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Where soft matter meets living matter -- Protein structure, stability, and folding in the cell

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Abstract

A protein is a biopolymer that self-assembles through the process of protein folding. A cell is a crowded space where the surrounding macromolecules of a protein can limit the number of ways of folding. These crowding macromolecules can also affect the shape and the size of a physically malleable, or "soft, squishy", protein with regulatory purposes. In this review, we focus on the *in silico* approaches of coarse-grained molecular simulations that enable the investigation of protein folding in a cell-like environment. When these simulation results were compared with experimentally measured properties of a protein, such joint effort has yielded new ideas on the specific function of a protein in cells. We also highlight the recent developments of computer modeling and simulations that encompass the importance of the shape of a macromolecule, the interactions between macromolecules, and the hydrodynamic interactions on the kinetics and thermodynamics of a protein in a high concentration of protein solution and in cytoplasmic environments.

Introduction -- Proteins are soft living matter

The concentration of a cell can reach up to 400mg/ml [1], which corresponds to a volume fraction of macromolecules of 20–40% of the total cellular volume [2]. These macromolecules exert volume exclusion [3] upon each other, which impacts the behavior of biopolymers inside a cell. This effect is the "macromolecular crowding effect" [4], which is a term coined by Minton [5]. It has been difficult to experimentally examine the protein dynamics in living species. Experimentalists have developed clever methods to mimic the macromolecular crowding effects by adding inert synthetic polymers (crowders) such as PEG, Ficoll, and dextran into a test tube [6]. Several theorists have used simple analytical calculations based on the scaled particle theory (SPT; references in reviews [7]) that model macromolecules as simple hard spheres or rods. The assumption of crowders as hard cores is an acceptable approach because experiments suggest that artificial crowders, such as Ficoll 70, can be modeled as semi-hard spheres [8]. SPT has provided the foundation of successful predictions on the thermodynamic behavior of a globular protein with high stability by assuming that the folded and unfolded states of a test protein are rigid objects in solution.

The scope of this article focuses on the polymeric behavior of a protein in a crowded medium. Starting from a pool of unstructured conformations, protein folding involves a large-scale structural fluctuation encompassing multiple orders of magnitude in both space

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and time. The complexity of this problem is beyond the scope of the analytical calculation such that the computer simulation must be used. Instead of the computationally demanding all-atomistic protein models, we focused on the simulations built on the coarse-grained protein models in which amino acids were represented by a few beads (reviews in [9–11]). Such a "low-resolution" polymeric model, which lacks the chemically atomistic details, can provide valuable insights into the analysis of the dominant influences on protein folding in a complex system.

We begin this review of recent *in silico* developments on protein structure, stability, and folding in the cell by asking several questions as the following: First, how can we extend the theories and the ideas for the understanding of protein folding *in vitro* (reviews in [12–14]) to protein folding *in vivo*? Second, how can we simulate and model a large and a physico-chemically complex system? Third, what are the prominent intracellular conditions that are not represented in dilute solution (reviews in [15])? To address these questions, the approaches that we have used are (1) to strip away the complex details of macromolecules through coarse-graining (Fig 1); (2) to collaborate with the experimentalists who can test our predictions by using different methods; and (3) to revisit the theories of protein folding and to include the necessary details wherever a prediction would fail, implying that a new algorithm or a physical model is required. It is important to understand the physical principle of how a protein folds from an unstructured conformation to a biologically meaningful one in a cell, because such knowledge will facilitate the manipulation of the specific functions of a protein through the change of its surrounding environment.

Simplicity out of complexity

Coarse-graining is a valuable approach because it provides an efficient venue to evaluate the dominant influence on a protein due to the macromolecular crowding effect. When we addressed the impact of the volume exclusion, we modeled the crowding agents or crowders as hard cores so that they cannot spatially overlap. The density fluctuation of the hard cores generates void spaces that can accommodate a compact protein. In the presence of these hard cores, an extended protein conformation is permitted but not statistically favored. This effect is referred to as the "deplete-induced attraction" [16–19], which the stability of a compactly folded protein is relevantly enhanced (Fig 2). In contrast, under confinement an extended configuration of a protein greater than the size of the pore is not allowed. This marks the distinction between the confinement and the macromolecular crowding effect.

Coarse-grained molecular simulations

The investigation performed by Cheung, Klimov and Thirumalai was the first to employ the coarse-grained molecular simulation on protein folding in a crowded medium [20]. A structure-based protein model (or G -like model [12]) that renders a minimally frustrated folding energy landscape was used to address the folding and the unfolding of a small protein in a medium of crowders that were modeled as hard spheres. They predicted the protein folding rates to be non-monotonically enhanced by the volume fraction of crowders. In addition, their study showed a rather modest enhancement of the folding stability due to the macromolecular crowding effect than the ones previously calculated according to the SPT formalism [21]. Later Cheung employed the coarse-grained molecular simulations on several large proteins. The range of the folding stabilities obtained from simulations agrees well with the experimentally measured values [22,23].

A thorough investigation that compares the coarse-grained molecular simulations with the SPT calculation was performed by Mittal and Best [24] on the study of three two-state proteins (folded-unfolded) with high stability. When the folded and unfolded states of a protein were both modeled as hard cores, it was found that the size of the folded state of an

all-β protein (Protein G) was independent on the content of crowders. However, a 20% reduction in the effective radius of a hard core for the unfolded states was required to match the calculation using Minton's SPT [21]. Cheung and Wittung-Stafshede have investigated the folding stability of cytochrome c in crowded media by a combined *in vitro* and *in silico* approach. The change in the enhanced folding stability ($\Delta\Delta G_{fu}$) in the presence of crowders was experimentally measured by the far-UV circular dichroism (CD). The changes in the folding temperature at a range between 5 and 10 °C were quantitatively in accordance with the results obtained by the coarse-grained molecular simulation [22]. They have also compared $\Delta\Delta G_{fu}$ with two prominent SPT calculations: It was found that Minton's SPT calculation overestimates $\Delta\Delta G_{fu}$ by an order of magnitude and Zhou's SPT calculation underestimates $\Delta\Delta G_{fu}$ by an order of magnitude. Such discrepancies are attributed to the insufficient modeling of the "softness" of the unfolded states. In Minton's SPT calculation, the unfolded state of a protein was treated as impenetrable hard cores, which results in a lower stability of the unfolded states. In Zhou's SPT calculation, the overly soft protein chains can overlap themselves and pass through the space of the crowders, leading to a greater stability of the unfolded state. To address an adequate configurational space of a protein ensemble in the presence of crowders, it is important to include the softness of a protein in a model by the consideration of the excluded volume of the polymeric chain that permits the structure of a protein to fluctuate in solution.

The energy landscape for protein folding in a cell-like environment

The configurational space of the unfolded and folded proteins can be described by an energy landscape [25] where a protein folds from a wide range of unfolded configurations on the top of the energy landscape to a few native states near the bottom of a global minimum. The folding energy landscape in a cell-like environment was explored for apoflavodoxin (an α/β protein) in the presence of Ficoll 70 by a combined approach of the far-UV CD experiment by the Wittung-Stafshede group and the coarse-grained molecular simulations by the Cheung group [23]. What was surprising is that the amount of experimentally measured helical content at a high concentration of Ficoll 70 was greater than that in aqueous solution. The computer simulation showed that more formation of native contacts appear at the loops, loose ends, and the regions between the helices and the central β sheet upon the addition of inert Ficoll crowders. The folded states of apoflavodoxin are better packed in the presence of crowders than in solution; they are structurally similar to the crystal structure.

The macromolecular crowding effect can also alter the folding routes of apoflavodoxin [26]. It is shown by the coarse-grained molecular simulations that the folding pathway of apoflavodoxin is topologically frustrated: this protein has an inherent packing problem during the course of folding. An addition of anisotropic crowders relieved the topological frustration at the early stage of folding by favorably populating the globular conformations over extended ones in the unfolded states. In a joint *in silico* and *in vitro* study, the kinetic folding experiments confirmed that the time-resolved folding pathways of apoflavodoxin were modulated by the geometry of crowders. This study suggests that the folding pathways of a protein under a heterogeneous intracellular condition may be distinct from those in a test tube.

Thermal denaturation is often used as a tool to populate the unfolded states of a protein in the computer simulations. However, the chemical denaturation is often used in experiments [27]. There is lack of a quantitative comparison of the energy landscapes acquired by the two different denaturation methods. This problem complicates the data analysis of protein folding in crowded conditions. The Cheung group has developed an *in silico* approach using the coarse-grained molecular simulations for the investigation of urea denaturation on protein folding [28]. The effect of urea was incorporated into an energy function derived

from the all-atomistic molecular dynamics simulations through a Boltzmann inversion method. This approach allows the development of a linear relationship between the concentration of denaturants and the temperature based on thermodynamics [29]. Experiments verified the *in silico* predictions by testing the thermal and chemical denaturation effects on three different proteins (apoazurin, cytochrome c and apoflavodoxin). Coarse-grained molecular simulations further produced the m-value of Trp-cage and created an *in silico* Chevron plot in a crowded medium that can be tested experimentally [30]. Coarse-grained molecular simulation provides a framework of understanding protein-folding dynamics and the folding energy landscape in a complex environment that involves both the chemical influences and the macromolecular crowding effect.

Effect of macromolecular crowding on the conformation and the specific function of a protein

The macromolecular crowding effect has been most studied on the proteins that can fold and unfold in two states. What happens to the anisotropic proteins whose folded conformations are structurally malleable (soft) and can appear in several compact states? Instead of a single basin on the energy landscape that represents a unique “native” conformation, there could exist several shallow basins separated by a low barrier that is within the same order of magnitude as the thermal fluctuation. A shift in the populations among these basins can occur upon a slight change in the environment as demonstrated through the simulations using a simple homopolymer model [31,32].

Modeling-wise, a protein model has to go beyond a “structure-based” one that has been useful for the investigation of protein folding toward a single folded state. The Cheung group used a statistical potential for the interactions among the amino acids that allows the contact formation according to their chemical characteristics. In collaboration with several experimentalists who employed a wide range of biophysics techniques, the folding thermodynamics of a few aspherical proteins with marginal stability, such as *Borrelia burgdorferi* VlsE (olive-shaped) [27], calmoduin (CaM, dumbbell-shaped)[33,34], and phosphoglycerate kinase (PGK, pacman-shaped) [35], was investigated under cell-like conditions. A short summary on the study of each protein is provided in the following paragraphs. These studies have yielded surprising results on protein folding in a crowded environment that are not found in dilute condition, which rendered indication on the specific function of a protein in cells.

Borrelia burgdorferi VlsE

The macromolecular crowding effect impacts the folding and the overall shape of the compacted folded states of VlsE [27]. In a crowded milieu, distinct conformations vary from an olive-shape, a bent, to a collapsed-spherical form, which are accompanied by the alteration of secondary structures that lead to the exposure of its hidden antigenic region.

CaM

The macromolecular crowding effect stabilizes several compactly folded conformations of CaM varying from a dumbbell-like shape to a globular one, which reflects its inherent plasticity in the structures [33]. A multiscale solution of charges was further developed to represent the charge distribution on CaM in the transition from apoCaM to holoCaM upon the calcium binding in a coarse-grained representation [34]. Increased levels of macromolecular crowding, in addition to calcium binding and ionic strength, can impact the conformation and the secondary structure of CaM, which may provide unique insight into understanding the promiscuous behavior of CaM in target binding and selection inside cells.

PGK

Experiments suggested that the appearance of several PGK conformations along the concentrations of Ficoll 70 is distinct from those in dilute solution [35]. The experimentally measured enzymatic activity of PGK is approximately 15-fold or greater in Ficoll 70 solution than in dilute solution. Coarse-grained molecular simulations suggested three possible compactly folded conformations of PGK in Fig 3. It was found by the *in silico* studies that the binding sites at the inner parts of two domains of PGK are already located in proximity in the compact forms of PGK under crowded conditions. Such a new finding obtained by a joint *in silico* and *in vitro* approach opposes a prior hypothesis that the enzymatic activity of PGK relies on a hinge motion that brings the two domains together.

Beyond artificial crowding agents

Several *in vivo* experiments observed a lower protein stability inside cells [36–38], which disagrees with other *in vitro* and *in silico* studies that assumed the dominance of the macromolecular crowding effect in a crowded environment. It implies that the inert artificial crowders are poor approximations to the protein crowders because the former lack the chemical details necessary for a correct description of macromolecular interactions in cells. Secondly, these artificial crowders are not adequate to represent the macromolecules in a poly-dispersive environment of a cell because their sizes are often much larger than the size of an average protein[22]. Therefore, there is a need to develop an *in silico* framework for the investigation of the impact of the intracellular environment on the macromolecular dynamics.

In the past two years, there has been a rapid growth in the modeling of the macromolecular dynamics in an intracellular environment. The Elcock group [39] created an atomistically detailed model of an *E. coli* cytoplasmic model that includes 50 of the most abundant types of macromolecules at experimentally measured concentrations. Although these macromolecules are rigid objects in the model, they parameterized the energy functions between the macromolecules (including electrostatic and van der Waals interactions) by matching the translational diffusion coefficients of a green fluorescence protein (GFP) in a cytoplasmic model with the experimentally measured values inside bacteria. Their simulations showed accordance with the experimentally measured folding stability of protein λ_{6-85} and CRABP in the cytoplasm of *E.coli*. The Skolnick group[40] included the hydrodynamic interactions (HI) into the simulations of diffusivity of a GFP in an *E. coli* cytoplasmic model that consists of 15 different types of most abundant macromolecules. They have shown that hydrodynamic interactions, together with the excluded volume effect, can account for a ~10-fold reduction in the GFP's translational diffusion coefficient without the need of non-specific interactions. The Wade group [41] has further pointed out that the weak protein-protein interactions, the shape of a protein, and the rotational and translational hydrodynamic interactions at a high concentration of protein solution can affect the rotational diffusion coefficient.

The number of atoms limits the capacity of the all-atomistic molecular dynamics simulation. The approach of coarse-grained molecular simulations is valuable because a low-resolution model offers a comprehensive understanding of the complex system, which provides a qualitative insight into problem solving. The Feig group used a mixed coarse-grained/all-atom model to create a protein solution with a box of 8 Protein G's [42]. Their peptide of interest is represented in an atomistic detail while Protein G's are represented by coarse-grained models. A Generalized Born (GB)-type implicit solvent is used to facilitate the dielectric investigation of the cellular content. They used this system as an example to show the significance of the enthalpic interactions (the van der Waals and the electrostatic interactions), which dominates over the volume exclusion in a protein milieu. The Cheung

group studied the folding of apoazurin in a coarse-grained *E coli* cytoplasmic model. They developed a self-assembled clustering algorithm (CGCYTO) [43] that uses the size of a test protein as a criterion (particle insertion method) to coarse-grain the macromolecules of an *E. coli* cytoplasm. The macromolecules represented by a few beads not only retain the geometry of a macromolecule, but also capture over 90 percent of the excluded volume effect relative to an all-atomistic model. It was computationally shown that the folding temperature of protein apoazurin in a polydisperse cytoplasmic model is higher than that of apoazurin in solution of monodisperse Ficoll 70 by approximately 5 degrees, indicating that the excluded volume effect due to macromolecules inside cells may not genuinely captured by inert artificial crowders.

Conclusion

We attempt to treat a protein-folding problem inside cells through the approach of coarse-graining from a physicist's perspective. The coarse-grained molecular simulations are able to reveal the interesting behavior of a protein in a complex system, particularly due to the physical softness of a fluctuating polymer. Together with experiments, it is possible to explore the connection between the protein conformations and their specific functions in a cellular content. One of the next challenges is to develop a comprehensive intracellular model that reflects the functional states of a cell for the investigation and prediction of protein folding *in vivo*. Coarse-grained molecular simulations can be useful for studying protein folding inside cells on other aspects involving post-translational modification [44], chaperone-assisted folding (reviews in [45]) and co-translational folding [46], which are beyond the length of this review. I apologize to any author whose work is not individually cited.

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Highlights

- Coarse-grained molecular simulations are used to investigate proteins under cell-like conditions.
- Macromolecular crowding effects can impact protein folding under cell-like conditions.
- Malleable proteins can sample several compact conformations driven by crowding effects.
- The function of a protein inside a cell may not be represented as that in a test tube.
- Modeling of cytosolic solution can be the next challenge.

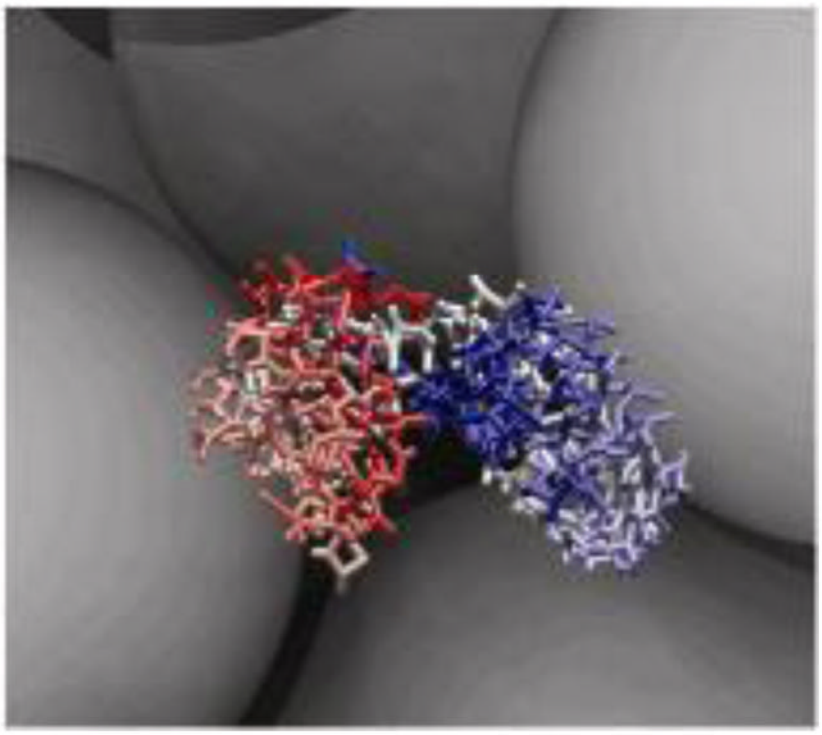

Fig 1.
Snapshot of phosphoglycerate kinase (PGK) and hard spheres of Ficoll 70 models in coarse-grained molecular simulations.

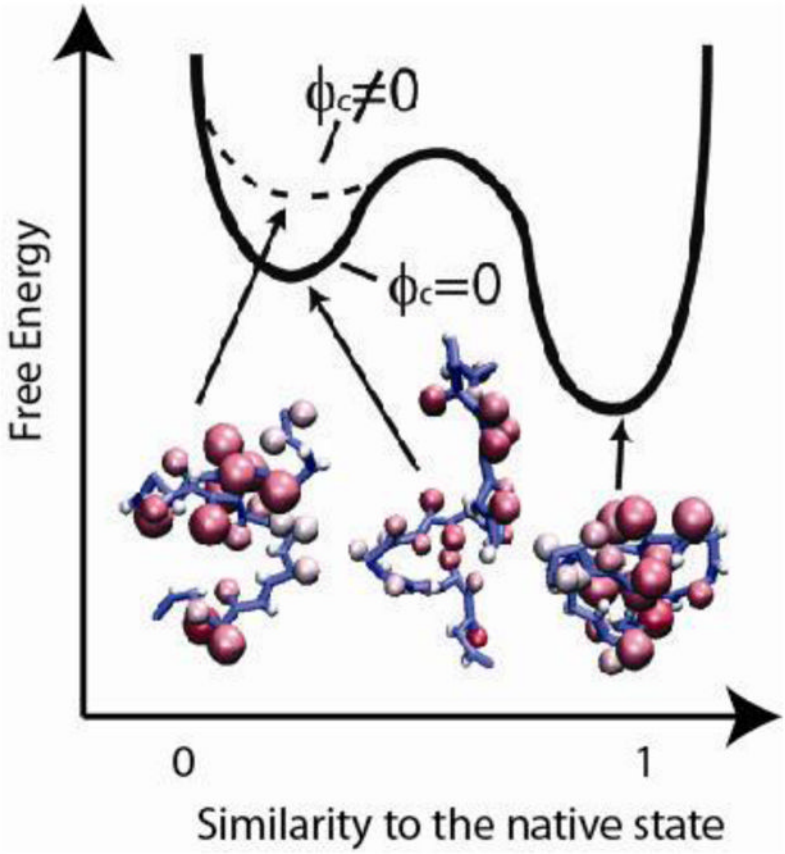

Fig 2.

Fig 2[20]. To illustrate the macromolecular crowding effect on the folding energy landscape, we plot a schematic diagram of the free energy against an order parameter, such as the fraction of native contact formation Q. When Q is close to l, the corresponding basin represents the folded state; when Q is close to 0, the corresponding basin represents the unfolded state. Upon the addition of hard-core crowders, the ensemble of unfolded states become compact and the free energy of the unfolded states are destabilized. As a result, the folded state of a protein is relatively stabilized. This shift in free energy is entropically driven.

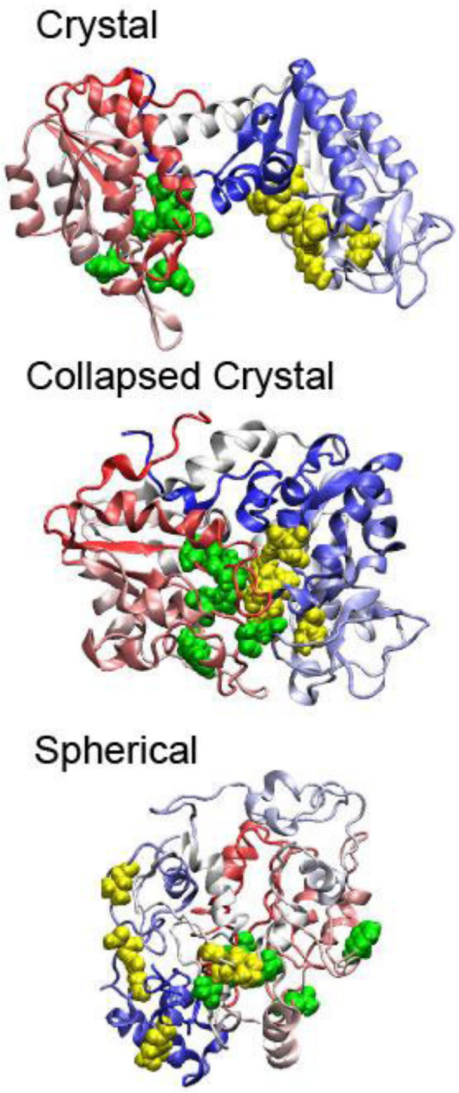

Fig 3.
Several dominant compactly folded structures of PGK in cartoon representation. (*A*) Crystal state, (*B*) collapsed crystal state, and (*C*) spherical state. The Mg-ATP and 3PG binding sites are colored in yellow and green.