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Exploring the top of the protein folding funnel by experiment

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While there have been impressive advances in understanding protein folding over the past few decades, we are still far from the goal of solving the protein folding problem: predicting the folding pathway and final structure entirely from the amino acid sequence. One reason for this shortcoming may be the lack of understanding of the complexity of the unfolded state before folding and earliest steps in the process. Recent technological advances and applications of cutting edge techniques in novel ways have begun to reveal this complexity. Comparing the kinetics with recent molecular dynamics simulations on the microsecond timescale may lead to more detailed and predictive folding models.

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Introduction

For twenty-five years, energy landscape theory has given us a theoretical framework to think about protein folding [1–3]. The famous folding funnel strongly suggests that there are many ways to fold a protein even if there is a single, global, free energy minimum, the folded structure. Unfortunately, energy landscape theory has had limited ability to make predictions and, with the exception of native-centric models that start with known structure of the folded state [4], few quantitative comparisons with experiment. On the other hand, the success of site-directed protein mutagenesis, stopped-flow mixing and various NMR and single-molecule methods [5–8], have produced a wealth of information about folding rates, residual structure in the denatured state, and the formation of structure on the millisecond timescale. The overwhelming conclusion of these various experimental studies has been very simple models of folding pathways such as two-state models or small numbers of obligate intermediates [9]. Experimentally, there has been very little evidence of multiple paths from the unfolded to the folded state.

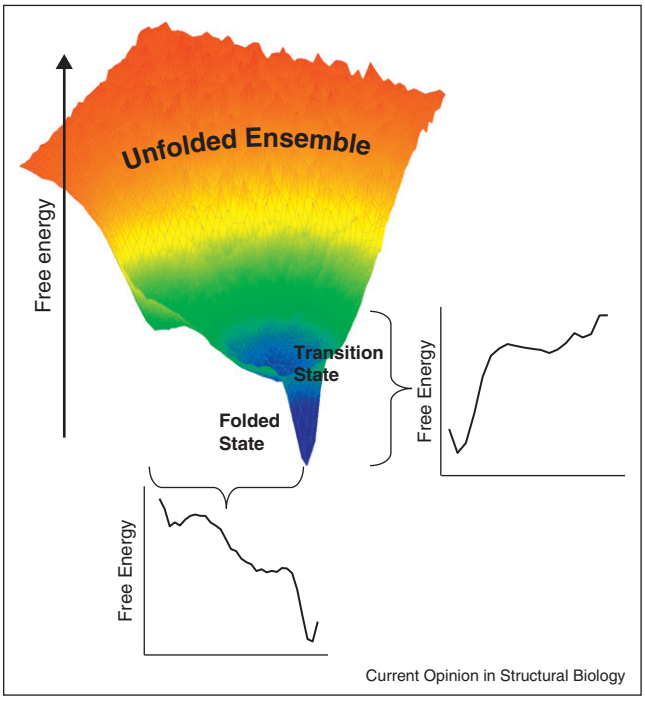
If heterogeneity of folding pathways exists, it stands to reason that such behavior would be easier to see at earlier stages of folding when there is no large energy barrier and when the ensemble of conformations is also broad. In terms of the energy landscape theory, the observed lack of frustration in foldable sequences leads to a landscape that is smooth and funnel-like. The depth is determined by the decrease in free energy upon folding, and the width of the funnel at any energy is determined by the entropy. In this picture the native state lies at the bottom of the funnel as does the transition state and native-like intermediates (see Figure 1) that are often discovered experimentally by methods such as hydrogen exchange and ϕ-value analysis. Thus most of the energy-space and entropy-space remain unexplored by conventional methods with millisecond time resolution.

Laser temperature-jump (T-jump)-induced folding or unfolding, which was developed fifteen years ago, provided researchers a tool to explore folding on the nanosecond to millisecond timescale on a wide range of proteins (i.e. any protein that can be folded or unfolded within the temperature range of water) [10–14]. Over the past ten years, we have seen development of several microfluidic mixers which allow the observation of an even wider range of proteins and also allow direct observation of the folding rate because the solution conditions can be rapidly switched from fully denaturing to almost fully physiological [15–22]. Over the past few years, these techniques have really come into their own by studying larger, more complex proteins, comparing experimental results between T-jump and mixing, between different folding probes and with simulation, which has also had several technological developments.

Heterogeneity in the earliest stages of folding

Given that the early stages of folding may be heterogeneous, how can this heterogeneity be probed? Obviously, an experiment that examines many single molecules may find heterogeneity and a few studies have begun to show this. For example, Yu *et al.* measured folding of the prion protein with single molecule force spectroscopy and found three off-pathway misfolded states accessible from only the unfolded state which likely are on pathway for aggregation [23]. Pirchi *et al.* have studied adenylate kinase using single molecule FRET methods and analyzed trajectories with a hidden Markov Model to identify metastable states on the landscape [24•]. At high concentrations of denaturant the flux between states was mostly sequential from low FRET states to high FRET states, or vice versa, suggesting a small number of similar pathways. But under very low denaturant concentrations in which folding is more likely, the flux was much more parallel. Adenylate kinase is a fairly slow folder so it was possible to capture these

Figure 1



View of protein folding from the top of the folding funnel. The protein can undergo a large free energy change as it folds, but most experiments on the millisecond timescale only capture the deepest part of the landscape (brackets). Kinetic measurements using a single probe may only probe the landscape along one coordinate and conclude the protein folds as a two-state (side inset) or three-state (bottom inset) system, depending on which part of the landscape is sampled. While the funnel is relatively smooth, avoiding frustration, roughness on the landscape is not insignificant (gray lines) producing more complexity in the folding paths because reconfiguration within the unfolded ensemble is fairly slow.

dynamics on the millisecond and longer timescale, but technological hurdles remain to watching such dynamics on faster timescales. The primary challenge of fast single-molecule fluorescence experiments is that at laser powers high enough to capture more than 5 photons per millisecond, photobleaching and long-lived triplet states of the fluorophores dominate the observations. Recently a new strategy of photo-protection has been demonstrated that works by rapidly quenching triplet states and scavenging oxygen singlet states and radicals that cause photobleaching [25]. This method allowed observation of dynamics with 50 μs resolution of the protein BBL and showed a single conformational ensemble at all denaturant concentrations, confirming earlier bulk measurements of downhill folding [26•]. However, since most proteins have a large energy barrier under folding conditions and few transitions from folded to unfolded states, single molecule microsecond dynamics of folding are still hard to observe.

There has been a long disagreement between experiments that probe unfolded conformations with single molecule FRET and small angle X-ray scattering (SAXS). Earlier measurements have shown that the radius of gyration (R_G) does not seem to change significantly with denaturant concentration, and a small number of mixing experiments show no change in R_G within the mixing time (or burst phase) of a stopped-flow mixer after dilution of denaturant. However, several single molecule studies in equilibrium show a significant compaction as denaturant is decreased, particularly below the denaturation midpoint. A recent paper from Yoo *et al.* [27] repeated SAXS measurements on the B1 domain of protein L, the subject of single-molecule studies by at least 3 groups [28–30], and still finds divergence in the measurements of the size of the protein by the different methods at a wide range of denaturant conditions. Thus the controversy has not been resolved, but adds evidence to extreme heterogeneity within the unfolded state.

Rapid folding kinetics

Observation of downhill folding in bulk also shows heterogeneity of folding pathways since the ensemble observations are not dominated by a large free energy barrier. The two best-known microsecond downhill folders, variants of BBL and lambda repressor (λ^{6-86}) have shown kinetics using different probes of folding that yield different rates using both T-jump and microfluidic mixing to prompt folding or unfolding (see Figure 2a) [31–33]. However, even NTL9, a two-state folder that folds in about 1 ms, shows differences in kinetics between ordering of the peptide backbone and one sidechain [34]. Some of these results have been analyzed by simple, one-dimensional energy landscapes in which each folding probe is represented by a unique switch along the reaction coordinate [35]. If the landscape has a large energy barrier switches at different locations will exhibit identical kinetics, but on a barrierless landscape probes with early switches will have faster kinetics than probes with later switches. Of course, it is possible that these downhill 1-d landscapes could be replaced with higher-dimension landscapes with barriers, and probe dependence could still be observed for different pathways [36]. This was the conclusion of another study of α_3D, a sequence designed to fold downhill [37•].

It is also possible to observe differences in folding kinetics with the same probe when folding is prompted by different methods. A hint of such behavior was seen in the FiP35 WW domain when slightly different kinetics were observed for different sized T-jumps ending at the same temperature [38]. A more recent study comparing villin headpiece subdomain (HP35) folding kinetics using T-jump and ultrarapid mixing found rates that were 5 times slower for mixing experiments ending at the same temperature and denaturant concentrations (see Figure 2b) [39]. This suggests that the unfolded ensembles sampled by these two experiments are distinctly different. The mixing experiment begins with proteins