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Proteins: 'Boil 'Em, Mash 'Em, Stick 'Em in a Stew'

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ABSTRACT Cells of the vast majority of organisms are subject to temperature, pressure, pH, ionic strength and other stresses. We discuss these effects in the light of protein folding and protein interactions *in vitro*, in complex environments, in cells, and *in vivo*. Protein phase diagrams provide a way of organizing different structural ensembles that occur under stress, and how one can move among ensembles. Experiments that perturb biomolecules *in vitro* or in-cell by stressing them have revealed much about the underlying forces that are competing to control protein stability, folding and function. Two phenomena that emerge and serve to broadly classify effects of the cellular environment are crowding (mainly due to repulsive forces) and sticking (mainly due to attractive forces). The interior of cells is closely balanced between these emergent effects, and stress can tip the balance one way or the other. The free energy scale involved is small, but significant on the scale of the "on/off switches" that control signaling in cells, or of protein-protein association with favorable function such as increased enzyme processivity. Quantitative tools from biophysical chemistry will play an important role in elucidating the world of crowding and sticking under stress.

1. Introduction

One of the fundamental tenets of physical chemistry is that one can learn much more about a system's equilibrium and dynamical properties by putting it under stress.[1] Time-resolved spectroscopy take molecules out of equilibrium and monitors recovery;[2] energy-resolved spectroscopy excites molecules to teach us about their structure;[3] studying phase diagrams away from ambient temperature, pressure or crowding quantifies the underlying molecular interactions.[4]

Proteins are particularly sensitive to stress because the characteristic free energy scale of protein-protein association, protein folding and other protein-biomolecular interactions is only a few k_BT_0 ($T_0 \sim 295$ K or 22°C is room temperature here).[5] Nonetheless, life thrives in environments that humans consider extreme: near-boiling water, rocks at high pressure, at low pH or extreme salinity (Table 1). What is thought of as normal temperature and pressure is not necessarily where most life on Earth exists.[6] Probably the vast majority of archaea and bacteria live in 'extreme environments.' How have proteins evolved to function in such extreme and complex environment?[7]

Table 1: Stress factors, known limits, and example organisms.[7]

Factor	Condition	Limits	Example
Temperature	High temperature	>110 to 121°C	*Pyrolobus fumarii*
	Low temperature	<-17 to -20°C	*Synechococcus lividis*
Pressure	High pressure	1100 bars	*Pyrococcus spheroides*
pH	Alkaline system	>11	*Psychrobacter*
	Acidic system	~0	*Natronobacterium*
Ionic strength	High salinity	2 to 5 M NaCl	*Halobacteriaceae*

In this feature article, we discuss how applying stress to proteins teaches us about their function and stability. In an earlier such article, we emphasized new in-cell methodologies.[8] Here the ongoing work is organized in terms of stressors such as temperature, pressure, and crowding,[9–11] or boiling, mashing and getting stuck in a stew, to paraphrase our titular quote from a screenplay based on a famous J. R. R. Tolkien novel.[12] We begin with *in vitro* studies and work our way through more complex environments to *in vivo*. Because stresses can lead to phase changes, including protein folding, which can be approximated as a first order or continuous phase

transition,[13] we will emphasize phase diagrams, and stress as motion between different areas in the phase diagram. We also emphasize the effect of these stresses on the quinary structure of the protein, which is the fifth level of organization present in protein structure. Quinary structure consists of weaker and more transient interactions between proteins and their surrounding which are highly perturbed by environmental stresses.[14,15]

2. Phase diagrams of proteins

The phase diagram for protein folding can be constructed to visualize how the transition between folded and unfolded state happens when temperature, pressure, pH or other stressors are changed.[16] Around a quarter of polypeptides in mammalian cells do not fold into compact structures.[17] However, even such disordered proteins (IDPs) can be placed in partially or highly unfolded regions of a phase diagram. Such IDPs or globular proteins can function at the verge of stability,[18–21] likely making them even more susceptible to their environment.

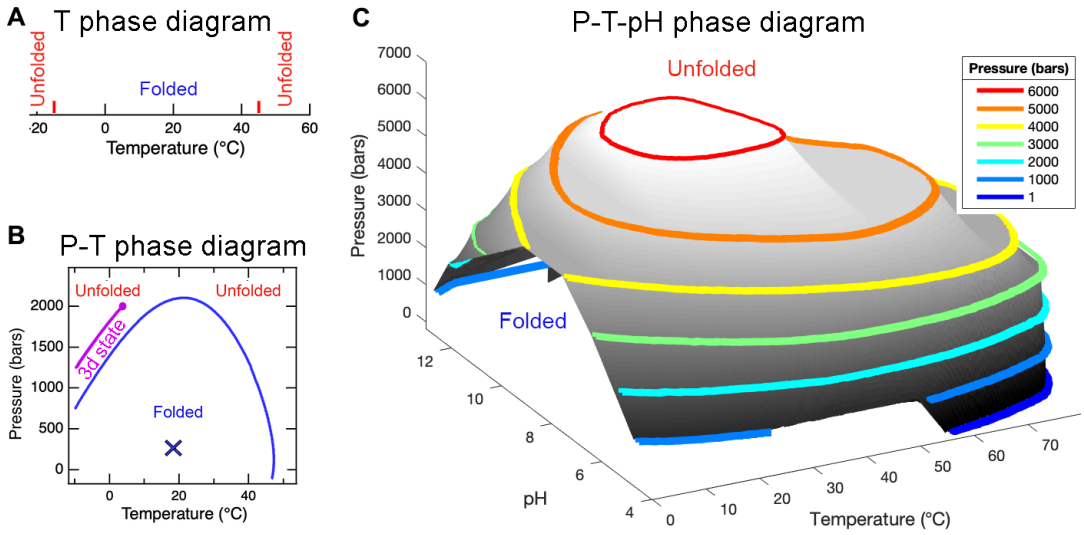

Fig. 1. 1D, 2D and 3D ellipsoidal protein folding phase diagrams. (A) Temperature as the only variable showing the cold denaturation and heat denaturation transition temperatures (red bars) and folded/unfolded temperature regions. (B) Schematic *P-T* phase diagram, showing the blue ellipsoid of stability where $\Delta G_{folding}=0$ and the unfolded and folded states have the same stability. The point of highest stability (marked x) is where $\Delta G_{folding}$ is most negative. (Typical data of this kind can be found in ref. 9.) In more complex situation, thermodynamically stable additional states (here a "third state") are possible; in the example here (purple curve), such a state is only stable up to a critical temperature marked by a dot. (C) *P-T-pH* phase diagram of metmyoglobin extending the ellipse to an ellipsoid in the third dimension. *P-T* data at constant pH (colored circles) are from ref. 22. The gray surface is a model 'ellipsoid.'

For now we consider globular proteins with well-defined secondary and tertiary structure. The native state under 'physiological conditions' (often taken to be room temperature 295 K (22°C), 1 atm and few 100 mM ionic strength *in vitro*) is generally near the center of an elliptical region where the free energy of folding $\Delta G_{folding}$ is less than 0. Hawley has described a simple thermodynamic formula for this elliptical region in terms of parameters such as heat capacity of folding, isothermal compressibility, etc.[4] When there is just one stressor (e.g. folding as a function of temperature T), the boundary consists of two points where $\Delta G_{folding}=0$ (e.g. the cold denaturation temperature, and the heat denaturation temperature in Fig. 1A). When more thermodynamic variables are added (e.g. P in Fig. 1B), the elliptical boundary of stability becomes an n-1 dimensional manifold for n variables (e.g. the surface of an ellipse for pH, T and P in Fig. 1C).[22]

Outside the elliptical boundary, a variety of non-native states will be most stable depending on the position of the point of interest in the phase diagram.[4,22,23] In the simplest case, it is believed that there is only one non-native state (so called two-state folding). An example would be the kinetic correspondence of heat- and cold-denatured states of the protein lambda repressor fragment.[24] Locally near a phase boundary the protein structural ensembles and differences between properties such as free volume are generally well defined, and we can treat the system by a two-state transition model.[9,25]

Two-state models break down near critical points in the phase diagram, where two free energy wells merge into a single free energy well,[13] and large fluctuations can occur (e.g. top left of Figure 1B). Downhill folding is an example where a barrierless transition separates initial and final conformational ensembles.[26–28] Such critical points influence fluctuations in other parts of the phase diagram. Therefore, even far from critical points it is important to understand how dynamical the structure a protein is and how the environment affects it.

Two-state models also break down when two phase boundaries lie close to one another (top left in Fig. 1B) and multiple states can co-exist. Such "3^d states" can lie on the pathway for folding,[29–31], or they can act as traps that hinder refolding because the protein must unfold again before re-sampling configurations to finally fold.[32,33]

Protein-protein surface interactions are in many ways analogous to folding: they are still driven by matching of hydrophobic surfaces, even though polar and electrostatic interactions also play a role;[34] and just as folding often produces marginally stable native states to preserve functionally

useful fluctuations, protein-protein interactions are not necessarily optimal for function when they are strongest. Strong binding and having many binding partners are mutually exclusive due to the limited information capacity of a protein surface to encode such interactions.[15] Thus multiple weaker interactions are often favored both in folding (> 2 secondary structure elements interact to form tertiary structure) and binding (>2 binding partners allows allosteric binding effects).

Protein-protein interactions will also lead to phase diagrams, including the formation of a great variety of protein-enriched phase-separated droplets.[35] Indeed, proteins rather easily salt out of solution or form inclusion bodies when overexpressed, so the real question is not how cells form droplets, but how they avoid being completely filled with phase-separated regions, and use membranous compartments for many separation tasks instead. The answer must be that protein surfaces co-evolve with the cell environment so that attractions (e.g. hydrophobic patches interacting) are balanced by repulsions (e.g. by charge distribution on the protein surface). Evidence for fine-tuned balance that can be disrupted by changing even one surface residue has been seen by in-cell NMR studies.[36]

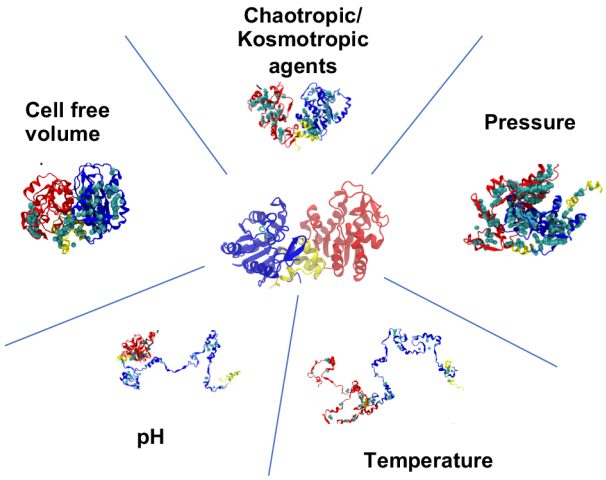

Fig. 2. Some stresses that affect folding *in vitro* and *in vivo*. Unfolded protein shapes adopted from ref. 37 are representative of what might happen in the presence of the corresponding stress.

3. Stresses that affect folding and binding *in vitro*

Temperature Temperature has been historically most commonly used to denature proteins. Proteins undergo both heat- and cold-denaturation,[38] indicating that folded states in nature are in a

delicate state of stability. Increasing the temperature strengthens hydrophobic interactions,[39] but breaks hydrogen bonds and increases backbone and sidechain disorder. This tug of war produces two phase transitions along the T axis in Fig. 1A. One study has demonstrated the kinetic equivalence of such heat- and cold-denatured states,[24] but there could be significant structural differences.

The stability of proteins is often quantified by their ability to withstand heat denaturation. A shift in melting temperature T_m indicates how much different conditions stabilize or destabilize a protein. The stability of multi domain proteins is complicated by domain interactions and different intrinsic stabilities of different domains.[40,41]

Pressure Pressure denaturation of proteins was first reported in 1914.[42] Since then, much work has been done to understand how pressure denatures protein.[4,22] Pressure denaturation of proteins occur when water is pushed inside protein cavities, destabilizing the hydrophobic core of the protein.[11,25,43] The net volume change upon unfolding is negative – the folded state has a larger molar volume than unfolded state. This seems counter-intuitive because the folded state is the most compact in terms of radius of gyration. Two changes contribute to the net volume change. While the change in hydration volume is positive, the change in void volume is even more negative.[11] Recently a computational study have been able to reproduce the sign and the magnitude of this volume change.[44] It is hypothesized that barophilic organisms (those living under high pressure conditions) might have evolved to have positive volume change upon unfolding, [44] which remains to be verified.

It is also possible to unfold proteins under hydrostatic tension, or 'negative pressure' (bottom of the ellipse in Fig. 1B). Experimental negative pressure conditions have been used to probe protein folding by NMR[45] using a Berthelot tube. These measurements complete the phase diagram on the negative pressure side, providing proof for the ellipsoid shape of the phase diagram.[4] Negative hydrostatic pressure experiments make a connection to the field of force-pulling experiments on proteins, where anisotropic force is applied via a tether and AFM tip, and reversible folding/unfolding can be observed.[46,47]

pH Proteins are generally most stable at their isoelectric point, where the net charge is close to zero.[48] Most proteins are stable around pH 7 and undergo partial denaturation at low pH (<2) or

high pH (>12).[49] It has been shown that at low pH some globular proteins have a propensity to form aggregates, moving away from the molten globule to the amyloid state.[50]

Many proteins can form partially structured acid-denatured states.[51] Such states often favor secondary structure that can be locally stabilized, i.e. helical structure.[52] Many folding kinetics experiments have shown that locally stabilized helical structure is also favored at early times during refolding before giving way to native-like secondary structure. [53–55] Indeed, older force fields such as CHARMM22 with excess helix-forming propensity[56] had to be corrected.

Changing pH can also allow some proteins to switch structures in a continuous fashion, acting as conformational rheostats.[57] This behavior is closely related to the continuous thermal phase transition, dubbed "downhill folding," that has been observed for some very fast-folding proteins.[28,39]

Osmolytes: chaotropic and kosmotropic agents Many *in vitro* studies have looked at the effect of small molecules on protein stability. These small molecules are called osmolytes and one way to classify them is as destabilizing proteins and hydrophobic interactions (chaotropes) or stabilizing (kosmotropes). This specific definition has over time been expanded to effects on solvent structure and chaotropes are 'structure breakers,' while kosmotropes are 'structure makers.[58–60]

The mechanism of this stabilization/destabilization pertains to the effect of these cosolutes on the hydrogen bond network of water.[59,61] Chaotropes break down the hydrogen bond network of water as they bind to the protein surface, while non-ionic kosmotropes are well-solvated and generally excluded from the macromolecular surface. The preferential solvation of kosmotropes in bulk water reduces the diffusion of water and makes protein surfaces less flexible.[62] Consequently, proteins are conformationally restricted, which could lead to reduction in enzymatic activity of a protein whose function requires the proteins to be flexible.

The conditions under which cosolutes act, and even whether they act as chaotropes or kosmotropes, depends on their concentration and the type of stress applied. For example, urea destabilizes proteins at high concentrations, but has been shown to stabilize protein at low concentrations.[63] Guanidinium chloride can be used to dissolve aggregates at high concentration,[64] but at 1 M induces extended (sheet-like) structure that accelerates aggregation.[28] Tri-methyl amine

oxide (TMAO) stabilizes proteins preferably against pressure denaturation compared to temperature denaturation, and is found in high concentration in some deep sea fish.[61,65] Dodine is a molecule that has both chaotropic and kosmotropic functional groups, and has been shown to insert into tertiary structure while retaining secondary structure.[66]

Macromolecular Crowding The presence of large macromolecules in the cell is one of the most important distinguishing features when compared to *in vitro* aqueous buffers. The steric effect of crowders is largely due to excluded volume, but depends on size and shape also.[67] Crowding is most effective when the size of the crowder matches the size of the protein. Small molecules lack significant excluded volume; oversize crowders have large interstitial spaces in which proteins are not crowded. At low concentration, crowders *in vitro* can actually destabilize proteins during temperature or pressure denaturation.[68] *In vitro* crowding by substances such as Ficoll, a cross-linked carbohydrate, can induce structural transitions in proteins and affect enzyme catalysis, and lead to the formation of entirely new structural ensembles in the protein's *P*-*T*-crowding phase diagram.[37]

4. Folding in complex environments

In-cell, proteins interact with a great variety of biomolecules. Some of these interactions are recapitulated in complex engineered environments, such as gels, polymer matrices or lysates that have applications in bio-sensitive devices, pharmaceutical delivery, or cell-free assays. These environments can induce significant shifts in protein stability or other properties.

Hydrogels These loose matrices of polymers are widely used in biomedical applications due to dielectric constants, polymer solvation, and hydrogen bonding properties being conducive to protein stability.[69] The crosslinks in the polymer create excluded volume effects. For example, the protein PGK is uniformly distributed in the 4% cross-linked gel but is preferentially present at the surface in the 10% cross-linked gel showing the effect of excluded volume.[70] There can also be hydrophobic or polar interactions between the protein and the polymer side chains. Increasing the cross-linking in polyacrylamide gels has been found to stabilize some proteins. Indeed, confinement as detected by the crowding sensor CrH2 increases significantly form 4% to10% cross-linking. The increase in confinement also increases FRET of fluorescent-labeled PGK in the

unfolded state, indicating a compactification of the unfolded protein in the hydrogel. Yet upon unfolding, the protein tends to aggregate more in the hydrogel compared to aqueous solution.

Polymers PEGylation of proteins (linking a protein to poly(ethylene glycol) is used as an industrial process to protect them against degradation in the human body. In one example studied at the atomistic level, the PEG chain interacted directly with lysine residues and a nearby hydrophobic patch. This shows the propensity of PEG chain to form both hydrogen bonds and hydrophobic interactions.[71,72] The PEG chain does not coat the protein surface, but rather interacts intermittently, sometimes forming a random coil in the solvent, and sometimes binding to the protein surface. PEG induces less crowding than a cross-linked polymer such as Ficoll.[73]

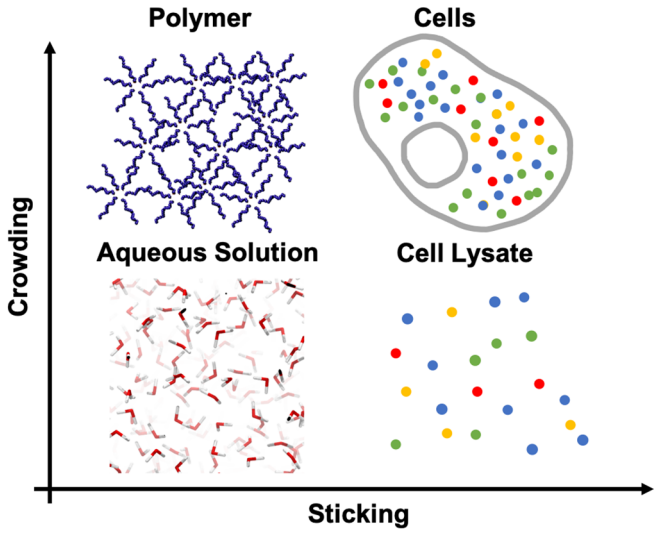

Fig. 3. Aqueous buffers, polymer solutions, lysate and live cells probe different quadrants of the "crowding" *vs*. "sticking" space, allowing steric or volume exclusion effects to be disentangled from non-specific binding.

Polyelectrolytes in solution have been generally thought to interact with proteins, though neutral polymers less so.[69,74] However, the effect that polyzwitterions like poly(sulfobetaine) (pSB) have on protein stability is protein-specific,[75] and pSB can destabilize proteins significantly due to surface-binding. Interestingly, pSB increases the cooperativity of the folding transition even while lowering stability, either because interaction of the polymer with the backbone or hydrophobic core is more "all or nothing" than for smaller guanidinium ions or water molecules, or because pSB perturbs the water molecular network. This in contrast with the stabilization effect seen from PEG solution.[75]

Cell lysate This material can be harvested by breaking open cells and removing membrane and membranous compartments. It can be used in cell-free assays,[76] and can act as a proxy for predicting the effect of the cytoplasmic environment on proteins.[77] Cell lysate is not as crowded as the cellular environment, and so it can be used to separate crowding from non-steric 'sticking' interactions in cells (Fig. 3). A combination of Ficoll and cell lysate, with Ficoll accounting for crowding and cell lysate accounting for non-steric interactions can act as mimics for cellular environment.[77]

5. Stresses that affect folding and binding in-cell and *in vivo*

We discussed stresses that affect protein folding *in vitro*, and one might think that many of these are not relevant to in-cell protein folding at 37 °C, 1 atm and with *ca.* 300 mM total ion concentration. For most experiments done *in vivo*, especially on human cell lines, the temperature is generally ~310K (37°C). Reality is different: many cells exist in extreme environments outlined in Table 1, and proteins have evolved to optimize function in these environments. Physico-chemical variables whose variation may not be obviously relevant to biomedical studies are highly relevant to how life evolved, and the conditions that life can exist in. In addition, a broader view of the phase diagram of proteins in-cell can teach us about the origin of protein properties, such as native state fluctuations, under the physiological conditions of interest.

Heterogeneous intracellular environment The cytoplasm is a heterogeneous and dynamic environment. 70% occupied by water, the cytoplasm also contains 20% proteins, as well as lipids, polysaccharides and nucleic acids.[78] The cell nucleus similarly contains a large fraction of macromolecules, with a larger component of nucleic acid.[79] Other organelles also offer a wide diversity of unique microenvironments with differing protein stability and folding kinetics.[80] For example, experiments in mammalian cells have shown that the enzyme PGK has higher stability and folding speed in the nucleus compared to the endoplasmic reticulum (ER) or cytoplasm.[81] Two general properties have been used to describe these environments: crowding and stickiness.

Crowding arises mainly from proteins in the 10-100 kDa mass range. Since protein radius R and mass m are related by $m \sim R^{2.7}$ due to imperfect packing of protein cores,[82] the dynamic range of proteins diameters is relatively small (2-3), so proteins can crowd each other very effectively.

Membranous organelles such as mitochondria or vesicles, the cytoskeleton and ribosomes also contribute to crowding. Crowding is generally thought to enhance globular protein stability because it reduces the conformational entropy of unfolded states, but not of compact native states.[83]

Stickiness mainly comes from electrostatic or hydrophobic (water-exclusion) attractive interactions between protein and/or nucleic acid surfaces. Stickiness actually reduces the effective binding strength (increases K_d) of complexes via competition from nonspecific binding, and can reduce protein stability by lowering the enthalpy of unfolded states via favorable intermolecular contacts.[5]

The increased stickiness of unfolded proteins in the cytoplasm has been observed directly by measuring diffusion as a function of temperature and comparing proteins that unfold above 35 °C *vs*. ones that do not (Fig, 4).[84] Proteins that do not unfold obey the Stokes-Einstein temperature dependence of the diffusion coefficient, whereas those that unfold show a large decrease in diffusion that cannot be explained by the increase of unfolded hydrodynamic radius alone. Maps of the cytoplasm at varying resolution show that protein diffusion is both anomalous and heterogeneous as a function of location in the cytoplasm.

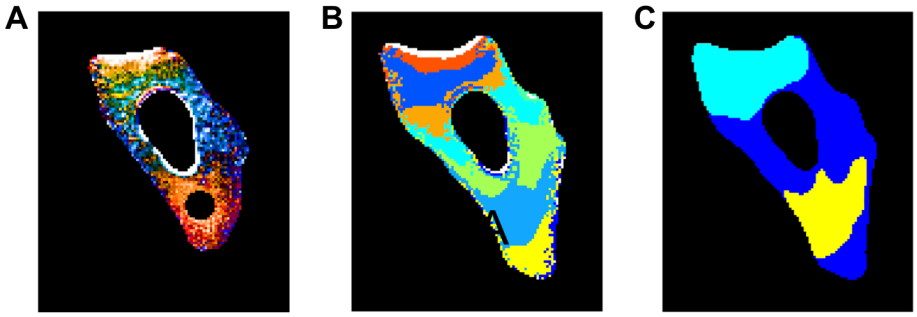

Fig. 4. Diffusion coefficient of PGK at increased levels of coarse-graining (A-C) in a U-2 OS cell, color coded from yellow (fast) to blue (slow). The data from ref. 84 (open access source) was obtained by bleaching fluorescent-labeled protein with a UV laser (small black circle in A) and monitoring the reduction of fluorescence throughout the cell by epifluorescence microscopy. The large oval area in the center of the cell is the nucleus, where PGK was not expressed.

In addition to utilizing basic physical mechanisms to control folding and function, the cytoplasm has also evolved machinery to protect against the perils of protein misfolding: molecular chaperones hold on to proteins during stress to promote refolding over aggregation, or even unfold

misfolded proteins to allow refolding to occur;[85–87] and the ubiquitin-proteasome system, which regulates degradation of misfolded proteins.[88]

Temperature Temperature is as fundamental to in-cell as *in vitro* protein folding studies. Many biomolecular reactions are temperature-sensitive (e.g. proteins unfold and complexes dissociate at higher *T*), and most cells (although not mammalian ones) are subject to large (20-30 K) temperature fluctuations even over the course of a day. Fast relaxation imaging (FReI) was developed in 2010 to study protein unfolding and refolding under sudden temperature stress in-cell,[89] and was recently extended to study folding in different animal tissues *in vivo*.[90] The temperature-dependence of PGK folding shows that PGK in-cell instability increases by about 6kJ/mol compared to 'physiological' buffer, but folding and unfolding rates otherwise parallel *in vitro* behavior.[91] Stabilization by crowding in that case exceeds destabilization by sticking. Not surprisingly, even greater stability has been observed in highly crowded (400 mg/ml) eye lens tissue of zebrafish.[90] However, sticking can also win out: the bacterial extracellular protein VlSE is destabilized in the cytoplasm,[92] and when human HAH1 is placed in a foreign bacterial cytoplasm, in-cell NMR reveals that the protein sticks and stops tumbling freely, whereas the homologous bacterial protein TT tumbles freely in bacterial cells, with similar observations for other proteins.[36,93]

Hydrostatic Pressure As discussed earlier, *in vitro* studies have confirmed that high pressure denatures proteins. A recent experiment on pressure denaturation of eukaryotic PGK in bacterial cells highlights the interplay of crowding and sticking mentioned above.[94] Relative to *in vitro*, yeast PGK is stabilized in bacterial cells under pressure denaturation, but destabilized under temperature denaturation. The latter is the opposite trend observed for yeast PGK in eukaryotic cells.[89]

This result demonstrates that temperature and pressure can be used to tease apart crowding and sticking effects. Simulations have shown that high pressure reduces protein structural fluctuations,[95] decreasing the dynamically accessible surface area of proteins. This could lead to reduced sticking of proteins in the cytoplasm, and explain why pressure denaturation favors stabilization by crowding over destabilization by sticking. Higher temperature on the other hand increases protein fluctuations and hydrophobic exposure, and strengthens the hydrophobic effect,[39,96] thus promoting sticking over crowding,

pH Intracellular pH is another important thermodynamic variable for cellular metabolic processes. There are many ways to determine intracellular pH such as microelectrodes, nuclear magnetic resonance spectroscopy (NMR), and radioisotopic assays.[97,98] More recently, pH-dependent fluorophores have been developed for *in vivo* measurements.[98,99] Compared to *in vitro*, pH in the cellular interior not only modulates protein stability and solubility,[100] but also affects quinary interactions between macromolecules.[101]

For example, Pielak and co-workers used in-cell NMR spectroscopy to study protein interactions in *E. coli* cells. The K10H mutant of protein GB1 has a high quality and pH-sensitive in-cell 15N–1H HSQC spectra. *E. coli* cells were stressed by pH changes in the range of 7.6 to 5.0. At lower pH, positive charge accumulates on the GB1 surface, which then interacts more strongly with negatively-changed proteins in the *E. coli* cytoplasm. GB1 then tumbles more slowly in the cell which leads to longer transverse relaxation time and a broader NMR peak. Thus protein-protein sticking in cells can be tuned by pH.[101]

Osmolytes: chaotropic and Kosmotropic agents Osmolytes such as TMAO are a natural protectant of cells against pressure or osmotic damage.[102] With the protection of osmolytes, protein folding processes can resist high pressure in deep sea organisms, or higher temperature in hot springs. Gierasch and coworkers used the fluorescent signal from fluorescein-based FlAsH dye to monitor the folding state and aggregation of proteins *in vivo*.[103] They studied three different osmoprotectants (proline, glycine betaine, and trehalose) and found them have different influences on protein folding and aggregation inside *E. coli* cells. The presence of proline can completely prevent CRABP from aggregation by effectively increase its stability and solubilization in *E. coli* cells.[104] The presence of glycine betaine retards protein aggregation by destabilizing the aggregation-prone intermediate, but cannot inhibit aggregation completely. The presence of trehalose cannot combat protein from aggregation in-cell.[103]

Finally, chaotropes such as urea have been used inside cells to reduce protein stability. It is remarkable that bacterial cells can survive several molar urea infusion, despite reduced stability of their proteome under those conditions.[105,106]

Macromolecular Crowding In the *in vitro* section, we discussed excluded volume and how *in vitro* studies are designed to change the macromolecular crowding with carbohydrates or other polymers. Crowding at the 200-400 mg/ml level is pervasive in cells. Conventionally

macromolecular concentration has been determined by bursting a cell and doing sedimentation studies.[107] More recently, it became possible to detect in-cell macromolecular crowding non-destructively using FRET-based approaches. For example, Poolman *et al.* developed the crowding sensor fCrH2,[108] and Ebbinghaus *et al.* developed a PEG-based crowding sensor.[109]

In-cell or *in vivo*, there are two ways to change macromolecular crowding: either by changing the free cell volume of a given cell, or by comparing different cell lines. Free volume is the cell volume occupied by water and other permeable solutes. To modulate the cell free volume, one perturbs the osmotic pressure to regulate water influx and efflux.[110–112] Modulation of kosmotropes such as mannitol/sucrose and ions such as Na^{+}/K^{+} has been used to control free cell volume by expulsion of water through membrane channels.[110] The above pairs have the useful property that the first does not enter the cell through channels under osmotic stress, but the second can. Therefore it is possible to control free volume and concentration of certain solutes inside the cell, effective making the cell a test tube for the study of chemical effects on proteins in-cell.

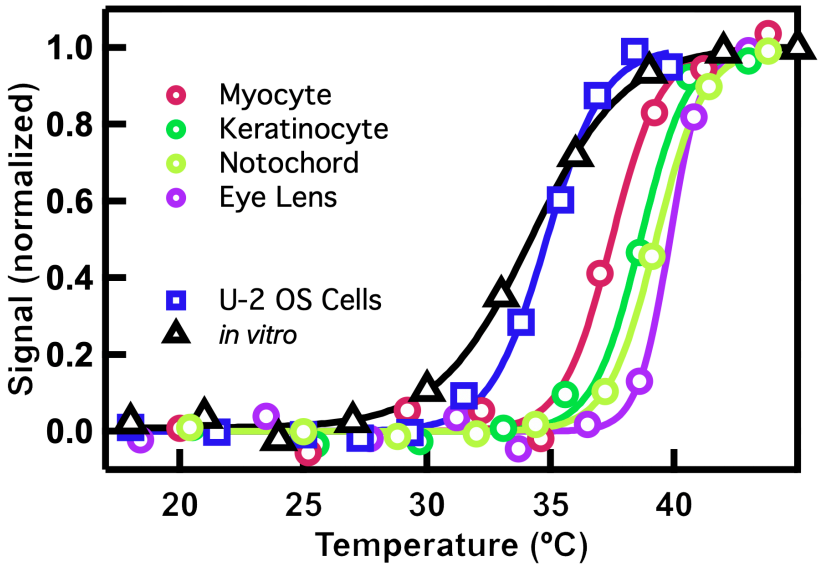

Fig. 5. Temperature unfolding of FRET-labeled enzyme PGK *in vitro* (least stable), cancer cell line U-2 OS, and several zebrafish tissues, including eye lens (most stable). FRET data normalized from 0 to 1 taken from ref. 90. All cell and tissue types show higher folding cooperativity (a steeper transition from folded at 0 to unfolded at 1) than *in vitro*.

For example, FRET-detected studies of PGK in cells have shown that as the cell free volume decreases, crowding compacts the unfolded protein (reducing its configurational entropy) and stabilizes the folded protein.[111] That is because the macromolecules have both steric interactions (repulsive interactions) and nonsteric interactions with proteins. Steric interactions are mainly due

to the volume exclusion and stabilize protein native states.[113,114] Nonsteric interaction are mainly due to the surface sticking and can either stabilize or destabilize proteins.[93,115]

Another way to adjust in-cell crowding is by looking at different cell types (Fig. 5) because macromolecular crowding varies across tissues.[83,116–118] Recently, *in vivo* studies of four zebrafish tissues showed that crowding affects protein stablity over a *ca*. 10 °C range, more than enough to unfold proteins near the brink of stability in one cell type while keeping them folded in another. Eye lens, keratinocytes, notochord, and myocytes were investigated by using fluorescence-detected temperature-jump microscopy.[90] Protein stability was enhanced to varying degrees *in vivo* over *in vitro.*

5. Outlook

Cells offer a natural and convenient laboratory for the quantitative study of protein folding and protein interactions. Recent work has shown that quinary structure between enzymes that may increase substrate processivity can be observed in-cell. One important question arising in that context is how much the cell interior hinders the navigation of proteins to find binding partners among the thousands of different macromolecules. The entropic effect of non-competitive binding (many weak non-functional sticking interactions competing with one stronger functional interactions) could lead to significantly smaller protein-protein or protein-RNA association constant (larger K_d) in cells than *in vitro*, where no competition exists. Thus interactions that seemingly over-evolved in strength when quantified *in vitro*, may come closer inside cells to the biologically useful 1% to 99% range when signals are recognized as "off" or "on" (corresponding to a free energy range of only $(\ln[0.99]-\ln[0.01])k_BT \approx 4.5\ k_BT$). Quantitative tools, rather than the traditional on/off tools will be useful in probing this range of weak functional interactions.

Extreme biological environments are another frontier for in-cell studies of protein folding and quinary structure. The thermodynamic parameters discussed here to interrogate cells vary widely in nature, with a large fraction of all organisms living very far from the 37 °C, 1 atm, pH 7, 0.3 M osmolyte conditions we consider 'physiological' for human cells. The new area of geobiochemistry[6] can adopt biophysical chemistry techniques such as the ones explored here to study protein dynamics in organisms from extreme environments. This will require significant technology development because these organisms often are unable to survive under 'physiological'

conditions,[119] making even relatively simple experiments quite challenging. But the rewards could be extreme, in terms of new phenotypes, enzymes useful for industrial processing, and of course understanding the origins of life on our planet.

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Biographies

Mayank Boob received his Integrated M. Sc. in Chemistry with a minor in Biomedical Engineering from Indian Institute of Technology Bombay in 2015. After graduation he worked as a Junior Research Fellow with Prof. Ravi Venkatramani at Tata Institute of Fundamental Research Mumbai for a year. Currently he is a fourth-year graduate student in Biophysics under Prof. Martin Gruebele and Prof. Taras Pogorelov at University of Illinois Urbana-Champaign. He is elucidating the effect of small molecules and extreme environments on protein folding *in vitro* and *in vivo* using fluorescence spectroscopy and molecular dynamics simulations. His hobbies include cooking, running, biking and photography.

Yuhan Wang was born in Yuyao, Zhejiang, China, in 1995. She received a B.S. in Physics from Beihang University in 2017, and she is currently a Ph.D. candidate in the Biophysics program at the University of Illinois at Urbana-Champaign. Under the supervision of Prof. Martin Gruebele, she studies protein folding, protein diffusion and protein-protein interaction inside the living cells by developing new fluorescence microscopy techniques.

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Notes

The authors declare no competing financial interest.

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TOC Figure