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What protein folding teaches us about biological function and molecular machines

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Protein folding was the first area of molecular biology for which a systematic statistical–mechanical analysis of dynamics was developed. As a result, folding is described as a process by which a disordered protein chain diffuses across a high-dimensional energy landscape and finally reaches the folded ensemble. Folding studies have produced countless theoretical concepts that are generalizable to other biomolecular processes, such as the functional dynamics of molecular assemblies. Common themes in folding and function include the dominant role of excluded volume, that a balance between energetic roughness and geometrical effects guides dynamics, and that folding/functional landscapes are relatively smooth. Here, we discuss how insights into protein folding have been applied to investigate the functional dynamics of biomolecular assemblies.

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A central tenet in the study of folding is that motion is accurately described as diffusion along an underlying energy landscape. With knowledge of the energy over the full range of 3N-dimensional phase space, all thermodynamic and kinetic properties may be directly evaluated. While, in principle, this would provide a complete quantitative description of the dynamics, such a complex characterization would be difficult, if not impossible, to conceptualize. Accordingly, there have been significant efforts to establish interpretable topographical metrics that properly account for the range of dynamic processes that span orders of magnitude in time and length.

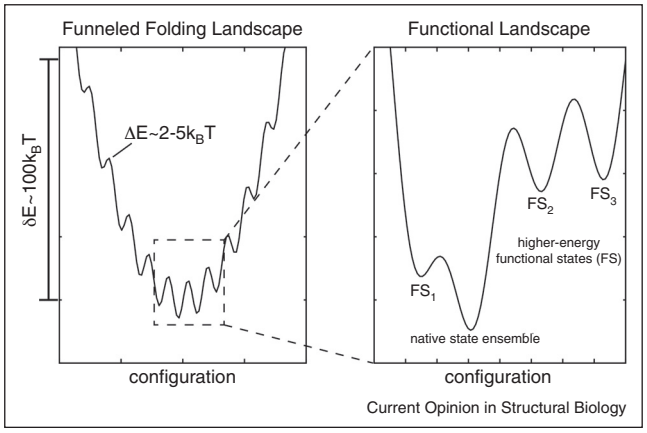
Two seminal advances in the description of protein folding landscapes were the Principle of Minimal Frustration [1] and the folding funnel [2]. Building on the theory of glass-forming liquids, Bryngelson and Wolynes showed that the most relevant quantities when describing these landscapes are the energy gap between the folded and unfolded ensembles δE and the scale of the energetic roughness ΔE (Figure 1). That is, for a protein sequence to fold on physiological timescales, $\delta E/\Delta E$ should be sufficiently large. Subsequently, Leopold *et al.* [2] used simulations to explore energy functions of varied roughness, which provided the first pseudoatomic description of the kinetics that result from minimally frustrated landscapes. They found that as folding proceeds, the number of accessible substates gradually decreases, until the low-configurational entropy folded ensemble is reached. Visually, the landscape adopted the shape of a funnel centered about a dominant basin of attraction. For the sake of clarity, it is important to note that while transitions between unfolded configurations are fast, the number of accessible unfolded states is orders of magnitude larger than the number of folded states. Thus, during each folding event, only a fraction of the unfolded ensemble is transited, and the full range of unfolded configurations is sampled in the long-time limit.

Since the development of the energy landscape theory of protein folding, these descriptions have been extended to biomolecular function, which is often associated with rearrangements between low-energy states (Figure 1). Below, we discuss how advances in protein folding have provided insights into functional dynamics.

The dominant role of sterics and native interactions

It is becoming clear that common physical properties guide folding and function. The Principle of Minimal Frustration indicates that a biomolecule's landscape may be described as being relatively smooth, where the native and functional configurations correspond to low-energy minima (Figure 1). This principle has been instrumental in the development of a broad range of models for biomolecular dynamics in complex environments, such as folding in the presence of membranes [3••]. One approach to studying minimally frustrated systems is through the use of 'structure-based' models, which span from coarse-grained [4] to all-atom [5] resolution. Since, traditionally, only native interactions are stabilizing in structure-based models, one naturally asks what is left to limit the kinetics? The answer is simple: The barriers,

Figure 1



The theory of protein folding has shown that the energy landscapes of proteins are relatively smooth, where there is a large energy gap δE between folded and unfolded ensembles and a small degree of roughness ΔE. At the bottom of these funnel-like landscapes are the native state ensemble and functional states. As we characterize biomolecular function, many of the concepts and tools developed for folding are proving to be effective at elucidating the properties of this subspace of the global landscape.

kinetics and accessible routes are determined by the connectivity of native contacts and atomic excluded volume interactions.

During folding, the atomic excluded volume and covalent geometry restrict many motions and give rise to 'Topological Frustration' [6]. In contrast to conventional frustration, where energetic roughness arises from stabilizing non-native interactions, topological frustration is associated with the formation of *native* contacts. If specific native contacts form early in the folding process, there can be insufficient space for the chain to reorganize, leading to large energetic barriers from excluded volume interactions. These large barriers can slow the kinetics, or make specific routes kinetically inaccessible. For example, in the protein IL-1β, early formation of a functional loop leads to a large barrier for folding, where part of the native structure disassembles and reforms before the protein reaches the folded configuration [6,7]. Perhaps the most dramatic examples of topological frustration can be found in knotted proteins, where early formation of a loop requires that the tail thread through a confined space [8,9] (Figure 2). While it may appear likely that slow folding kinetics could then result from the formation of non-native contacts, all-atom explicit-solvent simulations indicate minimal roughness is associated with the threading process [10•], consistent with the recent analysis of explicit-solvent simulations of folding for knot-free proteins [11]. In contrast to intra-protein excluded volume effects, which impede folding, steric contributions from crowding agents can stabilize the folded ensemble by reducing the phase space accessible in the unfolded ensemble [12–14], where the specific shape of the crowder affects its precise impact on folding [15••]. Together, these findings illustrate the critical role of excluded volume during folding in solution.

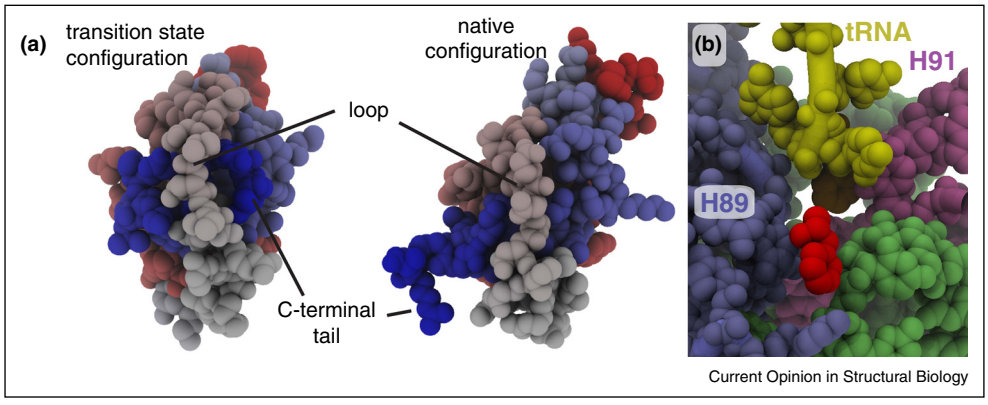
The effects of sterics during folding are echoed by observations in machines. For example, folding-inspired all-atom structure-based models [5] have shown that steric hindrance-induced barriers are also common in the ribosome. In our first application of these models to the ribosome [16], we found that the atomic excluded volume leads to at least three distinct barriers during aa-tRNA accommodation. Recently, these models suggested a similar sterically related intermediate ensemble during tRNA hybrid-state formation, as well [17]. From these simulations, each barrier is unambiguously attributed to a specific structural feature, similar to the identification of steric barriers in topologically frustrated proteins.

Folding, disorder, function and machines

Grounded in energy landscape theory, early studies of adenylate kinase predicted that the protein may undergo localized unfolded events during functional transitions [18], a process commonly referred to as 'cracking'. The ability of proteins to crack can be rationalized by the fact that the folded ensemble is composed of a diverse set of configurations that have varied degrees of native structure and stability. The diversity of the native ensemble of a protein is illustrated by simulations with structure-based models that predict 70–90% of the native contacts are formed in the native ensemble [4,5]. In addition, simplified models [5] and explicit-solvent models [19] predict that folding may be described as two partially separable processes: backbone collapse and side-chain ordering. Accordingly, when a protein interconverts between functionally relevant configurations, less-stable residues may spontaneously enter disordered states, while the molecule remains predominantly folded. Initially controversial, cracking has since been detected in simulations and experiments for many proteins, including adenylate kinase [20–22], kinesin [23], calmodulin [24], protein kinase A [25] and EGFR kinase [26•,27]. The ability of proteins to utilize unfolded phase space to regulate function indicates that there is a broad range of modes by which functionality is encoded in a specific sequence.

Cracking may be generalized as any conformational process where large changes in configurational entropy are present. For the ribosome, our early simulations with simplified models predicted a reduction in the available phase space as tRNA molecules accommodate (Figure 3b). More recently, it was demonstrated that this change in configurational entropy may contribute to a temperature dependence of the rates [28••]. This concomitant reduction in effective energy and configurational entropy is reminiscent of protein folding, where enthalpy stabilizes

Figure 2



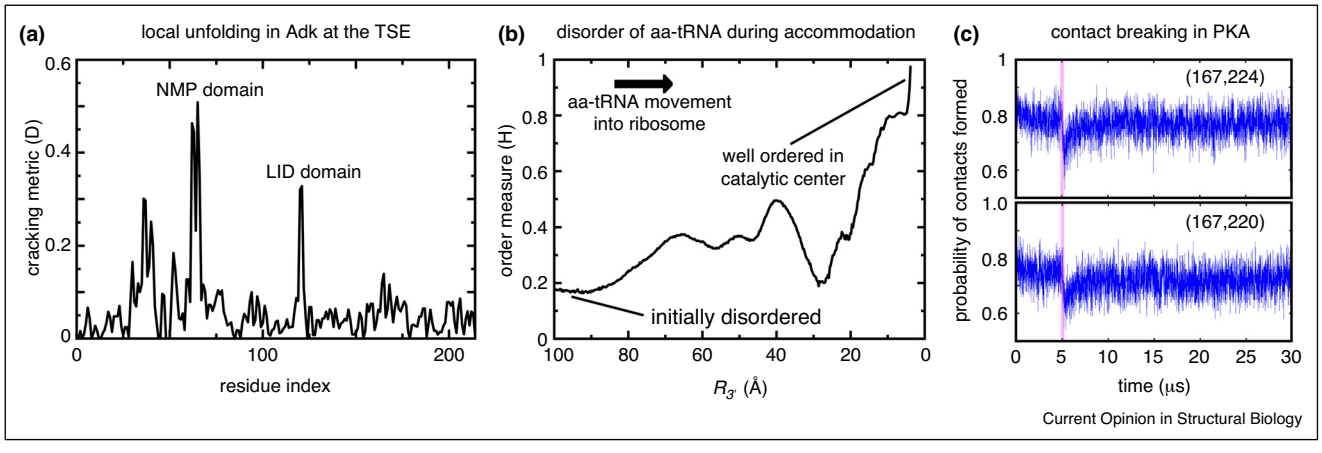
Steric interactions are indispensable contributors to folding and functional dynamics. **(a)** A prime example of the dominant role of sterics is provided by knotted proteins, where the rate of knotting through a compact loop (left) is governed by the precise molecular geometry. **(b)** As tRNA molecules (yellow) move through the ribosome, significant steric obstacles (H89 and H91) are imposed, which limit interconversion rates and accessible conformational routes. Structural snapshots taken from Refs [16,43].

the folded ensemble and entropy stabilizes the unfolded ensemble. While the biological implications of this balance are not fully understood, it is likely that it serves the purpose of delaying entry of the amino acid, thereby enabling the use of 'proofreading' mechanisms by the ribosome. This elegant connection between physical chemistry and biological function emphasizes the essential role that entropy has during folding and function.

Reaction coordinates for folding and function

To accurately describe the energy landscapes of folding, it is necessary to use an appropriate reaction coordinate ρ, or set of coordinates $\{\rho_i\}$. At the most fundamental level, a proper coordinate should describe movement along the lowest-free energy pathway/s connecting the endpoints. When describing folding, configurations identified as being in the transition state ensemble (i.e. $\rho = \rho^{\mathrm{TSE}}$) will then be equally likely to continue to the folded and unfolded ensembles, and the coordinate will be a monotonic function of the folding process. Strictly speaking, one may also desire that the projected dynamics is Markovian at all points along the coordinate [29]. However, for sufficiently large barriers, the overall kinetics are well described by the properties of the TSE, and the remaining coordinate space

Figure 3



During functional conformational rearrangements, fully folded biomolecules often transiently partially unfold, or crack. **(a)** Adenylate kinase (Adk) has been shown to crack during functional rearrangements before phosphoryl transfer [20]. **(b)** During accommodation, tRNA molecules enter a disordered state immediately preceding entry into the ribosome. Here, the Hellinger distance (*H*) of the dihedral orientation from a random distribution was calculated as a function of tRNA position, extracted from Figure 4 of Ref [16]. **(C)** Upon ATP binding (pink line) by protein kinase A (PKA), numerous native contacts (residue numbers in parentheses) transiently break and reform [25].

may be of secondary interest. Since the TSE is defined as configurations for which the committor probability P_{fold} is equal to 0.5, it may be argued that it is necessary to calculate P_{fold} for every candidate configuration. Cho *et al.* showed that such a calculation is unnecessary for many single-domain proteins, since geometrical coordinates (e.g. the number of native contacts, Q) can identify configurations for which $P_{\text{fold}} = 0.5$[30]. Alternate approaches to identifying reliable coordinates are to compare the projected barrier and diffusion coefficient with the global kinetics [31], or to design collective coordinates that are diffusive by construction [32]. A relatively straightforward method for optimizing coordinates was introduced by Best and Hummer, who showed that for coordinates that accurately capture the barrier, and along which the motion is Markovian, the probability of being on a transition path $P(\text{TP}|\rho)$ will reach a value of 0.5 at the TSE [33]. Consistent with Cho *et al.*, by evaluating $P(\text{TP}|Q)$ for explicit-solvent simulations of protein folding, Best *et al.* found Q is an accurate predictor for when the system is on a transition path [11]. In a clever implementation of Bayesian statistical analysis, they also demonstrated that formation of specific non-native interactions is not correlated with the folding process. Accordingly, these results indicate that native-centric structure-based models provide an energetic description for folding that is consistent with explicit-solvent simulations, as also observed for conformational transitions [27].

Identifying reaction coordinates that accurately describe the dynamics is necessary when studying any biomolecular transition. As discussed, it is well established that low-dimensional projections can accurately describe folding dynamics, where coordinates may be collective (e.g. Q), or simple distance metrics. For the ribosome, two recent studies have attempted to quantitatively identify appropriate low-dimensional coordinates. For subunit rotation, explicit-solvent simulations were used to identify sets of atoms that capture diffusive reorientation dynamics of large domains [34•]. A more rigorous analysis was performed for tRNA dynamics [28••], where the tRNA-ribosome complex was simulated and many barrier-crossing transitions were observed (≈100), which allowed the probability of being on a transition path $P(\text{TP}|\rho)$ to be calculated for over 200 candidate coordinates. To summarize the results, those calculations showed that changes in specific tRNA–tRNA atomic distances are highly indicative of when the system is undergoing a transition, just as Q is an accurate marker for folding events.

Effective diffusion and barrier-crossing events

In addition to elucidating mechanisms, energy landscape theory provides quantitative tools, such as effective diffusion along low-dimensional projections. That is, one may average over coordinates that are orthogonal to the folding process and consider the short-scale averaged free energy and diffusion along one dimension. The mean first passage time $\langle\tau\rangle$ between endpoint states (i.e. folded and unfolded ensembles) may then be expressed as [1]:

$$\frac{1}{k} = \langle\tau\rangle = \int_{\rho_{initial}}^{\rho_{final}} d\rho \int_{\rho_{\min}}^{\rho} d\rho\prime \frac{\exp[(G(\rho) - G(\rho'))/k_B T]}{D_\rho^{eff}(\rho)}, \quad (1)$$

where $D_\rho^{eff}(\rho)$ is the effective diffusion along the coordinate, $G(\rho)$ is the free energy and $\rho_{initial}$ and ρ_{final} are the endpoint values of the coordinate. One may compare the effective (or apparent) diffusion to the free diffusion, in order to infer the scale of the roughness from the relation:

$$D_\rho^{eff} = D_\rho^{free} e^{-(\Delta E/k_B T)^2}. \quad (2)$$

When D^{eff} and $G(\rho)$ are not available, it is convenient to approximate $\langle\tau\rangle$ as:

$$\langle\tau\rangle = C e^{\Delta G^{\text{TSE}}/k_B T}, \quad (3)$$

where the prefactor C is interpreted as the barrier-crossing attempt frequency and ΔG^{TSE} is the height of the free-energy barrier. Together, these three equations provide a relatively simple quantitative relationship between the short-scale roughness, large-scale barriers, diffusion and kinetics, which may be applied to most large-scale conformational transitions.

Many experimental and theoretical efforts have aimed to quantify the effective diffusion, short-scale roughness and exponential prefactors associated with folding. From a theoretical perspective, algorithms have been developed and employed to extract D_ρ from simulations, ranging from Bayesian analysis of equilibrium trajectories [31], to fitting dynamics to the short-time solution of the Fokker–Planck equation [35], to applying quasi-harmonic approximations [36]. Approaches have also been developed for extracting the scale of roughness from pulling experiments [37], and FRET measurements have implicated a dependence of D on protein compaction [38]. Overall, these studies have suggested that $\Delta E \approx 1$–$5 k_B T$, and that the solvent-averaged folding free-energy barriers are typically 2–$10 k_B T$. With regard to the exponential prefactor, Eaton and co-workers used experimental observations of the kinetics of secondary and tertiary rearrangements to argue that C is on the scale of 1 μs^{-1} [39]. Similarly, Thirumalai and Hyeon used theoretical arguments to show that C is a comparable value for small RNA folding [40]. In a purely computational approach, Shaw *et al.* performed folding simulations in explicit solvent and inferred an attempt frequency of ≈1 μs^{-1} [19]. These independent and complementary approaches are consistently indicating that the barrier crossing attempt frequency for folding is on the order of inverse microseconds.

While at a more nascent stage of development, similar questions have been posed for the ribosome. We attempted to describe diffusion of tRNA entry into the ribosome in explicit-solvent simulations (200–300 ns each) and estimated $D^{eff} \approx 1\ \mu m^2/s$ [41]. This is similar to experimental measures of tRNA diffusion in solution, suggesting $\Delta E < 1k_BT$. When tRNA moves inside of the ribosome, the D^{eff} decreases [34•], suggesting ΔE increases to $\approx 2–3k_BT$. Consistent with a broad range of studies on protein folding, these calculations suggest there is a relatively small degree of roughness associated with functional motions of the ribosome.

The future of function

Over the last ten years there has been a remarkable expansion in our understanding of biomolecular function. In contrast to earlier views, where functional rearrangements were largely described as discrete steps between well-defined endpoints, we now know that biomolecular stability and disorder are intimately related to kinetics. This broadened perspective has revealed that order–disorder transitions are a fundamental feature of many rearrangements, where some molecular machines transiently enter partially disordered transition states, and others exploit a delicate balance between order and disorder to signal for activity. With this plethora of newly identified disorder phenomena in biology [42], it should come as no surprise that the theoretical foundation established for folding has been instrumental in elucidating function. In the coming years, it will be exciting to see to what extent principles from folding can guide the design of more effective experiments that will ultimately enable quantitative control of biological machines inside of the cell.

Conflict of interest statement

Nothing declared.

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