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# Protein ensembles: how does nature harness thermodynamic fluctuations for life?:

## The diverse functional roles of conformational ensembles in the cell

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## Abstract

All soluble proteins populate conformational ensembles that together constitute the native state. Their fluctuations in water are intrinsic thermodynamic phenomena, and the distributions of the states on the energy landscape are determined by statistical thermodynamics; however, they are optimized to perform their biological functions. In this review we briefly describe advances in free energy landscape studies of protein conformational ensembles. Experimental (nuclear magnetic resonance, small angle x-ray scattering, single-molecule spectroscopy, cryo-electron microscopy) and computational (replica-exchange molecular dynamics, metadynamics, Markov state models) approaches have made great progress in recent years. These address the challenging characterization of the highly flexible and heterogeneous protein ensembles. We focus on structural aspects of protein conformational distributions, from collective motions of single- and multi-domain proteins, intrinsically disordered proteins, to multi-protein complexes. Importantly, we highlight recent studies that illustrate functional adjustment of protein conformational ensembles in the crowded cellular environment. We center on the role of the ensemble in recognition of small- and macro-molecules (protein and RNA/DNA), and emphasize emerging concepts of protein dynamics in enzyme catalysis. Overall, protein ensembles link fundamental physicochemical principles and protein behavior and the cellular network and its regulation.

## Graphical Abstract

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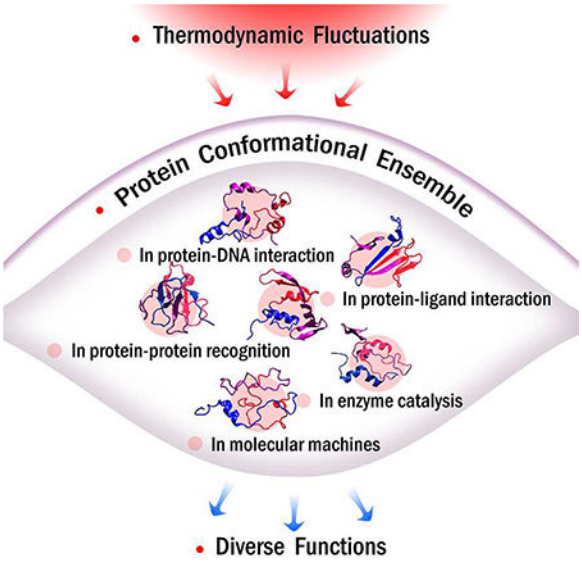


**Keywords**

Energy landscape; allostery; allosteric; conformational dynamics; protein; RNA; DNA; conformational selection; induced fit; enzyme catalysis

## 1. Introduction

In the cell, the conformational ensembles of all soluble proteins are in equilibrium around their native states.[1] The breath of the distributions of the ensembles varies across proteins; some with lower and some with higher structural variability as measured by root-mean-square deviation (RMSD) and other parameters[2]. Substates with larger RMSDs are separated by free energy barriers, which define the conformational exchange rates. Here our thesis is that the distributions of the states on the energy landscape are determined by statistical thermodynamics; however, they are also modulated by function which is optimized by evolution. Importantly, the two are intimately interconnected; more and more evidence indicates that the energy landscape dictates the conformational ensemble needed for the protein's biological function.[3]

X-ray crystallography has been the primary experimental method to identify the most populated average states in the ensemble under the crystallization conditions. Stable, folded proteins in crystal structures have well defined three-dimensional structures. As early as in the 1970s, dynamic protein conformations have been noticed from theoretical,[4–5] experimental[6–7] and molecular dynamics simulations studies.[8] It is now generally accepted that crystal proteins may still have different conformations in a single unit cell,[9] and there are multiple, populated substates that are not captured in the crystal state.[10] Conformational fluctuations of proteins in solution,[11] as revealed mostly by nuclear magnetic resonance (NMR) experiments and computations, make even stable proteins exist as conformational ensembles with highly populated substates whose populations follow thermodynamic distributions.[12–16] In natively disordered proteins some populations may be more evenly

distributed. NMR measurements are averaged over the ensemble; thus protein structures solved by NMR spectroscopy reflect the ensemble nature of protein conformations. Conformational differences can be small or large, with some conformations more populated than others. The more flexible are the proteins the larger the number of their populated states, with the disordered state being at the end of this spectrum.[17]

Characterization of the conformational ensemble and protein dynamics is important for deciphering the relationship between protein structure and dynamics and functional mechanisms. In this review, we focus on the fundamental nature of protein ensembles from their physical origins and chemical properties to their cellular functions and biological significance. We review state-of-the-art experimental and computational methods to help address the question of how nature harnesses the thermodynamic uncertainty principle as reflected in the ensemble properties for biological function.

Over the years we and others (e.g. references[18–67]) described macromolecular structures in terms of their fundamental ensemble properties. These works related to their roles in the cell and in organism life, detailed their relevance to enzyme catalysis,[3,68–74] DNA regulation,[75] cellular pathways (e.g. reference[76] and references therein) and elaborated on how evolution has manipulated ensemble properties for function through covalent (e.g. reference[77–79]) and non-covalent[80–86] interactions. Such descriptions are appealing in their simplicity and coherence; however, their aesthetics are not irrelevant in explaining reality, taking second place to cogency. Their strength is in their validity and ability to explain natural phenomena and in their predictive power. Allostery does not explain all biological phenomena and recognition events are not always allosteric events, as direct recognition of the post-translation modifications shows. Similarly, pharmacological actions can stem from orthosteric or allosteric drugs. A quarter of a century after publication of the landmark free energy landscape concept by Frauenfelder, Sligar and Wolynes,[1] which described proteins in terms of their statistics, the time is ripe to overview their implications to function. Nonetheless, as we have suggested early on, those implications required casting the statistics in terms of their dynamics.[87–88] The conformation of a single molecule changes with time and this is reflected in the dynamic conformational distribution of the ensemble. It is the dynamic change in the distributions – via population shifts – that portrays and captures the linkage to function and life.[89] The pivotal concept that all conformations pre-exist and are sampled by thermodynamic fluctuations – with function involving not new conformations but a shift in their relative concentration – underlies current approaches and interpretations. Where will the field take us? What more can it achieve? We believe that we are in a second molecular biology revolution where ideas from physics and chemistry are being imported into molecular biology.[90] These may restructure molecular biology inspiring deeper understanding of cellular processes and deciphering the mysteries of life.

In this review we first describe the physical principle governing protein conformational behavior and within this framework discuss advances in free energy landscape studies. We review the ensembles of classes of protein structural modules ranging from single to multiple domains, complexes, and ensembles in the crowed cellular environment. We next discuss recent progress in experimental and computational methods to characterize the

ensemble, analyze the diverse functional roles of conformational ensembles in the cell and through some examples chronicle how nature harnesses thermodynamic fluctuations for life.

## 2. Thermodynamic principle of protein molecules

Fluctuations of protein structures, like those of any molecular system, are intrinsic thermodynamic phenomena. Fluctuations in e.g. energy and volume can be revealed by macroscopic properties which give us quantitative perception of their orders of magnitude. These aspects are briefly described below along with the free energy landscape which yields a common ground.[91] All contribute to a physical perspective of the conformational ensembles of proteins.

### 2.1 Intrinsic thermodynamic fluctuation of protein and water

Protein structures must be considered as dynamic objects at the molecular level due to their intrinsic thermodynamic fluctuations[16,92] as well as those of water.[93–94] Any particular state $i$ of a system can be defined by the set of quantities $X_{ji}$, where, for example, $X_{1i}$=total energy ($E$) of the system in state i, $X_{2i}$=volume (V), and so on. For any given physical variable $X_{ji}$, the mean square fluctuation is defined by: $\langle \delta X_j^2 \rangle = \sum_i P_i (X_{ji} - \langle X_j \rangle)^2$, where $P_i$ is the probability distribution of $X_i$. For example, the mean square fluctuations of energy $E$ ($X_j = E$) and volume $V$ ($X_j = V$) are respectively:[4]

$$\overline{\Delta E^2} = \left\langle E^2 \right\rangle - \langle E \rangle^2 = k_B m T^2 C_v \quad (1)$$

$$\overline{\Delta V^2} = \left\langle V^2 \right\rangle - \langle V \rangle^2 = k_B m T V \beta_T \quad (2)$$

The fluctuation of the entropy $S$ is written as:[95]

$$\overline{\Delta S^2} = k_B m C_p \quad (3)$$

Here $C_v$ *and* $C_p$ are the heat capacity of the system at constant volume and at constant pressure, and $k_B$, $m$, $T$, $V$ and $\beta_T$ *is* Boltzman constant, mass, volume, temperature, and isothermal compressibility of the system, respectively.

For a typical protein of 25k daltons (about 240 amino acids), the mass is ~ $4 \times 10^{-20}$ $g$ and the volume ~ $3 \times 10^{-20}$ $cm^3$, the heat capacity is ~$C_p = 1.3 J g^{-1} K^{-1}$, $\beta_T = 0.2$ $Pa$, $k_B = 1.38 \times 10^{-23}$ $J K^{-1}$. One could estimate that the root mean square fluctuations of $E$ and $V$ are respectively:[16] $\sqrt{\langle \Delta E^2 \rangle} \approx 38$ kcal mol$^{-1}$, $\sqrt{\langle \Delta V^2 \rangle} \approx 80$ Å$^3$. The volume fluctuation is approximately the volume of three water molecules[16], which is considerable. The large protein energy fluctuation can be suppressed by protein folding. However, the intrinsic protein energy fluctuation coupled with the local energy fluctuations in water with a range of

10 to 20 kcal/mol,[93–94] is enough to perturb a well folded protein to a vast number of states. Proteins with very flexible conformations, such as intrinsically disordered proteins (IDP), are expected to have larger heat capacity than well-defined proteins.[96–98] Levitsky et al. studied the intrinsically disordered small heat shock protein Hsp22.[96] During the thermally-induced unfolding process, the protein revealed a larger heat capacity than a globular protein with the same molecular mass. The heat capacity of IDP could be 2 to 4 times larger than globular proteins with the same molecular mass.

At thermodynamic equilibrium, structural fluctuations of proteins are not only induced by the fluctuations of intra-molecular interactions of proteins, but also by the fluctuations of the energy of the surrounding water molecules. Kim and Hirata developed a statistical mechanics model to treat the conformational fluctuations of proteins around their native states and the correlated water molecules.[99] This first principle formulation is based on the generalized Langevin equation. It describes the motions of proteins in continuum solvent and provides the theory of the 3D-reference interaction site model (3D-RISM/RISM) which could evaluate the free energy surface around the thermodynamic equilibrium and its first derivative.[100] The second derivative of the free energy surface is calculated by the Hessian matrix term. They extended the model with the linear response theory so that the effect of the conformational fluctuations of proteins and the influence of solvent could be taken into account. The new model helps to evaluate the dynamic binding process of molecular recognition instead of the previous static models. Based on the same model, a recent work focused on the induced structural fluctuations by thermodynamic perturbations such as pressure.[101] This model was extended to describe the influence of macroscopic perturbation (such as pressure), on the fluctuations of proteins and proved useful in explaining variable-pressure NMR experiments. The model was also used in studies of the fluctuating thermodynamics of amyloid peptides.[102] The key role of solvent-averaged effective energy during the dimerization process implies a hydration water-centric perspective of aggregation.

The protein compressibility $\beta_T$ and heat capacity $C_v$ or $C_p$ are directly related to the conformational fluctuations of proteins. The volume fluctuations of a protein are intimately related to pressure. Over the past years, pressure perturbation has been increasingly used to study protein dynamics in combination with NMR spectroscopy[103] and X-ray crystallography.[104] High-pressure techniques have a broad range of applications in thermodynamics and kinetics of macromolecules, such as proteins and protein complexes.[105–112] High-pressure techniques are widely used not only in protein dynamics and folding[113] but also in amyloid aggregation,[114–115] crowding effects,[116] and more. The effects of hydration and cavities on compressibility-structure-function relationships were recently reviewed by Gekko.[117] High pressure induced conformational changes (like unfolding) are distinct from those induced by urea.[118–119]

There are two mechanisms for a protein in solution to attain a lower volume in response to pressure: ‘elastic response’ by general compression within the sub-ensemble of the conformers and a shift of the conformational equilibrium from a high-volume to a low-volume ensemble. In a typical case, the partial molar volume change is about −20 to −100 mL/mol, which means the free energy change is on the order of −0.5 to −2 kcal/mol per 1 kbar which may be sufficient to shift state B as the dominant species under elevated pressure

in place of state A. A protein molecule in solution exists in a dynamic equilibrium mixture of sub-ensembles of conformers differing in partial molar volumes.

The second mechanism is external cavities change under pressure. In conformations with lower volume the cavities are filled with solvent.[120–121] The lower the volume, the more rigid the conformation would be. Changes in volume and SAS (solvent accessible surface area) are positively correlated. Chalikian and Filfil[122] developed a volumetric model to determine the contributions of changes in volume (in internal protein cavities) to protein folding and binding events. During formation of protein-ligand complexes or enzymatic catalysis, external cavities change. In principle, large changes in compressibility parallel enzyme activity. However, in practice, the volumetric and fluctuation change of specific proteins are complex. T4 lysozyme and its mutants have shown this mechanism of volume change.[123–127] Beside water molecules, the cavities may be filled or partially filled with side chains of proteins which would reduce the volume under high-pressure.[124] Correspondingly, ligand binding to cavities would compete with side chains instead of solvent. A protein could rearrange its core to fill a cavity with protein atoms or hydrated cavity to respond to pressure.[125]

## 2.2 Thermodynamic fluctuations and the energy landscape theory

The statistical energy landscape theory was developed to study protein folding.[1,128–131] Mapping the folding landscape to a single coordinate, such as the fraction of native contacts, the energy landscape can be visualized as a funnel,[132] elegantly resolving Levinthal's paradox.[11] Compared to random amino-acid sequences, the energy landscapes of natural proteins are smoother, in line with the funnel description. In small proteins, domains, and disordered states, protein folding is often a two-state process[132] separated by an energy barrier. Consequently, the functional energy landscape has multiple minima, bridged by complex transition states.[133] These can be observed with different experimental methods (Fig. 1), reviewed in section 4.

The energy landscape concept was extended to protein-ligand binding and function[87–89,134–141] and statistical approaches were developed to describe the protein binding energy landscape.[142–158] The energy landscape concept provided statistical information about the conformational ensemble and their thermodynamic fluctuations. The landscape can be probed by statistical mechanics methods, including atomic level simulations. Even the simplest 2D lattice model was shown to be helpful in understanding the energy landscape of protein folding and the ensemble of transition states.[159] Although such model does not necessarily generate a funnel-like folding landscape, insights can be obtained. Even with a funnel shape, the protein folding and interaction energy landscape is necessarily rugged since a vast number of conformational states and substates exist along the folding/binding pathway as well as thermodynamic fluctuations around native state. The statistical treatment of the protein folding/interaction energy landscape usually uses the thermodynamic functions of the thermal average energy $\langle E \rangle$, the ruggedness $\sqrt{\Delta E^2}$, the density of states or equivalently the entropy S, and the glass transition temperature $T_G$.[128] The energy landscape ruggedness is directly measured by thermodynamic fluctuations in equation 1. On the folding/binding pathways, the ruggedness/fluctuation affect kinetics, and

the bottom ruggedness/fluctuations decide the conformational ensemble of the protein and its complexes. Therefore, on the energy landscape, the protein native state is not a single structure but a collection of conformations fluctuating at the bottom of the funnel with comparable energy and small energy barriers.[160–161] The width and ruggedness of the unique global energy minimum determine the conformational entropy of the native state.[133] The shallower the global energy minimum, the larger the protein fluctuation is likely to be. As indicated in Fig. 2, intrinsically disordered proteins generally have lower energy barriers. The energy landscape of IDP folding and binding is a key to their structure-dynamics-function relationship. Chu and coworkers developed a structure-based method to quantify the topography of the energy landscape to describe the thermodynamics and kinetics of flexible biomolecular recognition.[158] By performing replica-exchange molecular dynamic (REMD) simulations of 15 homodimers, the global intrinsic energy landscape has been quantified by the density of states. The folding and binding kinetics can have different scenarios such as ‘coupled binding-folding’ or ‘folding prior to binding’. The recognition mechanism depends on the topography of the intrinsic energy landscape.

The local glass transition temperature $T_G$ is proportional to the fluctuation energy, and therefore the protein folding frustration $T_F/T_G$ is inversely proportional to the fluctuation energy. Here, $T_G$ and $T_F$ denote respectively the glass transition temperature and the folding temperature of proteins. Since $T_F/T_G$ must be greater than 1 for fast folding, rugged energy landscape is more frustrated. A smooth energy landscape has lower fluctuation energy. $T_F/T_G$ as a measure of frustration is borrowed from statistical mechanics of spin glasses.[128] As compared to crystal, the energy landscapes of proteins in solvent are similar to those of glass and supercooled liquid.[163] Polymorphs of glass[164] are similar to protein conformational substates.[165] Unlike the glass, protein motion is intrinsically coupled to the solvent. Exploiting the concept of glasses and supercooled liquids,[166] protein motions could be classified by the α fluctuation (primary) and $\beta_h$ fluctuation (secondary).[167–168] The slower α relaxation motions are inversely controlled by the solvent viscosity[169–170] and the faster $\beta_h$ fluctuations are largely due to the hydration shell of the proteins.[171] Indeed, recent NMR studies revealed that the small globular protein GB1 has a hierarchical distribution of protein-solvent motions over a temperature range of 105 to 280 K.[172] A recent Mössbauer effect and quasi-elastic neutron scattering study of the dynamics and the free-energy landscape of proteins also observed β fluctuations in the hydration shell.[173]

The minimum frustration principle implies that nature has optimized protein folding,[129,174] thus the effect of point mutations on the energy landscape is analogous to thermal perturbations.[175] However, the ruggedness and frustration are indispensable components of the protein energy landscape, partially due to the intrinsic thermodynamic fluctuations of proteins. Nature has taken advantage of the ruggedness of the energy landscape to optimize proteins for their functions. Proteins can fold on rugged energy landscapes through conformational diffusion.[176] Even though below we review recent work related to the topic, here we highlight few studies to illustrate the functional significance of the ruggedness. For example, the energy landscape roughness slows down dissociation kinetics and can contribute to streptavidin-biotin interaction dynamic strength.[176] In the study of large-amplitude fluctuations of allosteric proteins, Li and coworkers developed a multiscale molecular dynamics approach and applied it to 71 allosteric proteins.[143] They used the

atomic interaction-based coarse-grained (AICG) model to predict the native basin mean fluctuations and the orientation of conformational changes. Their work confirmed that hinge regions are located near regions of high frustration, which enables allosteric proteins to exhibit rare and large-amplitude fluctuations nearly up to the opposite state basin.[143] Energy landscape studies using various other models also illustrated the importance of pre-existing fluctuations and coupling in allosteric transitions.[177–179] The free energy landscape revealed that allostery can be derived from the large entropy which decreases the free energy barrier of allosteric states.[177]

Electrostatic interactions have strong effects on the protein energy landscape. One can design surface charge-charge interactions to speed up protein folding by reducing the frustration of the folding landscape and increasing the native-like contacts in the transition-state ensembles.[180] However, nature can use electrostatic interactions in different ways. Wolynes and coworkers have developed a coarse-grained (CG), associative memory, water-mediated, structure, and energy model (AWSEM)[181] to investigate protein energy landscapes.[144,182] Electrostatic interactions in protein folding and binding were investigated with this model.[146] The energy landscapes of thirteen monomeric proteins and four dimers have been calculated. Surprisingly, adding electrostatic interactions does not improve the prediction of protein structures but the folding stability may depend on electrostatic strength. In binding, the electrostatic interactions may enhance the stability by providing direct charge-charge interaction. In other binding cases, such as the protein FIS-DNA complex, electrostatics caused frustration instead of enhancement. Energy funnels of complexes, such as protein-protein associations, are much more complicated.

The protein energy landscapes described above are based on the statistics of ensemble averages. To understand single molecule behavior of dynamic protein energy landscape, Chien and coworkers developed a theoretical method to describe the dynamics of enzyme with embedded active sites and stochastic surrounding.[183] Considering the coupling of electronic structure and protein motion during photon emission of the photon-activated fluorescent protein KFP1, the dynamics of an active center cannot be described as a Markovian process. Instead, the generalized Born-Markov approximation was used to split the complex environment into a direct sum of subreservoirs. Using stochastic matrix to describe the transitions between basins on the energy landscape, this approach revealed that slow conformational fluctuations caused by the environment could regulate the stochastic evolution of the protein active center.[183] The non-Markovian dynamics was observed in the single-molecule enzymology study on a flavoenzyme by Lu and Xie, in which enzymatic turnover was not independent of its previous turnovers because of slow fluctuations of the protein conformation.[184]

## 3. Structural (and sequence) modules in protein conformational ensembles

Because the majority of proteins are multi-domain, two thirds in prokaryotes and eighty percent in eukaryotes,[185] considerable attention has focused on linkers' properties and roles.[29,186] Here we distinguish between those with hinge motion (with short linkers) and those

with long flexible linkers, enabling large domain fluctuations. In addition to large scale domain motions in multi-domain proteins, single domain proteins also have significant local conformational dynamics from side chains, loops, and synchronized backbone motions.

### 3.1 Single domain proteins

Proteins are often treated as rigid molecules in their crystal conformation. But the conformation observed in the crystal is not necessarily the most populated one in solution,[87,187–189] and the crystal structure is likely affected by crystallization conditions.[189] It further does not capture the ensemble exploited for function. The crystal structure presents a fairly homogeneous population often of one conformer, whereas other conformers are not accounted for. Three structural elements underlie the conformational ensemble of single domain proteins: side-chain rotamers, loop movements, and collective movements of connected parts. The hierarchical stability and combination of the conformational diversities of these structural elements can lead to complex energy landscapes, even for a small protein like gpW.[190]

A study combining MD simulations and temperature-dependent X-ray diffraction data of proteins observed that proteins can be classified as surface-molten solids since the interior of native proteins is solid-like, while their surface is liquid-like.[191] However, when analyzing over longer time scales, side-chains within protein cores also have liquid-like behavior. Bowman and Geissler used Markov state models to describe the thermodynamics and kinetics of proteins.[192] Examination of the side-chain degrees of freedom revealed that almost every residue visits at least two rotameric states over hundreds of microseconds time frame, with rotamer transition rates spanning a wide range of time scales (from nanoseconds to tens of microseconds). They also reported substantial backbone dynamics on time scales longer than are typically addressed by experimental measures of protein flexibility, such as NMR order parameters.[192] The liquid-like behavior of side chains within protein cores may explain the limited entropy loss in ubiquitin folding. For ubiquitin, the total change in entropy is $T\Delta S_{total}$ = 1.4 kcal/mol per residue at 300 K with only 20% from the loss of side-chains entropy.[193] While sidechain motion may be affected by local packing density, however, the sidechain dynamics also correlate with allosteric motion.[194] The conservation of side-chain dynamics within a protein family supports the proposition that the side-chain motions correlate with protein functions.[194–195] Side-chain rotamers influence salt bridge formation, which in turn modulate the overall protein conformation. In a study of eleven proteins with NMR structures, each containing at least 40 conformers,[196] the stabilizing/destabilizing effects of salt bridges were extensively analyzed. Electrostatic interactions of a total of 1249 ion pairs have shown that most ion pairs can change from stabilizing to destabilizing the structure depending on the side-chain distances between the ion pairing residues. It has also been shown that salt bridges may exist in some conformers in the ensemble but not in others due to side-chain movements.[196]

Loop fluctuations provide large scale local conformational change. Loops' dynamics can have two roles: (1) allow multiple ligands binding via direct recognition; and (2) correlated loop fluctuations help in transmitting signals across proteins and their assemblies. For example, based on loop conformations near the binding site, the EphA4 ligand binding

domain (LBD) structures can be classified into open and closed states, indicating highly dynamic receptor conformations. Protein conformational dynamics can be characterized by MD simulations and NMR experiments.[197] The heterogeneous ensemble and loop dynamics explain how EphA4 is able to bind multiple A- and B- ephrin ligands and small molecules. The observation[197] that the heterogeneous free EphA4 conformations (including both open and closed loop conformations) already exist before binding to the ephrin ligands provide experimental support for the role of the ensemble in function.

The collective movements of proteins correspond to low frequency vibrational motions. A comprehensive study of the vibrational properties of natively folded as well as random coil structures of more than 60 polypeptides indicated that compared with random coil structures, both alpha-helices and beta-hairpins are vibrationally more flexible in terms of collective motions.[198] Ubiquitin is one of the best studied proteins with extensive coupled motions of backbone and side-chains.[199–204] Even though ubiquitin is a relatively rigid molecule, it still has a highly diverse dynamic ensemble. Residual dipolar couplings (RDCs) revealed that the structural ensemble of ubiquitin in solution covers the complete structural heterogeneity observed in as many as 46 ubiquitin crystal structures. Interestingly, a large part of the solution dynamics is concentrated in one concerted mode, which accounts for most of ubiquitin's molecular recognition heterogeneity and ensures a low entropic complex formation cost.[200] NMR dipolar coupling experiments revealed that backbone motion in ubiquitin corresponds closely to the amplitude, nature, and distribution of motion found in a 400 ns molecular-dynamics trajectory of ubiquitin.[202] Micro-heterogeneity within ubiquitin's conformational states was revealed by high resolution trapped ion mobility spectrometry, which indicates that within a conformational family the relative state-to-state abundance can be altered by solvent memory, energetic, and kinetic effects.[203] Characterized collective motions in ubiquitin span four β-strands separated by up to 15 Å, and the correlations link molecular recognition sites.[201] Interestingly, the collective motions can extend into the surrounding solvent on a 10 Å length scale.[204] However, direct coupling of internal and global motion of a ubiquitin-like RhoGTPase binding domain of plexin-B1 was not observed in a MD simulation study.[205]

Post translation modifications can modify protein motions and functions. Using nuclear magnetic resonance relaxation, Kern and coworkers characterized the motions of a single domain signaling protein, NtrC in three functional states, the inactive unphosphorylated state, the phosphorylated active state and an unphosphorylated state of a mutant which is partially active. They found a strong correlation between phosphorylation-driven activation of NtrC and microsecond time-scale backbone dynamics.[206] The structural states of NtrC, and its backbone interconversion between active and inactive states are consistent with biochemical data.[207] By combining multiple computational enhanced sampling methods with new NMR data, Kern and coworkers further explored the free energy landscape of NtrC and found that functional states are defined purely in kinetic and not structural terms. They also showed that the transition between inactive and active states occurs through multiple pathways, with both entropic and enthalpic (nonnative transient hydrogen bonds) contributions decreasing the transition barrier.[208]

### 3.2 Multi-domain proteins with hinge motions or linkers

Large-scale flexibility within a multidomain protein often plays an important role in its function. Hinge-bending involves movement of relatively rigid parts of a protein about flexible joints. In hinge bending motions, structural units move with respect to each other. While the packed arrangement within the protein subunit is conserved, the packing at their interface is disrupted. The parts move as relatively rigid bodies, swiveling on their hinge. The motion observed can be roughly perpendicular to the interface.[209] Various computational methods have been developed to analyze the hinge motion. [210–213] Many multi-domain proteins are connected by linkers with different secondary structures and lengths (typically ~5–25 amino acids).[29,214–217] Multi-domain proteins with linkers usually populate an ensemble with large conformational heterogeneity,[214] with the highly flexible linkers having low transition barriers between the states. For example, the modular xylanase Cex has an N-terminal catalytic domain and a C-terminal cellulose-binding domain, joined by a glycosylated proline-threonine (PT) linker. The PT linker is a random coil without any predominant structure, and there are no noncovalent interactions between the two domains of Cex or between either of the domains and the linker.[218]

Hinge sites with multiple conformations are often the binding sites for multiple-ligands,[219] and mutations at the hinge regions could allosterically affect the binding-site dynamics or induce alternative binding modes by modifying the ensemble of accessible conformations[220]. Ligand binding sites which closely neighboring catalytic sites may enjoy moderate flexibility which accommodates ligand binding.[221] For example, the interface between the N- and C-terminal domains of bacteriophage T4 lysozyme (T4L) is the hinge region that is accessible to the substrate with the help of the equilibrium dynamics of domain motions. Correlation analysis of fluorescence indicates that T4L populates multiple intermediate states.[222] A combination of simulations and experiments revealed details of the conformational ensemble of the T4L.[223–224] Analysis of topologically-related structures has also indicated that the hinge-bending motions are at similar locations. Depending on the distributions of the conformers and their conformational variability, they provide a gradient of binding site conformations of different shapes and volumes. These can potentially favorably interact with ensembles of ligands of variable sizes.[219,225] The Lys63-linked ubiquitin chain with multiple conformational states for specific target recognition provides an example. Analysis of inter-subunit paramagnetic NMR data showed that free K63-Ub2 exists as a dynamic ensemble comprising multiple closed and open quaternary states. One of the preexisting quaternary states can be selected and stabilized by a target protein. Quaternary dynamics enables K63-Ub2 to be specifically recognized in a variety of signaling pathways.[226]

Biological signal integration through the inter-domain linkage often does not display simple additive responses to activating inputs; instead, the linker provides synergistic activation effects.[217] Linkers encode multiple states in the ensemble, where each state may relate to allosteric response. Different sequences in the linker control the linker conformation and dynamics, and linker conformational changes can propagate to the whole protein and influence the transition pathway. This mechanism can be also illustrated by multi-scale ensemble modeling of p53 proteins with intrinsically disordered linker regions.[227] All-atom

molecular dynamics simulations of the explicitly solvated p53 linker region aimed to find long-range contacts within the linker. The linker conformational ensemble was then fed into a CG model to extract an optimal set of contact potentials by reproducing the contact probability map from the all atom MD simulations. Finally, CG MD simulation of the tetrameric p53 fragments including the core domains, the linker, and the tetramerization domain was performed to obtain an ensemble of the p53 tetramer. The calculated SAXS profile agrees well with the experiment. However, long-range contacts in the p53 linker region were required to reproduce the experimental SAXS profile, indicating allosteric communication.[227] The propagation of conformational change at certain locations[228–230] can lead to large amplitude fluctuations of the linked domains.[231] The low barrier heights between subsequent functionally-relevant states allow fast time scale response. Stabilizing such linkers may abolish function. Using SAXS and microsecond atomistic MD simulations, Strieter and coworkers compared the structural properties of ubiquitin dimers connected by native and non-native linkages. They found that SAXS profiles for the two types of dimers are similar. The MD results also reveal similar conformational ensembles for the native and non-native ubiquitin dimers. It is noted that due to the low resolution nature of SAXS data which prevents a precise determination of relative orientation of the two monomers, the MD generated conformations differ somewhat from those fitted with the experimental structural library.[232]

The sequence of the linkers and of residues in contact between linkers and adjoining domains may encode successive or parallel states through which signals travel.[29] While there is experimental and computational evidence that validate the allosteric behavior of linkers, the concept that the sequences encode a series of states is more difficult to validate experimentally.[233] Recently, Kukic et al determined the individual roles of linker residues in the interdomain motions of calmodulin using NMR chemical shifts as replica-averaged structural restraints in molecular dynamics simulations.[234] They identified 10 residues in the interdomain linker region that change their conformations upon substrate binding, including five flexible residues (Met76, Lys77, Thr79, Asp80 and Ser81) and five rigid residues (Arg74, Lys75, Asp78, Glu82 and Glu83). The results indicated that the linker sequence is not randomly distributed; the resulting conformational ensemble of the linker must be optimized for calmodulin binding.[234]

Ribosomal protein L12 is a two-domain protein with a 20-residue long linker separating the N- and C-terminal domains (Fig. 3). Interestingly, the N-terminal domains of L12 form dimers. The L12 dimer has a flexible three-lobe topology. The ensemble of L12 conformations reveals that the two C-terminal domains sample a large volume and extend further away from the ribosomal anchor than expected for a random-chain linker, indicating that the flexible linker has residual order. It was suggested that anti-correlation of the distances between each C-terminal domain and the anchor promotes the function of L12 to recruit translation factors and control their activity on the ribosome.[235]

Linkers sometimes serve to constrain conformational change, as in the case of procaspase 3, the primary executioner in apoptosis.[236] The native conformational ensemble of inactive procaspase 3 is constrained by its intersubunit linker (IL). Cleavage of the linker activates procaspase 3. However, releasing the strain of the short IL is not enough to sufficiently

increase the population of the active conformer in the native ensemble. The combination of optimal length, IL flexibility, and specific contacts between the IL and interface are needed to stabilize the active state. Interestingly, increasing the IL length by introducing 3–5 alanines can lead to constitutively active procaspases.[236]

### 3.3 Disordered proteins

Many proteins either contain at least one intrinsically disordered region (IDR) (such as intrinsically disordered domains and linkers in multidomain proteins)[234,237] or are completely disordered (such as IDPs which are highly flexible and dynamic).[238–239] Here we focus on proteins with large disordered portions. 'Disordered' or 'intrinsically unstructured' proteins lack a stable, well-defined structure under physiological conditions, existing in a continuum of conformations from the less to the more structured states.[240–243] Intrinsically disordered linkers connecting folded domains and intrinsically disordered domains usually encode the degrees of conformational flexibility essential for protein function,[17,29,244–250,239,247,249–253] including regulation of transcription and translation, cellular signaling, phosphorylation, regulation of large multi-molecular self-assemblies and small molecule storage.[249] Even though they appear to challenge the paradigm of structural biology, that function requires distinct 3D structure, this is not the case, since function involves a specific selected state, which may have marginal stability and low barriers, thus short residence time which may evade detection. The active state of a specific function is a unique state, which is not the case for the inactive states; its stability (population) may increase through a shift of the ensemble, e.g. via binding.

Disordered proteins account for a large fraction of all cellular proteins.[248] In many cases, the disordered regions constitute only certain parts or domains of the whole protein. The regulation of unstructured proteins in the cell can occur at multiple levels of mRNA transcription and degradation; protein translation and degradation; and fidelity control of transcription and translation, including post-translational modifications in functional/degradation control.[254] Such regulation of intrinsically disordered proteins at nearly every stage during transcription and translation may be warranted to ensure precision, speed, membrane anchoring, flexibility in biological control.[255]

Intrinsically disordered proteins are on average twice more likely to be substrates of kinases, highlighting the importance of post-translational modifications in fine-tuning function.[254] Post-translational modifications are key modulators of the conformational energy landscape regulating IDP's (as well as stable states) binding. One such example is the p53 protein, which has more than a dozen phosphorylation and acetylation sites with different biological signals.[256] A post-translational modification (PTM) can bias the conformational distribution, increasing the population time of a cluster favored for binding a specific partner[77]. Allosteric post-translational modification is a signal switch, which can turn on or off IDP's binding potential with a consequent binding and population shift. Post-translational modifications of IDPs may similarly serve as signals to their own degradation, although to date there are virtually no experimental structures where the PTMs are allosteric. In the case of p53, phosphorylation at Ser20 turns off p53-MDM2 binding, with a consequent increase in p53 concentration; while phosphorylation at Thr155 targets p53 to degradation by the ubiquitin

system (reviewed in[256]). Here, however, as in many other cases it is a direct recognition rather than an allosteric PTM functioning through a population shift. Combinations of PTMs can constitute an allosteric code.[77] Disordered proteins provide evidence that the function of a protein and its properties are not only decided by its static folded three-dimensional structure; but by the distribution and redistribution of the conformational ensemble.

Recently, a distance matrix-like approach was used as conformational vector to quantitatively measure the heterogeneity of the unbound ensemble of IDPs.[2] The structures of disordered proteins are not 'random'. Rather, the disordered state has significant metastable structures.[240–243,257] The ensemble may encode dominant conformations which may be functionally relevant. For example, n16 is a framework protein family associated with biogenic mineral stabilization, thought to operate at three key interfaces in nacre: protein/β-chitin, protein/protein, and protein/CaCO3. The n16N protein lacks a well-defined secondary structure, both in the presence and absence of calcium ions. However, a combination of replica exchange molecular dynamics simulations with NMR experiments showed that in the equilibrium conformational ensemble of the intrinsically disordered peptide the dominant structures present the key residues in locations thought to be critical for selective binding to β-chitin surfaces.[258] The PaaA2 antitoxin is another example of intrinsic disorder with dominant structures. The conformational ensemble of PaaA2 is highly compact and in solution the protein exists as two preformed helices, connected by a flexible linker where the helices may act as molecular recognition elements for toxin inhibition.[259] IDPs are not only involved in a wide variety of physiological processes, but also involved in pathological aggregation processes associated with many human diseases such as Alzheimer' and Parkinson's.[260–263] Therefore, IDPs have also become the focus of studies of molecular mechanisms of amyloid aggregation, characterized by conformational transitions from intrinsic disorder in the soluble monomeric/oligomeric form to ordered self-assembled amyloid fibrils of the same proteins.[262–263] Due to the relevance of IDPs both in physiological and pathological processes, many studies have been conducted to characterize their conformational properties.[264–265]

### 3.5 Multi-protein complexes

Oligomeric proteins with two or more subunits comprise about one third of the cellular proteins,[266–267] most of them symmetrical.[266] Symmetrical oligomeric complexes are evolutionarily selected by functional, genetic, and physicochemical needs. Nearly all complexes have different side chain conformations, generating ensembles at the local level. Some asymmetric complexes have reciprocal mechanisms in which all subunits cycle through the same set of conformations[266] and create global conformational ensembles. Cooperative functions, such as allosteric regulation and multivalent binding require conformational ensemble of multi-protein complexes. Oligomerization and nanocluster organization can render specificity in protein interaction.[268]

For large proteins, the local conformational change is often coupled with large domain motion, as in the case of the conformational flexibility of the myosin loops.[269] Large collective motions often regulate the functional properties of the ensemble of the oligomers,[270] and protein flexibility facilitates quaternary structure assembly and acquiring new

functions.[271] Flexibility is conducive to formation of heterologous (i.e., asymmetric) intersubunit interfaces, thus relating subunit flexibility to homomeric complexes with cyclic and asymmetric quaternary structure topologies.[271]

αB-crystallin is a molecular chaperone able to interact with unfolded proteins, and to inhibit further unfolding. αB-crystallin forms large oligomeric complexes, containing up to 40 or more subunits, which in vivo consist of heterooligomeric complexes of αB-crystallin and of other small heat shock proteins (sHsps).[272] As shown in Fig. 4, the human sHsp αB-crystallin is highly dynamic.[273] The polydispersity and quaternary dynamics of αB-crystallin are intrinsically inter-twined, and the αB-crystallin solution ensemble is governed by molecular motions of varying amplitudes and time-scales spanning several orders of magnitude. The ensemble of oligomeric and monomeric conformational states is required for the αB-crystallin's chaperone function.[274–275] Here too, the local conformational change is coupled with the quaternary dynamics of αB-crystallin, which is a direct consequence of localized tertiary fluctuations in its C-terminus.[276]

### 3.6 Conformational ensemble in cellular environment

In the heterogeneous cellular environment, protein conformations including those of symmetric oligomers may differ across time and space. The extracellular protein VlsE can be destabilized inside cells.[277] The protein energy landscape can be adjusted in space and time with the fluctuations in the intracellular environment; for example the rate of folding and the thermodynamic stability of yeast phosphoglycerate kinase (PGK) are cell cycle-dependent.[278] The stability and folding kinetics of the PGK in the nucleus and endoplasmic reticulum (ER) of eukaryotic cells are different, and the nucleus increases PGK stability and folding rate over the cytoplasm and ER.[279] Cell signaling can also be shaped by a network of multiprotein assemblies. Besides the homo-oligomers, the transiently-associated multiprotein complexes are often mediated by scaffolding proteins. Scaffolding proteins have an essential function in actively controlling regulation of signaling of multienzyme complexes and branching points in cellular pathways.[280–281] Scaffolding proteins often integrate flexible modules, which are frequently disordered domains/regions.

In the cellular environment, the high concentration of macromolecules creates crowding effects. Macromolecular crowding decreases the diffusion rate, shifts the equilibrium of protein-protein and protein-substrate interactions, and changes the protein conformational dynamics. Crowding may bias the conformational change and dynamics of enzyme populations and affect catalysis. Experimental studies revealed a correlation between protein dynamics and function in the crowded environment. HIV-1 protease is an example of the effects of crowding on internal protein dynamics. The conformational ensemble with different flap orientation is important for interacting with other molecules.[282] The distance between a pair of flaps in the HIV-1 protease can vary from 5 Å in the closed form to 22 Å in the open form. Molecular dynamics simulations indicated that flap opening is significantly suppressed in a highly crowded environment, leading to a more compact conformational ensemble[283–284]. In a coarse grain simulation of the effects of molecular crowding on protein conformational dynamics and transport properties of adenylate kinase, the system is crowded by a random stationary array of hard spherical objects. Protein

dynamics was investigated as a function of the obstacle volume fraction and size.[285] With three domains, adenylate kinase undergoes large scale hinge motions in the course of its enzymatic cycle. The simulations show that the protein prefers a closed conformation for high volume fractions. The crowding effect becomes more pronounced as the obstacle radius decreases for a given volume fraction since the average void size in the obstacle array is smaller for smaller radii.[285] Another crowding effect can be seen in α-chymotrypsin. α-chymotrypsin undergoes a reversible conformational change between inactive and active conformations. Interestingly, for α-chymotrypsin activation conformational dynamics is more important than sequence differences since active bovine and inactive rat chymotrypsin explore different regions of conformational space,[286] and there are multiple pathways for chymotrypsin activation.[287] The hydrodynamic diameter of α-chymotrypsin decreases considerably with increasing temperature, indicating that the enzyme is more compact at higher temperatures.[288] With a correlation between dynamics and activity, macromolecular crowding should decrease the structural dynamics and α-chymotrypsin activity; however, for α-chymotrypsin, crowding could either increase or decrease the catalytic rate, depending on the crowding molecules used.[289–291]

The above situation reflects uniform crowding effects, whereas synthetic particles with a narrow size distribution create random crowding conditions. In the highly coordinated cellular environment, proteins and other macromolecules are clustered and organized, and crowding is more structured,[68] as illustrated by the simulations of large collections of proteins.[292–293] For example, the folding mechanism of PGK can be modified by intracellular compartments. The density of crowders in the nucleus is no greater than in the ER or cytoplasm; however, PGK folds fast in nucleus, where it has a more homogeneous crowding and chemical environment.[279] In structured crowded environments, the perturbation of protein thermal stability may be lower; however, it may still be able to effectively dynamically modulate function. The crowding effect may stabilize the folded state; however, the effect can be counterbalanced by the favorable energetic interactions which take place in unfolded conformations.[293] Crowding perturbations are lower for proteins than for synthetic particles[294]. Moving closer to a real crowded environment in the cell, hen egg white has been used to study the dynamics and stability of several proteins.[295] While the dynamic parameters of the studied protein are clearly affected by the crowded medium, the thermal stability of the protein is similar to that in buffer.[295] In the structured crowded environment, the protein energy landscape may be more similar to that in buffer solution. Overall, the dynamics of globular proteins may be more sensitive than the dynamics of intrinsically disordered proteins, since crowding causes limited structural changes of IDPs.[296–297]

In the crowded cellular environment, it is of paramount importance to prevent disease-causing nonspecific interactions. While it is possible to achieve near-absolute specificity,[298] the collective evolution of the amino acid sequences of protein binding interfaces leads to the optimization favoring networks in which a few proteins have many partners, while most proteins have few partners.[299] Computational methods have been developed to design protein interface sequences to maintain correct and avoid unwanted interactions by optimizing promiscuous protein interfaces.[300] Apparently, flexible protein make promiscuous protein interfaces possible.

## 4. Experimental and computational methods to characterize protein ensembles

Proteins in aqueous solution often populate highly flexible, heterogeneous ensembles of conformations. Therefore, it becomes challenging to determine their structures using standard high-resolution biomolecular structure determination techniques alone such as X-ray crystallography. Different ensemble approaches have been developed to study the protein conformations and dynamics, including both experiment-based and computation-based methods. Major experimental methods include but are not limited to nuclear magnetic resonance (NMR),[301–302] small-angle X-ray scattering (SAXS),[303] single-molecule spectroscopy,[304–306] and cryo-electron microscopy.[307–309] These experiments are often combined with computational algorithms to map the conformations of proteins, in which experimental data are used as structural constraints.[310–311] Among the physical-model-based computational methods are replica-exchange molecular dynamics simulations,[312–313] metadynamics,[314] steered[315] and accelerated molecular dynamics[316–317] and Markov state models.[318–319] Due to limited space, we only discuss some of the abovementioned methods and their applications.

### 4.1 NMR/SAXS spectroscopy experiments

NMR spectroscopy has emerged as the most promising tool for the characterization of large-amplitude conformational dynamics of proteins (including single-domain proteins, multi-domain proteins and IDPs) at amino acid resolution, and even at atomic resolution.[200,320–321] NMR signals from disordered regions of proteins exhibit the spectroscopic features of small molecules, making resonance assignment possible even for very large IDPs,[322] which can provide both short-range and long-range conformational information.[242,323–324] The NMR parameters providing short-range and long-range structural information include chemical shifts (CSs), residual dipolar couplings (RDCs), and paramagnetic relaxation enhancements (PREs). The chemical shift of a nucleus depends on its local physicochemical environment and is highly sensitive to the presence of secondary structure in both folded and disordered proteins.[325] RDCs report on the distribution of relative orientations sampled by different structured domains[324] as well as the conformational propensities of disordered regions.[326] PREs detect the change in the relaxation rate of a nuclear spin induced by the presence of a distant paramagnetic group to infer the distance between the two centers, which can be used to monitor long-range contacts in protein−protein complexes or in IDPs.[327–328] The long-range conformational change is especially valuable for allosteric proteins. Recent applications of NMR in studies of protein dynamics in allostery have been reviewed.[18] SAXS, on the other hand, provides complementary information concerning the overall dimension (or radius of gyration) of proteins.[326,329] Similar to NMR, SAXS is increasingly employed in studies of flexible systems such as IDPs and multi-domain proteins with unstructured regions.

Different ensemble fitting (or selection) algorithms have been developed to fit the NMR and SAXS data. The Flexible-Meccano[330–331] and the Ensemble Optimization Method (EOM)[310–311] are respectively the first approaches introducing the concept of ensemble fitting of the NMR and SAXS data from flexible macromolecules. A number of other

ensemble approaches have followed, including ASTEROIDS[332] BSS-SAXS,[333] ENSEMBLE,[334] and EOM 2.0 (an enhanced version of EOM).[335] These ensemble approaches, as well as MD simulations, rely on a set of structural constraints determined by RDCs and CSs, and distance restraints from PRE and SAXS. In each method, an ensemble of conformations is generated and iterated to match the experimental restraints as closely as possible. The NMR/SAXS-based ensemble approaches have been summarized in several excellent recent reviews.[264,321,335] Most excitingly, the recently developed in-cell NMR spectroscopy allows the study of protein folding and binding dynamics in living cells at atomic resolution.[336–337] Recent progress of in-cell NMR spectroscopy has been summarized in a recent review.[337] These NMR/SAXS-driven ensemble approaches have enabled effective characterization of the dynamics and conformational ensembles of proteins that were not possible by X-ray crystallography.

Several selected proteins, such as ubiquitin,[200] calmodulin[335], and flexible multidomain splicing factor U2AF65,[251] together with the NMR/SAXS parameters and the ensemble fitting approaches are given in Table 1. For these works, we can see that NMR and SAXS are often combined, sometimes additionally also with MD simulations. For example, the available degrees of conformational freedom of U2AF65 are initially sampled using statistical coil model based program, flexible-meccano,[330–331] and the sampled conformational ensemble is then elucidated by the ASTEROIDS ensemble selection approach with the NMR and SAXS data as restraints. The spatial distribution of U2AF65 conformations is found to be highly anisotropic, comprising of significantly populated inter-domain contacts that are electrostatic in origin. SAXS data with amplified collective motions (ACM) were used to elucidate T4L structures and tandem WW domains of the formin-binding protein 21. The conformations generated by ACM are significantly better at reproducing the SAXS data than those from MD simulations due to the larger conformational space explored in the ACM sampling.[223] However, the MD results still provide a large conformational ensemble comparable to the distribution of crystallographic conformations of T4 Lysozyme.[224] Essential dynamics analyses of the collective fluctuations from both simulated trajectories and distribution of crystallographic conformations indicated that the main collective fluctuations are the domain motions. For the closure mode, the difference in hinge-bending angles between the most-open and most-closed X-ray structures along this mode is 49 degrees, comparable to a rotation of 45 degrees in MD simulations.[224]

### 4.2 Single-molecule spectroscopy experiments

Single-molecule spectroscopy methods include force-based spectroscopy methods such as atomic force microscopy (AFM) and optical tweezers,[342] and fluorescence-based spectroscopy methods such as single-molecule fluorescence resonance energy transfer (smFRET).[343] These approaches have become widely employed for quantifying the conformational heterogeneity and structural dynamics of biomolecules both *in vitro*[344–345] and *in vivo,*[346–347] allowing the observation of transient intermediates as well as static and dynamic heterogeneity.

AFM and optical tweezers are used to apply a stretching force between two points of a protein, unfolding the protein to an extended state. Two different protocols are commonly used in these experiments: constant velocity and constant force modes. Both protocols provide similar information, although constant force experiments are slightly more intuitive and simpler to interpret. Constant velocity experiments are useful for quickly assessing the force scales for folding and unfolding. By measuring the unfolding and folding trajectories of individual proteins, insight can be gained into the folding energy landscape, mechanical properties and conformational flexibility of globular proteins and IDPs.[342,348–349] An earlier AFM study on the folding of a two-domain protein, the calcium-sensing protein calmodulin, reported near-equilibrium two-state folding/unfolding transitions of the individual domains.[350] Later, folding of calmodulin within a 6-state network involving two off-pathway intermediates was observed in optical tweezers experiments.[344] AFM can also be used in an ultrafast scanning mode, which can provide the 'movies' of the conformational transitions of proteins such as that of myosin V motor proteins moving along actin tracks enabled by disordered linker regions. The 'movies' provide direct evidence of dynamic molecular behavior, resulting in a detailed understanding of the motor mechanism.[351] A recent optical tweezers study has directly shown that molecular shredding machines ClpX and ClpP proteases generate mechanical forces to unfold and translocate their protein substrate GFP.[352]

Single-molecule FRET allows distances and distance dynamics to be monitored in a range from about 2 nm to 10 nm, which has been applied successfully to the study of the conformational dynamics of globular proteins[353] and IDPs.[343] An important strength of single-molecule FRET is the separation of folded and unfolded populations of proteins,[354–355] which allows the structural properties of the unfolded state to be quantified even under conditions where the majority of the molecules are folded. Therefore, FRET is able to reveal hidden complexity in a protein energy landscape.[356] For example, in the structural distribution and dynamics study of p53, it was found that its N-terminal domain has multiple preferred conformations, with some of them interacting with the DNA-binding domain.[357] Single-molecule FRET even enabled studies of structural stability and conformational dynamics of proteins in live cells, as demonstrated in recent studies of several proteins such as individual SNARE proteins,[346] prothymosin α (an IDP), yeast frataxin homolog Yfh1, and IgG-binding domain of protein G (GB1).[347] Recently, a combined optical tweezers and smFRET study reported direct observation of the connection between the conformational states and the unwinding versus rezipping activity of UvrD, a DNA repair helicase.[358] In that study, the unwinding activity and the conformation of the UvrD helicase were measured simultaneously. UvrD has two conformational states: open state and closed state (see Fig. 5 A). In the smFRET experiment, UvrD was labeled with donor and acceptor fluorophores, thus high or low FRET efficiency revealed the closed or open states, respectively (Fig. 5 A). An example data trace of UvrD conformational states and unwinding activity measured simultaneously (Fig. 5 B) show that when a monomer is in the closed conformation (Fig. 5 B, shaded intervals), the DNA duplex unwinds, whereas the duplex rezips upon switching to the open conformation (Fig. 5 B, unshaded intervals). These observations demonstrate that the two conformational states and the interconversion between the two states correlate strongly with UvrD rezipping and unwinding activities. The average speed versus FRET

efficiency plot for many individual FRET-determined time intervals (Fig. 5 C) supports the finding that unwinding (positive velocity) and rezipping (negative velocity) correspond to high (closed state) and low (open state) FRET states, respectively.

### 4.3 Replica-exchange molecular dynamics simulations

Replica-exchange molecular dynamics (REMD) simulation[312–313] is one of the widely employed physical-model-based simulation methods. It is an enhanced sampling method, in which several identical copies (replicas) of the system are run in parallel at different temperatures and exchanges are periodically attempted using Monte Carlo criteria. [312–313,359–360] This allows enhanced sampling of the conformational ensemble. [361–366] While computationally expensive, the REMD method is able to explore broad, biologically significant conformational space. For example, conformational ensembles of a 16-residue human islet amyloid polypeptide (hIAPP—an IDP associated with type 2 diabetes) fragment (hIAPP(11–25)) monomer (for which solution NMR data are available[367]) and dimer have been studied by all-atom explicit-solvent REMD simulations with the aim to understand the atomic details of α-helical intermediates and the mechanism of α-helix to β-sheet transition during the aggregation process.[365] The converged 200-ns REMD simulation shows that hIAPP(11−25) monomers can transiently sample both α-helical and β-hairpin structures in solution at 310 K. Small disordered histone tails (14~38 residues), with similar size as hIAPP(11–25), have also been simulated by microsecond-long all-atom explicit-water REMD simulations by Potoyan and Papoian. The simulations demonstrated that the conformational ensembles of histone tails are composed of states with various degrees of residual order and most tails are not fully disordered, but show distinct conformational organization, containing α-helical elements and β-hairpins.[368] Conformations with a high α-helical propensity in two regions of the histone tail peptide were also reported in a recent millisecond atomistic MD study by Zheng and Cui.[369]

The conformational space of large sizes of IDPs--K18 (130 aa) and K19 (99 aa), two truncated tau constructs from the microtubulin (MT)-binding domain, have been investigated recently by us using REMD simulations.[370] We take the simulation results of K18 monomer as an example. Representative conformations of the top eight most-populated RMSD-based clusters (Fig. 6 A, B) reveal the both ordered and disordered nature of the K18 monomer. The REMD-predicted and experimentally measured Cα SCSs show a Pearson correlation coefficient of 0.614 (Fig. 6 C), consistent with previous NMR data.[371] This correlation is encouraging for such a large system with 130 amino acids (aa) in explicit water. A recent REMD study of a small 20-aa IDP fragment produced Cα SCSs having a correlation coefficient of 0.73 with experimental values.[364] The calculated Cα CSs from recent REMD simulations on α-synuclein multimers have a correlation coefficient of 0.991 with NMR values.[372] An excellent agreement with NMR Cα CSs was also obtained for K18 with a correlation coefficient of 0.989 (Fig. 6 D). These good correlations between the REMD- and NMR-derived Cα chemical shifts indicate that the REMD-generated conformational ensemble of K18 monomer is consistent with previous NMR data.[371]

The importance of metastable conformations in intrinsically disordered proteins can be well illustrated by the tau protein. Tau proteins possess intrinsic enzymatic activity capable of

catalyzing self-acetylation mediated by a pair of catalytic cysteine residues residing within the microtubulin (MT)-binding domain.[373] Acetylation of tau inhibits its function and promotes pathological aggregation.[373–375] An interesting question is how the conformational ensembles of IDPs are related to function, aggregation, and disease pathogenesis.[376] IDPs adopt multiple conformations for function;[377–378] that is, the multiple conformational ensembles collectively determine functions. In order to address these challenging questions, Luo *et al.* characterized the atomic structures of two truncated tau constructs, K18 and K19, consisting of, respectively, only the four- and three-repeats of tau protein, providing structural insights into tau's paradox.[257] The results of the analysis of the conformational ensembles of the tau proteins support the view of structured disorder and conformational selection as functional mechanism for IDPs.[377] Our REMD-generated data of K18 monomer show that cysteine-containing sequences $^{283}$DLSNVQSKCGS$^{293}$ in R2 and $^{314}$DLSKVTSKCGS$^{324}$ in R3 transiently sample α-helical structure (Fig. 7 A). These two regions are highly similar to the catalytic regions in MYST-family acetyltransferase Esa1 and Tip60.[379] Enzymatic activity requires structural stability and conformational dynamics.[69] Cys322 is located in a well-formed helix, which is critical for catalysis. The lysine −cysteine distance distribution curve in Fig. 7 (B) displays peaks between 5 and 10 Å, indicating that the two residues are close enough as in acetyltransferase with a Cα−Cα distance of 8.4 Å between Lys262 and Cys304.[380] The close contact between lysine and cysteine residues can be seen in representative conformations of C5 and C7 (Fig. 7 C). The close lysine-cysteine contacts in K18 may facilitate tau's self-acetylation activity mediated by cysteine residues as reported recently.[379]

### 4.4 Metadynamics simulations

Similar to the REMD method, metadynamics is also an enhanced sampling method which is widely used to explore the conformational space of biomolecules.[314,381] The enhancement is obtained by adding a history-dependent bias potential (which is a function of a few selected collective variables (CVs)) to the Hamiltonian of the system. This potential is constructed as a sum of Gaussians deposited along the system trajectory in the CV space, which can force the system to explore the conformations that have not yet been sampled. Thus, metadynamics can greatly enhance the sampling of rare events by pushing the system away from local free-energy minima. Next, the bias potential provides an unbiased estimate of the underlying free-energy surface.[382] Often a reweighting scheme based on configurational populations is needed to recover the Boltzmann statistics.[383]

Several improved metadynamics variants have been developed in recent years, including well-tempered metadynamics,[384–385] bias-exchange metadynamics,[386] and ensemble-biased metadynamics (EBMetaD).[387] Well-tempered metadynamics is a particular form of metadynamics, in which the energy is used as a collective variable. Bias-exchange metadynamics involves a combination of replica exchange[312–313] and metadynamics, in which a set of CVs are chosen and several metadynamics simulations are performed in parallel on different replicas of the system at the same temperature, each replica biasing a different CV. This allows complex free-energy landscapes to be explored with high efficiency. The ensemble-biased metadynamics biases a MD simulation to sample a conformational ensemble that is consistent with one or more probability distributions known

*a priori*, e.g., experimental intramolecular distance distributions obtained by spectroscopic techniques such as double electron-electron resonance.[387]

These methods alone, or in combination with high-resolution experimental techniques such as x-ray crystallography and NMR, have been successfully applied to the study of the free energy landscape of flexible proteins and protein oligomers,[36,388–389] peptide folding,[390–392] ligand and selective ion permeation through cell membrane protein channels.[393] A recent interesting study on the gating and permeation of poorly ion-selective cyclic nucleotide-gated (CNG) channels, using bias-exchange metadynamics with a combination of X-ray crystallography and electrophysiology, showed that (1) the selectivity filter can adapt to large and small ions with a different geometry (see the snapshots and the free energy landscape in Fig. 8) and (2) the pore diameter critically depends on the ion inside the pore. Based on these results, the authors concluded that the pores of CNG channels are highly flexible and that this flexibility underlies the poor selectivity of CNG channels and their strong coupling between gating and permeation.

### 4.5 Markov state models

Enhanced sampling methods such as REMD and metadynamics can provide the structural and thermodynamic properties of proteins. However, they do not reproduce kinetic rates and conformational dynamics. A Markov state model (MSM), known as a stochastic model, provides an approach which can be used to identify the kinetically relevant states and the rates of interconversion between these states. MSMs can predict kinetic quantities on long timescales (e.g. milliseconds) using a set of much shorter MD simulations.[395–396]

MSMs are employed to analyze simulation trajectories, determine microstates and calculate the transition state probabilities. To build a MSM, conformational space needs to be explored first, and subsequently discretized into microstates from which transition probabilities can be calculated, and finally refined and validated.[397–399] Before constructing a MSM, enhanced sampling techniques such as replica exchange,[312–313] metadynamics,[314] and coarse graining[400] are often used to provide an initial sampling of the configuration space, followed by short MD simulations in order to capture the correct underlying thermodynamics. Transition probabilities between microstates are calculated from the short MD trajectories and the MSM is generated. Improvements on the initial MSM can be made through adaptive sampling. The procedure for adaptive sampling contains iteration of three steps: running a series of short MD trajectories from previously collected conformations, constructing an MSM based on the accumulative data, and seeding new MD trajectories based on the sampling criterion.[398–399] Coarse-graining models can be further used to lump these kinetically relevant microstates into intuitive macrostates.[397–398] An example of microstate- and macrostate-MSM network can be seen from 3000- and 10-state MSM network of agonist-bound GPCR given in Fig. 9 (a more detailed description is given below).

Markov state models have been applied to a wide variety of problems, spanning protein folding,[402] protein-ligand binding process,[403] protein conformational change,[404–405] the dynamics and kinetics of IDPs,[369,406–407] and ATP permeation through membrane protein channels.[408] In the study of the histone tail, based on 75.6 μs long implicit solvent

simulations and 29.3 μs long explicit solvent simulations, both the free energy landscape and the underlying kinetics have also been analyzed with the MSM and different computational techniques, reaching qualitatively similar results.[369] Several recent studies have reported the application of MSMs to the study of the activation pathways of Src-kinases,[409] GPCR,[401,408] protein kinase A,[410] and bacterial signaling protein NtrC.[411] We take the abovementioned agonist-bound GPCR system as an example. The kinetic network representation of the 3000-state MSM built from the simulations of agonist bound GPCR is given in Fig. 9 A. Such a detailed picture of β2AR kinetics is useful for illustrating activation pathways at atomistic resolution. MSMs also provide a way to simplify this network by discarding fast conformational dynamics to obtain an intuitive picture of receptor dynamics consisting of lumped states. This lumping procedure is used to reduce the 3000-microstate model to a simplified 10-macrostate model of β2AR dynamics (Fig. 9 B). This macro state model of β2AR reveals two highly connected states, which are identified as inactive state (R) and the intermediate state (R'), and several states with fewer connections, including the active state (R*). Overall, these studies revealed the potential of MSMs to identify putative allosteric binding sites or to differentiate between agonists and antagonists using conformational information along putative functional pathways. A detailed description of the application of MSMs to biological systems has been presented in two recent excellent reviews.[398–399] We will not go into the details.

## 5. Protein interactions and reactions under ensemble control

Proteins function through their interaction with other molecules. Intuitively, two strongly interacting molecules would have more negative enthalpy change ΔH, which could lead to more favorable ΔG. The traditional 'lock and key' mechanism states that a protein has to have an exact match with its ligand to form a functional complex. Apparently, this mechanism overlooked the protein ensemble and entropy contributions. A lock-and key match of protein-ligand interaction is hard to achieve. The 'induced fit' hypothesis[412] suggests that the bound conformation can be 'induced' by the binding partner when the conformation in the complexes differs from that in the unbound form. The 'conformational selection and population shift' model[87–89,134] provides a more realistic description of the molecular mechanism considering the ensemble nature of protein molecules. Proteins and their binding partners are flexible and exist in ensembles with certain conformational distributions. During binding, higher energy (lower population) conformers which are most complementary to some pre-existing ligand conformations can be selected and the equilibrium shifts toward these conformers.[87–89,134] The mechanism partially accounts for entropic effects. Here we examine the effects of protein conformational ensemble on protein interactions.

### 5.1 Protein-small molecules interactions

Protein dynamics have been shown to be universally important for protein-ligand interaction.[219] A protein binds small molecules through distinct conformations; all of which may differ from its prevailing or crystal unbound or its protein-bound states. For example, the mouse major urinary protein (MUP-I) functions as carrier of volatile effectors of mouse physiology. Crystal structures of MUP-I complexed with two synthetic pheromones, 2-sec-butyl-4,5-

dihydrothiazole and 6-hydroxy-6-methyl-3-heptanone have shown that the ligands differ in their orientations within the MUP-I β-barrel pocket.[413] In this case, the ability of MUP-I to bind different lipophilic ligands derives from a limited extent of conformational flexibility and unoccupied space within the hydrophobic interior of the β-barrel.[413]

Different protein conformations often correspond to different biological functions. Estrogen receptors are classic examples of close couplings of protein conformational change and selective transcriptional activities. It has been shown that ligands can interact with similar targets in different conformations, and that the biological outcomes like ERβ selective agonist depend on the relative affinities of a ligand to ensembles of protein conformations.[414] The conformational ensemble may determine the ability of a drug to compete with a native ligand for a receptor target. In the case of estrogen receptor alpha (ERalpha) and estrogen receptor beta (ERbeta), the functional outcome of ligand binding can be inferred from its ability to simultaneously bind both ERalpha and ERbeta in agonist and antagonist conformations.[414] G protein-coupled receptors (GPCRs) are also good examples of couplings of protein conformational change and functions. GPCRs exhibit multiple inactive and active conformations, and the population balance between these conformations is altered upon binding of ligands.[415] The effect of various ligands binding on the ensemble of conformations sampled by human β2-adrenergic receptor (β2AR) also corresponds to different pharmacological reaction. The binding of agonist norepinephrine or partial agonist salbutamol leads to the selection of a subset of conformations including active and inactive state conformations, while inverse agonist carazolol selects only inactive state conformations. Therefore, receptor activation depends on both low energy states and the range of the conformations sampled by the receptor.[416]

Many computational methods have been developed to consider the ensemble nature of protein conformations and their interactions with ligands, mostly small molecules.[414–415,417–418] Nunes-Alves and Arantes parametrized a linear interaction model for implicit solvation with coefficients adapted by ligand and binding site relative polarities in order to predict ligand binding free energies. They proposed approximations to average contributions of multiple ligand-receptor poses built from a protein conformational ensemble and find that exponential averages require proper energy discrimination between plausible binding poses and false-positives (i.e., decoys).[417] Dong, Abrol, and Goddard used a modified GPCR Ensemble of Structures in Membrane BiLayer Environment (GEnSeMBLE) to predict ensemble of low-energy 3D structures. Using the predicted binding sites for a series of five known antagonists, they predicted binding energies consistent with experimental results reported in the literature for Human somatostatin receptor subtype 5 (hSSTR5).[418]

Many docking methods and tools take into the accounts of protein conformational ensembles using either experimental or computational ensemble structures.[419–428] A database of binding site ensemble can provide a fourth dimension to the otherwise three dimensional data.[420] For example, Pocketome allows searching for sites of interest, analysis of conformational clusters, important residues, binding compatibility matrices and interactive visualization of the ensembles.[421] In a simulation of Bcl-2 family proteins, Kalenkiewicz, Grant, and Yang have demonstrated that structural ensembles derived from either accelerated MD or MD in the presence of an organic cosolvent generally give better scores than those

assessed from analogous conventional MD.[419] Unfortunately we are not able to exhaustively list all the docking approaches. Instead, here we provide three examples of ensemble docking. One algorithm simultaneously docks a ligand into an ensemble of protein structures and automatically selects an optimal protein structure that best fits the ligand by optimizing both ligand coordinates and the protein.[425] The docking algorithm was validated on 10 protein ensembles containing 105 crystal structures and 87 ligands and achieved a success rate of 93%, significantly better than single rigid-receptor docking (75% on average).[425] In the Surflex-Dock, protein pocket similarity was used to choose representative structures for ensemble-docking. The docking protocol made use of known ligand poses prior to the cutoff-date, both to help guide the configurational search and to adjust the rank of predicted poses.[423] ReFlexIn (Receptor Flexibility by Interpolation) combines receptor flexibility with potential grid representation of receptor molecules has been evaluated on the retroviral HIV-1 protease system, with good agreement with experimental results.[427] Overall, ensemble receptor-based protocols display a stronger discriminating power between active and inactive molecules as compared to its standard single rigid receptor counterpart.[426] However, the prospective selection of optimum ensembles is a challenging task.[428]

As compared to globular proteins with flexible binding sites, the interactions of intrinsically disordered proteins with small molecules are much harder to track either experimentally or computationally. Several intrinsically disordered proteins such as α-synuclein, tau and the Aβ peptide are implicated in neurodegenerative diseases like Parkinson's and Alzheimer's diseases. Therapeutic targeting of the monomeric state of such intrinsically disordered proteins by small molecules has been a major challenge.[429–431] Targeting the intrinsically disordered structural ensemble of α-synuclein by small molecules as a potential therapeutic strategy for Parkinson's disease has been shown to be promising.[429] Interaction of small molecules with Aβ can significantly alter properties of monomeric Aβ via multiple routes of differing specificity,[431] and it may be possible to identify small-molecule binding pockets in the soluble monomeric form of the Aβ42 peptide.[430]

The disordered state has a significant metastable structure character[240–243,257] and functionally relevant conformations may have population times higher than of other conformations.[240–242] In a study of the impact of small molecule binding on the energy landscape of intrinsically disordered protein c-Myc, a transcription factor that is overexpressed in a broad range of cancers, a small molecule was found to perturb the composition of the apo equilibrium ensemble and to bind weakly to multiple distinct c-Myc conformations. Comparison of the apo and holo equilibrium ensembles reveals that c-Myc binding conformations are already partially formed in the apo ensemble, suggesting a conformational selection mechanism.[432]

### 5.2 Protein ensemble in enzyme catalysis

Enzymes selectively and efficiently catalyze biochemical reactions. The catalytic power of enzymes largely derives from their ability to stabilize transition states, lowering the barrier that reactants have to pass to reach productive states. Enzymes are optimized by evolution to exploit conformational ensembles to recognize their substrates and stabilize the transition states,[70,209] and both experimental and theoretical evidence indicates that enzyme

conformational transitions are highly organized which increases enzyme specificity and efficiency.[133,399,433–435] Yang and Bahar have systematically analyzed the type and level of coupling between catalysis and conformational mechanics of 98 enzymes with the Gaussian network model (GNM) and compared these with experimental data. They found that in more than 70% of the examined enzymes, the global hinge centers predicted by the GNM are colocalized with the experimentally identified catalytic sites. These hinge region ligand binding sites are usually nearby catalytic sites and have a moderate flexibility to accommodate the ligand binding.[221] For example, combined experimental and computational approaches revealed conformational substates along the reaction trajectory of adenylate kinase.[436] Fluctuations in hinge regions of the adenylate kinase generate the conformational ensemble that contains a catalytically competent state.[436]

In most cases, a single fluctuating enzyme can either follow or be reconciled with ensemble-averaged Michaelis-Menten steady-state kinetics.[437–438] The contribution of conformational ensembles in enzymes[70,87,438] can be described by including the interconverting conformers at each step via ‘catalytic networks[438–439]. In catalytic networks, each reaction step in parallel reactions has multiple enzyme conformers in equilibrium, even though the fs time scale for the transition state motions does not necessarily permit thermodynamic equilibrium between the transition state and stable enzyme states.[440]

Conformational ensembles enable enzymes to function through multiple steps. In most situations, the multiple enzyme conformers exist as open and closed conformations of the binding site or catalytic centers. Phosphoenolpyruvate carboxykinases (PEPCK) is a representative enzyme in which the transition state between the open and closed conformations occludes the active site from the solvent.[441] As in the case of ribonuclease AlkB, the open state is highly populated in the AlkB/Zn(II) complex, and the closed state is highly populated in the AlkB/Zn(II)/cosubstrate/substrate complex.[442] In the AlkB/Zn(II) co-substrate complex, the two conformations have approximately equal populations. The conformational ensemble and population shift allow the enzyme to bind the co-substrate prior to the substrate, and can also limit premature release of substrate.[442] Conformational sampling for bacterial phosphotriesterase,[443] maltose binding protein,[444] and choline oxidase,[445] cytochrome *bc*1 complex,[446] and many other lid-gated enzymes indicated that the closed and open conformations are in equilibrium prior to ligand binding, permitting a conformational selection pathway at different catalytic steps.[447] In the bacterial phosphotriesterase, with both closed and open conformations present in the apo state, the closed conformation is ideally preorganized to lower the reaction barrier, but it is not compatible with product release. In contrast, the open conformation is better organized for product release but not for chemical reaction.[443]

The rate by which the enzyme converts substrates into products is not the speed of the chemical step.[448] Instead, for efficiency, all steps need to be well executed. The protein conformational ensemble not only allows enzymes to catalyze multiple chemical steps, but accommodates the conformational reorganization required to stabilize the transition states.[449] For example, the two monomers in homing endonuclease I-AniI dimer have different specificities: one for substrate binding, the other for transition state stabilization.[450] MD simulations and free energy calculations revealed the crucial role of protein flexibility in

formation of a stable reaction transition state in α-amylase catalysis.[451] The transition state region is often energetically flat, with a range of structures which are very close in energy.[70,452] Substates with a large population may lead to transition state ensemble.[70,453–454] Thermodynamically, a barrier could decrease due to an entropy compensation effect.[70,455] Therefore, entropy can have a larger role in the conformational ensemble than in direct energy transfer.[456] Conformational dynamics may greatly facilitate the positioning of the substrate toward barrier crossing or product release through a pre-organization mechanism.[457–459] Coupled motions spanning femtoseconds to milliseconds in DHFR catalysis were proposed to promote catalysis.[460] NMR studies found that each intermediate in the catalytic cycle of DHFR samples low-lying excited states whose conformations resemble the ground-state structures preceding and following the intermediates. The structural ensemble in DHFR is exquisitely optimized for every intermediate in the catalytic cycle.[461] Ensemble-averaged QM/MM kinetic isotope effects have already been shown in small molecules, like S(N)2 reaction of cyanide anions with chloroethane in DMSO solution.[462] The populations of reactant-state (RS) and transition-state (TS) ensembles also influence the kinetic isotope effects for reactions catalyzed by enzymes;[462] that is, the change in the rate of the reaction when one of the atoms in the reactants is substituted with one of its isotopes. Using an average over an ensemble of transition state structures, the variational transition-state theory with multidimensional tunneling (EA-VTST/MT) successfully incorporates thermally fluctuating environments into enzyme kinetics for studying chemical steps of the catalytic cycles of the DHFR and several other enzyme systems.[433]

It is easy to understand that conformational ensemble heterogeneity promotes enzyme promiscuity. Evolution may select flat energy landscapes to promote multifunctionality, which can be found in the immune system, enzymatic detoxification, signal transduction, and the evolution of new functions from an existing pool of folded protein scaffolds.[463] It has been suggested that detoxification enzymes have been optimized to have a conformational ensemble with broad, barrierless transitions between states.[464]

Enzyme specificity can be controlled through multiple conformational selection steps. To allow active site substrate selectivity, transition state stabilization, and product release, enzymes have to balance specificity and efficacy. A ‘lock-and-key’ mechanism could provide ‘perfect’ selectivity. However, enzymes classified as following a lock-and-key mechanism also present a dynamic conformer selection process. Serine protease is conventionally regarded as fitting the rigid lock-and-key model. However, nanosecond timescale binding loop movement was observed to select an inhibitor conformation.[465] The flaviviral nonstructural 3 protease (NS3pro), a chymotrypsin-like serine protease also presents conformational selection to facilitate substrate binding and product release, promoting the formation of the catalytically-competent oxyanion hole.[466] Recently, it has been found that ATP turnover by individual myosin molecules uses two conformers of the myosin active site, one that allows the complete ATPase cycle and one that dissociates ATP uncleaved.[467]

Human cyclophilin A (CypA) catalyzes cis-trans isomerization of the prolyl peptide ω-bond in proteins. An earlier QM/MM study[468] demonstrated that R55K substitution at the active site of CypA leads to a significant decrease in catalysis, indicating that the active site

stability likely plays an important role in the chemical step of catalysis. More and more studies show that enzyme motions are also necessary for catalysis. Using NMR relaxation experiments, the dynamics of the prolyl cis-trans CypA isomerase were studied in the enzyme's substrate-free state and during catalysis. Characteristic enzyme motions detected during catalysis were observed in the free enzyme state with frequencies corresponding to the catalytic turnover rates. This correlation could suggest that protein motions necessary for catalysis are an intrinsic property of the enzyme and might even limit the overall turnover rate.[469] The direct link between the intrinsic motions and the catalytic turnover rate was revealed using ambient-temperature X-ray crystallographic data collection and automated electron-density sampling of interconverting substates of the human proline isomerase CypA.[470] A computational study of CypA also provided evidence of the intricate coupling dynamics and substrate turnover.[471] Molecular dynamics simulations of CypA show that the ensembles of enzyme conformations recognize diverse inhibitors and bind different configurations of the peptide substrate.[3] Nagaraju et al. found that small nonpeptidomimetic inhibitors with varying activity are recognized by enzyme ensembles that are similar to those that tightly bind the transition state and cis configurations of the substrate.[3] They suggested that functionalizing lead compounds to optimize their interactions with the enzyme's conformational ensemble bound to the substrate in the cis or the transition state could lead to more potent inhibitors of the cyclophilin A.[3]

Ensemble-averaged states sometime are not applicable to enzymes with distinct functional states. For example, H-Ras exchange factor Son of Sevenless (SOS) is an important hub for signal transduction. SOS samples a broad distribution of turnover rates through stochastic fluctuations between distinct, long-lived (more than 100 seconds), functional states. It has been shown that SOS functional output may be determined by the dynamical spectrum of rates sampled by a small number of enzymes, rather than the ensemble average.[472] Similarly, in the energy landscape of the Michaelis complex of lactate dehydrogenase the rate enhancement can arise from a stochastic search through available phase space that involves a restricted ensemble of more reactive conformational substates as compared to the same chemistry in solution.[473]

### 5.3 Protein-protein recognition

Protein-protein interactions and other cellular networks have the ability to adjust their internal states to incoming stimuli. Cellular heterogeneity is connected with high entropy of a network ensemble,[474] and protein conformational ensembles are among the important features that modulate the protein-protein interaction network.[475–477] Using experimentally known protein conformational ensembles in modeling protein-protein interactions on the proteome scale can boost the percentage of successfully predicted interactions from ~26 to 66%.[478] Inclusion of the orientational entropic effect was also found to improve the prediction of protein-protein interactions.[479]

Above, we have already discussed ensembles of protein oligomers. Here we discuss three aspects of protein conformational ensembles and protein-protein recognition: (1) structural features, (2) 'promiscuous' and specific protein-protein interactions, and (3) disordered proteins.

How interacting proteins recognize each other[480] relies on their structural features, concentration and the cellular environment.[476,481] Structural features of protein-protein interactions may be characterized by interface area, geometrical shape and the physicochemical nature of the interface. A number of chemical aspects contribute to protein-protein associations.[481–490] These range from shape complementarity to organization[491] and the relative contributions of the physical/chemical components to their stability. Protein-protein interfaces are characterized by the existence of hot spots or 'hot regions' in interacting regions instead of an even contribution across all contact areas.[371,476,487,492–495] Studies of protein binding hot spots[487,496–497] have illustrated that conserved residues at protein-protein interfaces correlate with residue hot spots identified by alanine scanning mutagenesis.[498] For example, several hot spot residues in Src SH2 interact with the phosphotyrosine and contribute about one-half of the binding free energy.[499]

Hot spots are often conserved in protein-protein interfaces. The mobility of amino acids in dimeric interfaces is generally lower than other amino acids on the protein surface.[500–501] However, the more rigid environment of hot spots can be coupled either locally or allosterically with other flexible regions. In addition, a rigid hot spot region can be flexible before forming the complex. Evidence of conformational selection driving the formation of ligand binding sites in protein-protein interfaces has been shown in one study which focused on ensembles of ligand-free protein conformations obtained by NMR. The identification of hot spot interactions was used as the measures for structure comparison. The interface binding hot spots were mapped through docking small probe molecules on the protein surface, which is independent of the ensembles generation by NMR. Interestingly, even though the unbound conformational ensemble was used in screening, the method selects binding site conformations that are similar to some peptide-bound or ligand-bound structures, supporting the conformational selection model of molecular recognition. Subsequent induced fit would shift the interaction toward the complete, optimized bound structure.[502]

Coupling of rigid hot spot and flexible protein regions can be an efficient way to adjust protein-protein interaction energy. Hot spot residues at binding interfaces confer rigidity to minimize the entropic cost of binding, whereas the residues surrounding the conserved residues may form a flexible cushion.[496] Depending on the function of the complex, protein-protein interactions may have a broad range of binding energies. Free energy changes ($\Delta G_a$) of −6 to −19 kcal/mol correspond to the range of the dissociation constants $K_d$ of protein-protein interactions between mM to less than pM. Some associations are obligatory where one protein is bound to another throughout its functional lifetime, whereas others are transient, continuously forming and dissociating[503–510]. Often, weak complexes have smaller contact area and the interfaces are more planar and with more polar residues,[511] but the large interfaces area does not necessarily equate to high affinities. For example, with similar interactions, the binding affinities of EphA4 with ephrin-A1, ephrin-A2, ephrin-A4, ephrin-A5, and ephrin-B2 are 1.2 μm, 2.3 μm, 36 nm, 360 nm, and 10.8 μm, respectively,[512] indicating varied selectivity towards various ligands.

Eph-Ephrin recognition is a good example for the coupling of rigid and flexible protein binding sites to adjust 'promiscuous' and specific protein-protein interactions. Eph-ephrin

interactions control a subtle signal transduction between cells and play an important role in carcinogenesis and other diseases. Several hot spots in the Eph binding pocket were identified as important for the binding of several peptides.[475] Conformational dynamics and the distribution of the conformational ensemble are important in Eph-Ephrin recognition.[9,475,513–516] In a comprehensive study of the energy landscapes of Eph-Ephrin recognition, the conformational ensembles and recognition energy landscapes were generated starting from separated Eph and ephrin molecules and proceeding up to the formation of Eph-ephrin complexes.[514] Dynamic conformational changes during the Eph-ephrin recognition were found to be stepwise conformational selection and population shift events, with two dynamic salt bridges between EphB4 and Ephrin-B2 contributing to specific recognition. The results indicated that the specificity is not only controlled by the final stage of the interaction across the protein-protein interface, but also contributed by dynamic intermediate stages along the pathway from the separated Eph and ephrin to the Eph-ephrin complex.[514]

Antibody-antigen interactions optimize the coupling of rigid and flexible protein binding sites for specific antigen binding. Antibody variable regions are necessarily flexible to enable recognition of tremendously diversified targets. In an in-depth analysis of subclass-specific conformational preferences of IgG antibodies, the SAXS data of identical variable regions from IgG1, IgG2 and IgG4 antibodies were thoroughly analyzed.[517] The ensembles were optimized through shape clustering, revealing distinct subclass-specific conformational preferences. The specific differences in the linker region correlate with the solution structure of intact antibodies.[517]

Even though antibody-antigen interfaces have fewer hot spot residues,[496,518] Tyr is a preferred hot spot residue for immunoglobulins.[119a] Antibody evolution may constrain conformational heterogeneity by tailoring protein dynamics.[519] Controlled evolution of the antifluorescein antibody 4–4-20 localized the Ab-combining site from a heterogeneous ensemble of conformations to a single conformation by introducing mutations that act cooperatively and over significant distances to rigidify the protein.[519] Clearly, nature utilized conformational selection to fit specific targets. The change and optimization of the conformational ensemble also allow allosteric antibody interaction. In comparing two scFv mutants with similar thermodynamic stability, local and long-ranged changes in backbone flexibility are observed.[520] It was also found that correlated flexibility may increase during antibody complex formation.[520] Antibody evolution and conformational changes are similar to point mutations within the ubiquitin core, which changes the binding specificity allosterically by shifting the conformational equilibrium of the ground-state ensemble between open and closed substates that have similar populations in the wild-type protein.[521]

The wide distribution of the conformational ensemble of flexible protein enables hub proteins to bind a large number of partners with different binding modes. For example, the interactions of nidogen-1 with laminin variants indicate the existence of a conformational ensemble of both individual proteins and complex, implying different modes of interaction through distinct protein-protein interfaces.[522] To examine promiscuous binding sites and their dynamical properties, Fornili et al. simulated the intrinsic dynamics of a large protein data set and generated conformational ensembles for the isolated proteins.[523] They found that promiscuous residues tend to be more flexible, and this additional flexibility permits a

broader range of organizations which could take place in different conformers in the ensemble.[523] This mechanism appears to be in addition to other mechanisms adopted by hub proteins, such as covalent linkage to protein interaction domains, alternative splicing variants, etc.[524]

Obviously, the most flexible proteins are the disordered proteins, with some more folded than others.[525] Highly fluctuating conformations of intrinsically disordered proteins offer functional advantages in protein-protein interactions. Upon binding their partner, natively disordered proteins can form ordered complexes. In cases of multiple-partner binding, or in altered homo-multimeric organization, the different binding modes are accomplished through distinct conformers from the ensemble, explaining the so-called 'promiscuous' binding. Human centrosomal proteins are one example.[526–528] Bioinformatics analysis indicated that human centrosomal proteins have a significant bias to be both unstructured and with coiled-coil regions with respect to generic human proteins. Centrosomal proteins tend to be larger than a control set of human proteins, and are rich in predicted disordered regions, which cover 57% of their length, compared to 39% in the general human proteome.[526] This bias implies that they adopt an ensemble of disordered and partially helical conformations, with the latter becoming stabilized when these proteins form complexes, depending on pH and concentration.[527] Interestingly, protein disorder in the centrosome correlates with complexity with the number of cell types, and the structural heterogeneity conferred by the disordered regions and phosphorylation playing an important role in its mechanical properties and regulation in space and time.[528] Cell-signaling proteins also have disproportionately intrinsically unstructured regions. KID interacts with the CREB binding protein KIX domain. When unphosphorylated and unbound, KID is disordered. Cooperative folding and binding occur upon pKID–KIX interaction, forming two α-helices kinked near the phosphorylated site.[529] KIX is an allosteric domain able to bind two other proteins cooperatively. Different partners binding at the second site can modulate the conformations and thus the affinity of pKID-KIX interaction, regulating the transactivation complex.[530]

### 5.4 Protein ensembles and molecular machines

Conformational ensembles are essential for molecular machine tasks requiring high specificity. Molecular motors are classic protein machines in cell.[531] Thermodynamically, both enzymes and molecular motors can be described by ensembles of a discrete set of states.[532–533] Since signaling and other regulatory complexes typically consist of highly dynamic molecular ensembles a 'conventional' mechanical description of protein complexes that requires well-defined quaternary structures is misleading.[534–535] In an elegant work, Suderman and Deeds simulated the yeast pheromone signaling network. They compared mechanisms aiming to identify the more effective MAPK signaling through heterogeneous sets of protein complexes.[535] They found that the ensemble model generated reliable responses that match experimental observations. In contrast, the model that employs hierarchical assembly pathways to produce scaffold-based signaling machines could not replicate experimental observations. The results illustrated on the cellular systems level that ensembles are able to signal effectively through a multiple-conformations combinatorial scheme that represents a form of weak linkage. Such a cellular strategy facilitates variable response to the environment and gain of function in network evolution.[535] Ensembles

conceptualize parallel signaling pathways whose abundance reflect concentrations of cofactors and second messengers and cell states, including interplay with post-translational modifications.

Yu et al. discussed intersubunit coordination and control in simple biomolecular machines that transform chemical free energy from NTP hydrolysis to mechanical work. They focused on (1) how the machinery coordinates essential degrees of freedom during the mechanochemical coupling process, and (2) how the coordination and control are manifested in experiments, and how they can be captured in modeling and computations.[536–538] In the classic example of muscle contraction as biomolecular machines, the release of chemical energy is often accompanied by thermal fluctuation, which can transform muscular force generated in response to external stimuli through cyclical interactions between myosin and actin.[539]

### 5.5 Protein-RNA/DNA interactions

Protein-protein and protein-RNA/DNA interactions should follow the same physico-chemical principles. Both RNA and DNA are dynamic molecules. Since RNA and DNA carry generic information, protein-RNA/DNA interactions should ensure correct processing of genomic information. RNA and DNA also exist as conformational ensembles, no matter whether in freely diffusing state,[540] in crystal,[541] or in large nucleosomes,[542] ribosomes, or spliceosomes.

The complexity of gene regulation requires a combination of high and low affinity DNA binding.[543] Thermodynamic state ensemble models are needed to describe DNA regulation, including protein-DNA interactions.[544] Protein conformational ensembles should correlate with gene regulation. For example, binding of transcriptional control proteins to their cognate DNA response elements with different DNA sequences will lead to different transcription factor conformations which can be reflected in altered binding sites to their co-regulators.[49,545]

DNA interacting-proteins are enriched by highly flexible ordered or disordered proteins or domains. For example, intrinsically disordered C-terminal tails of E. coli single-stranded DNA binding protein regulate cooperative binding to single-stranded DNA via conformational ensembles.[546] Flexible conformational ensembles allow proteins to diffuse on DNA in chromatin-unpacked regions, in search for binding sites[547] (although the length of the DNA over which they diffuse and the mechanism are still open questions considering that the DNA is bound to proteins), to lock DNA binding,[548] or to repair DNA damage.[549] Conformational ensembles can also safeguard against errors in DNA replication.[550–555] For example, DNA polymerase I samples open and closed conformations in millisecond timescale to select substrates.[550] Conformational dynamics of the Y-family DNA polymerase Dpo4 was also shown to control its selectivity.[551–552] DNA polymerase μ (polμ) has a rate-limiting ‘pre-catalytic translocation step’ to ensure accuracy and retain efficiency.[553] The flexible region surrounding the H-helix of the thumb domain, which selects the correct Watson-Crick base pair[551] can distinguish among small differences.[554] In a detailed study of the structural factors that determine selectivity of a high fidelity DNA polymerase for deoxy-, dideoxy-, and ribonucleotides, Wang et al. analyzed 10 high resolution crystal

structures and enzyme kinetic of Bacillus DNA polymerase I large fragment variants. They found that intermediate conformations of the O-helix (a part of taq polymerase suggested to play an important role in the enzyme fidelity) between extreme open and closed states creates an ensemble of binding sites that trap and misalign non-cognate nucleotides.[555] This study illustrated a particular advantage in ensemble control in gene information processing. The conformational ensemble allows the protein to recognize large number of the DNA conformations with non-cognate nucleotides and to clear them. Such a mechanism could also apply to RNA polymerase.[556]

Conformational ensembles also allow proteins to fit into various RNA functional machineries. For example, while ribosome-bound elongation factor G (EF-G) predominantly adopts an extended conformation, the ribosome-bound EF-G may also occasionally sample at least one compact conformation.[557–558] A number of experiments provide details of how conformational ensembles of U2AF(65) facilitate molecular recognition of diverse RNA sequences in the spliceosome.[559–564] The tandem RNA recognition motif (RRM) domains of U2AF(65) have two different domain arrangements in the absence and presence of a high affinity ligand. RRMs exhibit a broad range of conformations in the solution ensemble[563] (Fig. 10), with the U2AF(65) ensemble of closed and open conformations accounting for recognition of sequence variability.[560,562–563] The local structural changes suggest that the N-terminal RRM1 is more promiscuous, i.e. with a broader ensemble with preexisting complementary conformations, toward binding of cytosine-containing pyrimidine tracts than the C-terminal RRM2, with conformational selection acting as a universal 3’ splice site recognition by U2AF(65).[562] Interestingly, another protein hnRNP A1 can help proofreading the 3’ splice site recognition by U2AF in the selection of AG-containing/uridine-rich RNAs.[564]

## Conclusions: Evolution selected protein ensembles for function

Flexibility is one of the strategies embraced by evolution to adapt to more complex functions at reduced costs.[524] Evolution not only encodes states for direct function; but also propagation pathways for cellular action. These include enzyme catalysis,[69–70,565] recognition of specific DNA regulatory elements by transcription factor binding,[49,414,545] and even harnessing it to stabilize the hyperthermophilic protein well above ambient temperature;[566] ensembles have been exploited for functions of folded and disordered states.[567–568] They allow competent biological responses to the changing environment; they can also trap DNA conformations with non-cognate nucleotides providing a yet another mechanism with evolutionary advantage. Conformational flexibility provides a practical solution for a cell. It may address the need to not only recognize correct binding partners, but also disfavor unwanted interactions. Flexibility can encode (1) preferred sampling of conformations which are functionally-relevant; (2) short time scales from the triggering event to the response; this is particularly the case for enzyme metabolic reaction efficiency; (3) temperature-sensing mechanism to adjust to environment;[569–571] and (4) the crucial allosteric response. Allostery is regulation at a distance by conveying information from one site to another. The effector perturbs the structure of the first site and thereby leads to altered activity in a distant second.[22,61,572–573] Allostery is of paramount importance to the cell, harnessing a fundamental a macromolecular physical property for cell life. Allostery is

based on the free energy landscape;[1] however, rather than the ensemble being static, function is based on the redistribution of states following some structural perturbation. Allostery directly relates the ensemble to dynamic energy landscapes, where there is a shift in the distribution of the *pre-existing* conformational states.[22,25–26,572,574]

The evolution of protein structural ensembles is under functional constraints.[575] Broadly distributed ensembles can help quaternary structure assembly; in support of this, evolutionarily more recent subunits are generally more flexible than older subunits.[271] Along similar lines, p53 appears to have consistently increased its disordered contents during evolution, and cancer-related mutations may have reversed this trend.[576] Apparently intrinsically unstructured proteins with simple sequences evolve more rapidly than those of ordered proteins.[577] Within this framework, there is evolutionary evidence for the importance of linker flexibility.[578] A study that compared the evolvability of weakly active ordered and disordered variants of dihydrofolate reductase by genetic selection observed that scaffolds evolved at similar rates and to similar extents, reaching near-native activity after three rounds of evolution. Evolution of both the ordered and disordered states improved catalytic efficiency indirectly by bolstering the network of dynamic conformational fluctuations that productively couple with the reaction coordinate.[579]

Appreciation of the importance of protein ensembles and allostery – viewed as dynamic shifts of the free energy landscape – is on the rise. This view links biology with chemistry and physics, and provides a basis for a ‘second molecular biology revolution’ which is the energy landscapes of biomolecular function.[90] The free energy landscape is not merely a metaphor; it brings forth new theoretical and a panoply of new experimental approaches for characterizing the key ensembles in both biomolecular assembly and function. The landscape way of thinking is now bearing fruit, helping to understand the chemical basis of biological phenomena, and by so doing, leading the way in prediction and design. The structure-function paradigm that now dominates molecular biology was inspired by the notion that even living things must conform to the laws of quantum mechanics and structural chemistry. The powerful idea that energy landscapes and their dynamic change with the environment can capture the essence of molecular behavior in the cell and in life has far reaching implications in biology. It is challenging to translate it to the gigantic range of scenarios in biological actions. Biomolecular behavior should be described statistically. The immense range of conformational states and substates contain all the possibilities for function in living matter. Biomacromolecules are dynamical objects; they continuously interconvert between structures with varying energies. These fluctuations encode current – and future – functions to be gained whether via evolution to increase organism complexity and diversity or via mutations in disease. Mutations do not lead to new conformations; rather, they too work by shifting the landscape.

Here we only touched the surface with a few examples. Among these, two functions standout: enzyme catalysis and recognition carried by disordered protein states. Disordered states are sometimes still viewed as ‘plastic’. However, they too consist of ensembles – albeit broader and with metastable states with no sufficiently-stable dominant conformation. Importantly, the disordered state acts via the same physicochemical principle: conformational selection of favored (complementary) pre-existing states, which results in

shifting and redistributing the (dynamic) landscape, followed by minor induced fit. In catalysis, enzyme dynamics point to stepwise or combinatorial conformational selection – through ensembles. Conformational ensembles and conformational selection and population shift provide the basic mechanism.[69–70] Since its inception, the free energy landscape theory has transformed approaches to protein folding. Currently, the focus has been shifting from folding to function. The principles are unaltered: rather than consider the entire protein conformational landscape as in folding, attention centers on the ensemble around the bottom of the folding funnel and its dynamics. Population shift is the origin of allostery, and thus of signaling; it crosses protein interfaces,[580] influences multimolecular associations, and signaling pathways across the cell.[34] It exists in proteins, RNA, DNA[581] and membrane lipids,[582–583] including cholesterol.[584] It explains cooperativity. Protein ensembles link fundamental physicochemical principles and protein behavior - and the cellular network and its regulation.

Within this broad framework, here we aimed to highlight the critical importance of a statistical inclusive view anchored in dynamic interconverting ensembles. We believe that it foments powerful biological research under normal physiological conditions, dysfunction in disease, and the evolving molecular translational science. Insight into the hallmarks of the cellular network and its regulation would come from such physicochemical ‘second molecular biology revolution’.

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## Biography

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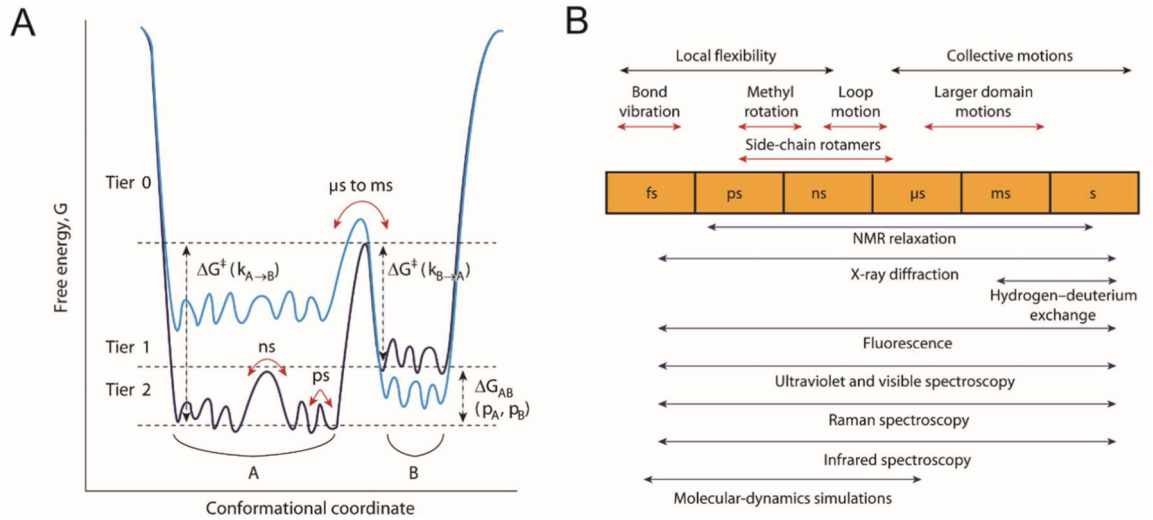


**Figure 1.**

The energy landscape defines the amplitude and timescale of protein motions. (A), One-dimensional cross-section through the high dimensional energy landscape of a protein showing the hierarchy of protein dynamics and the energy barriers. Each tier is classified following the description introduced by Frauenfelder, Sligar and Wolynes and co-workers.[1] A state is defined as a minimum in the energy surface, whereas a transition state is the maximum between the wells. Lower tiers describe faster fluctuations between a large number of closely related substates within each tier-0 state. (B), Timescale of dynamic processes in proteins and the experimental methods that can detect fluctuations on each timescale.[133] (Adapted with permission from reference [133]. © 2007 Macmillan Publishers Limited).

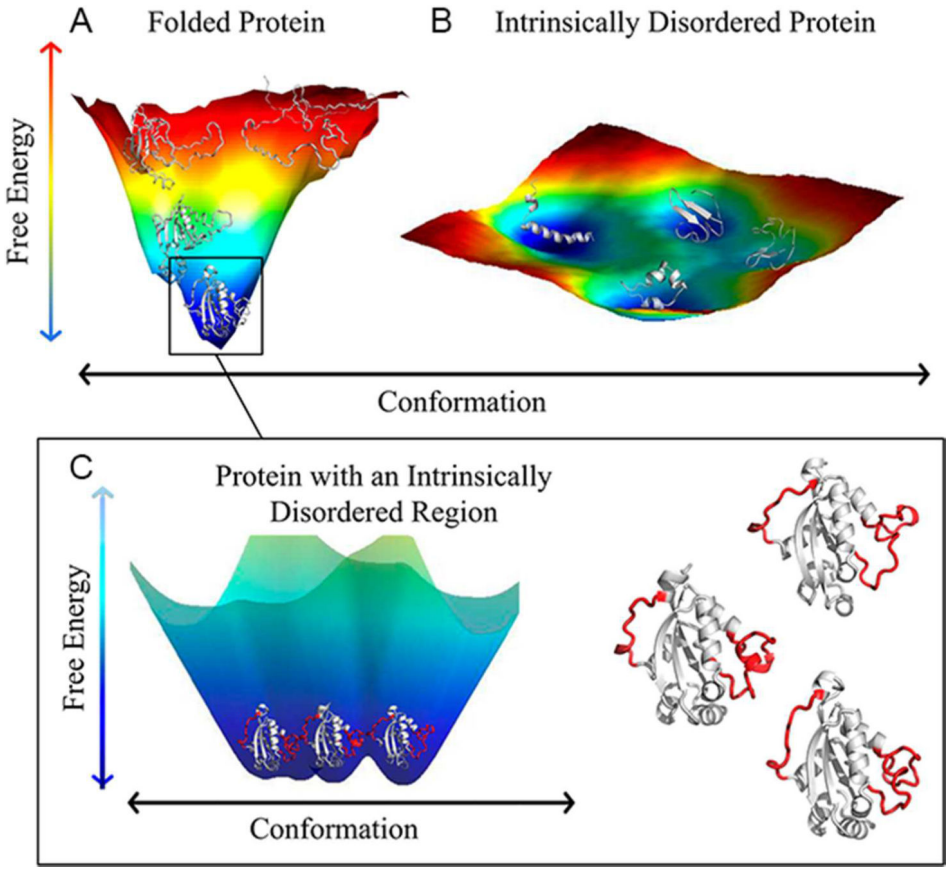


**Figure 2.**

Schematic of energy landscapes.[162] (a) A folded protein (human nucleoside diphosphate kinase (NDPK), PDB ID: 1nsk) and (B) an intrinsically disordered peptide (CcdA C-terminal, PDB ID: 3tcj); (C) close-up of the minimal free energy well in (A), where IDRs are shown in red and ordered regions are shown in white. The example NDPK conformations are shown again enlarged to the right for better visualization. In (C) lower free energy (dark blue) represents more probable conformations. Representative protein conformations were generated with molecular dynamics simulations in CHARMM using coordinates from the 1nsk and 3tcj PDB structures as initial states. Reprinted with permission from reference [162]. 

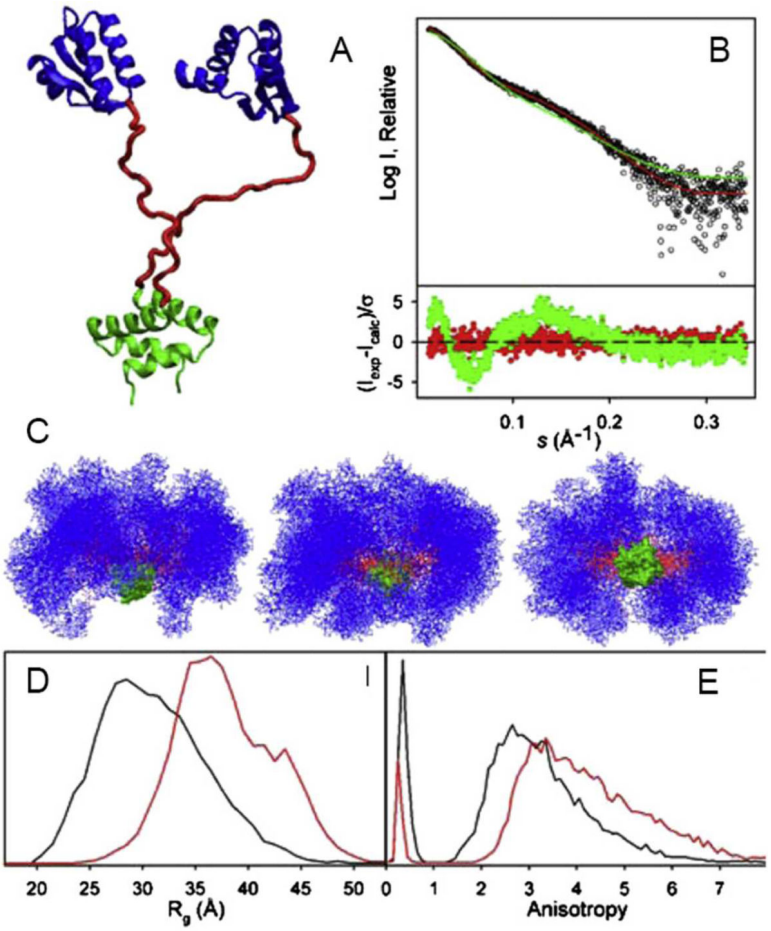


**Figure 3.**

Ensemble optimization analysis of the SAXS profile measured for L12. (*a*) Cartoon of a single L12 conformation, 1rqu, showing the NTD dimer (*green*), the CTD (*blue*), and the linker (*red*). (*b*) Logarithm of the scattering intensity (*black dots*) as a function of the momentum transfer, $s = 4\pi\sin(\theta)/\lambda$. The fitted scattering profile of the optimized ensemble (*OE*), obtained by the Ensemble Optimization Method (EOM) approach, is shown in red. The theoretical scattering curve of the random ensemble (*RE, green line*) is shown for comparison. The bottom panel displays the point-by-point error function for the two ensembles using the same color code. Both ensembles contain 10,000 independent conformers. (*c*) Three orthogonal views of a random subset ($N = 50$) of the OE; color code as in panel *A*. The orientation in the side view (*left*) is the same as in panel *A*. (*d*) Radius of gyration ($R_g$) and (*e*) anisotropy ($A$) distributions for the RE (*black lines*) and the OE (*red lines*). The sharp peaks at $A < 1$ correspond to oblate conformers with populations of 4.8% and 14.2% for the OE and RE, respectively. Reprinted with permission from reference [235]. © 2015 Elsevier B.V.

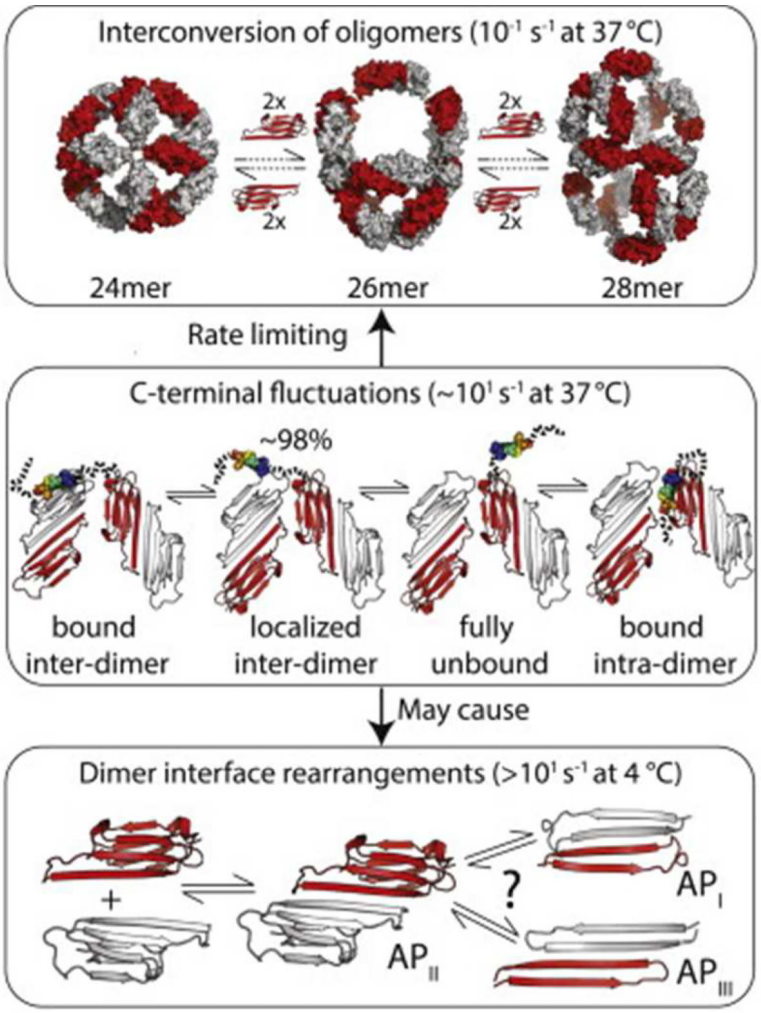


**Figure 4.**

Dynamic movements of αB-crystallin in solution. A model of how the dynamic motions of αB-crystallin at three different time scales are inter-related. The C-terminus is localized to an adjacent dimer with the IXI unbound for the majority of time, but converts on the millisecond time scale into a bound conformation that can be either inter- or intra-molecular (middle panel). This tail-binding may induce distortions in the dimer interface that lead to rearrangements including breaking of the dimer interface or registration shifts (lower panel). Together these two effects determine the rate of subunit exchange between higher-order oligomers, which is ultimately rate-limited by C-terminal fluctuations (upper panel). Reprinted with permission from reference [273]. © 2015 Elsevier B.V.

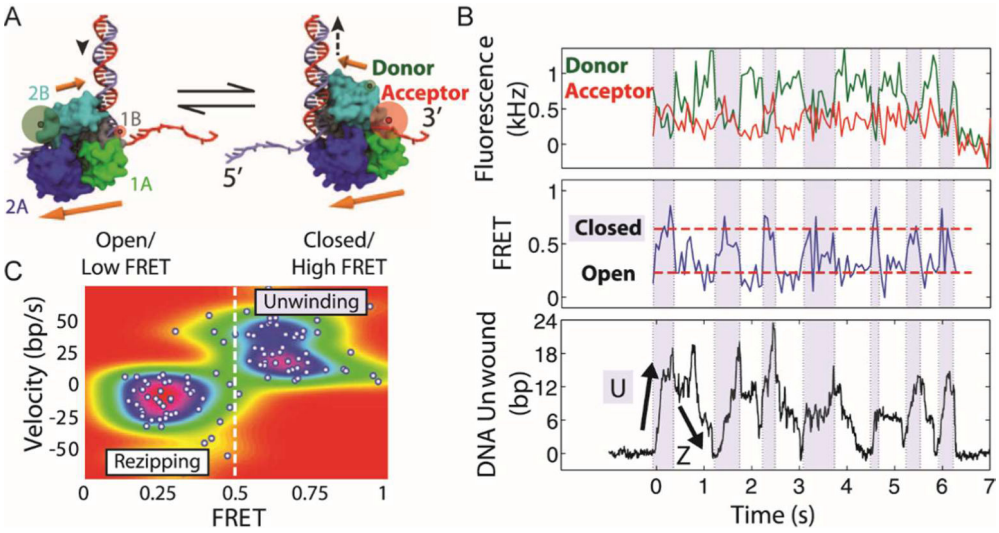


**Figure 5.**

Effect of open and closed UvrD conformation on unwinding and rezipping activity. (A): location of donor and acceptor fluorophores for smFRET measurement and model of UvrD conformational switching. Upper (and lower) orange arrows denote 2B (and 1A-2A) domain orientation. (B): a representative time trace of monomeric UvrD conformation and activity. (C): correlation between UvrD activity and conformation. The color map represents the probability distribution of FRET state and velocity. Adapted with permission from reference [358]. © 2015 American Association for the Advancement of Science.

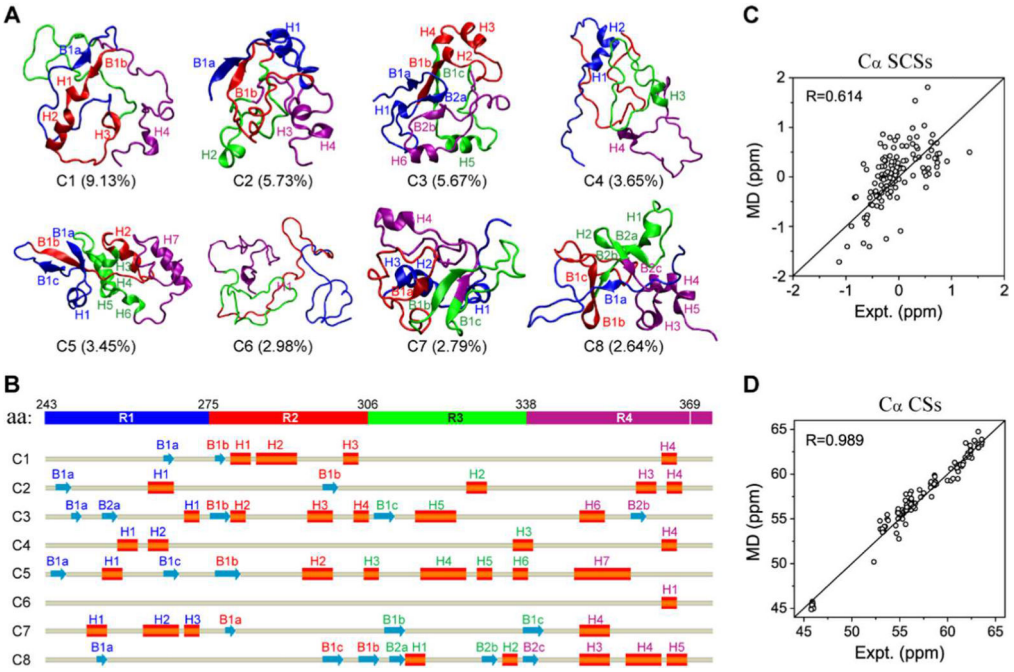


**Figure 6.**

Structural analysis of K18 monomer in aqueous solution at 310 K. (A) Representative conformations for the top eight most-populated clusters (labeled by "Cn", n=1~8) along with their corresponding probabilities. Secondary structures are displayed in new-cartoon style, with different colors representing different repeats, blue for R1, red for R2, green for R3, and purple for R4 and the last four residues after R4. For each structure, helices are indicated with H1, H2, …, and β-sheets are labeled with B1, B2, …; β-strands in the same sheet are labeled with B*n*a, B*n*b, B*n*c...(*n* = 1, 2, …). Two adjacent β-strands (for example, a and b) are labeled using two neighboring letters in the alphabet. (B) Sequence views of the eight clusters. The amino acid (aa) residue numbering is based on the full-length 441-aa tau protein. The β-strand is shown with a blue arrow and the helix with a red cylinder. Each helix/β-strand is labeled using the same label and color as used in (A). (C, D) Scatter plots comparing experimental (Expt.) and SPARTA-predicted (MD) chemical shifts (CSs) and secondary chemical shifts (SCSs) of the Cα atom. The Pearson correlation coefficients (R) between experimental and MD-generated CSs and SCSs are indicated. Adapted with permission from reference [370]. © 2015 American Chemical Society.

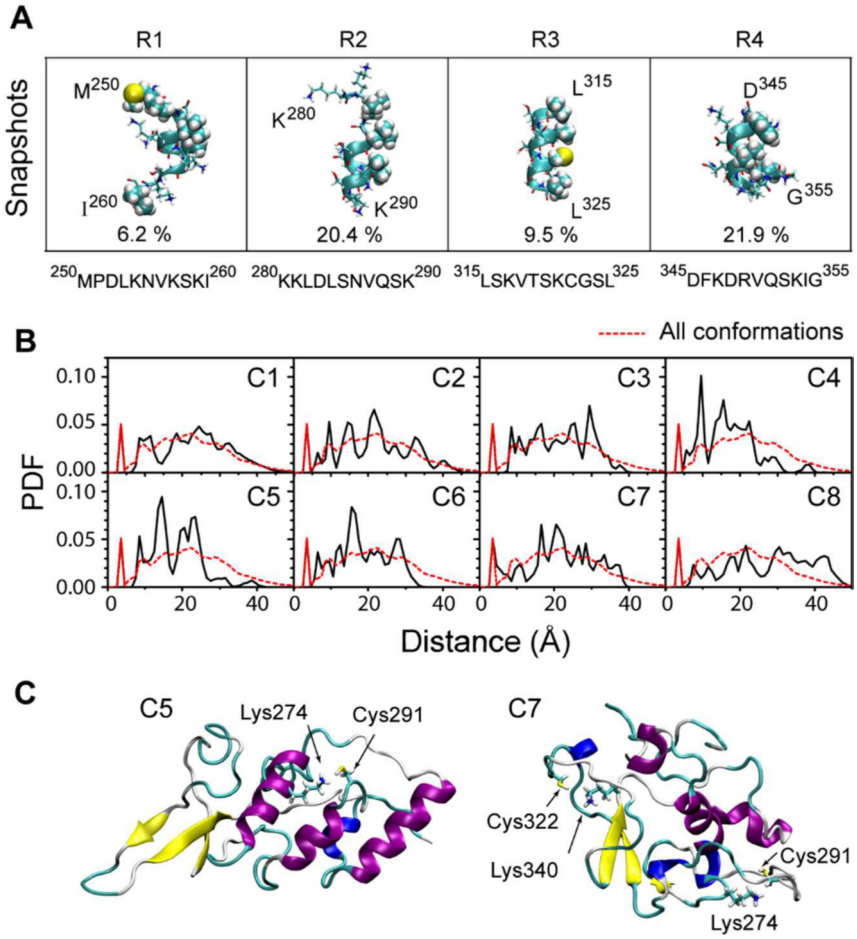


**Figure 7.**

Analyses of conformations of middle segments in each repeat and of the contacts between lysine and cysteine residues in K18. (A) Representative structure of the most populated helical conformation in each repeat. Helical structures are mainly located in the middle region of each repeat: i.e. $^{250}$MPDLKNVKSKI$^{260}$ in R1, $^{280}$KKLDLSNVQSK$^{290}$ in R2, $^{315}$LSKVTSKCGSL$^{325}$ in R3, and $^{345}$DFKDRVQSKIG$^{355}$ in R4. The most populated helix in each repeat was identified by performing a RMSD-based cluster analysis using a backbone-RMSD cutoff of 3 Å. (B) Lys-Cys minimum-distance probability density function (PDF) for conformations in the top eight most-populated clusters (C1~C8). (C) Representative conformation of C5 and C7 showing the close contact between lysine and cysteine residues. Adapted with permission from reference [370]. © 2015 American Chemical Society.

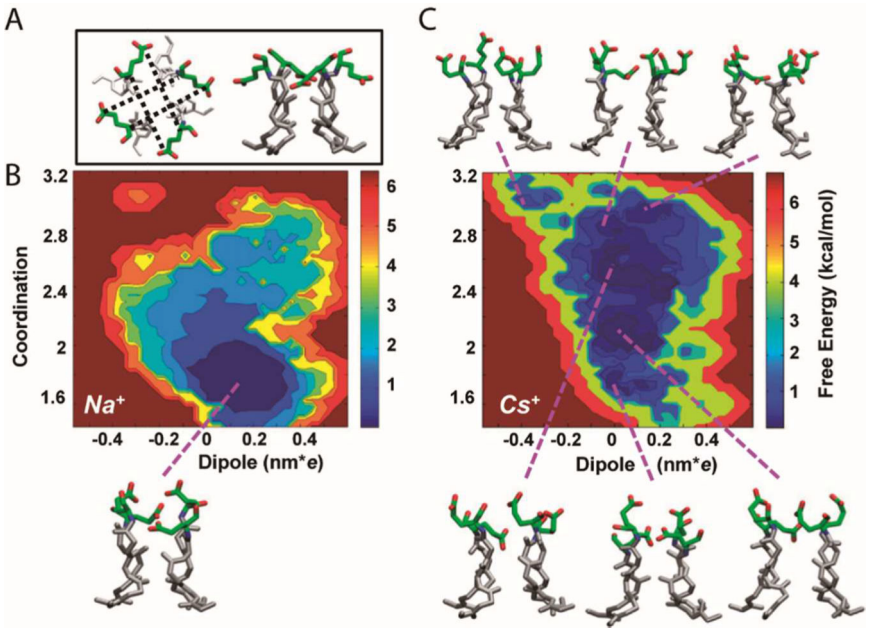


**Figure 8.**

Free energy landscape of the Na+ and Cs+ systems. (A) Top and side views of the selectivity filter in the crystal structure are shown (Glu66 in green sticks; oxygen atoms are colored red). In the top view (Left), the distances between the carbonyl and carboxylic group of Glu66 in different monomers are shown as black dotted lines. (B, C) The free energy landscape as a function of the z dipole [nanometers for electron charge (nm*e)] and of the Glu66-Coordination variable for the Na+ (B) and Cs+ system (C). The variable Glu66-Coordination counts the number of carboxylate and carbonyl groups of Glu66 in opposite monomers whose distance is larger than 8 Å. Possible conformations of Glu66 residues corresponding to different minima are shown for both the Na+ and the Cs+ systems. This figure is adopted from reference [394] with permission. Adopted with permission from reference [394]. © 2015 National Academy of Sciences.

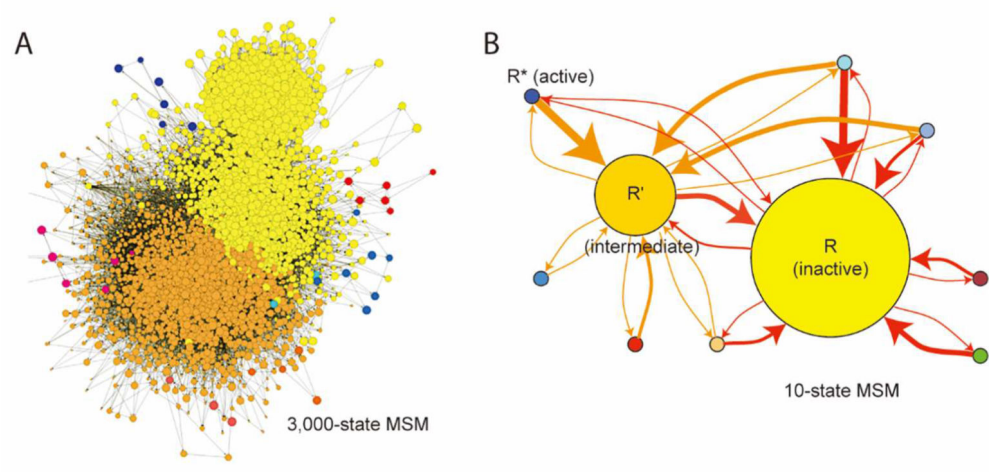


**Figure 9.**

(A) Network representation of the 3000-state MSM built from the simulations of agonist-bound GPCR with each circle representing an individual conformational state. (B) 10-state MSM built from the 3000-state MSMs using spectral clustering methods to identify kinetically relevant states. The circles in the 3000-state MSM are colored according to their membership in the coarse-grained 10-state MSM. The weight of arrow indicates the transition probability between states. Reproduced with permission from reference [401]. © 2013 Macmillan Publishers Limited.

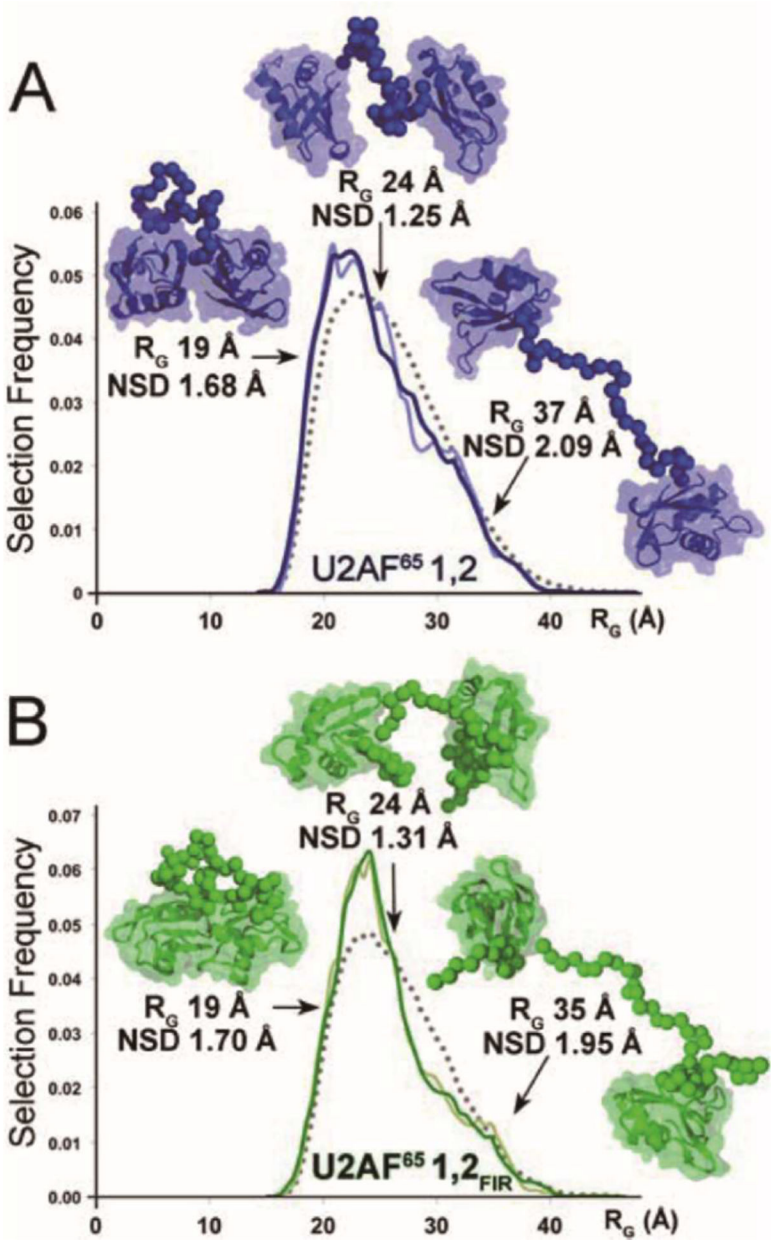


**Figure 10.**

The 20-PDB (light color) or 50-PDB (dark color) ensemble fits of (A) U2AF$^{65}$1,2 (blue) and (B) U2AF$^{65}$1,2$_{FIR}$ (green) SAXS data. The radii of gyration ($R_G$) are plotted on the x-axis, and the frequency of a structure with a given $R_G$ on the y-axis. Gray dashed lines plot the randomized starting pool; Solid lines the selected pool. The most typical or divergent selected structures are inset. Reproduced with permission from reference [563]. © 2015 American Chemical Society.

**Table 1.**

Selected proteins whose dynamics and conformational ensembles have been characterized by NMR/SAXS experiments. This table gives the NMR/SAXS parameters along with the combined ensemble selection methods used to describe conformations of these proteins.

| NMR/SAXS parameters | Ensemble fitting approaches | Protein type | Protein name | Refs. |
|---|---|---|---|---|
| RDCs | MD simulations | Single-domain protein Multi-domain protein | Ubiquitin Calmodulin-IQ complex | 200 338 |
| SAXS | EOM EOM 2.0 | Multi-domain protein | BTK Calmodulin | 311 335 |
| SAXS | amplified collective motions (ACM) | Multi-domain protein with hinge motion | T4 lysozyme, formin-binding protein 21 | 223 224 |
| RDCs, SAXS | Flexible-Meccano, MD simulations | IDP | p53 | 339 |
| CSs, RDCs, PREs, SAXS | ENSEMBLE | IDP in protein complex | Sic1 in Sic1-Cdc4 complex | 340 |
| PREs, RDCs, SAXS | Flexible-Meccano ASTEROIDS | IDP Multi-domain protein IDP in protein complex | Tau U2AF65 MKK7 in MKK7–JNK signaling complex | 323 251 341 |