

Effects of External Perturbations on Protein Systems: A Microscopic View

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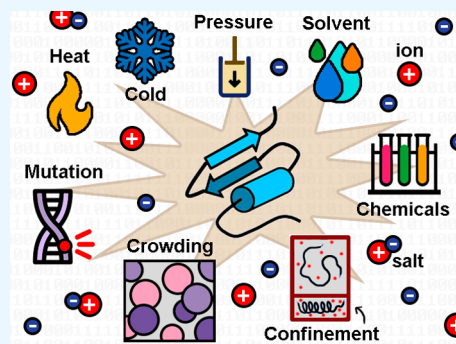
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ABSTRACT: Protein folding can be viewed as the origami engineering of biology resulting from the long process of evolution. Even decades after its recognition, research efforts worldwide focus on demystifying molecular factors that underlie protein structure–function relationships; this is particularly relevant in the era of proteopathic disease. A complex co-occurrence of different physicochemical factors such as temperature, pressure, solvent, cosolvent, macromolecular crowding, confinement, and mutations that represent realistic biological environments are known to modulate the folding process and protein stability in unique ways. In the current review, we have contextually summarized the substantial efforts in unveiling individual effects of these perturbative factors, with major attention toward bottom-up approaches. Moreover, we briefly present some of the biotechnological applications of the insights derived from these studies over various applications including pharmaceuticals, biofuels, cryopreservation, and novel materials. Finally, we conclude by summarizing the challenges in studying the combined effects of multifactorial perturbations in protein folding and refer to complementary advances in experiment and computational techniques that lend insights to the emergent challenges.



INTRODUCTION

Proteins are the ubiquitous workhorses of cellular processes. Facilitated by the coherent construction of interaction networks, their functions range from executing external stimuli to processing genetic material.^{1–6} In most cases the attainment of a native tertiary structure from a nascent polypeptide chain is a requisite for its specific cellular functions. Interestingly, understanding the process of achieving the functional fold of proteins still stands as a grand challenge in biology several decades after its inception.^{7–9} As described by Levinthal's paradox, it is practically impossible to reach the native fold of a protein by a random unbiased conformational search.^{10–12} Experimental studies led by Christian Anfinsen on the reversible denaturation of ribonuclease indicated the dominant role of thermodynamics in the folding/unfolding process.¹³ Variability in the solution and thermal conditions in this experiment further reveals the contribution of physicochemical conditions in biasing the folding process toward the lowermost Gibbs free energy state of the whole system. These insights fueled the development of free energy surface models for thermokinetic explanations of spontaneous protein folding.^{14,15} The folding process is mediated through an ensemble of intermediates and generally aided by the formation of a hydrophobic core at the expense of water entropy, resulting in organization of a well-defined tertiary structure with the alignment of secondary structures in a specific arrangement.^{16–18} However, functional diversity in proteins often precludes the existence of a single native conformation.

Experimental evidence and the development of suitable methods for elucidating the dynamic picture of proteins broaden the single native structure dependent “thermodynamic hypothesis” toward the “conformational ensemble” of a native structure (Figure 2).¹⁹

Structure-dependent intrinsic motion and the cooperative nature of interactions within proteins separate the multitude of conformers into accessible substates within the folding basin. Thermally induced inherent motions in a protein induce a hierarchy of dynamics over the femtosecond to the millisecond time regime.^{20,21} Importantly, these motions are required to overcome variable kinetic barriers over the free energy landscape resulting in hierarchical dynamics ranging from local side-chain movements to global domain rearrangements (Figure 1).^{22,23} Despite the existence of immense variety in the protein dynamics, the thermodynamically accessible conformational ensembles are distributed along a set of “principal modes of motion” or “preexisting paths”.^{25,26} Differential conformation of a protein while binding to its substrates can be sampled along a few principal axes of motion shared by its ligand free

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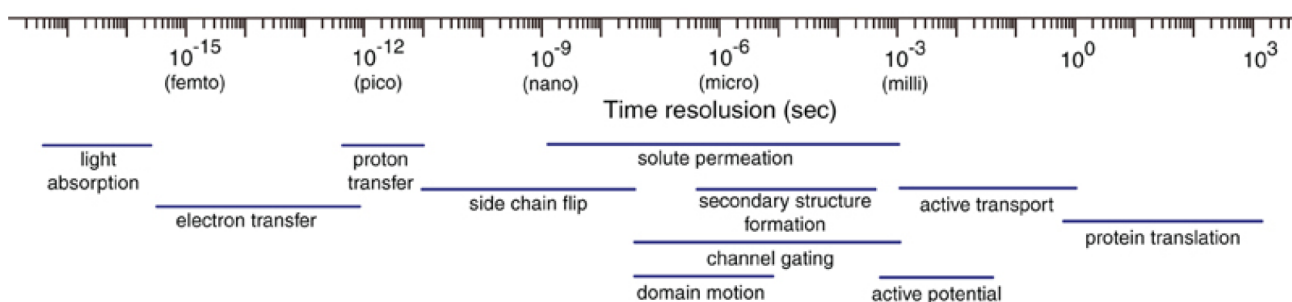


Figure 1. Spatiotemporal view of protein inherent motion. From ref 24. CC BY 3.0.

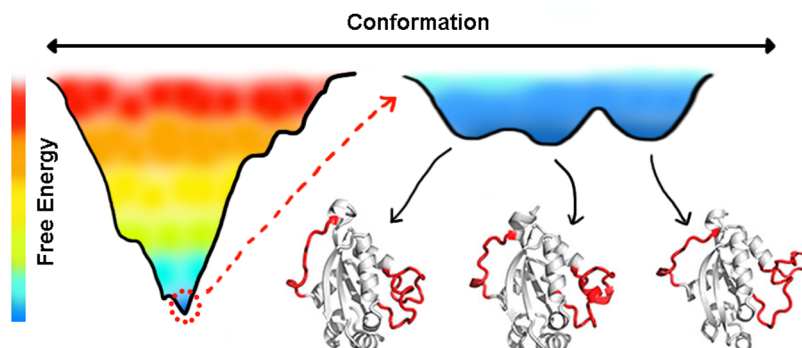


Figure 2. Schematic representation of protein folding and ensemble nature of the native state at global energy minima of folded globular proteins. Adapted from ref 19. Copyright 2016 American Chemical Society.

state.^{27,28} While an optimal structural arrangement according to the funnel shaped free energy landscape demands minimal overall structural frustration, intrinsic fluctuations driven by localized surface frustrations often extend the protein folding energy landscape for the binding kinetics. This has crucial implications in recognition mechanisms including in “coupled folding–binding” or “folding prior to binding”. Along with the energetic frustration, interaction fuzziness (structural multiplicity or dynamic disorder) allows different ordered structures of the protein to populate within its bound state.^{29–31}

Perturbations in environmental conditions surrounding a protein system can alter the relative probabilities of its substates leading to “conformational shifts” that may contribute to the impairment or enhancement of biomolecular function or binding specificity.³² Biological systems are susceptible to fluctuations in the thermodynamic conditions of temperature and pressure. Although water is globally present as protein solvents, heterogeneous cellular environments add complexity with the existence of ions, metabolites, and other cosolvents. Different cellular compartments and organelles introduce drastic variations in the concentration of these cosolvents and the effective solvent pH.^{33,34} The presence of cosolvent with variable concentrations, sizes, and chemical properties can further give rise to crowding environments for proteins. Macromolecular crowding at different levels can induce changes in solvent viscosity and effective volume for the system of interest.^{35,36} Another perturbative effect, namely confinement, is also often introduced as a result of macromolecular crowding. The polarity of the confining object may further enhance the perturbative effect.³⁷ In the folded form of a protein, mostly the surface residues are able to interact with the external environment or other biomolecules. Typically, such residues are free to interact with water molecules, lipid membranes, nucleic acids, ligands, or other protein surfaces.³⁸

Mutations and post-translational modifications that introduce residue level structural changes in protein sequences can be considered as perturbative factors that function in combination with the forces discussed earlier.

Protein folding at varying temperature and pressure conditions has been extensively studied with both experimental and computational techniques. The variations in these thermodynamic factors have direct implications on the stability and dynamics of proteins. The presence of solvents with unique properties can introduce sharp dynamical alterations at extreme thermodynamic conditions. Solvent level fluctuations are found to dominate the dynamics of proteins and thereby their functions.^{39,40} Studies on the effect of the addition of metal ions and salts reported the role of non-native electrostatic interactions in perturbing or stabilizing the native fold of the protein.^{41,42} Calorimetry and spectroscopic experiments have studied protein denaturation/renaturation behavior as an effect of further heterogeneity in the microenvironment contributed by different sizes and types of cosolvents.^{43,44} The dynamics and stability of the native fold of a protein also depends on its level of protonation. Proteins are reported to remain stable in an optimum range of pH, beyond which their propensity for denaturation increases significantly. Laser-induced rapid pH jumps in a poly-L-glutamate system have shown a slowdown in its folding kinetics by introducing kinetic traps in the system free energy landscape.⁴⁵ Effects of macromolecular crowding on protein folding dynamics have also been greatly explored with spectroscopic methods including NMR and CD. Surprisingly, crowding has shown opposite effects on the folding of large proteins and small peptide sequences.^{46,47} Experimental studies on confinement have revealed a significant increase in the stability of the protein conformation indirectly by influencing solvent dynamics.^{48,49} Moreover, mutations and post-translational

modification experiments have shown changes in the protein structure and stability by altering the intramolecular interaction pattern or through allostery.⁵⁰

Understanding the molecular basis of perturbative effects on proteins requires molecular level introspection. Such studies, including detailed investigations and correlative origins of multifactorial phenomena, are significantly facilitated by modern day computational methods, i.e., by leveraging the “computational microscope”. Recent advancements in experimental techniques^{51,52} are constantly accompanied by developments in computational architectures, molecular simulation engines, and advanced algorithms.^{53–57} Together, they allow the estimation of structural dynamics and exchange rates in complex biomolecular transitions. Enhanced sampling techniques facilitate the computation of protein folding free energy landscapes over reaction coordinates derived with fully atomistic protein models.^{58–62} However, these strategies of atomistic molecular simulations are solely founded on the form of the potential energy function or the nature of the force field. In proteins, this empirical function is generally modeled as

$$\begin{aligned}
 U(\vec{R}) = & \frac{1}{2} \sum_{\text{bonds}} k_b (b - b_0)^2 + \frac{1}{2} \sum_{\text{angles}} k_\theta (\theta - \theta_0)^2 \\
 & + \sum_{\text{dihedrals}} k_\phi [1 + \cos(n\phi - \delta)] \\
 & + \sum_{\text{LJ}} \epsilon_{ij}^{\text{min}} \left[\left(\frac{R_{ij}^{\text{min}}}{r_{ij}} \right)^{12} - 2 \left(\frac{R_{ij}^{\text{min}}}{r_{ij}} \right)^6 \right] + \sum_{\text{Coulomb}} \frac{q_i q_j}{4\epsilon_0 \epsilon r_{ij}}
 \end{aligned} \quad (1)$$

The first three terms in eq 1 represent bonded interactions, while the remaining terms represent nonbonded interactions. The bonded interactions are described with a harmonic function, whereas nonbonded interactions are described by the Coulombic and Lennard-Jones (LJ) 12–6 potentials. Improvements in force-field parameters for disordered protein sequences, polarizability, and other challenging regimes in the chemical space for studying protein dynamics under different environments amount to valuable contributions.^{63–66} For a more detailed understanding and development of a protein force field, the reader is referred to other recent reviews.^{62,63,67–71} Development of explicit solvent constant-pH simulations has significantly overcome the limitations of implicit models that were inadequate in computing desolvation energies for buried charged residues and solvent dynamics around membrane proteins and ion channels.⁷² Coarse-grained models for molecular crowders at different resolutions have been developed over the years to study the effects of such factors on protein stability.⁷³ Brownian dynamics simulation or postprocessing of trajectories with the particle insertion method can provide further knowledge in protein diffusivity.⁵⁸ Advancement in the usage of mixed atomistic–coarse-graining simulations adds further value to such studies.^{58,59} Extension of coarse-grained force fields for carbon nanotubes helps streamline protein stability study under confinement.⁷⁴ Computational studies on the effect of post-translational modifications in protein folding are significantly aided with the development of specific force fields and systematic MD simulation protocols.^{75,76}

In the current review, we have focused on the effects of different physicochemical perturbations and cellular micro-environments on the dynamics of protein folding. The review

is organized as follows. We begin by reviewing key computational contributions that study the influence of major perturbative forces on protein structure and/or dynamics. In line with previous discussion, we add the biological consequences for each of these factors. Next, we highlight the possibilities of harnessing these perturbative factors advantageously. Finally, we conclude by looking at the current limitations and future aspects of computational methods that can provide insights that complement experimentally observed phenomena, collectively leading to enhanced understanding.

■ PERTURBATIVE EFFECTS AND BIOLOGICAL CONSEQUENCES

Temperature and Pressure. The modulation of environmental factors such as the temperature, pressure, and radiation can be a suitable strategy to explore protein conformational dynamics. Conventional molecular dynamics (MD) simulations are restricted to physiological temperatures while studying the properties of the proteins under standard conditions. Classical analysis of a long trajectory shows that the thermal fluctuations at room temperature allow local side-chain movements in picosecond time scales and global domain movements in the time range of microseconds and beyond. Intrinsic fluctuations within a protein system are a manifestation of the prevalent thermal conditions. Proteins are prone to thermal denaturation at sufficiently high temperatures. Analysis of atomistic protein trajectories simulated at high temperatures shows a significant increase in their radius of gyration, exposing the hydrophobic core to the solvent.^{77–79} Although decreasing temperature causes compactification in proteins, beyond a critical temperature the dynamics get reversed. Simulations using the explicit water model shed light on the molecular mechanisms underlying such dynamics. In such scenarios water molecules were observed to penetrate the protein core to passivate the nonsaturated waters in the hydration shell by forming hydrogen bonds, thereby destabilizing hydrophobic contacts.^{80–82} This phenomenon of wetting the core is known as hydrophobic hydration in cold denaturation. Importantly, the presence of a glasslike transition was observed in the internal dynamics of hydrated proteins at cold temperatures of ~200 K.⁸³ In order to obtain mechanistic insights into this observation, MD simulation studies using dual thermostats were designed where the protein and the solvent were simulated at different temperatures.^{84,85} Interestingly, the results show that the protein dynamics are independent of self-temperature, and the glass transition is highly correlated to the solvent in the hydration layer. Unlike the common cold denaturation behavior in native proteins, experiments have disclosed amyloid-specific cold denaturations for fibril structures.⁸⁶ Furthermore, the cold thermal response to amyloid assemblies was studied using exhaustive simulations, unlike protein monomers, which show almost a linear increase in oligomeric stability with decreasing temperature below ~250 K (Figure 3B).⁸⁷ Signatures of the transitions were also observed in the dynamical behavior of the hydration layer.⁸⁸

Molecular simulations have revealed that the high-temperature stability in thermophilic proteins are attained with a combination of high secondary structure propensity, with a higher number of salt bridges, disulfide bonds, and intramolecular hydrogen bonds contributing in a more compact hydrophobic core formation in comparison to the mesophilic proteins.^{90–93} In contrast, psychrophilic proteins that are stable

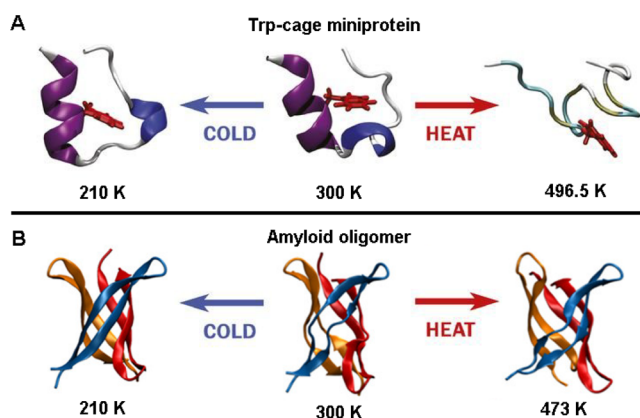


Figure 3. Heat and cold thermal responses of (A) Trp-cage miniprotein where denaturation happens in both cases. Adapted with permission from ref 89. Copyright 2016 National Academy of Sciences. (B) Amyloid oligomer where decreasing temperature increases system stability. Adapted from ref 87. Copyright 2019 American Chemical Society.

in cold temperatures share an overall unstable fragile structure that is stabilized with a small number of weak intramolecular interactions. Furthermore, to overcome the rate-limiting peptidyl-prolyl isomerizations in low temperature, psychrophilic proteins are reported to show less proline content than mesophilic proteins in their sequences, which becomes the opposite in the case of thermophiles.^{94,95}

Hydrostatic pressure plays a significant role in altering the protein dynamics and reveals an atypical conformational change in contrast to the temperature effect.⁹⁶ The ensemble nature of proteins at the global energy minima consists of pressure-sensitive conformations characterized by dynamically activated side-chain motion with “dry-molten-globule”-like features.⁹⁷ High temperature causes a cooperative change in protein conformations by altering total energy, where thermal expansion plays a vital role by shifting water away from the protein surface. In contrast, at high pressure, volumetric change is the predominant contributor to protein conformational instability.⁹⁸ In solution the partial molar volume (\hat{V}) of a protein is comprised of three terms:

$$\hat{V} = \hat{V}_{\text{atom}} + \hat{V}_{\text{cavity}} + \Delta \hat{V}_{\text{hydration}} \quad (2)$$

The first and second terms in eq 2 relate to the actual protein volume and that of the spatial cavity with it, respectively; the last term denotes changes of volume due to protein–solvent interactions. Molecular level understanding of the volume decrease in high pressure reveals a multitude of solvent effects with significant roles in the unfolding process. Other than alteration of hydration water (higher density along with increased solvation), structural change of bulk water leads to weakened hydrophobic interactions.^{99,100} Molecular simulations constrained with high-pressure NMR data revealed that increasing density and structural changes in the hydration layer induces denaturation.⁹⁶ This study further reports a major contribution of cavities in the folded protein during the change of molar volume upon unfolding. It appears that high-pressure-induced changes are specific to a protein’s conformation and its internal packing density as the MD study reveals stabilization of an isolated α -helix at a pressure of ~ 100 MPa.¹⁰¹ Moreover, atomistic simulations have revealed that the high-pressure condition introduces water into the core

region or cavity of a protein, contributing to nonpolar hydration which is also common in cold denaturation.¹⁰² In contrast to at high temperature, a protein’s intrinsic fluctuation decreases with an increase in pressure leading to kinetic slowdown of the unfolding process. Altogether these factors help shift the native population toward the unfolded or intermediate state of compact shape with decreased conformational entropy than that of the low-energy state.^{103–105} Fluorescence spectroscopic studies on the effect of pressure provides important insights based on the relaxation time. The trend of pressure and relaxation time in a logarithmic scale highlights the emergence of midpoint pressure effects in the unfolding process.¹⁰⁶ In the context of the volume effect, folding and unfolding undergo large positive and small negative activations of volume change, respectively.^{107,108} We note here that, in comparison to high-pressure effects, the response to negative pressure on protein systems remains sparsely explored. Combination of experiments and MD simulation showed destabilization of ubiquitin between 1 and -114 atm. Interestingly, MD simulations suggest that increasing negative pressure induces protein stabilization. This unique phenomenon can be described as a reentrant phase transition reaching the “island of stability” in P – T space.¹⁰⁹ As compared to direct pressure effects, the addition of membrane effects such as lipid rafts can further shift the population to more solvent exposed states but with smaller effective volume. Such effects are not observed at ambient pressure conditions.¹¹⁰

The cumulative effect of both intensive thermodynamic variables, temperature (T) and pressure (P), is more complicated. For this, an analytic theory is developed by considering a simple two-state model of protein folding–unfolding that shows the change of volume and conformational entropy within the T – P space. In this line, an interesting feature emerged via NMR, which showed that T and P factors together cause stabilization of some class of protein’s internal hydrogen bonds.^{111,112} In the case of mature fibrillar assemblies, experiments have reported high-pressure stability, while a high temperature–pressure stability is shown for catalytically active fibrils.^{113–115} Simulation studies using coarse-grained physicochemical models attempted to obtain a phase diagram for the protein system over the T – P plane.^{116,117} For the purpose of study, the coarse-grained protein force field AWSEM was modified to incorporate the effects of nonphysiological temperature and pressure on the protein free energy landscapes.¹¹⁶ Modest changes in free energy and entropy in water were observed to induce characteristically different denatured ensembles at different parts of the phase space. Therefore, the folded proteins are predicted to display almost a close elliptical region of stability in the phase diagram when modeled with the heteropolymer collapse theory generalized for a T – P plane.¹¹⁸ The Gibbs free energy difference in between the native and denatured states is defined as

$$\Delta G = G_{\text{unfolded}} - G_{\text{folded}} \quad (3)$$

A second-order Taylor series expansion of ΔG with respect to T and P around T_0 and P_0 is derived:

$$\begin{aligned} \Delta G = & \Delta G_0 + \frac{\Delta\beta}{2}(P - P_0)^2 + \Delta\alpha(P - P_0)(T - T_0) \\ & - \Delta C_p \left[T \ln \left(\frac{T}{T_0} - 1 \right) + T_0 \right] + \Delta V_0(P - P_0) \\ & - \Delta S(T - T_0) \end{aligned} \quad (4)$$

where Δ is the change in the corresponding parameters; β denotes the compressibility factor and is defined as $\beta = (\delta V / \delta P)_T$. α is the thermal expansivity factor: $\alpha = (\delta V / \delta T)_P = -(\delta S / \delta P)_T$. $C_p = T(\delta S / \delta T)_P$ is the heat capacity, and all other symbols hold their usual meanings.^{117,119} An analytical solution for the transition line that separates the folded and unfolded protein conformations is given by $\Delta G = 0$. Considering the first-order terms, it generates the following mathematical constraint which ensures an elliptical shape.

$$\Delta\alpha^2 \geq \frac{\Delta C_p \Delta\beta}{T_0} \quad (5)$$

Other than low-temperature and high-pressure conditions, molecular simulations of the activated rhodopsin channel reported water penetration inside the binding pocket upon photoisomerization of the retinal.^{120,121} Unfolding simulations of mammalian rhodopsin disclose a highly connected network core comprising long-range interactions unlike bacteriorhodopsins.^{122,123}

Crowding and Excluded-Volume Effects. It is apparent that the cellular milieu is not a dilute medium but rather is a crowded environment owing to the interplay of myriad macromolecular interactions.¹²⁴ It is worthwhile noting that macromolecular crowding within the cytoplasm is directly associated with the excluded-volume effects, and this is a profound effector for viscoelastic and colligative properties.¹²⁵ Consequently, the thermodynamic activity of a given solute in a crowded environment depends not on its nominal concentration but on the available volume and the effective concentration. One predicted outcome of macromolecular crowding is the promotion of protein compaction, which may stabilize globular proteins (Figure 4) or, conversely, enhance protein–protein associations that may, in turn, lead to conformational collapse and aggregation.¹²⁶ Similar arguments suggest that, under spatial constraints, unfolded protein states will be disfavored owing to high entropic penalties. This is consistent with faster protein folding and aggregation due to

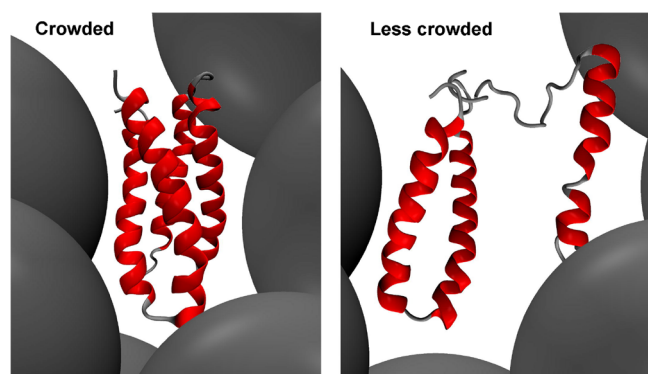


Figure 4. Stability of folded protein under varying crowded environment. Compaction is preferred with higher crowding. Reprinted with permission from ref 140. Copyright 2013 Elsevier.

lower reaction barriers in crowded environments; this is referred to as Hammond's principle.¹²⁷ Earlier work further illustrates that the physicochemical properties of cytoplasm resemble a colloidal suspension, and extreme crowding can trigger a glass transition.¹²⁸ However, it should be noted that most of the studies reporting protein stabilizing effects upon crowding were performed using synthetic polymers as crowders. Experiments using FLAsH labeling have challenged the viewpoint of the excluded-volume principle by reporting a small destabilization effect caused by crowding agents. This observation was further supported by both in vitro and in vivo urea titrations.¹²⁹ A similar mild destabilization also emerged from NMR studies using protein molecule crowders.¹³⁰

Molecular simulations that mimic the cellular crowding by means of coarse graining can reproduce the excluded-volume effect and resultant thermodynamic stability of a protein by considering purely steric intermolecular interactions. However, when the "full" energetic model was used, a decrease in the protein stability was observed.^{131,132} In general, the excluded volume v can be derived as

$$v = - \int \left(\exp \left[- \frac{U(r)}{k_B T} \right] - 1 \right) d^3r \quad (6)$$

where $U(r)$ is the interaction energy as a function of the distance r between the particles.¹³³ Exclusive hard core steric repulsions lead to clear excluded-volume effects. On the other hand, inclusion of "soft" chemical interactions can either enhance or diminish this effect. Explicit solvent models with atomistic MD simulations were further used for a detailed exploration of the interactions that result in crowding. While explaining the effect of crowding, these studies showed the major contribution as variable interactions between crowders and solute, in contrast to the traditional volume exclusion concept. In these studies, the noninteracting crowders only moderately reduced solute diffusion rates while strong interactions with crowders introduced structural destabilization along with a significantly low diffusion rate.^{58,134} The reduction in the overall free diffusion of protein further induces the formation of transient protein clusters through nonspecific binding.^{135,136} The volume exclusion effect of molecular crowding has been recently studied again, using simulations of a structure-based protein folding model. The results indicate that both the size and shape of the crowders influences the volume exclusion event.¹³⁷ In general, lengthy polymeric crowders stabilize the native protein folds better than the small and spherical ones. Binding of small molecule inhibitors and catalytic activity of enzyme may also get altered under crowding pressure. For instance, both coarse-grained and atomistic MD simulations have shown an alteration in the relative population of enzyme active and inactive states and therefore a change in the catalysis.^{138,139}

Apart from short-range repulsive effects, cellular crowders such as proteins and ribonucleic acids (RNA) bear surface charges that are involved in electrostatic, hydrophobic, hydrogen bonding, and long-range interactions. Cumulatively, these factors may drive nonspecific effects that lead to sticking or quinary interactions of the cellular milieu. Such weak interactions can manifest as transient assemblies.¹⁴¹ The low stability and rates of association or dissociation of such complexes are considered the markers for the sticking interaction, a phenomenon led by entropic forces. All-atom computational models for bacterial cytoplasm that include the

most abundant proteins, other macromolecules, waters, and ions are expected to provide a more realistic understanding of a cell. These models consider both hydrophobic and electrostatic interactions and therefore allow the sticking property of cytoplasm via protein surface charges.^{142,143} Quinary interactions, the fundamental to the fifth level of organization of the protein interaction network, have been described as constructive “underneath forces” for exhibiting the multifunctionality, and accumulation of such forces can create “metabolon” (supramolecular complex of sequential metabolic enzymes and cellular structural elements)-like functional assemblies.^{141,144,145} In some cases, quinary interactions in vast numbers can generate membraneless organelles or “liquid droplets” with specific composition and functions: some of the examples are Cajal bodies, stress granules, and p-bodies.^{141,144} Due to the diffusive and short-lived properties, quinary interactions enable cellular cross talk over longer ranges.¹⁴⁵ It is to be noted that molecules and complexes need to be in (nearly) equienergetic states to facilitate such transient interactions; a high kinetic barrier (or stable state) would lead to the complex being trapped (or visited more often) in one state for a longer time and reduce the transient nature. However, within the heterogeneous cellular interior, quinary interactions depend on inherent properties of proteins.¹⁴⁶ With the development of high-end computational resources and efficient sampling algorithms, such transient short-lived protein–protein interactions have been modeled in recent years.^{147,148} The effect of such interactions over the mutants of a protein was also studied combining NMR and MD simulation data.¹⁴⁹ These studies indicate that the mutational perturbations of proteins can manifest alteration of their surface electrostatics, leading to either amplification or reversion of the effects of quinary interactions. It is noteworthy that altered quinary interactions can impact protein aggregation.^{149,150}

Moreover, macromolecular crowding within the cellular milieu causes local nanoviscosity via hydrodynamic interactions. The viscosity (η) of a solvent reduces the self-diffusion of solute molecules and can potentially affect enzymatic reaction kinetics.^{151,152} At low solvent viscosity, protein fluctuations are independent of viscosity, but its incremental changes generate a power law dependence (as an inverse first power or a proportionality with $\eta^{1/3}$) in the first hydration shell along with localized variation due to protein structure.^{153–155} It has been suggested that, unlike low temperature effects, viscosity does not “freeze” protein motions; it arrests conformational changes.¹⁵³ MD simulation studies designed to investigate the effect of solvation layer mobility on protein dynamics have reported an excellent correlation between the local solvent viscosity and the protein backbone fluctuations.¹⁵⁶ Another computational study has recently shown that, in concentrated solutions, the proteins diffuse as constantly exchanging members of transient clusters. Their nonspecific interactions and cluster formation contribute to the high viscosity in the crowded solution.¹⁵⁷ The molecular level understanding through computational studies further shows that the presence of a crowder or a cosolvent (effector of solvent viscosity) significantly changes the structure and dynamics of the hydration shell and can reduce the diffusivity and the dielectric constant of solvent waters.^{158–162} It is reported that, due to crowding effects, peptide interfacial waters can behave as a glue (or adhesive) for assembly with the

increase in the lifetimes of water–water hydrogen bonds.^{163–168}

Surface Interactions and Confinement Effects. Within the cellular milieu, proteins functionally explore various biomolecular surfaces.^{169–171} Such surfaces, categorized on the basis of their chemical nature or topology, have immense influence in tuning protein stability and dynamics. Incorporating natural, biomimetic, and artificial surfaces, several studies have attempted to probe surface influence on protein conformation, aggregation propensity, and coupling with the local solvent environment.^{172–174} Signatures of surface and confinement effects can be found in the vicinal water molecules.¹⁷⁵ In general, different experimental measurements show that most of the cytoplasmic water displays bulk water character, whereas less than half of cell water is quantified as “slow water”.^{176–178} Such a dramatic slowdown results from the strengthening of heterogeneous water hydrogen bonds at protein surfaces and the reduction of fragmentation of water–water hydrogen bonds.¹⁷⁹ Extensive molecular dynamics simulation studies show the degree of dynamical retardation of different types of hydration water in the order bulk < surface < interstice < bridging water.^{180,181} Moreover, similar studies in combination with analytical modeling revealed that a protein’s topology and secondary structure profoundly influence heterogeneous structure–dynamics associations in the hydration water; for example, the β -sheet hydration is characterized by a thicker layer and rigid hydration network in comparison to α -helical motifs.^{81,182–186} All-atom explicit solvent simulations along with hydrophobic free energy calculations have further reported that the surrounding nearest water also signifies a crucial role in protein–ligand or protein–protein interactions or protein aggregation via polarization, charge transfer, or long-range electrostatic effects.¹⁸⁷

Gene expression, a multistep process, requires the simultaneous involvement of proteins to walk over the nucleic acid surface.¹⁸⁸ Predominantly, the electrostatic interactions strengthen the protein–DNA (or RNA) complexes but are mediated through counterion charges.¹⁸⁹ However, the three-dimensional structural organization and, further, supercoiling in nucleic acids promote a greater degree of motional variation in interactions with proteins.¹⁹⁰ Overall, the physicochemical nature of the nucleic acid surface modulates solvent reorganization, the interplay of enthalpic and entropic factors, and thereby the spontaneity of protein–nucleic acid recognition in a number of key subcellular processes including supercoiling, replication, and translation.^{190–192} Although experimental observations expected a noticeable displacement of eukaryotic DNA clamp along a double-stranded DNA in a microsecond long MD trajectory,¹⁹³ in reality the mean squared displacement was significantly underestimated by simulations.¹⁹⁴ Recent improvements in the force-field parameters considering protein–nucleic acid interactions have, however, reproduced the experimental results.¹⁹⁵ The new modification in the pair-specific Lennard-Jones parameters incorporating experimental benchmarks corrected the charge–charge interaction parameters which are known to be overestimated by traditional force fields and can strengthen the protein–DNA interactions. MD simulations along with free energy perturbation calculations complementing a isothermal titration calorimetry have recently reported variable contributions of enthalpy and entropy while binding a transcription factor with two different nucleotide sequences.¹⁹⁶ Moreover, attempts toward the estimation of binding free

energies of transcription factors using alchemical free energy calculations and potential of mean force are appreciable.^{197–199}

The lipid membrane is involved in crucial protein-mediated cellular processes that include protein sorting, transport, and signaling.^{200–202} Structurally, the lipid membrane is categorized as a surface characterized by a strong hydrophobic core and electrostatic surface that can constitute heterogeneous microstructures including lipid rafts.^{203–205} Such chemical demarcation influences a protein's dynamical interaction with lipid membrane and, further, alters protein conformations along with membrane topology.^{206–210} Generally, the dynamics of a membrane protein is greatly reduced as compared to cytosolic proteins. Again, membrane anisotropy causes slower rotational diffusion in proteins than translational diffusion. In a recent computational study, the dynamics of four different proteins were observed within a coarse-grained phospholipid membrane using the latest force-field parameters.²¹¹ These developments include the usage of fragment-based topologies with the classical residue-based parameters in the SIRAH force field for separate descriptions of lipid head and tail groups.²¹¹ These simulations have reported a drop in the β -structure propensity in the case of β -barrels embedded in membranes. However, for a protein containing predominantly α -helix, the conformations could retain the structure and native contacts; moreover, a tight packing was noticed in the membrane environment. Furthermore, advancements in enhanced sampling algorithms along with powerful computational architectures are allowing the computation of free energy landscapes for membrane protein dynamics, helping in understanding their mechanisms at molecular details.^{209,212}

Volume exclusion, compartmentalization of cellular components, and organization of organelles are the manifestations of cellular confinement.^{213–215} In biology, confinement has significant effects on phenomena ranging from protein folding to viral replication. Spatial confinement can be considered as another physical force that can perturb the classical protein folding pathway. Thermodynamically, confinement can be defined as the free energy requirement for shifting a biomolecule from a set of conformations in dilute solution to a probable conformation allowed in the bounded volume.¹⁴¹ The required free energy in this process can be defined as

$$\Delta F^{\text{confine}} = -k_{\text{B}}T \ln \left(\frac{W_{\text{allowed}}}{W_{\text{all}}} \right) \quad (7)$$

where W_{allowed} is the number of allowed states in the confined environment and W_{all} refers to the number of total conformations allowed in the dilute solution. The interactions underlying confinement can be repulsive or attractive, and most of the effectual induction requires a compartment of 10–100 Å.^{48,216} Like crowding, confinement also triggers the compactification of biomolecules at the expense of the entropic force by restricting the possible conformations accessible by intrinsic protein motions.⁴⁹ Confinement induced by crowder concentration generates an altering effect in protein fluctuations. Interestingly, a combination of experimental and computational approaches revealed a general slowdown in the protein-folding kinetics, independent of crowder concentration, but molecular insights show a distinct origin: stabilization of unfolded conformations and higher viscosity at low and high concentrations than optimal, respectively.²¹⁷ Earlier computational studies of proteins under polymeric confinement reported significant stabilization of the folded

state and elimination of expanded unfolded conformations.²¹⁸ However, unlike the spatial confinement models (using carbon nanotube), the previous models are limited in considering the dynamics of protein due to solvent interactions. Generally these carbons in nanomaterials are modeled using the van der Waals parameters for sp^2 carbons.²¹⁹ The Lennard–Jones parameters are suitably modified to appropriately model the weak interaction between nanosurface and water.³⁷ Application of spatial confinement on dry protein molecules destabilizes the unfolded state, whereas in the presence of solvent a compact unfolded ensemble different from the standard extended state is stabilized.²²⁰ A similar effect is reproduced when one regulates the distance between the protein and the surface hydration shell by introducing polar and nonpolar confinements with a fullerene ball.³⁷

In hard confinement with a proper geometric shape, a protein usually adopts the confinement shape.²²¹ But such dynamics vary depending upon the conformational properties, the role of water, and the chemical nature of the confining wall.^{222–224} A study using an Ising-like model and Monte Carlo simulation elicits the role of confining (radius) length scales on protein thermal stability and conformation alteration.²²⁵ A gradual decrease of the confining cavity radius increases the protein stability as long as the compact structure is spatially accommodated. In the presence of crowder or particularly denaturant, confinement provides stability to the protein against unfolding.²²⁶ Furthermore, the computational approach using the hard particle partition theory further added other complexities to confinement. According to this study, the open or closed nature of a pore also has significance to biomolecular stability and aggregation.²²¹ In the case of open confinement, the solute molecules exist in equilibrium with the bulk liquid phase, and upon reduction of the pore size, the solute concentration reduces due to the altered exchanges. Interestingly, in the case of closed confinement, protein aggregation adapts to the shape of the confinement. This behavior highlights the complex manifestations of nonspecific interactions in proteins causing misfolding.²²¹ Furthermore, these features also have biological relevance vis-a-vis interstitial spaces between large fibrous elements or membrane enclosed microscopic regions.^{227,228}

Chemical Factors. Perturbation of physical factors can bring about global conformational change in the protein, whereas a variable effect is observed for chemical modulation. A change in the surrounding medium like pH, ion concentration, or cosolvent can access the entire region of the unfolded protein state, while in the case of the folded state only the surface area is mostly available. Complementing experimental information, constant pH simulations have shown the reduction of the free energy barrier for unfolding upon deviations of pH from the optimal range.^{229,230} Disruption of native electrostatic interactions takes place when a change in pH alters the degree of ionization of the charged groups.²³¹ According to a MD simulation study, K^+ channel proteins in extreme acidophiles are reported to hide their ionizable amino acids to reduce protonation.²³² Other than pH, another crucial role in protein folding is played by metal ions. Based on the initial position of the Na^+ ion, the β -hairpin showed different folding pathways, whereas multivalent ions can make the folding energy landscape rugged or funnel-shaped by nonspecific coordination and a specific binding pose, respectively.^{233,234} However, heavy metal ions are also

known for their toxicity that promotes misfolding and restricts refolding in proteins.^{235–237}

Perturbation of the native folding pattern in a protein system can also be influenced by the electrostatic interactions and nonpolar Hofmeister effects resulting from the variation in salt identity and concentration in the environment.^{238,239} MD simulations with increasing salt concentration were reported to induce compaction in the protein structure for a folded ensemble, whereas “coil-to-globule”-like transitions are observed for the unfolded state in the presence of highly stabilizing salts.^{41,240} These changes are also subject to the protein sequence and its tertiary topology in its stable states.²⁴⁰ Existence of cosolvent in the media might unfold (denaturant) or stabilize (protecting osmolytes) proteins. Extensive molecular simulations using variable cosolvents and their mixtures offered insights into the molecular mechanisms. In the presence of different polyols, a protein is found to be stable even at a higher temperature than its melting point in pure water. An increase in stability is obtained with a higher molecular volume of the polyol.²⁴¹ Similarly, the addition of methanol with ubiquitin protects the secondary structures but has an weakening effect on tertiary interactions.²⁴² On the other hand, urea as chemical denaturant exerts both direct and indirect effects at the molecular level. Direct interaction with the protein involves hydrogen bonding leading to solvation of the hydrophobic core via the influx of water followed by urea. This leads to stabilization of non-native contacts over the transition state, thereby promoting unfolding. Indirectly, alteration of the hydration structure and dynamics results in a diminishing hydrophobic effect that enhances hydrophobic core exposure.^{243–245} Unlike force-dependent unfolding, urea-mediated chemical denaturation does not lead to fully extended conformations.²⁴⁶ Interestingly, denaturant dependent protein conformational stability is observed in lysozyme unfolding in the presence of urea and guanidium chloride (GdmCl).²⁴⁷ However, in general, the pathway of unfolding goes through a two-stage kinetic process mediated by a “dry molten globule”.^{248,249} The extensively studied protein protective agent trimethylamine *N*-oxide (TMAO) is known to influence the structure and dynamics of the hydration water, and it also shows a counteracting effect against urea-induced denaturation in mixed solvent (Figure 5).^{250,251} In general, an “indirect hypothesis” of slower solvent rotational dynamics surrounding the protein and the exclusion of osmolytes is considered as the molecular mechanism underlying the behavior of protecting osmolytes.²⁵²

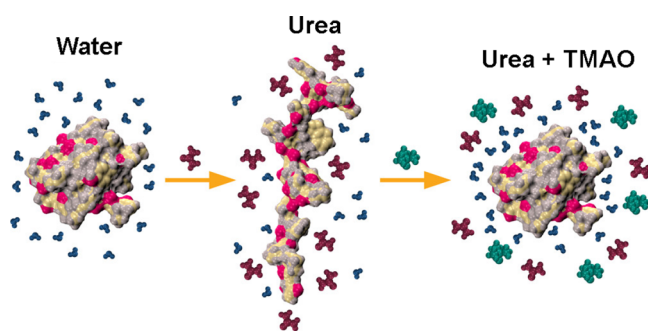


Figure 5. Effects of solvent (water) and cosolvents (urea and TMAO) on stability of folded protein. Adapted from ref 250. Copyright 2020 American Chemical Society.

Mutations and Post-translational Modifications. Intramolecular or structural change (by changing the amino acid composition) in proteins mostly gives rise to localized effects, and cooperative interactions within proteins transmit them to distal regions; this is referred to as allostery. Substitution of an active site residue or ligand binding causes faster rearrangement of the nearest residues within ~ 7 Å of the active site and slowly extends up to ~ 15 – 20 Å by the conformational selection model.²⁵³ However, in some proteins, the localized effect can be manifested as a global change. Mutational strategies can provide significant insights about protein conformational dynamics and the sensitivity of subtle alteration. Depending upon the location of the mutation, its effect varies: mutation of a surface residue is generally localized, whereas a protein core region mutation can generate a drastic conformational change. The interfacial residue mutation or alteration of electrostatic interactions can hinder the protein’s binding mechanism.²⁵⁴ Stabilizing effects of variable surface charged mutations were further reported with an MD simulation using the explicit solvent model; however, they share different mechanisms in each case.²⁵⁵ Computational substitution of a core proline residue in fibroblast growth factor receptor with serine showed high fluctuations due to a reduction in the total number of hydrogen bonds compared to the wild type structure. Moreover, this mutation was reported to be the most deleterious using a sequence homology-based prediction tool.²⁵⁶ Recently, a Monte Carlo simulation based site-directed mutation study revealed the existence of a critical number of hydrophobic residues required for the core stability of proteins. The protein stability was subjected to a sharp decrease upon reduction of the critical hydrophobicity with mutations. Moreover, this study suggests a limitation of protein surfaces to accommodate hydrophobic residues given a large number of hydrophilic residues are present in the surface and critical core hydrophobicity is maintained.²⁵⁷

During protein folding, post-translational modification like N-site or O-site glycosylation or disulfide bond, etc. formation plays a crucial role in altering the dynamics.^{258–260} For instance, N-glycosylation does not have an effect on the local or global protein conformation, yet it reduces the overall protein dynamics compared to the nonglycosylated form.²⁶¹ O-Glycosylation, on the other hand, can be stabilizing or destabilizing depending on the interactions it introduces or impairs. A recent MD simulation study also supported these findings by discovering glycan-involved hydrogen bonds inducing proteolytic stability in O-glycosylated insulin.^{262,263} Other attempts toward streamlining force-field parameters and a computational platform for studying post-translational modifications are also equally appreciable.^{75,76} Recently, free energy calculations by combining molecular dynamics with the generalized Born and surface area continuum solvation methods were performed to study the effects of acetylation and phosphorylations over yeast proteins. While acetylation induced a locally stabilizing effect on protein–protein interactions, phosphorylations played an opposing role; their co-occurrence was discussed to be more complicated than the sum of individual effects.²⁶⁴

Harnessing Perturbative Effects Advantageously. Engineering proteins that have biotechnological applications often requires tuning of function optimally at extreme conditions. For instance, thermostable enzymes including extremozymes can catalyze specific reactions at high temperatures and therefore have high industrial importance.²⁶⁵

However, due to the challenge in replication of exact host conditions, it is challenging to use these thermophiles at industrial levels.²⁶⁵ Molecular level understanding of thermophilic systems can help in designing an appropriate mutation that introduces additional surface H-bonds, salt bridges, and disulfide bridges or stabilizes the hydrophobic core for the rational design of such enzymes.²⁶⁶ Similarly, acid-stable enzymes are greatly used in multiple industries including biomineralization from low-grade ores, food processing, and pharmaceuticals.^{267,268} Incorporation of the common features of acid-stable proteins such as reduced charged density at protein surfaces can help in the design of industrially important enzymes that are stable and functional at low pH conditions.²⁶⁷ Sustainable production of biodiesel by lipase-based enzyme catalysis is gaining attention among biofuel production techniques. Such a production system requires methanol in a high amount that can result in reduction in activity and stability of the lipases.²⁶⁹ However, studies including MD simulation have confirmed that the N-glycosylation of these enzymes can improve both their activity and stability in the presence of organic solvents through the formation of non-native H-bonds.²⁶⁹ Such mutations were utilized to significantly enhance the production of biodiesel from colza oil and waste soybean oil.

Rational and semirational protein design methods have major implications in biomedical applications as well. Although cryopreservation can help in storing biosamples for medical demands, it is challenging to effectively avoid the risks of freezing and cryoprotectant toxicity. Therefore, antifreezing proteins that help organisms to survive in low-temperature conditions have high technological significance.²⁷⁰ Computational studies supporting experimental observations have revealed the alteration of long-range water dynamics as a ice-inhibition mechanism of glycoproteins and their mimics.²⁷¹ Synthesis of such bioinspired cryoprotectants are increasingly considered for efficient cryopreservation. Other usage of post-translational modifications can be noticed in pharmaceutical industries.²⁷² Computational design of stereoselective enzymes and ranking their substrate-binding efficiency with molecular simulations can replace a significant amount of laboratory work before experimental screening.²⁷³ Modern usage of highly purified protein molecules as antigens are found to be safer for vaccine preparation than the old techniques using killed/inactivated or live-attenuated pathogenic organisms. To induce a high level of immunity, however, these proteins are required to be assembled properly to mimic the pathogen surface. Design of an interaction that forms self-assembling protein nanoparticles needs to mention this direction.²⁷⁴

Understanding protein–protein interface interactions in atomistic details allows engineering of bionanomaterials by utilizing the self-assembly mechanism of proteins.^{275,276} These nanomaterials can have multiple applications ranging from photosynthetic apparatus, drug encapsulation and delivery, bioimaging, biocatalysis, biosensors, vaccine and antibody design, etc. For instance, ferritin has been explored for drug encapsulation and delivery in cancer treatment, due to its high selectivity to the cancer cells overexpressing TfR-1. However, its assembly was limited to work for a specific drug size. Recently, by controlling intra- and intersubunit disulfide bonds, protein nanocages of different geometries have been fabricated for encapsulating larger proteins including enzymes.²⁷⁷ Another key example of material bioengineering is the design of silk proteins for multipurpose applications.^{278,279}

Silks are biopolymers that offer high mechanical strength and extensibility, promoting their usage in textile industries and in producing medically important wound dressings and sutures. In general, these silks are comprised of protein fibrils aligned along the fiber axis. Modifications of these proteins are helping in engineering recombinant silk polymers having commercial applications in cosmetics, regenerative medicine, textile fabrication, and customized material design.²⁸⁰ Furthermore, the design of smart biopolymers related to scaffoldlike materials has great utility in tissue engineering and regenerative medicine.²⁸¹

■ SUMMARY AND CONCLUSIONS

Proteins can be viewed as polymers of amino acids; however, when folded in their optimal tertiary forms they can perform diverse functionalities required for a cell to survive. Obtaining an optimal fold is dependent not only on the internal polypeptide sequence but also on the external environment that is composed of different physiological forces, solvents, other chemical factors, macromolecular crowding, etc. Perturbations in one or more of these factors can introduce diverse changes in the protein stability, dynamics, and interactions. In the current context, we have reviewed major computational approaches that provide a microscopic view complementary to experimental observations into environmental perturbations to protein folding and stability. This review summarizes that the role of temperature changes in either direction from the physiological condition may have different consequences on the protein monomer and amyloid fibrils. We have seen that proteins under high-pressure conditions, macromolecular crowding, or confinement may exert a common volumetric effect on the solute, yet they can ramify distinct protein responses depending on solvent properties and molecular elements composing the perturbative factor. Moreover, we have discussed chemical factors and post-translational modifications of individually opposing effects that can either win over the other or introduce an altogether different phenomenon more complex than the sum of single factor contributions. The review also contains responses of protein surfaces to different kinds of biomolecules such as water, nucleic acid, other proteins, and lipid bilayer. However, owing to limitations in scope, we have not included a detailed discussion on membrane–protein interactions. For better insights on the same, the reader is referred to other comprehensive reviews related to the subject.

The discussion on technological advancements in protein engineering, cryopreservation, material designs, and related industries has summarized the applications of the insights on protein behavior upon environmental perturbations, derived from extensive experiments and theoretical research. Whereas these environmental effects are easy to study individually, in cells, proteins need to face much more complicated and multifactorial perturbations that can be challenging to incorporate both in experiments and in computational models. Interestingly, a recent effort has reported a protein phase diagram over the temperature–pressure–crowding space, combining results from fluorescence spectroscopy and molecular simulation techniques.²⁸² Other challenges lie in appropriately modeling the cellular crowding via computation. Atomistic models that can probe the results of crowding beyond the simplistic volume exclusion viewpoint requires high-end supercomputers; therefore, it is always a compromise between available resources and required accuracy.²⁸³ How-

ever, the developments of advanced algorithms and models that allow mixed resolution (partially atomic and coarse-grained) simulations can provide a satisfactory trade-off.^{58,136} Studying the stability, dynamics, and interactions of membrane embedded proteins poses a similar difficulty related to modeling. Further complexities are introduced while considering the heterogeneity and protein–protein interactions in a membrane environment.²⁸⁴ Besides the improvements in coarse-grained models and enhanced sampling techniques, the concept of hydrogen mass repartitioning in accelerating membrane simulations is quite appreciable.²⁸⁵ Moreover, an advanced X-ray crystallographic technique recently offered a real-time perspective in the investigation of membrane–protein interactions.^{209,286} Therefore, in conclusion, the advancements in computational techniques and architectures along with the developments of time-resolved crystallographic and spectroscopic methods are underpinning a platform for better understanding of protein biophysics in real time and with real environmental conditions.

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Notes

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