange). The H/D exchange is initiated when bacteria are transferred to a deuterated medium. SUPREX data showed that the monomeric λ repressor fragment, a viral protein that occurs in *E. coli*, has the same stability inside *E. coli* as *in vitro*.⁷⁸

FlAsH as an in-Cell Protein-Folding Probe. 4',5'-Bis(1,3,2-dithioarsolan-2-yl) fluorescein (FlAsH) had been used to study protein stability inside cells.⁵⁴ Fluorescence of this dye is turned on when FlAsH is incorporated into a protein by binding to the genetically encoded tetracysteine motif C–C–X–X–C–C.⁷⁹ To study protein folding using FlAsH, the tetracysteine is incorporated in the hydrophobic core of the protein. Thus, when the protein unfolds, the tetracysteine motif is exposed, FlAsH binds to it, and the fluorescence signal is enhanced. Using this method, CRABP1 showed an apparent destabilization inside *E. coli* when urea was present.⁵⁴ Also, the glutamine repeats in the exon 1 of huntingtin were shown to destabilize flanking proteins.⁸⁰

NMR Studies in Live Cells. Except for the need for high sample concentrations, NMR is a biocompatible technique. Changes in a protein NMR structure from *in vitro* to the cell can be extrapolated to changes of the protein folding energy landscape from *in vitro* to the cell. This was the approach first used by Serber et al. to study the structural changes of Tn501 mercuric ion reductase (MerA) inside *E. coli* using high-resolution NMR.⁸¹ Additionally, utilizing ¹⁵N-labeling to single out FlgM against the background of other cellular biomolecules, it was found that this disordered protein gains structure inside *E. coli*.³⁹ Equilibrium folding studies also have been performed; for example, a mutant of protein L was not able to fold inside *E. coli* cytoplasm.⁸²

NMR studies inside eukaryotic cells were the next obvious step. *Xenopus laevis* oocytes microinjected with ¹⁵N-labeled GB1 showed that GB1 undergoes structural changes related to the higher viscosity and crowding inside cells.⁸³ In another study, a cell-penetrating peptide linked to ¹⁵N-labeled human ubiquitin (Ub-3A-CPP) was incubated with HeLa cells with a translocation mediator; this first study of protein folding in mammalian cells found that Ub-3A is destabilized inside HeLa cells.⁸⁴

In-Cell TOOL Studies. Temperature oscillation optical lock-in (TOOL) microscopy was first used to study DNA hybridization kinetics, but modulation techniques can also be applied to study protein folding inside cells.⁸⁵ In this experiment reminiscent of phase-sensitive fluorescence lifetime detection,⁸⁶ a perturbation of variable frequency (e.g., pH or temperature modulation) is applied to the cell. When the frequency applied has a period greater than the reaction time τ of interest, the reaction follows the perturbation, but when the period is smaller than τ , the reaction lags behind the perturbation, creating a phase shift. The frequency-dependent signal amplitude and phase shift are used to infer τ for the reaction.

4. THE FAST RELAXATION IMAGING (FREI) INSTRUMENT AND PROCEDURE

FREI allows the study of fast biomolecule dynamics (currently milliseconds, potentially microseconds) inside cells, by first applying a sudden small temperature up- or down-jump to a cell and then imaging the whole cell's response. A FRET-labeled protein, pair of proteins, or other biomolecule combination such as protein/RNA is detected inside the cell as it responds to the perturbation.⁵⁶ Thus, FREI allows us to investigate protein association, folding kinetics, and stability inside cells by monitoring proteins in real time with spatial localization, as well as cell temperature and cell viability simultaneously.

Instrument and Experimental Improvements. Our laboratory had been using laser-induced temperature jumps, based on the relaxation idea by Manfred Eigen,^{87,88} for nearly two decades to study fast protein folding in aqueous buffer. To bring relaxation experiments into the cell, FREI as shown in Figure 3 was developed.

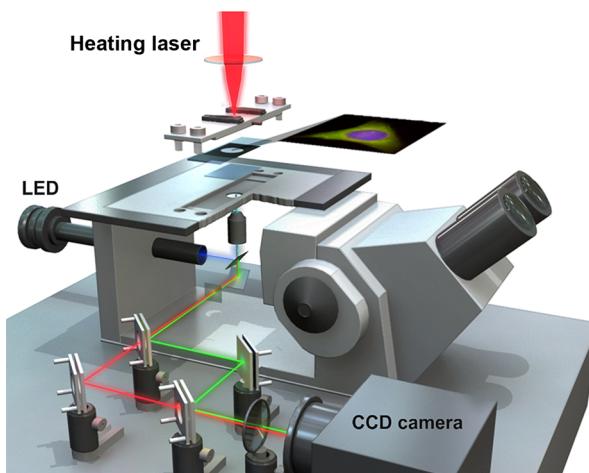


Figure 3. Temperature-jump fluorescence imaging microscope utilized for FREI experiments. To perform equilibrium experiments, a resistive heated stage is used to increase the temperature, and for relaxation experiments, the heating laser is used to initiate the temperature jump. The FRET donor is excited with a blue light-emitting diode (LED), and a camera is used to record fluorescence of the donor and acceptor. The figure was adapted from ref 56 with permission. Copyright 2010 Nature Publishing Group.

FREI experiments are performed on an epifluorescent microscope where changes in FRET are monitored after a small mid-infrared laser creates a temperature jump. FREI is only limited by the time needed to induce the relaxation kinetics, and can be used to study protein folding in a variety of cells with a diffraction-limited spatial resolution. A full description of the FREI instrument setup and data collection is given elsewhere.⁵⁶

To perform a FREI experiment, the protein or biomolecule of interest needs to be labeled with two fluorescent probes to monitor FRET changes. We have been using terminal AcGFP as a donor and mCherry as an acceptor because they can be conveniently expressed together with the target protein inside the cell. However, any other combination of FRET pairs, combined with cell injection, can be used. The major consideration for the FRET pair selection is that it should be fully functional over the 20–50 °C temperature range. It is worth noting that the presence of two large protein labels already “crowds” the target protein sandwiched between them. Therefore, we compare the same construct *in vitro* and *in vivo*; the actual crowding effect in-cell relative to unlabeled protein *in vitro* may be even larger than what we measure.

Since our first publication on FREI,⁵⁶ we have added several instrument improvements that allow us to have more flexibility and versatility. For example, increasing the stage temperature of the microscope slowly to reach the desired base temperature killed the more sensitive cells. Thus, we developed a fast laser stepping method to measure thermodynamic traces in a fraction of the time, yielding data from more viable and healthier cells.⁵⁷ For this method, the infrared T-jump laser (2200 nm, water extinction coefficient ca. 50 cm⁻¹) is focused to a 1 mm diameter spot on the slide holding the cells to provide uniform heating in our 40× objective field of view. The laser then steps up in power spiking briefly at each step to reach the desired temperature rapidly before settling into a plateau where the measurement is taken. A full temperature titration with less protein aggregation is observed, as shown in Figure 4.

The stepping idea can be further extended to any programmable heating laser waveform, with a response time limited by the heat capacity of water and thermal conductivity of the sample. We also applied more general modulation waveforms to PGK-FRET to learn more about its folding kinetics.⁸⁹

In Vitro and in Cell Sample Preparation. Detailed procedures for *in vitro* and in-cell sample preparation are

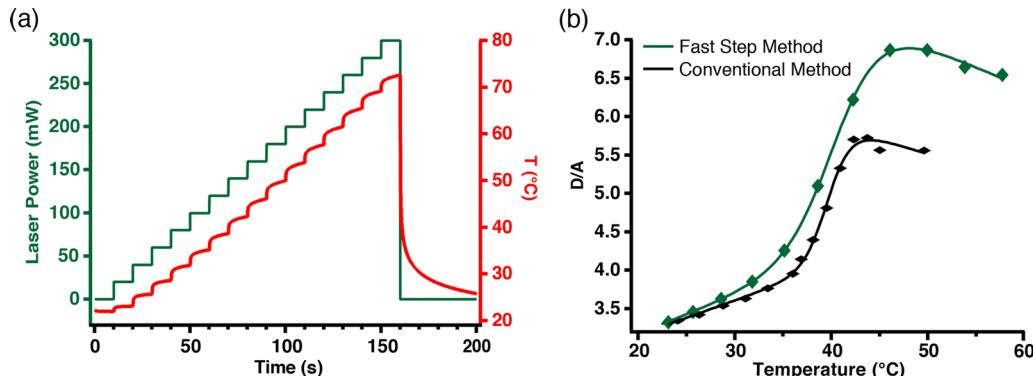


Figure 4. (a) The stepping of a 2200 nm heating laser in black and mCherry temperature response in red. (b) Equilibrium traces of PGK-FRET *in vitro* unfolding using the fast temperature stepping method in green and the conventional resistive element method in black. Adapted from ref 57 with permission. Copyright 2012 National Academy of Sciences.

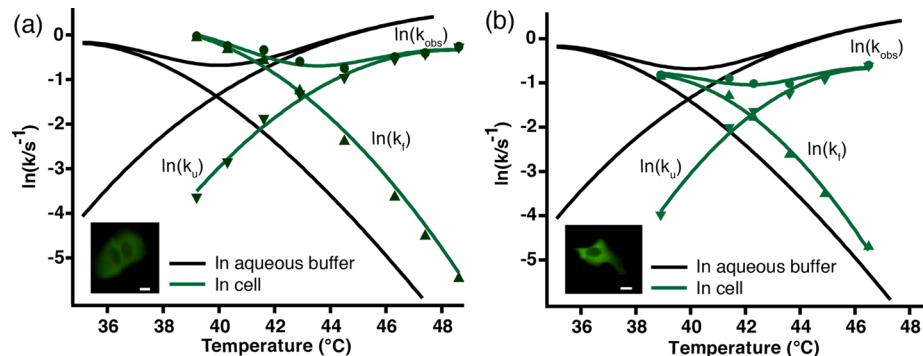


Figure 5. PGK-FRET temperature dependence inside two different cells showing the cell to cell kinetic variability in green versus aqueous buffer in black. Adapted from ref 57 with permission. Copyright 2012 National Academy of Sciences.

published;^{44,56} here we present a general summary. FRET-constructed proteins are usually expressed in BL21 competent cells and pDream 2.1 is used as an expression vector. After expression, the cells are lysed and proteins are purified using a Ni-NTA column. Characterization and purity of the proteins are assessed through SDS-PAGE and low-resolution electrospray ionization mass spectrometry.

In vitro experiments are generally performed in one of two media: (1) in aqueous buffer containing the FRET-constructed protein or (2) in the same solution but with macromolecular crowder agents added. Aggregates are removed from both types of *in vitro* solutions with a 20 μm filter, and then, the solution is added onto a glass coverslip with a 120 μm spacer. The spacer depth is limited by the extinction coefficient of water at the chosen heating laser wavelength. Subsequently, the glass coverslip is positioned on top of a microscope slide, creating a chamber that is ready to be imaged. For some applications, *in vitro* experiments are performed in PEGylated or quartz imaging chambers to reduce surface fouling of the protein during measurements. The concentration of the FRET-constructed protein varied from 1 to 5 μM depending on the desired FRET pair, and to check for concentration-dependent aggregation. Micrometer-sized dye beads are used in the imaging chamber to focus the objective on the surface of the slide by measuring at the same z position. Also with the dye beads, temperature variations due to heating and heat dissipation gradients can be reduced to as little as 0.1 °C from sample to sample.

Our *in-cell* experiments usually are performed with cancer mammalian cell lines. The majority of the experiments so far were done in human osteosarcoma (U2-OS) cells. We have published a protocol that describes in detail the preparation of cells for experiments.⁵⁹ Basically, around 24 h after transfection of the cells with a plasmid expressing the FRET construct of interest, the microscope coverslip containing the cells is used to prepare an imaging chamber similar to the one described above.

Data Collection and Analysis. When *in vitro* and *in-cell* protein unfolding is monitored by FREI, the donor fluorescence intensity (D , AcGFP) increases and the acceptor (A , mCherry) fluorescence intensity decreases. Therefore, upon protein unfolding, there is a decrease of FRET efficiency $E = D/(D + A)$ and an increase of the D/A ratio. The reverse would be observed in a protein association experiment. The fluorescence from the cell is split into donor and acceptor channels by a dichroic mirror, and the donor and acceptor channels are imaged side by side onto a CCD or similar camera. Depending on the experiment, fluorescence is imaged from 24

to 100 frames per second. Experiments are done in a temperature range from 20 to 50 °C. Throughout the course of the experiments, the morphology of the cell is observed closely for viability.

Thermodynamic data is analyzed by plotting the donor over acceptor fluorescence intensity ratio D/A , which reduces the temperature-dependent baseline of the FRET signal because both the donor and acceptor have a quantum yield that linearly decreases with temperature. A more complete cancellation of the temperature baseline can also be achieved by explicitly including the temperature-dependent quantum yield of the acceptor (see Figure 2C of ref 44). Other reaction coordinates such as FRET efficiency, $E = A/(D + A)$, or the difference $D - A$ can also be plotted. As the protein unfolds and the donor and acceptor move further apart on average, the donor fluorescence increases and the acceptor fluorescence decreases. Therefore, the D/A ratio increases with temperature. To fit the D/A ratio as a function of temperature, we often use a Taylor expansion $\Delta G(T) = C_T^{(1)}(T - T_m) + C_T^{(2)}(T - T_m)^2 + \dots$ to describe the temperature dependence of the protein folding reaction free energy. In this equation, $\Delta G(T)$ is the free energy, T_m is the midtransition point or melting temperature of the protein, and $C_T^{(n)}$ are expansion parameters. Another common model is to let $dH = Cp dT$ and $dS = (Cp/T)dT$ express the dependence of free energy on temperature.

For kinetic analysis, the data is plotted as $D - aA$ because it is linearly proportional to the folded population. In this equation, a is the initial donor to acceptor ratio, which mostly eliminates the temperature dependence of quantum yield from the signal. We use a stretched exponential function $D(t) - aA(t) = S(t) = Ae^{-(t/\tau)^\beta}$ to fit the kinetic data. In the previous equation, β is a stretching factor ≤ 1 that reports the deviation from the single exponential decay expected for a simple two-state reaction between the unfolded and folded states. We also developed a method to extract thermodynamic parameters from kinetic data.⁵⁸ This can be useful when cell viability does not allow the entire folding/unfolding transition to be traced out and fast measurement improves cell viability.

5. A VERSATILE LOOK AT IN-CELL PROTEIN FOLDING WITH FREI

PGK-FRET in-Cell Stability and Folding Kinetics. PGK-FRET was the first protein to be studied by FREI because of two major reasons: we already performed extensive *in vitro* protein folding studies of PGK, and PGK was large enough to yield a large FRET signal change upon unfolding. We also thought such a large protein would be less perturbed by the

attached labels when comparing *in vitro* and in-cell studies. Initial experiments were performed in U2-OS cells, and confirmed in HeLa cells, and established a higher protein stability in the cytoplasm than for the same construct *in vitro* ($\Delta T_m = 3 \pm 0.5$ °C, see Figure 5). Thanks to the fluorescent bead method of aligning the imaging region, relative temperatures can be determined to about 0.5 °C. Absolute readings vary by as much as 2 °C depending on where the measurement in the chamber is taken. The higher in-cell stability results from the crowded environment inside the cell that favors a more compact or native-like structure. As seen in the Introduction, and discussed later on, proteins are not always stabilized because crowding can be overcome by electrostatic or hydrophobic interactions (e.g., Figure 2).

We subsequently measured the full temperature dependence of the equilibrium constant $K_{eq} = k_f/k_u$ (two-state model), observed rate coefficient $k_{obs} = k_f + k_u$ (two-state model), and thus the folding and unfolding rate coefficients k_f and k_u .⁵⁷ This analysis confirmed an ~6 kJ/mol stabilization of PGK-FRET in the cytoplasm, and highlights cell-to-cell variations of stability and kinetics. In Figure 5, $K_{eq} = 1$ at the point where the unfolding and folding rate coefficients intersect ($\ln(k_f) = \ln(k_u)$). This point averages 3 °C higher in-cell than *in vitro*, but it varies from cell to cell. Likewise, while the folding rate in the cell is always faster than *in vitro*, and the unfolding rate is always slower, both rates vary from cell to cell (Figure 5).

The simplest crowding model, which neglects viscosity changes, predicts that the folding rate should speed up as observed experimentally in Figure 5.^{41,63} The speed-up of refolding is caused by entropic destabilization of the large unfolded state in the small space between crowders. The same models predict that the unfolding rate should remain constant, but Figure 5 shows that it slows down. We concluded that the unfolding rate decreases because of increased viscosity in the cell, in agreement with crowding models that take solvent and protein self-friction into account.⁹¹ We showed that the rate returns to the aqueous buffer value when the cell is immersed in hypotonic solution, so its cytoplasm swells with water due to osmotic pressure.⁹² The transition state for folding is generally more compact than the unfolded state but more expanded than the native state, and thus is subject to destabilization by crowding, which could also slow down unfolding kinetics. We ruled out a large contribution of the activation barrier to the reduced unfolding rate inside cells as follows: We started by measuring the spread of folding free energies within and among different cells.⁹² Next we assumed that the m^\dagger value upon crowding is similar to the one caused by chemical denaturation (typically 1/3). We then calculated the shift and spread of the activation energy as $\approx 1/3$ times the shift and spread in folding free energy. The small increase of the average activation barrier and of its spread thus calculated does not account for the reduced unfolding rate and the large spread of relaxation times shown in Figure 6.

The Intracellular Environment Affects Protein Structure. The radius of gyration and the N-to-C terminus distance are often used as rulers for protein compactness and as coordinates on protein folding energy landscape.^{60,93} Protein compactness and structure are affected by several factors such as the chemical environment, macromolecular crowding, and solvation.^{63,94,95} Thus, protein structure could be significantly different inside cells than in aqueous buffer.

In-cell FRET studies of PGK-FRET have shown that cells modulate the compactness of proteins.^{55,56} Comparison of

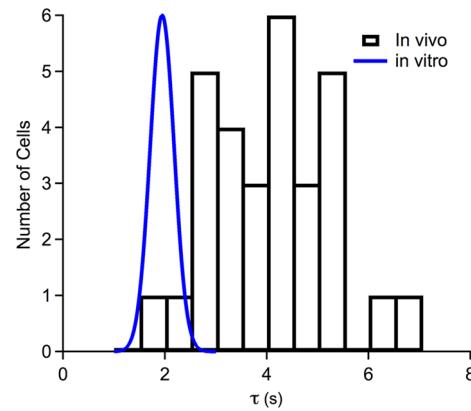


Figure 6. Histogram of PGK-FRET relaxation times in aqueous buffer (blue) and in cells (black): the relaxation time variability is wider and slower inside cells. Adapted from ref 92. Copyright 2010 Cell Press.

PGK-FRET in aqueous buffer, crowded by 200 mg/mL of Ficoll 70 (a carbohydrate crowder), and in-cell shows that both the native and unfolded states have a smaller D/A ratio when crowded.^{60,92} Using D/A as a readout of N-to-C terminus distance, we found PGK-FRET to be more compact in the cytoplasm than *in vitro*. Folded PGK-FRET has a D/A ratio of about 10 *in vitro*, 7 in 200 mg/mL Ficoll crowder, 6.6 ± 0.3 in the cytoplasm, 5.8 ± 0.5 in the ER, and 6.1 ± 0.7 in the nucleus.⁴⁴ Additionally, the final D/A after unfolding is up to 1.5 times larger in mitotic cells than in interphase cells, which implies PGK-FRET is more expanded upon unfolding in mitotic cells.⁴⁵

Simulations by the Cheung group propose a new “spherical” native state under such crowded conditions, in which the two lobes of PGK come together to form an enzymatically more active state.⁶⁰ Thus, the native and unfolded states in cells are not expected to be identical to their aqueous solution counterparts but rather more compact. For example, an unfolded state *in vivo* could resemble a compact folding intermediate seen *in vitro*. In cells, even more so than *in vitro*, characterizing unfolded states will be very important: Compact denatured states in the cell may be more prone to misfolding than *in vitro* unfolded states, making the evolution of protective mechanisms such as chaperones key to cell survival.

As a word of caution, although the greater acceptor fluorescence in our data is consistent with more compact protein, the FRET labels themselves could interact differently in a crowded environment than *in vitro*, so it will be very important to verify these studies with chemically different labels, such as dye molecules instead of fluorescent proteins.

Effects of Subcellular Compartments on PGK-FRET Folding. Dhar et al. performed thermodynamic and kinetics studies in different cellular compartments.⁴⁴ Each cellular compartment is a different microenvironment that can modulate the energy landscape, affecting the stability and function of interacting macromolecules. Localization tags were used to introduce FRET-PGK into the cell nucleus and the endoplasmic reticulum.

The melting temperatures T_m in aqueous buffer for these two constructs do not differ significantly from the untagged PGK-FRET, and an average T_m of 38.5 °C was observed for the three constructs. Figure 7 shows that PGK-FRET was more stable in the nucleus than in the endoplasmic reticulum (ER). In the nucleus, PGK-FRET is stabilized by ~4.5 °C and in the ER by ~2.5 °C relative to aqueous buffer.

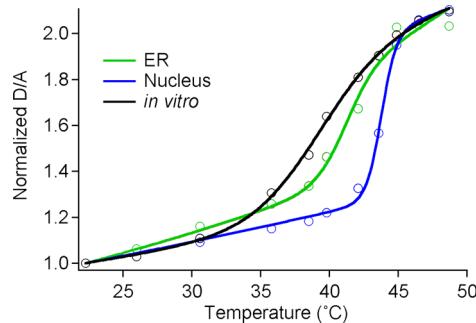


Figure 7. Representative equilibrium experiment traces showing PGK-FRET stability in different subcellular environments. Adapted from ref 44 with permission. Copyright 2011 Cell Press.

The kinetics also shows differences between the two compartments. The average folding relaxation time was twice as fast in the nucleus. The variation of rates from cell to cell was larger in the ER. However, the ER kinetics in individual cells could be fitted with $\beta \approx 0.9$ in the unimolecular rate law $\exp[-(kt)^\beta]$ (the nucleus required $\beta = 0.6$). Therefore, the reaction has a smaller spread of rates in the ER than in the nucleus. PGK-FRET is not a two-state folder in aqueous buffer ($\beta \sim 0.6$). Thus, we believe that the ER makes the folding mechanism of PGK-FRET more two-state-like: If a two-state folder has kinetics with $\beta < 1$ inside the cell, it could be due to a change in mechanism, or it could simply be that the mechanism remains two-state, but the rate coefficient varies between different regions of the cell, yielding a nonexponential rate law when averaged over the whole cell. However, if a protein is not a two-state folder in aqueous buffer (like PGK-FRET) but the rate law approaches $\exp[-kt]$ in the ER ($\beta \approx 1$), the simplest explanation is that the mechanism has become more two-state-like, and that the folding rates are all similar within the ER. It is important to reiterate that the two states interconverting in the cell may not be structurally the same ones as *in vitro*. We discussed above that the intracellular environment affects protein structure, generally making proteins more compact. Thus, the unfolded state inside the cell could resemble a folding intermediate in aqueous solution, for example.

Modulation of PGK-FRET during the Cell Cycle. The cell cycle is a highly organized temporal regulator of macromolecule expression, which allows checkpoints to modulate the proteome during cell growth and division. We

investigated the temporal variation of PGK-FRET folding as a model system to study proteome dynamics during the cell cycle.⁴⁵ In particular, we compared the mitotic and interphase stages of the cell cycle⁴⁵ to make a connection with the nucleus-cytoplasm studies discussed above. We hypothesized that exposure of PGK-FRET to nuclear material during mitosis would stabilize and speed up PGK-FRET folding, just as the nucleus had done. Indeed, PGK-FRET was stabilized by ~ 5 kJ/mol (~ 2.5 °C) in mitotic cells relative to interphase (Figure 8a). The relaxation kinetics was also a little faster in mitotic cells $k_{\text{obs}} = 0.62 \pm 0.004$ s⁻¹ instead of 0.44 ± 0.01 s⁻¹ during interphase (Figure 8b). Phi-value analysis showed that mitotic cells have larger diffusion coefficients as well as larger activation barriers than interphase cells, but faster diffusion overcomes the increased barrier increase to speed up the rate.⁴⁵

The structural cause of the faster folding in mitotic cells is not as simple as we hypothesized. When we measured the folding kinetics of PGK-FRET only in the DNA-rich areas (located with Hoechst 33258 in mitotic cells), the rate increase was very modest over the DNA-poor areas. If DNA-PGK interaction is at all responsible for the observed faster folding kinetics in the nucleus, some other aspect of the nuclear environment not present during mitosis must assist this interaction.

Folding of an Extracellular vs an Intracellular Protein. PGK-FRET had been the golden model system for FRET studies inside cells. However, how typical is it of proteins in general, when we use the cell as a test tube for folding experiments? After all, experiments by Giersch and co-workers or Oas and co-workers did not necessarily show intracellular protein stabilization.^{54,78} We decided to look for an extracellular protein that was similar in size and surface charge to PGK, but being extracellular had not particularly evolved to be compatible with the cytoplasmic environment. VlsE was the perfect candidate because its size and *in vitro* stability and kinetics, determined by Wittung-Stafshede and co-workers,⁵² are similar to PGK. Like PGK, VlsE is stabilized by addition of a simple crowder like the 70 kDa carbohydrate Ficoll.⁹⁶ We designed VlsE-FRET by cloning GFP at the N-terminus and mCherry at the C-terminus of a truncated construct of VlsE that cannot be translocated to integrate into the extracellular membrane,⁵⁵ forcing it to stay in the cytoplasm.

VlsE-FRET was destabilized inside cells by ~ 3 °C (Figure 9a) relative to aqueous buffer, which is the opposite behavior of PGK-FRET, although both proteins are stabilized when

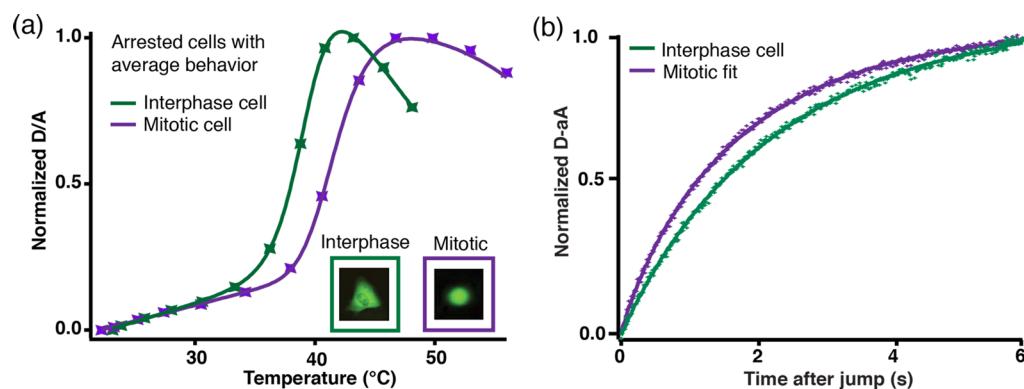


Figure 8. (a) Sigmoidal curves of temperature titration of PGK-FRET in nine randomly selected mitotic and interphase arrested cells. (b) Average kinetic traces for PGK-FRET in mitotic and interphase arrested cells. Adapted from ref 45 with permission. Copyright 2013 American Chemical Society.

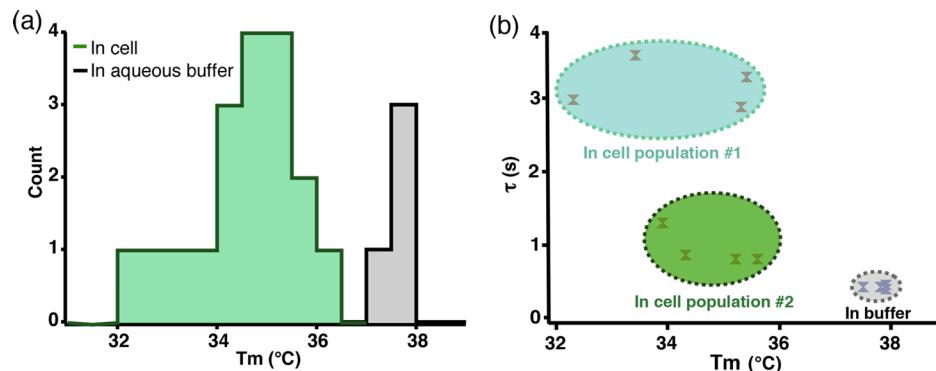


Figure 9. (a) Average traces of *in vitro* and *in cell* temperature titrations of VlsE-FRET. (b) VlsE-FRET has distinct kinetic populations inside cells. Adapted from ref 55 with permission. Copyright 2014 Elsevier Ltd.

crowded by Ficoll 70. The average relaxation time inside the cell was 1.9 ± 1.2 s, which is 4.5 times slower than the relaxation in aqueous buffer (0.4 ± 0.1 s), and even more than the factor 2 slow-down observed for PGK-FRET.

Although the distribution of VISE stabilities from cell to cell had a single peak (Figure 9a), two distinct kinetic populations were observed inside cells (Figure 9b). By the Kolmogorov–Smirnov statistical test, we concluded that these populations were kinetically distinct with 97% probability, as shown in Figure 9b. (The Kolmogorov–Smirnov test is used for two sequences of data, to disprove the hypothesis that two data sets are drawn from the same distribution functions.⁹⁷) We proposed two different scenarios to explain VlsE-FRET destabilization in the cell: (1) In the “long-range scenario”, interactions of the type highlighted in Figure 1 overcome the crowding effect inside cells by stabilizing the unfolded state, which can stick to other molecules in the cell. (2) In the shape-dependent crowding scenario, a less stable but more compact native state is favored inside cells. We cannot presently distinguish between these scenarios, highlighting the need for more *in vitro* experiments with asymmetric or multiple-size crowders to determine the effect of shape and size. Theoretical models certainly indicate that shape can play a role.⁴⁰

Protein Folding in the Presence of Molecular Chaperones. Nucleoplasmin was the first chaperone discovered to be involved in protein assembly, and gave birth to a new paradigm for *in-cell* prevention of misfolding or protein aggregation. Nucleoplasmin was found to be crucial for the assembly of histone proteins in the nucleus.⁵⁰ The term chaperone was initially used to name a new class of proteins required for protein assembly.⁵⁰ Later this term was generalized to include any protein that protects the unfolded state of other proteins from aggregation and helps them achieve the native structure in high yield, without itself forming part of the final structure.⁹⁸ There are several classes of chaperones, and they are usually classified on the basis of their molecular weight and the stages of protein metabolism that are involved.

PGK-FRET unfolding is not completely reversible after a temperature jump *in vitro* (e.g., Figure 4b). Since molecular chaperones are known to improve folding inside the cell, one might expect to see more complete refolding of PGK-FRET inside cells. Figure 10 shows that this is the case:⁵⁶ When PGK-FRET is first unfolded in the cell by an upward temperature jump, and the temperature is jumped back down 15 s later, the native D/A ratio is recovered. Such recovery could be an indication of chaperones at work but certainly shows that the

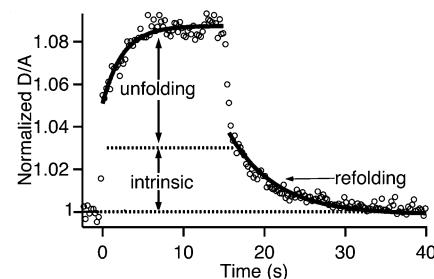


Figure 10. Kinetic trace of PGK-FRET unfolding and refolding profile inside cells upon consecutive temperature upward and downward jumps separated by 15 s. After the heating laser is turned off, the protein refolds, which demonstrates complete reversibility (within experimental uncertainty) in the intracellular environment. *In vitro* data such as Figure 4 do not show such reversibility. Adapted from ref 56 with permission. Copyright 2010 Nature Publishing Group.

cytoplasm is a more conducive environment for folding than the test tube after a temperature stress.

Several chaperone classes have been extensively studied. For example, hsp70 reduces misfolding after proteins have left the ribosome, and during translocation across the mitochondria as well as in proteostasis control.⁹⁹ hsp70 proteins function through an ATP-dependent reaction cycle and are regulated by nucleotide-exchange factor and hsp40 at different stages.^{100,101} The β -sandwich subdomain in the C-terminus of hsp70 recognizes approximately seven hydrophobic residues of a partially unfolded substrate. This is the result of an allosteric process controlled by the binding and further hydrolysis of ATP to the N-terminus of hsp70.⁹⁸ Another extensively studied chaperone is the chaperonin system, which is a large complex of ~ 900 kDa that encapsulated unfolded proteins inside its double ring to enhance folding.⁹⁹ Each chaperone system has obligatory client proteins like actin and tubulin, but chaperones can also protect or hold other proteins during stress conditions.⁹⁸

6. THE FUTURE OF IN-CELL PROTEIN FOLDING STUDIES

There is no question that quantitative protein folding science has evolved much since Anfinsen started it on a physicochemical basis with his provoking studies on bovine pancreatic ribonuclease A.^{17,102,103} During the *in vitro* era, basic concepts such as a funneled energy landscape¹⁰⁴ and off-pathway intermediate¹⁰⁵ were established. Also, fast experiments and simulations met on the microsecond time scale to yield a

structurally resolved and experimentally verified picture of the folding process. The in-cell protein folding field was unexplored because so many fundamental questions had to be answered first.

From biochemical assays to time-resolved microscopy, in-cell protein folding has rapidly evolved in less than a decade. Many of the questions in this field need to be answered quantitatively because small energy differences can have large effects on populations. Thus, physicochemical studies will be important. We have only studied the tip of the iceberg of in-cell protein folding. There is still a long way to go with many open questions about how quinary interactions affect the energy landscape of proteins and other biological macromolecules.

Since the importance of in-cell protein folding studies to gain more information about aggregation and misfolding diseases has been well-documented,¹⁰⁶ we highlight some of the other questions. How important is the interaction of chaperones with nonobligatory clients in controlling their folding and degradation? How much does the cell use compartmentalization and heterogeneity to control the activity and folding of proteins, and how much is it just random noise without functional consequences? How is protein shape and dynamics affected by crowding (entropy) and sticking (enthalpy) inside cells? What is the full range of measures the cell evolved to suppress undesirable signaling ("short circuiting" of signaling networks)?

Intrinsically disordered proteins (IDPs) relate to the last two questions. Many proteins that are fully or partly unstructured *in vitro* undergo allosteric regulation and acquire structure when they are bound to other macromolecules.^{107,108} For example, the apoptotic p53 protein is a partial IDP that not only gains more structure upon binding but is also regulated by other IDPs. p53 is inhibited by BCL-xL, and is released to bind pro-apoptotic proteins when BCL-xL binds to PUMA. This binding event unfolds BCL-xL, which releases p53.^{109,110} We do not yet fully understand the process by which PUMA induces BCL-xL unfolding. Additionally, the N-terminus of p53, known as TAD, gains structure by binding to regulatory proteins. Figure 1 (white proteins at the top right) shows how the TAD region gains more α -helical structure when it binds to the regulatory protein Mdm2.

Consider the endoplasmic reticulum (ER), where newly translated proteins undergo post-translational modification. These modifications are capable of changing the hydrophobicity and electrostatic properties of proteins, which can lead to unexpected dynamics and cellular relocalization. For example, a single phosphorylation in Ser193 of KSRP protein (an important protein in mRNA metabolism) starts its unfolding process, which then exposes a binding site that results in the relocalization of KSRP to the nucleus.^{111,112} Here, partial unfolding/site exposure is coupled to protein transport. What is the mechanism of this phosphorylation-mediated unfolding process, and how much do the stability and Ser193 exposure of KSRP change when KSRP is localized to the nucleus? The ER also contains an advanced machinery of chaperones such as the disulfide isomerases, prolyl *cis*–*trans* isomerases, and others that work in close proximity during vectorial and luminal protein folding.¹¹³ This machinery catalyzes chemical modifications, regulates the ER environment, fosters binding interactions, and applies mechanical force. These physicochemical processes are all linked to folding.

As a final example, there are proteins with two distinct and functioning structures. The transcription factor RfaH can be

transformed into a translation factor when the carboxy-terminal domain switches from α -helix to β -barrel.¹¹⁴ Also, two distinct structures have been characterized for lymphotactin in thermal equilibrium at two different temperatures: one is a dimeric all- β -sheet and the other a monomeric three-stranded β -sheet. The first structure is a regular chemokine and binds XCR1 agonist, and the second structure binds glycosaminoglycans.¹¹⁵ Such "Janus" proteins are candidates for structural triggering by small fluctuations in the intracellular environment. Thus, cellular control of protein folding is not just a question of on–off but a question of one functional state vs another in some cases.

Summary. Using two model proteins and two cell lines so far, we were able to study several aspects of protein folding dynamics in the cell. Macromolecular crowding explains increased PGK-FRET stability inside cells, as expected from theoretical and *in vitro* experiments. Stickiness and transient interactions with other cellular components had the opposite effect on VlsE-FRET, which was found to be less stable inside cells (Figure 8). The cell has the capability of modulating the stability of folding and binding processes by compartmental and temporal localization, and even within different parts of the cytoplasm.¹¹⁶ The impact of the cell on the protein folding energy landscape is at most a few kT , and the effect on the diffusion is a factor of 2–10. However, many biomolecular processes are highly cooperative, so this small range of quinary interaction energy and of diffusion coefficient is enough to modulate populations and mobility of proteins substantially. The cell does not alter folding and binding processes fundamentally from the *in vitro* result but probably enough that it can control important signaling or metabolic processes on or off, and even switch structure and function from one type to another.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 001-217-333-1624. E-mail: mgruebel@illinois.edu.

Notes

The authors declare no competing financial interest.

Biographies

Irisbel Guzman was born in Caguas, Puerto Rico, in 1986. She received a B.S. in Industrial Chemistry from the University of Puerto Rico in 2009, and she is currently a Ph.D. candidate in the Biochemistry program at the University of Illinois. Under the supervision of Prof. Martin Gruebele, she has utilized equilibrium and perturbation methods to study protein folding and protein–RNA interaction dynamics inside living cells with fluorescence microscopy. She is currently a NSF graduate research fellow.

Martin Gruebele was born in Stuttgart, Germany in 1964. He obtained his B.S. in 1984 and his Ph.D. in 1988 at UC Berkeley. He worked as a postdoc in the lab of Ahmed Zewail at Caltech and then moved to the University of Illinois in 1992. He is currently the James R. Eiszner Professor of Chemistry, Professor of Physics, and Professor of Biophysics and Computational Biology. He is a Fellow of the American Physical and Biophysical Societies as well as a recipient of the Sackler International Prize in Biophysics, the Coblenz Award, and the Wilhelm Bessel Prize, among others. He is a member of the German National Academy of Sciences, the American Academy of Arts and Sciences, and the National Academy of Sciences. He has served as Senior Editor at the *Journal of Physical Chemistry* and serves as Associate Editor of the *Journal of the American Chemical Society*. His research includes protein and RNA folding, fast dynamics in live cells, vibrational energy flow in molecules, quantum computing and

quantum control of reactions, optically assisted STM, glass dynamics, and vertebrate swimming behavior. The work is published in over 200 papers and reviews. Martin Gruebele is married to Nancy Makri, with two children, Alexander and Valerie.

ACKNOWLEDGMENTS

I.G. thanks the NSF Graduate Research Fellowship under grant number DGE-1144245. M.G. thanks the Eiszner family for support through the James R. Eiszner Chair. This work was supported by National Science Foundation grant MCB 1019958. We also acknowledge Prof. Adrian Elcock and Prof. Taras Pogorelov for their contribution to Figure 1 and Dr. Seung Joon Kim for fitting the data shown in Figure 2.

ABBREVIATIONS

FREI, fast relaxation imaging; FRET, Förster resonance energy transfer; FlAsH, 4',5'-bis(1,3,2-dithioarsolan-2-yl) fluorescein; SAXS, small-angle X-ray scattering; SUPREX, stabilities of unpurified proteins from rates of H/D exchange

REFERENCES

- (1) Englander, S. W. A Hydrogen Exchange Method Using Tritium and Sephadex: Its Application to Ribonuclease. *Biochemistry* **1963**, *2*, 798–807.
- (2) Matouschek, A.; Kellis, J. T., Jr.; Serrano, L.; Fersht, A. R. Mapping the Transition State and Pathway of Protein Folding by Protein Engineering. *Nature* **1989**, *340*, 122–126.
- (3) Krantz, B. A.; Dothager, R. S.; Sosnick, T. R. Discerning the Structure and Energy of Multiple Transition States in Protein Folding Using Psi-Analysis. *J. Mol. Biol.* **2004**, *337*, 463–475.
- (4) Donne, D. G.; Viles, J. H.; Groth, D.; Mehlhorn, I.; James, T. L.; Cohen, F. E.; Prusiner, S. B.; Wright, P. E.; Dyson, H. J. Structure of the Recombinant Full-Length Hamster Prion Protein Prp(29–231): The N Terminus Is Highly Flexible. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 13452–13457.
- (5) Hilser, V. J.; Dowdy, D.; Oas, T. G.; Freire, E. The Structural Distribution of Cooperative Interactions in Proteins: Analysis of the Native State Ensemble. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9903–9908.
- (6) Schuler, B.; Hofmann, H. Single-Molecule Spectroscopy of Protein Folding Dynamics—Expanding Scope and Timescales. *Curr. Opin. Struct. Biol.* **2013**, *23*, 36–47.
- (7) Chung, H. S.; Eaton, W. A. Single-Molecule Fluorescence Probes Dynamics of Barrier Crossing. *Nature* **2013**, *502*, 685–688.
- (8) Prigozhin, M. B.; Gruebele, M. Microsecond Folding Experiments and Simulations: A Match Is Made. *Phys. Chem. Chem. Phys.* **2013**, *15*, 3372–3388.
- (9) Lapidus, L. J.; Eaton, W. A.; Hofrichter, J. Measuring the Rate of Intramolecular Contact Formation in Polypeptides. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7220–7225.
- (10) Vu, D. M.; Peterson, E. S.; Dyer, R. B. Experimental Resolution of Early Steps in Protein Folding: Testing Molecular Dynamics Simulations. *J. Am. Chem. Soc.* **2004**, *126*, 6546–6547.
- (11) Whitford, P. C.; Onuchic, J. N.; Sanbonmatsu, K. Y. Connecting Energy Landscapes with Experimental Rates for Aminoacyl-TRNA Accommodation in the Ribosome. *J. Am. Chem. Soc.* **2010**, *132*, 13170–13171.
- (12) Craig, P. O.; Latzer, J.; Weinkam, P.; Hoffman, R. M.; Ferreiro, D. U.; Komives, E. A.; Wolynes, P. G. Prediction of Native-State Hydrogen Exchange from Perfectly Funneled Energy Landscapes. *J. Am. Chem. Soc.* **2011**, *133*, 17463–17472.
- (13) Zagrovic, B.; Snow, C. D.; Khalil, S.; Shirts, M. R.; Pande, V. S. Native-Like Mean Structure in the Unfolded Ensemble of Small Proteins. *J. Mol. Biol.* **2002**, *323*, 153–164.
- (14) Eliezer, D.; Yao, J.; Dyson, H. J.; Wright, P. E. Structural and Dynamic Characterization of Partially Folded States of Apomyoglobin and Implications for Protein Folding. *Nat. Struct. Biol.* **1998**, *5*, 148–155.
- (15) Oliveberg, M.; Wolynes, P. G. The Experimental Survey of Protein-Folding Energy Landscapes. *Q. Rev. Biophys.* **2005**, *38*, 245–288.
- (16) Ghosh, K.; Ozkan, S. B.; Dill, K. A. The Ultimate Speed Limit to Protein Folding Is Conformational Searching. *J. Am. Chem. Soc.* **2007**, *129*, 11920–11927.
- (17) Anfinsen, C. B. Principles That Govern the Folding of Protein Chains. *Science* **1973**, *181*, 223–230.
- (18) Jäger, M.; Zhang, J.; Bieschke, J.; Nguyen, H.; Dendle, G.; Bowman, M.; Noel, J.; Gruebele, M.; Kelly, J. The Structure-Function-Folding Relationship in a Ww Domain. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 10648–10653.
- (19) Weinkam, P.; Zimmermann, J.; Romesberg, F. E.; Wolynes, P. G. The Folding Energy Landscape and Free Energy Excitations of Cytochrome C. *Acc. Chem. Res.* **2010**, *43*, 652–660.
- (20) Go, N.; Abe, H. Randomness of the Process of Protein Folding. *Int. J. Pept. Protein Res.* **1983**, *22*, 622–632.
- (21) Cho, S. S.; Weinkam, P.; Wolynes, P. G. Origins of Barriers and Barrierless Folding in Bbl. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 118–123.
- (22) Dumont, C.; Emilsson, T.; Gruebele, M. Reaching the Protein Folding Speed Limit with Large, Sub-Microsecond Pressure Jumps. *Nat. Methods* **2009**, *6*, 515–519.
- (23) Schonbrun, J.; Dill, K. A. Fast Protein Folding Kinetics. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 12678–12682.
- (24) Walters, B. T.; Mayne, L.; Hinshaw, J. R.; Sosnick, T. R.; Englander, S. W. Folding of a Large Protein at High Structural Resolution. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 18898–18903.
- (25) McGuffee, S. R.; Elcock, A. H. Diffusion, Crowding & Protein Stability in a Dynamic Molecular Model of the Bacterial Cytoplasm. *PLoS Comput. Biol.* **2010**, *6*, e1000694.
- (26) Wagner, A. Energy Constraints on the Evolution of Gene Expression. *Mol. Biol. Evol.* **2005**, *22*, 1365–1374.
- (27) Wilkinson, A.; Gozani, O. Histone-Binding Domains: Strategies for Discovery and Characterization. *Biochim. Biophys. Acta* **2014**, *7*.
- (28) Flotho, A.; Melchior, F. Sumoylation: A Regulatory Protein Modification in Health and Disease. *Annu. Rev. Biochem.* **2013**, *82*, 357–385.
- (29) Lothrop, A. P.; Torres, M. P.; Fuchs, S. M. Deciphering Post-Translational Modification Codes. *FEBS Lett.* **2013**, *587*, 1247–1257.
- (30) Price, C. E.; Driessens, A. J. Biogenesis of Membrane Bound Respiratory Complexes in Escherichia Coli. *Biochim. Biophys. Acta* **2010**, *1803*, 748–766.
- (31) Saraogi, I.; Shan, S. O. Co-Translational Protein Targeting to the Bacterial Membrane. *Biochim. Biophys. Acta* **2013**, *9*.
- (32) Harashima, H.; Dissmeyer, N.; Schnittger, A. Cell Cycle Control across the Eukaryotic Kingdom. *Trends Cell Biol.* **2013**, *23*, 345–356.
- (33) Amm, I.; Sommer, T.; Wolf, D. H. Protein Quality Control and Elimination of Protein Waste: The Role of the Ubiquitin-Proteasome System. *Biochim. Biophys. Acta* **2014**, *1843*, 182–196.
- (34) McConkey, E. H. Molecular Evolution, Intracellular Organization, and the Quinary Structure of Proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 3236–3240.
- (35) Giersch, L. M.; Gershenson, A. Post-Reductionist Protein Science, or Putting Humpty Dumpty Back Together Again. *Nat. Chem. Biol.* **2009**, *5*, 774–777.
- (36) Wirth, A. J.; Gruebele, M. Quinary Protein Structure and the Consequences of Crowding in Living Cells: Leaving the Test-Tube Behind. *Bioessays* **2013**, *35*, 984–993.
- (37) Gruebele, M. Downhill Protein Folding: Evolution Meets Physics. *C. R. Biol.* **2005**, *328*, 701–712.
- (38) Uversky, V. N. Natively Unfolded Proteins: A Point Where Biology Waits for Physics. *Protein Sci.* **2002**, *11*, 739–756.
- (39) Dedmon, M. M.; Patel, C. N.; Young, G. B.; Pielak, G. J. Flgm Gains Structure in Living Cells. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12681–12684.

- (40) Ellis, R. J.; Minton, A. P. Cell Biology: Join the Crowd. *Nature* **2003**, *425*, 27–28.
- (41) Cheung, M. S.; Klimov, D.; Thirumalai, D. Molecular Crowding Enhances Native State Stability and Refolding Rates of Globular Proteins. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 4753–4758.
- (42) Minton, A. P. The Effective Hard Particle Model Provides a Simple, Robust, and Broadly Applicable Description of Nonideal Behavior in Concentrated Solutions of Bovine Serum Albumin and Other Nonassociating Proteins. *J. Pharm. Sci.* **2007**, *96*, 3466–3469.
- (43) Miklos, A. C.; Sarkar, M.; Wang, Y.; Pielak, G. J. Protein Crowding Tunes Protein Stability. *J. Am. Chem. Soc.* **2011**, *133*, 7116–7120.
- (44) Dhar, A.; Girdhar, K.; Singh, D.; Gelman, H.; Ebbinghaus, S.; Gruebele, M. Protein Stability and Folding Kinetics in the Nucleus and Endoplasmic Reticulum of Eucaryotic Cells. *Biophys. J.* **2011**, *101*, 421–430.
- (45) Wirth, A. J.; Platkov, M.; Gruebele, M. Temporal Variation of a Protein Folding Energy Landscape in the Cell. *J. Am. Chem. Soc.* **2013**, *135*, 19215–19221.
- (46) Ebbinghaus, S.; Kim, S. J.; Heyden, M.; Yu, X.; Heugen, U.; Gruebele, M.; Leitner, D. M.; Havenith, M. An Extended Dynamical Hydration Shell around Proteins. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 20749–20752.
- (47) Ebbinghaus, S.; Kim, S. J.; Heyden, M.; Yu, X.; Gruebele, M.; Leitner, D. M.; Havenith, M. Protein Sequence- and Ph-Dependent Hydration Probed by Terahertz Spectroscopy. *J. Am. Chem. Soc.* **2008**, *130*, 2374–2375.
- (48) Kim, S. J.; Born, B.; Havenith, M.; Gruebele, M. Real-Time Detection of Protein-Water Dynamics Upon Protein Folding by Terahertz Absorption Spectroscopy. *Angew. Chem., Int. Ed. Engl.* **2008**, *47*, 6486–6489.
- (49) Kim, S. J.; Dumont, C.; Gruebele, M. Simulation-Based Fitting of Protein-Protein Interaction Potentials to SAXS Experiments. *Biophys. J.* **2008**, *94*, 4924–4931.
- (50) Ellis, R. J. Molecular Chaperones: Assisting Assembly in Addition to Folding. *Trends Biochem. Sci.* **2006**, *31*, 395–401.
- (51) Braselmann, E.; Chaney, J. L.; Clark, P. L. Folding the Proteome. *Trends Biochem. Sci.* **2013**, *38*, 337–344.
- (52) Homouz, D.; Perham, M.; Samiotakis, A.; Cheung, M. S.; Wittung-Stafshede, P. Crowded, Cell-Like Environment Induces Shape Changes in Aspherical Protein. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 11754–11759.
- (53) van den Berg, B.; Ellis, R. J.; Dobson, C. M. Effects of Macromolecular Crowding on Protein Folding and Aggregation. *EMBO J.* **1999**, *18*, 6927–6933.
- (54) Ignatova, Z.; Giersch, L. M. Monitoring Protein Stability and Aggregation in Vivo by Real-Time Fluorescent Labeling. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 523–528.
- (55) Guzman, I.; Gelman, H.; Tai, J.; Gruebele, M. The Extracellular Protein Vlse Is Destabilized inside Cells. *J. Mol. Biol.* **2014**, *426*, 11–20.
- (56) Ebbinghaus, S.; Dhar, A.; McDonald, J. D.; Gruebele, M. Protein Folding Stability and Dynamics Imaged in a Living Cell. *Nat. Methods* **2010**, *7*, 319–323.
- (57) Guo, M.; Xu, Y.; Gruebele, M. Temperature Dependence of Protein Folding Kinetics in Living Cells. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 17863–17867.
- (58) Girdhar, K.; Scott, G.; Chemla, Y. R.; Gruebele, M. Better Biomolecule Thermodynamics from Kinetics. *J. Chem. Phys.* **2011**, *135*, 015102.
- (59) Dhar, A.; Gruebele, M. Fast Relaxation Imaging in Living Cells. *Curr. Protoc. Protein Sci.* **2011**, Chapter 28, Unit 28.1.
- (60) Dhar, A.; Samiotakis, A.; Ebbinghaus, S.; Nienhaus, L.; Homouz, D.; Gruebele, M.; Cheung, M. S. Structure, Function, and Folding of Phosphoglycerate Kinase Are Strongly Perturbed by Macromolecular Crowding. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 17586–17591.
- (61) Minton, A. P. How Can Biochemical Reactions within Cells Differ from Those in Test Tube? *J. Cell Sci.* **2006**, *119*, 2863–2869.
- (62) Beardsley, D. S.; Kauzmann, W. J. Local Densities Orthogonal to Beta-Sheet Amide Planes: Patterns of Packing in Globular Proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4448–4453.
- (63) Zhou, H. X.; Rivas, G.; Minton, A. P. Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences. *Annu. Rev. Biophys.* **2008**, *37*, 375–397.
- (64) Hammond, G. S. A Correlation of Reaction Rates. *J. Am. Chem. Soc.* **1955**, *77*, 334–338.
- (65) Denos, S.; Dhar, A.; Gruebele, M. Crowding Effects on the Small, Fast-Folding Protein Lambda6-85. *Faraday Discuss.* **2012**, *157*, 451–462 (discussion 475–500).
- (66) Hong, J.; Giersch, L. M. Macromolecular Crowding Remodels the Energy Landscape of a Protein by Favoring a More Compact Unfolded State. *J. Am. Chem. Soc.* **2010**, *132*, 10445–10452.
- (67) Zhou, H. X. Polymer Crowders and Protein Crowders Act Similarly on Protein Folding Stability. *FEBS Lett.* **2013**, *587*, 394–397.
- (68) Ziv, G.; Haran, G.; Thirumalai, D. Ribosome Exit Tunnel Can Entropically Stabilize Alpha-Helices. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 18956–18961.
- (69) Demirci, E.; Junne, T.; Baday, S.; Berneche, S.; Spiess, M. Functional Asymmetry within the Sec61p Translocon. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 18856–18861.
- (70) Baumketner, A.; Jewett, A.; Shea, J. E. Effects of Confinement in Chaperonin Assisted Protein Folding: Rate Enhancement by Decreasing the Roughness of the Folding Energy Landscape. *J. Mol. Biol.* **2003**, *332*, 701–713.
- (71) Zhou, H.-X. Protein Folding in Confined and Crowded Environment. *Arch. Biochem. Biophys.* **2008**, *469*, 76–82.
- (72) Jonas, J. High-Resolution Nuclear Magnetic Resonance Studies of Proteins. *Biochim. Biophys. Acta* **2002**, *1595*, 145–159.
- (73) Ravindra, R.; Zhao, S.; Gies, H.; Winter, R. Protein Encapsulation in Mesoporous Silicate: The Effects of Confinement on Protein Stability, Hydration, and Volumetric Properties. *J. Am. Chem. Soc.* **2004**, *126*, 12224–12225.
- (74) Peterson, R. W.; Anbalagan, K.; Tommos, C.; Wand, A. J. Forced Folding and Structural Analysis of Metastable Proteins. *J. Am. Chem. Soc.* **2004**, *126*, 9498–9499.
- (75) Patel, K.; Goyal, B.; Kumar, A.; Kishore, N.; Durani, S. Cured of “Stickiness”, Poly-L Beta-Hairpin Is Promoted with Ll-to-Dd Mutation as a Protein and a Hydrolase Mimic. *J. Phys. Chem. B* **2010**, *114*, 16887–16893.
- (76) Persson, E.; Halle, B. Cell Water Dynamics on Multiple Time Scales. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 6266–6271.
- (77) Luong, T. Q.; Verma, P. K.; Mitra, R. K.; Havenith, M. Do Hydration Dynamics Follow the Structural Perturbation During Thermal Denaturation of a Protein: A Terahertz Absorption Study. *Biophys. J.* **2011**, *101*, 925–933.
- (78) Ghaemmaghami, S.; Oas, T. G. Quantitative Protein Stability Measurement in Vivo. *Nat. Struct. Biol.* **2001**, *8*, 879–882.
- (79) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. Specific Covalent Labeling of Recombinant Protein Molecules inside Live Cells. *Science* **1998**, *281*, 269–272.
- (80) Ignatova, Z.; Giersch, L. M. Extended Polyglutamine Tracts Cause Aggregation and Structural Perturbation of an Adjacent Beta Barrel Protein. *J. Biol. Chem.* **2006**, *281*, 12959–12967.
- (81) Serber, Z.; Keatinge-Clay, A. T.; Ledwidge, R.; Kelly, A. E.; Miller, S. M.; Dotsch, V. High-Resolution Macromolecular Nmr Spectroscopy inside Living Cells. *J. Am. Chem. Soc.* **2001**, *123*, 2446–2447.
- (82) Schlesinger, A. P.; Wang, Y.; Tadeo, X.; Millet, O.; Pielak, G. J. Macromolecular Crowding Fails to Fold a Globular Protein in Cells. *J. Am. Chem. Soc.* **2011**, *133*, 8082–8085.
- (83) Selenko, P.; Serber, Z.; Gadea, B.; Ruderman, J.; Wagner, G. Quantitative Nmr Analysis of the Protein G B1 Domain in Xenopus Laevis Egg Extracts and Intact Oocytes. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 11904–11909.
- (84) Inomata, K.; Ohno, A.; Tochio, H.; Isogai, S.; Tenno, T.; Nakase, I.; Takeuchi, T.; Futaki, S.; Ito, Y.; Hiroaki, H.; Shirakawa, M.

- High-Resolution Multi-Dimensional Nmr Spectroscopy of Proteins in Human Cells. *Nature* **2009**, *458*, 106–109.
- (85) Igmar Schoen, H. K. and Dieter Braun Hybridization Kinetics Is Different inside Cells. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 21649–21654.
- (86) Lakowicz, J. R.; Cherek, H. Phase-Sensitive Fluorescence Spectroscopy: A New Method to Resolve Fluorescence Lifetimes or Emission Spectra of Components in a Mixture of Fluorophores. *J. Biochem. Biophys. Methods* **1981**, *5*, 19–35.
- (87) Eigen, M. Methods for Investigation of Ionic Reactions in Aqueous Solutions with Half Times as Short as 10–9 Sec. *Angew. Chem.* **1954**, *66*, 720–720.
- (88) Eigen, M. Über Die Kinetik Von Ionenreaktionen an Wassriger Losung. *Angew. Chem.* **1954**, *66*, 143–143.
- (89) Gelman, H.; Platkov, M.; Gruebele, M. Rapid Perturbation of Free-Energy Landscapes: From in Vitro to in Vivo. *Chemistry* **2012**, *18*, 6420–6427.
- (90) Nishii, I.; Kataoka, M.; Goto, Y. Thermodynamic Stability of the Molten Globule States of Apomyoglobin. *J. Mol. Biol.* **1995**, *250*, 223–238.
- (91) Yuan, J. M.; Chyan, C. L.; Zhou, H. X.; Chung, T. Y.; Peng, H.; Ping, G.; Yang, G. The Effects of Macromolecular Crowding on the Mechanical Stability of Protein Molecules. *Protein Sci.* **2008**, *17*, 2156–2166.
- (92) Dhar, A.; Ebbinghaus, S.; Shen, Z.; Mishra, T.; Gruebele, M. The Diffusion Coefficient for Pgk Folding in Eukaryotic Cells. *Biophys. J.* **2010**, *99*, L69–71.
- (93) Chowdary, P. D.; Gruebele, M. Molecules: What Kind of a Bag of Atoms? *J. Phys. Chem. A* **2009**, *113*, 13139–13143.
- (94) Stigter, D.; Alonso, D. O.; Dill, K. A. Protein Stability: Electrostatics and Compact Denatured States. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4176–4180.
- (95) Perez-Sayans, M.; Supuran, C. T.; Pastorekova, S.; Suarez-Penaranda, J. M.; Pilar, G. D.; Barros-Angueira, F.; Gandara-Rey, J. M.; Garcia-Garcia, A. The Role of Carbonic Anhydrase IX in Hypoxia Control in Oscc. *J. Oral Pathol. Med.* **2013**, *42*, 1–8.
- (96) Fissell, W. H.; Hofmann, C. L.; Smith, R.; Chen, M. H. Size and Conformation of Ficoll as Determined by Size-Exclusion Chromatography Followed by Multiangle Light Scattering. *Am. J. Physiol.: Renal Physiol.* **2010**, *298*, F205–208.
- (97) Peacock, J. A. Two-Dimensional Goodness-of-Fit Testing in Astronomy. *Mon. Not. R. Astron. Soc.* **1983**, *202*, 615–627.
- (98) Hartl, F. U.; Hayer-Hartl, M. Converging Concepts of Protein Folding in Vitro and in Vivo. *Nat. Struct. Mol. Biol.* **2009**, *16*, 574–581.
- (99) Hartl, F. U.; Bracher, A.; Hayer-Hartl, M. Molecular Chaperones in Protein Folding and Proteostasis. *Nature* **2011**, *475*, 324–332.
- (100) Mayer, M. P. Gymnastics of Molecular Chaperones. *Mol. Cell* **2010**, *39*, 321–331.
- (101) Kampinga, H. H.; Craig, E. A. The Hsp70 Chaperone Machinery: J Proteins as Drivers of Functional Specificity. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 579–592.
- (102) Anfinsen, C. B.; Haber, E. Studies on the Reduction and Re-Formation of Protein Disulfide Bonds. *J. Biol. Chem.* **1961**, *236*, 1361–1363.
- (103) Anfinsen, C. B.; Haber, E.; Sela, M.; White, F. H., Jr. The Kinetics of Formation of Native Ribonuclease During Oxidation of the Reduced Polypeptide Chain. *Proc. Natl. Acad. Sci. U.S.A.* **1961**, *47*, 1309–1314.
- (104) Onuchic, J. N.; Luthey-Schulten, Z.; Wolynes, P. G. Theory of Protein Folding: The Energy Landscape Perspective. *Annu. Rev. Phys. Chem.* **1997**, *48*, 545–600.
- (105) Sosnick, T. R.; Mayne, L.; Hiller, R.; Englander, S. W. The Barriers in Protein Folding. *Nat. Struct. Biol.* **1994**, *1*, 149–156.
- (106) Valastyan, J. S.; Lindquist, S. Mechanisms of Protein-Folding Diseases at a Glance. *Dis. Models & Mech.* **2014**, *7*, 9–14.
- (107) Cortese, M. S.; Uversky, V. N.; Dunker, A. K. Intrinsic Disorder in Scaffold Proteins: Getting More from Less. *Prog. Biophys. Mol. Biol.* **2008**, *98*, 85–106.
- (108) Motlagh, H. N.; Li, J.; Thompson, E. B.; Hilser, V. J. Interplay between Allostery and Intrinsic Disorder in an Ensemble. *Biochem. Soc. Trans.* **2012**, *40*, 975–980.
- (109) Chipuk, J. E.; Bouchier-Hayes, L.; Kuwana, T.; Newmeyer, D. D.; Green, D. R. Puma Couples the Nuclear and Cytoplasmic Proapoptotic Function of P53. *Science* **2005**, *309*, 1732–1735.
- (110) Follis, A. V.; Chipuk, J. E.; Fisher, J. C.; Yun, M. K.; Grace, C. R.; Nourse, A.; Baran, K.; Ou, L.; Min, L.; White, S. W.; Green, D. R.; Kriwacki, R. W. Puma Binding Induces Partial Unfolding within Bcl-XL to Disrupt P53 Binding and Promote Apoptosis. *Nat. Chem. Biol.* **2013**, *9*, 163–168.
- (111) Diaz-Moreno, I.; Hollingworth, D.; Frenkel, T. A.; Kelly, G.; Martin, S.; Howell, S.; Garcia-Mayoral, M.; Gherzi, R.; Briata, P.; Ramos, A. Phosphorylation-Mediated Unfolding of a Kh Domain Regulates Ksrp Localization Via 14–3–3 Binding. *Nat. Struct. Mol. Biol.* **2009**, *16*, 238–246.
- (112) Gherzi, R.; Trabucchi, M.; Ponassi, M.; Ruggiero, T.; Corte, G.; Moroni, C.; Chen, C. Y.; Khabar, K. S.; Andersen, J. S.; Briata, P. The Rna-Binding Protein Ksrp Promotes Decay of Beta-Catenin Mrna and Is Inactivated by Pi3k-Akt Signaling. *PLoS Biol.* **2006**, *5*, e5.
- (113) Braakman, I.; Hebert, D. N. Protein Folding in the Endoplasmic Reticulum. *Cold Spring Harbor Perspect. Biol.* **2013**, *5*, a013201.
- (114) Burmann, B. M.; Knauer, S. H.; Sevostyanova, A.; Schweimer, K.; Mooney, R. A.; Landick, R.; Artsimovitch, I.; Rosch, P. An Alpha Helix to Beta Barrel Domain Switch Transforms the Transcription Factor Rfah into a Translation Factor. *Cell* **2012**, *150*, 291–303.
- (115) Tuinstra, R. L.; Peterson, F. C.; Kutlesa, S.; Elgin, E. S.; Kron, M. A.; Volkman, B. F. Interconversion between Two Unrelated Protein Folds in the Lymphotactin Native State. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 5057–5062.
- (116) Ebbinghaus, S.; Gruebele, M. Protein Folding Landscapes in the Living Cell. *J. Phys. Chem. Lett.* **2011**, *2*, 314–319.