How does solvation in the cell affect protein folding and binding?
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The cellular environment is highly diverse and capable of rapid changes in solute composition and concentrations. Decades of protein studies have highlighted their sensitivity to solute environment, yet these studies were rarely performed in situ. Recently, new techniques capable of monitoring proteins in their natural context within a live cell have emerged. A recurring theme of these investigations is the importance of the often-neglected cellular solvation environment to protein function. An emerging consensus is that protein processes in the cell are affected by a combination of ionic and non-ionic interactions with this solution. Here we explain how protein surface area and volume changes control these two interaction types, and give recent examples that highlight how even mild environmental changes can alter cellular processes.

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Introduction

The cell’s interior is a dynamic and heterogeneous environment: in it, metabolites, ions, small solutes and macromolecules such as proteins and nucleic acids are unevenly distributed, and are constantly in flux due to both internal and external changes. Internally, a cell may morph and shift shape during motility or as part of its replication cycle, causing volume changes, internal water movement and concentration gradients [1–3]. Membrane bound organelles display distinct solution compositions from other cellular regions [4*], and membrane-less, phase-separated regions within the cytoplasm create regions of high solute concentration [5]. The cell cycle involves the breakdown of the nuclear envelope, releasing large amounts of charged species that were previously contained in the nucleus [6]. Externally, single-cell organisms, plants, and insects are susceptible to environmental changes in osmotic pressure, temperature, and water content [7–9]. Multi-celled organisms capable of homeostasis provide a more stable environment for most of their cells, but certain cell types (e.g. kidney, gut, skin) are nonetheless exposed to environmental changes [10]. In addition, pathological conditions such as fevers, diabetes, and other metabolic diseases or therapies will induce various environmental stresses even in multi-cell organisms [11].

Even in vitro, proteins display a remarkable sensitivity to their solvation environment, as evolution for function in many cases seems to favor marginally stable proteins [12]. It is therefore reasonable to expect proteins to be sensitive to the dynamic cellular environment. While studies of protein function in the cell are technically challenging to perform and difficult to interpret, they are necessary to advance our understanding of how proteins interact with their natural environment. Specifically, live-cell NMR and fluorescence microscopy have emerged as two complementary techniques that can detect protein dynamics within the cellular environment. NMR reports on protein dynamics of isotopically labeled overexpressed proteins at the single atom level [13,14**]. Microscopy of fluorescently tagged proteins provides high temporal resolution of protein dynamics, and reveals the context of their function within the cell, though such experiments suffer from limited structural resolution [15,16]. Such studies complement in vitro studies with well-defined crowders or cell lysates.

Here we consider recent findings from NMR and fluorescence microscopy, and present a framework with which to understand how the cellular environment affects protein processes. It is important to note that many factors beyond solute composition alter protein dynamics inside the cell. Perhaps most importantly, we will not discuss interactions with chaperones or other post-transcriptional regulating proteins, such as kinases [17], and refer the interested reader to other reviews in this issue [18]. We begin by discussing the composition of the cell’s internal solution. Next we present a model for translating protein thermodynamics in vitro to the cellular solvation environment. Finally, we highlight experimental data that
shows how altering the environment in which a protein is observed affects the studied process.

**What does the cellular environment look like?**

On average, ~60–70% of a cell’s volume is composed of water [19], with the rest being a combination of electrolytes, small organic molecules and metabolites, nucleic acids, and proteins [20]. The relative concentrations of many molecular species have been characterized using biochemical assays [21] to determine multi-cell averages. Absolute numbers in a single cell depend on organism, cell type, and volume [22], and even then different assays give concentrations that can vary by over an order of magnitude [23]. With the exception of cases like acidity in lysosomes, little is known about how these solutes (e.g. Mg2+ [24]) are distributed spatially in the cell. Localization of proteins is a well-known phenomenon, but only recently have experiments revealed differences in smaller solute composition between cytoplasm [25], membrane-bound organelles [4*,26] and membrane-less microenvironments within the cytoplasm [5].

Recently, concentrations derived from these biological assays have been used to generate all-atom molecular dynamics simulations of the cytoplasm [32*,33]. These simulations provide a dynamic view of the interaction between the cell’s internal solution and its proteome. Each protein in the cell is separated from other proteins by only a few layers of water (Figure 1a). Small molecules in the cellular milieu, which often exist in mM concentrations (Figure 1b), form transient interactions with protein surfaces. These solutes can alter the structure of interfacial water layers, effectively changing their interaction with protein surfaces [34]. Together with the limited volume of the cell’s interior [35], these interactions change protein structure, activity, and interactions in cell compared to the dilute solutions of in vitro experiments.

Despite their highly confined heterogenous native environment, many proteins studied in idealized in vitro solutions, where water is abundant, solution properties are uniform, and binding partners are all but lacking, successfully recapitulate processes as they occur in the cell. This should not be taken to mean that protein studies can be conducted solely in the test tube; rather, it attests to the robustness of many cases of protein dynamics. Other proteins participate in new and unexpected behaviors depending on their environment, including phenomena such as protein moonlighting [36], intracellular phase separation [37], intrinsic disorder [38], and functional and pathological protein aggregation [39,40].

**How does the cellular environment affect protein thermodynamics?**

In this review, we highlight the interplay of steric and non-steric interactions and how they scale differently: volume versus surface area. Our definition of ‘steric’ is in line with molecular crowding, an idea pioneered by

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**Figure 1**

The composition of a cell. (a) A simulation box containing characteristic *E. coli* cytoplasmic concentrations [27] of proteins and nucleic acids within a slice of solvent (top). Removing the large macromolecules shows the sparsity of water molecules between adjacent proteins (bottom left), and the high concentration of small solutes and electrolytes in the regions surrounding macromolecular surfaces (bottom right). Image courtesy of Y. Zhang. (b) Characteristic concentrations of small molecules, metabolites (obtained using mass-spectrometry [4*,27]), and ions (obtained using biochemical methods [28–31]) inside *E. coli* (left) and HeLa cells (right). In both cases external osmolarity is ~0.3 Osm [30]. For reference, the total concentration of all proteins in the cell is in the 1–10 mM range. The reported concentrations include components that are bound to cellular species (i.e. not labile), and ion contributions in the ‘other’ category may be double-counted since they are also charged solutes. Note the large difference between the *E. coli* and HeLa absolute concentrations, which is in part due to differences in measurement methods, but also highlights the stark differences in the intracellular environments of different species.
Minton and co-workers [41]. Steric interactions stem from the rigid volume a protein takes up in an already crowded environment composed of other rigid bodies (Figure 2a). In such an environment, any reaction that increases the translational and rotational freedom of other solvents will result in entropic gain. Steric interactions rarely occur independently of non-steric interactions [42].

Recently, attention has turned to non-steric interactions. Soft, or non-steric interactions describe all interactions a protein may have with its environment aside from molecular crowding (the steric interaction discussed above). Electrostatic interactions with cellular electrolytes [44] and other non-covalent interactions with cellular solutes fall under the broad definition of non-steric interactions. One way to differentiate non-steric interactions from steric ones is that non-steric interactions are dominated by the solvent-accessible surface area (SASA) of the protein(s) of interest, shown in Figure 2b, while steric interactions depend on the volume excluded by proteins and surrounding molecules.

Quinary interactions, proposed to describe certain non-steric interactions of proteins with their surroundings almost four decades ago [45], have been the subject of renewed interest [14**,46*,47,48]. Since primary, secondary, tertiary, and quaternary structures are all derived from evolutionary constraints, we define quinary interactions as the collection of non-steric interactions that stem from evolutionary pressure exerted by the cellular environment. Thus, quinary interactions are those non-steric interactions that have, over time, caused a protein to adapt to its native environment [49]. This definition fits with the observation that the mobility of a single protein is decreased upon expression in an organism in which it is not endemic [14**], or its stability is decreased when transferred from its endogenous extra-cellular environment into the cell’s cytoplasm [50]. Recent evidence has suggested that electrostatic interactions play a major role in quinary interactions [14**,44], though these are by no means the only interactions that will exert evolutionary pressure on a protein. While an important class of quinary interactions is the weak, transient protein-macromolecule interactions cells evolved to improve survival, quinary interactions also heavily depend on small solutes and water molecules [14**,44,51], just as the folding free energy of protein secondary and tertiary structure is strongly influenced by small solutes.

To help us understand the crowding and quinary effects of the cellular environment on protein structure and dynamics, we describe them thermodynamically as contributions to the free energy of reaction, $\Delta G_r$. The term reaction is used in the broad sense to cover any protein process. In the case of monomolecular protein folding, the initial reactants are the unfolded ensemble and the final product is the folded state. For protein–ligand or protein–protein binding, the reactants are the monomers and the product is the complex. To highlight the importance of both crowding (scales approximately with volume) and quinary interactions (scales approximately with exposed
surface area of biomolecules), we describe \( \Delta G' \), the reaction free energy in the cell, as a thermodynamic cycle, shown schematically in Figure 2c:

\[
\Delta G_{r,u/f}^{'} = \Delta G_{r,u/f}^{\text{cb}} + \Delta G_{r,b,c}^{\text{cf}} - \Delta G_{r,b,c}^{\text{cb}}.
\]

Here \( \Delta G_{r,u/f}^{'} \) is the free energy of the reaction from reactant \( u \) to product \( f \) in either buffer \( b \) or cell \( c \). \( \Delta G_{r,b,c}^{\text{cf}} \) is the transfer free energy from dilute buffer \( b \) to the cell \( c \) for either reactants \( u \) or products \( f \). We make several assumptions to facilitate discussion of in-cell protein reactions: that the cellular environment is uniform and that an additive energy term \( \Delta G_i \) can translate from in cell to in vitro observations. This means that the ensembles of reactants and products are the same in both environments (Figure 2c). These assumptions may be an adequate approximation in certain cases, but are by no means always valid [52]. Another inherent problem with the thermodynamic treatment of in cell proteins is that measurements are obtained from finite sections of a micron-sized environment, while thermodynamic systems must tend to infinite size to avoid surface terms that make energies non-additive. Despite these caveats, this simple picture can help us think about how the cellular environment affects protein processes.

The key to understanding the effect of the cellular environment on the reaction of interest lies in the transfer free energies, \( \Delta G_i \). This is the free energy associated with moving an ensemble from one environment to another [53]. Here we focus on the transfer from a dilute buffer to the cell’s cytoplasm, but transfer free energies can also occur when a protein changes its localization, for example, from the cytoplasm to the ER [54]. As alluded to earlier, contributions to \( \Delta G_i \) can be divided into two types, steric and non-steric interactions:

\[
\Delta G_i = \Delta G_{\text{steric}} + \Delta G_{\text{non-steric}}
\]

The contributions to \( \Delta G_{\text{steric}} \) are mainly dependent on the volume of the protein(s) of interest and the volume of the surrounding solute, as indicated by the red ‘hard sphere’ interaction potential in Figure 2a:

\[
\Delta G_{\text{steric}} \propto \frac{\bar{\nu}}{1 - \bar{\nu}},
\]

where \( \bar{\nu} \) is the volume fraction of the protein in the cell, assuming the interactions between all solutes are steric repulsions (Figure 2a, inset). Steric repulsion will always be a factor when transferring a reaction from dilute \textit{in vitro} to crowded in cell environments, with a few caveats recently discussed [55]. While steric interactions often accurately model \( \Delta G \) [56,57], live cell experiments have shown that \( \Delta G_{\text{steric}} \) alone often fails to account for the observed effect of the cellular environment [50,58].

The non-steric transfer free-energies, \( \Delta G_{\text{non-steric}} \), are formulated by summing over all possible interactions between surface type and solute [59,60],

\[
\Delta G_{\text{non-steric}} \propto \sum_{i,j} \gamma_{ij} S_i
\]

where \( i \) and \( j \) subscripts denote surface and solute type, respectively. \( \gamma \) describes the free energy per surface-area for the interaction between surface-type \( i \) and solute \( j \), and \( S \) is the total SASA of type \( i \). \( S \) changes for example when a protein folds, or two proteins bind (both reactions decrease SASA). In the above equation, we neglect any non-linear terms resulting from coupling of surface types or cosolutes, though these will also contribute to this term. Surface types can be grouped by charge, hydrophobicity, or similar chemical attributes (Figure 2b) [59,60]. Changes in exposed surface area upon structural change will dominate \( \Delta G_{\text{non-steric}} \) in protein folding. For protein binding and interactions, it is primarily the surface area characteristics at the interface that determines whether and how a complex will form. Unlike steric interactions, non-steric interactions can have both stabilizing and destabilizing effects on the protein reaction and depend on the cellular conditions as much as they do on the specific sequence and structure of the studied protein.

\section*{How does the environment affect protein structure and interactions?}

In general, crowding and quinary interactions exert relatively small changes to the total free energy of a protein reaction. At biological temperatures, this amounts to no more than \( \approx 20 \text{ kJ/mol} \) — small contributions when compared to tight ligand binding, which can be an order of magnitude larger. However, even small contributions to the free energy add up, and mild structural changes at the single protein level can translate into large phenotypic changes in the cell [61]. Furthermore, multi-protein interactions can amplify small energetic and structural fluctuations because such interactions may be correlated, and thus not add up randomly [62]. It is therefore important to study proteins in their natural habitat.

Fluorescent proteins are one of the most convenient ways to monitor protein processes in the cell [63]. Often fluorescent proteins are used to report on other proteins, but they themselves are known to form homo-oligomers [64]. Their similar sequence and structural homology makes it possible that different fluorescent proteins may hetero-oligomerize. Indeed, we found that the Aequorea victoria FRET 2a-pair exhibits a \( K_d \) of 20 \( \mu \text{M} \) and associates at a 1:1 ratio \textit{in vitro} [65]. We developed a fluorescence microscopy technique for determining
and stoichiometry in cells that employs osmotic stress to initiate a re-equilibration of the free and bound complex. Inside cells we find $K_d$ is reduced by an order of magnitude compared to in vitro and that the fluorescent proteins bind, on average, with a higher order 2:2 stoichiometric ratio [65]. This indicates that crowding or quinary interactions in cells may perturb binding affinity and promote the formation of higher order complexes.

Cellular environments differ between organisms and cell lines (Figure 1b), and quinary interactions are expected to follow suit. A recent study by the Oliveberg group measured the motions of three proteins from evolutionarily divergent organisms in the Escherichia coli cytoplasm by in cell NMR [14**]. Bacterial proteins moved freely in the E. coli cytoplasm, whereas proteins that evolved in eukaryote (in this case human) cells stuck to the foreign bacterial environment. These quinary interactions were found to be critically sensitive to surface mutation. The desired in-cell mobility was tuned by protein surface engineering: the bacterial proteins were made to stick and the human proteins made to move freely by a structurally benign surface mutation. These results demonstrate that differences between in-cell and in vitro do not arise only from self-interactions, but also from protein interactions with cytoplasmic material. This highlights the importance of evolution-directed quinary interactions to protein function.

As an example of a mass quinary interaction, membraneless organelles are comprised of heterogeneous mixtures of proteins and nucleic acids that assemble through liquid–liquid phase separation. One challenge is to understand the structural and dynamic basis of phase separation in this complex environment [66]. In vitro work has shown that small molecules, specifically adenosine triphosphate (ATP), regulate phase separation; ATP is both critical for processes that occur in liquid compartments (transcription, DNA repair and RNA biogenesis) and capable of preventing formation of or dissolving previously formed droplets [67**]. ATP was also found to solubilize intrinsically disordered proteins that are prone to aggregation. Modulating the ionic strength of the solution around physiological conditions was unable to reproduce the ATP results, indicating that electrostatic interactions have a negligible effect on this phase separation. Since ATP concentrations are known to fluctuate as cells experience stress or aging, ATP induced phase separations exemplify phenotypical response to the cell’s solute composition. Understanding the complicated relationship between metabolites, salts and proteins is critical to resolve complex cellular processes.

Conclusions
Recent investigations have shown that the cellular environment plays an important role in regulating many protein processes. This regulation is facilitated by the marginal stability of many proteins and protein complexes. Fluorescence microscopy and in-cell NMR have emerged as powerful methods for monitoring how the cellular environment alters protein processes. Quinary interactions are responsible for differences in protein binding affinity and dynamics in the cell. Continued in vitro experiments are important to interpret complex cellular processes, and to tease apart contributions due to excluded volume or quinary interactions, including electrostatic or hydrophobic sticking. While crowding and quinary interactions exert relatively small changes on the free energy of a protein, they can have large physiological effects. Weak interactions that are seemingly random on small length scales but are correlated could play an important role in such ‘emergent’ physiological effects. It is increasingly clear that these in-cell interactions, dependent on the cytoplasmic concentrations of ions, metabolites, and cellular macromolecules, play key roles in regulating protein processes during the cell cycle and in different environments.

Conflict of interest statement
The authors declare no conflicts of interest.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest
5. In this paper the authors combine biochemical purification methods and high-throughput mass spectrometry to quantify the metabolic contents of an entire cell as well as its mitochondria, highlighting differences in composition between the membrane-bound organelle and the whole cell.


This paper uses live cell NMR to show that transferring a protein to a non-endogenous organism will alter its interaction with the cellular solvent.


This paper details all-atom simulations of a large section of bacterial cytoplasm. The simulations beautifully highlight the numerous interactions between numerically abundant small solutes and protein surfaces.


46. Cohen RD, Pielaik GJ: A cell is more than the sum of its (dilute) parts: a brief history of quinary structure. Protein Sci 2017, 26:403-413.

This recent review provides an in depth discussion and further experimental examples of quinary interactions and their importance to the cell’s metabocial pathways.


Here, the authors combine in vitro and in vivo cross linking with in vitro NMR to show that a temperature induced, small structural change to the heat-shock protein Hsp33 facilitates its binding to client proteins.


The paper demonstrates that physiological concentrations of the ubiquitous ATP molecule can prevent intrinsically disordered proteins from forming phase separated globules.