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Protein folding and surface interaction phase diagrams *in vitro* and in cells

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Protein stability is subject to environmental perturbations such as pressure and crowding, as well as sticking to other macromolecules and quinary structure. Thus, the environment inside and outside the cell plays a key role in how proteins fold, interact, and function on the scale from a few molecules to macroscopic ensembles. This review discusses three aspects of protein phase diagrams: first, the relevance of phase diagrams to protein folding and function *in vitro* and in cells; next, how the evolution of protein surfaces impacts on interaction phase diagrams; and finally, how phase separation plays a role on much larger length-scales than individual proteins or oligomers, when liquid phase-separated regions form to assist protein function and cell homeostasis.

Keywords: protein folding; quinary structure; *E. coli*; mammalian cell; liquid–liquid phase-separated regions

Proteins (and other biomolecules) rarely exist under conditions that maximize their stability [1]. For one thing, functional motions require flexibility, and extreme stability can be counterproductive in that regard. For example, enzymes in thermophilic organisms have evolved to function at high temperature, but often function less well at room temperature because they become too rigid [2].

One way to think about protein adaptation to different environments is to think in terms of the rich phase diagrams that proteins have [3], and how they can be altered by protein evolution [4] subject to physico-chemical constraints. These phase diagrams (Fig. 1 left) contain folded regions 'F' of stability where the folding free energy $\Delta G_f < 0$, surrounded by boundaries where activated unfolding to 'U' occurs, similar to a first-order phase transition [5]. There may be several such boundaries, and between them lie folding intermediates 'I' that are neither fully folded nor maximally unfolded. Phase boundaries also can disappear, in analogy to critical point of water, where thermodynamic transitions go downhill in free energy instead of being activated. For example, the acid-denatured state of apomyoglobin simply disappears above pH 5, and the three-state transition F-I-U turns into a two-state transition F-U [1,6].

While intermediates may simply signal separate steps in folding, for example, if two domains in a protein have different stabilities [7], intermediates can also be signatures of function. For example, an intermediate may not be significantly populated in the folded state, but its existence at a fairly low free energy facilitates fluctuations in the folded part of the phase diagram, particularly near the folded-to-intermediate phase

Abbreviations
ANS, 8-anilinonaphthalene-1-sulfonic acid; BSA, bovine serum albumin; CD, circular dichroism; *E. coli*, *Escherichia coli*; FRET, Förster resonant energy transfer; GAPDH, glyceraldehyde phosphate dehydrogenase; LLPSR, liquid–liquid phase-separated region; ParB, chromosome partitioning protein B; PEG, poly(ethylene glycol); PGK, phosphoglycerate kinase; PSD-95, postsynaptic density protein, 95 kilodaltons (also known as SAP-90 = synapse-associated protein 90); RNA, ribonucleic acid; TMAO, trimethylamine oxide; UCST, upper critical solution temperature; YAP, Yes-associated protein.

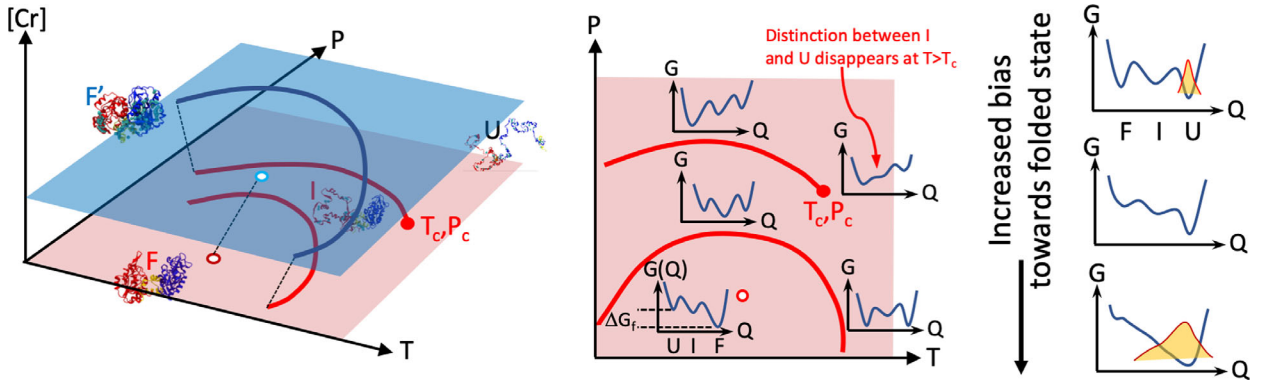

Fig. 1. Phase diagrams and free energy surfaces for protein folding. *(Left)* Phase diagram as a function of temperature T, pressure P, and crowder concentration [Cr] provides an example of variables (pH, denaturant concentration, and other variables are also possible). The red and blue transition curves separate the different phases (or states) F, I, and U. The red curves on the red plane are at low crowding, and the blue curve on the blue plane is at high crowding. Sample protein structures for folded (F), intermediate (I), and unfolded (U) states are shown in the corresponding parts of the phase diagram; note that the folded state can change structure when crowded (F′ ≠ F) or other variables. The most stable state (red and blue circles) is not necessarily at body temperature or ambient pressure. The intermediate disappears above the critical temperature T_c (red dot). *(Middle)* Phase diagram showing the free energy surfaces for folding in various locations on the phase diagram. In the small plots, Q is a folding coordinate (left = unfolded, right = folded). Although the protein enthalpy ΔH is funnel-shaped, the free energy G has local minima at the folded, intermediate, and unfolded states. Near the point of greatest stability (red circle inside the red F-to-I transition curve), F is lowest in free energy and $\Delta G_f < 0$. Near the critical point at the end of the I-to-U transition curve (red dot), the free energy of state I increases to where the state I disappears, and only a two-state transition between F and U exists. *(Right)* Free energy of a three-state protein as the bias toward the native state increases. Under extreme bias (bottom), folding occurs downhill. Although the state I no longer exists, its signature remains: A much broader free energy well in the folded state allows the folded population (probability as a function of Q potted in orange) to explore far more structures than with low folding bias (probability in orange in the top plot).

boundary (Fig. 1 middle). Such excited states of protein thus leave their mark on protein dynamics and function even in the native basin of the phase diagram [8]. In extreme cases, such as near critical points, the fluctuations can become large in amplitude and continuous, allowing proteins to act like rheostats to sense the environment continuously [9] (Fig. 1 right). In even more extreme cases, there is no folded local minimum in the phase diagram unless a binding partner is added, as is the case for some disordered proteins [10].

Quinary structure can extend beyond a few proteins and ultimately leads to macroscopic liquid–liquid phase-separated regions (LLPSRs). Phase separation can be driven by electrostatics, hydrophobicity, domain swapping, or hydration, and of course, these mechanisms can act simultaneously [11,12]. Among other functions, it can be useful for the storage of nucleic acids or proteins that can be toxic at high concentration, but need rapid deployment, such as spliceosomal components [13]. Over the last few years, the study of LLPSRs has seen a renaissance in cell biology, in the form of many membraneless organelles, although Cajal bodies, speckles, and other examples have been known for over a century [14].

Disordered proteins are just one example where the environment is critical for function. Inside cells, proteins are in constant contact with other biomolecules [15], and while crowding generally enhances folding or binding, sticking can reduce diffusion and can be detrimental to folding [16], or it can evolve into quinary structure, when proteins interact weakly and transiently to the advantage of enzyme processivity (metabolons) or signaling [17,18]. Quinary structure relies on interactions not far above the thermal energy k_BT and is therefore particularly sensitive to stress, but redundancy (both multiple protein–protein interactions for a given protein, and analogous interactions in different protein pairs) creates robust signaling and metabolic networks [19], rather than the cell just being a 'bag of proteins' [20].

Protein phase diagrams and quinary structure in the context of evolution or extreme environments have been reviewed recently [21–23,24], and here, the focus is on work from the last few years in the areas of folding phase diagrams under extreme conditions, thermodynamic tuning by sticking and quinary structure, and phase separation in cells. A few interesting cases studies are discussed in a nutshell, and the reader may find more related work in those references.

Folding phase diagrams under extreme conditions

Environmental fluctuations can have unexpected effects on proteins. For example, temperature

fluctuations can speed up folding by stochastic resonance, a process whereby noise accelerates a process or enhances a signal [25]. Other effects are more expected such as crowding inside a cell, which produces a native state of higher compactness, due to the increased free energy cost of states with high configurational entropy [26].

The pressure–temperature–crowding phase diagram of the enzyme phosphoglycerate kinase (PGK), represented schematically in Fig. 1, reveals that while the intermediate state disappears above the critical point at high temperature, it is robust at lower temperature [8]. The large fluctuations near the critical point at about 110 kPa facilitate large amplitude motions even at lower pressure and temperature, and indeed, PGK is known to switch to a compact 'spherical state' folded structure very different from the crystal structure under high crowding conditions (Fig. 1 right) [28]. Note that while the appearance of intermediates leads to multiphasic folding kinetics, due to the free energy constraints (e.g., $\Delta G_f = \Delta G_{UF} = \Delta G_{UI} + \Delta G_{IF}$ in Fig. 1, middle), such intermediates do not substantially speed up folding rates compared with direct two-state kinetics between U and F.

The environment can reach the extremes used in temperature–pressure denaturation experiments discussed above, for instance in deep-sea organisms that use trimethylamine oxide to stabilize their proteins [29]. Experiments have shown that trimethylamine oxide (TMAO) acts as a stabilizer under extreme conditions [30]: in simulations [31], TMAO herds water molecules toward the protein surface (Fig. 2), and at sufficiently high temperatures (large enough hydrophobicity of the protein core), this reduces the tendency to unfold. At low temperatures, this mechanism fails and cold denaturation takes over (Fig. 1 left, left side of temperature axis).

Proteins have been imaged directly in yeast and bacterial cells under pressure by microscopy in a square capillary [32]. The work has shown that binding equilibria of ParB shift to alter nucleoid condensation and thus potentially affect gene expression. Other recent work has shown that a eukaryotic probe protein introduced into *E. coli* will be less destabilized by pressure than temperature denaturation in *E. coli*, relative to *in vitro* pressure and temperature denaturation results [33]. Thus, destabilization of the protein by 'sticking' to the cytoplasmic matrix is mitigated by high pressure (better crowding), but enhanced by high temperature (stronger hydrophobic effect [34]). It thus is plausible that thermophiles would use electrostatic interactions more than hydrophobic interactions to stabilize proteins, whereas in piezophiles, hydrophobic interactions are more likely to assist stability [35].

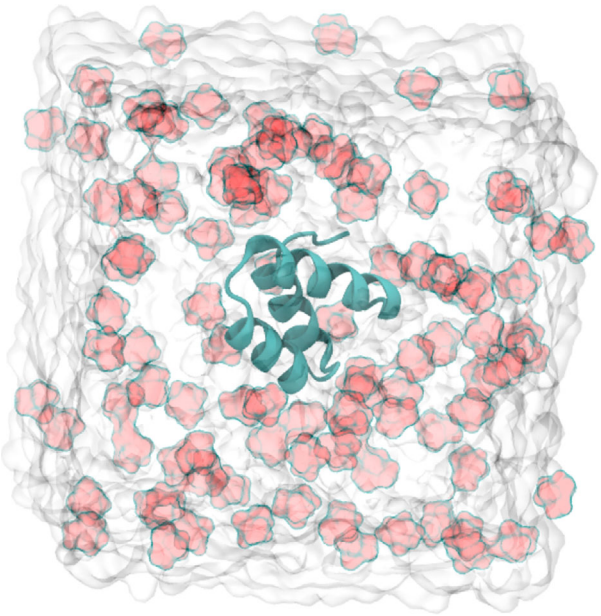

Fig. 2. Snapshot from a molecular dynamics simulation. The triple-helix protein B is shown in cyan, TMAO in red, and water in gray, filling the simulation box with periodic boundary conditions. While TMAO generally avoids the protein surface, there is a significant peak in the radial distribution function right at the surface, corresponding to interaction of the negatively charged oxygen with basic side chains such as arginine or lysine. This observation agrees with experiments that show the methyl groups on TMAO pointing away from the protein surface [27].

Thermodynamic tuning of sticking and quinary structure

Why are enzymes so big? One reason is that nature works with only 20 amino acids, each a 2-nm-size building blocks, yet requires sub-0.01-nm positioning of atoms for catalysis at active sites. Accurate positioning can be achieved by having sufficiently long and flexible loops between key residues to enable induced fit or conformational selection, very different from the 'local design' approach chosen by synthetic chemists who make catalysts [36]. Perhaps equally importantly, enzymes do not live in a vacuum. They can form interacting metabolons to speed up substrate processing [37]. The interaction between GAPDH and PGK in cells is an example [38], and the preceding pair aldolase–GAPDH has been studied by simulation [39]. Enzyme association and any other type of protein signaling require surface area to encode information for either a few strong or many weak binding interactions, while avoiding unproductive 'sticking' [4]. Protein interaction networks are a delicate balance between quinary structure (transient productive interactions) and sticking (transient unproductive interactions) [40].

In essence, evolution against sticking is as critical as evolution for productive binding. Metamorphic proteins that can display more than one surface [41], disordered proteins that can acquire alternative folds [42], or simply large surface area are all mechanisms to maximize information encoding.

Protein surfaces have been neglected for a long time, the idea being that they are generally hydrophilic but not highly conserved by evolution. Recent work, for example, in-cell NMR of bacterial vs. eukaryotic proteins [16,43], has shown that protein diffusion and interactions are very sensitive to whether a protein is in the proper organismal cytoplasm. Protein diffusion itself is highly sensitive to folding state of the protein and position in the cytoplasm due to sticking [44]. And of course, protein interactions, including longer-range ones to draw encounter complexes together [45], are very sensitive to the protein surface also.

Due to differences in crowding and sticking, protein stability varies greatly by organelle or tissue type [46,47]. Dissolving the cytoskeleton with specific drugs reduces the stability of PGK, even though PGK is not associated directly with microtubules or actin filaments [48]. Sticking (and presumably also quinary structure) can be quite nonadditive in free energy: For example, the stability of PGK is affected much less when two sticking partners are present *vs.* just a single one because the two sticking proteins can interact with each other instead of PGK [49]. This hints at the importance of three-body effects in sticking and quinary structure.

Protein interaction ranges from the pairs discussed above to very large assemblies, such as virus capsids or cytoskeletal tubules. Such interactions can be highly cooperative and sensitive to stresses in the environment, as in the following three examples. Hepatitis B virus capsids assemble from 120 dimers of a core protein, so the dissociation constant K_d scales with the 120th power of the dimer concentration. Enthalpy and entropy are both positive and nearly cancel out in the free energy, such that assembly increases at higher temperature [50]. More stable assembly at high temperature is counterintuitive, but analogous to cold denaturation of proteins. Some bacteria produce tubulins that assemble into microtubules and even show dynamic instability to allow growth and shrinkage [51], a highly cooperative process. These molecules acquired by horizontal gene transfer have a very different surface amino acid composition to adapt to the bacterial cytoplasm, and do not form quinary structure with mammalian tubulins [52]. Antibodies can assemble into clusters whose size and morphology (random-walk-like *vs.* collapsed) are highly dependent on the viscosity of the surrounding environment [53]. In the cell, local diffusion is very shape-dependent [54], and when estimated by protein folding relaxation rates (a local phenomenon) [55], local diffusion is also much faster than long-range diffusion.

Atomistic simulations have also entered the fray of new tools to study the stress on protein interactions inside cells [56]. Recent results show that sticking times obey a power law and occur over a wide range of timescales [57] and that unfolded proteins in the cytoplasm can actually unfold other proteins in a cascade of failure of proteostasis [58].

Phase separation *in vitro* and in the cell

Quinary structure generally refers to the assembly of a few proteins or other biomacromolecules in the cell, but cooperativity can lead to much larger-scale liquid–liquid phase separation [11,12]. Recently, there has been a resurgence of interest in such liquid–liquid phase-separated regions (LLPSRs) *in vitro* and in-cell [59]. In-cell, protein and/or nucleic acid condensates are often referred to as membraneless organelles. They are much more sensitive to environmental stress than low-order quinary structure or protein folding, and regulating them could turn out to be a limiting factor for organisms in extreme environments, such as under kilobar pressure in the deep sea [60].

Figure 3 shows a typical phase diagram for two large biomolecules dissolved in water with an upper critical solution temperature (UCST), above which the biomolecules are freely miscible. It is also possible for such systems to have lower critical solution temperatures, or even form multiple phases rather than the two shown here [61]. In our example, at high enough concentration of both biomolecules (blue plane) a phase boundary appears, and liquid droplets enriched in one of the two biomolecules are suspended in a solution enriched in the other. At even higher concentrations typically inside cells (>300 mg·mL^{-1} macromolecules), additional phases may appear such as tubular-shaped lamellae. Above a critical temperature (the top of the 'parabolas' in Fig. 3), everything is well mixed irrespective of concentration. Transitions between mixing and demixing are also important in two-dimensional systems such as membranes.

A few examples will illustrate the relation between LLPSRs, folding, and function in terms of phase behavior. Pressure perturbation of BSA binding to ANS in a phase-separating Dextran/PEG aqueous polymer mixture shows that binding depends strongly on the phase the BSA is in, likely due to competition

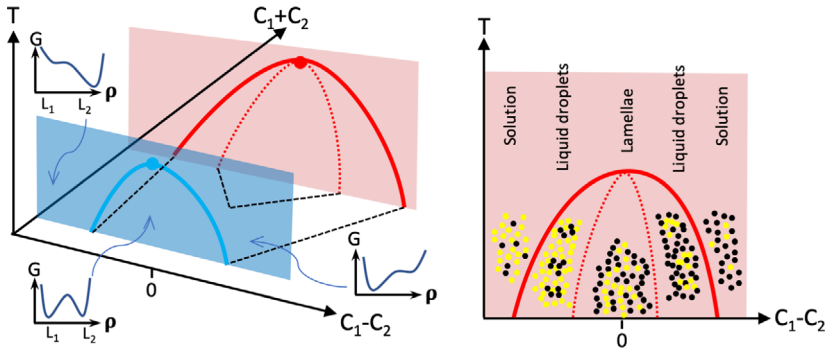

Fig. 3. Typical simple phase diagram. The example shows a solution of two macromolecules at concentrations C_1 and C_2 with an upper critical solution temperature (UCST). (Left) At low total concentration $c_1 + c_2$, both components dissolve uniformly in the solvent. But as total concentration begins to increase (blue plane), a critical line appears in the phase diagram (blue curve), within which two liquid phases separate, one enriched in one of the two components. The free energy diagrams as a function of an order parameter ρ (e.g., $\rho = [c_1 - c_2]/[c_1 + c_2]$) show both dilute phase L_1 and enriched phase L_2 at low free energy in the middle, but the uniform solution is more stable on the right or left. Above a critical temperature Tc (blue dot), the solution becomes uniformly mixed again. At even higher total concentration, an additional phase appears between the dotted line. (Right) Phase diagram at high concentration showing the structure of the solution (solvent not shown, solute 1 in black, solute 2 in yellow). When $[c_2]$ is much larger than $[c_1]$ (left) both solutes dissolve evenly. As concentration of '1' increases, phase-separated droplets enriched in '1' form, and the remaining solution is depleted in '1'. Moving to the right, a lamellar phase forms when $c_1 \approx c_2$, then droplets form again with the rest of the solution depleted of '2'. Finally, a well-mixed single liquid phase is again obtained when $[c_1]$ becomes large relative to $[c_2]$.

of ANS with the polymers for the binding sites at the BSA surface [62]. Thus, LLPSRs can regulate binding and signaling by affecting accessibility of protein surfaces. Phase-separated condensates of SynGAP and PSD-95, important for synaptic communication, are also very sensitive to pressure and could play a role in decompression sickness [63], when divers return from a high-pressure environment.

Reversibility is a hallmark of true thermodynamic phase transitions, and this property is shared by many LLPSRs. For example, the spliceosome is a biological machine whose components continually cycle between the nucleus and cytoplasm and assemble/reassemble to splice premessenger RNA into messenger RNA [64]. The components concentrate in nuclear membraneless organelles such as speckles [65]; among other reasons, storage of spliceosomal components in LLPSRs reduces their toxicity, while making them rapidly available when replication is up-regulated and a lot of RNA needs to be processed. Spliceosomal components U1A (protein) and SL1 (RNA) have been shown to form LLPSRs at low temperature, which dissolve during high temperature stress as indicated at the top of the phase diagram in Fig. 3. As another example, the transcriptional coactivator YAP will reversibly form a LLPSR when crowded by a single polymer component, PEG. Similar behavior was observed for YAP inside cells under hyperosmotic stress [66]. Thus, reversible phase transitions can be important for gene regulation. YAP is particularly notable because it has the intrinsic ability to phase-separate when crowded. In the *in vitro* experiments with PEG as a crowder, no nucleic acids were required for droplet formation.

Conclusions and perspectives

Crowding, temperature, pressure, pH, and ionic strength are just a few of the variables that can induce phase changes in proteins. Phase transitions control the equilibrium and dynamics of proteins on many length and time scales. At the small-size, high-speed end of this spectrum, protein folding itself can be viewed as a phase transition that is first order for two-state folding and critical for downhill folding. At intermediate time- and length-scales, quinary structure ranging from dimers to organelles with hundreds of components is controlled by concentration-dependent phase diagrams. And on the largest and slowest time-scales, phase separation in liquid- or gel-like environments enables storage of biological machinery and its control.

For disordered proteins or once a protein has folded, much of this phase control occurs via protein surfaces, the evolution and dynamics of which have not been sufficiently investigated. The problem is that except for exposed active sites, surfaces do not pack like a protein's core and, being highly variable except for their hydrophilicity, were considered relatively unimportant. Yet, surfaces are the protein's display to the external world, and control sticking, quinary

structure, and function. As an eye-opener, 'sequence differences between consensus and extant homologues are predominantly located at weakly conserved surface residues' [67]. Protein surfaces are well adapted to their specific cellular environment, and surface interactions influence phase transitions from droplet formation to folding (which of course also relies on a hydrophobic core). There is evidence for both electrostatic (screened charges) and hydrophobic (solvent entropy) interactions playing an important role [16,56,57], although hydrophilic residues dominate at surfaces. Nature has evolved interesting ways to maximize the available surface area of proteins for quinary structure, from simply making proteins large, to proteins with metamorphic structure, or even a large fraction of disordered proteins that bind upon folding (~ one third of proteins in mammalian cells).

The study of protein surface dynamics, and its interplay with phase transitions and protein homeostasis [68] will be a fruitful area for investigation in biophysics and quantitative cell biology for years to come.

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