REVIEW

How cooperative are protein folding and unfolding transitions?

Pooja Malhotra and Jayant B. Udgaonkar*

National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bengaluru 560065, India

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Abstract: A thermodynamically and kinetically simple picture of protein folding envisages only two states, native (N) and unfolded (U), separated by a single activation free energy barrier, and interconverting by cooperative two-state transitions. The folding/unfolding transitions of many proteins occur, however, in multiple discrete steps associated with the formation of intermediates, which is indicative of reduced cooperativity. Furthermore, much advancement in experimental and computational approaches has demonstrated entirely non-cooperative (gradual) transitions via a continuum of states and a multitude of small energetic barriers between the N and U states of some proteins. These findings have been instrumental towards providing a structural rationale for cooperative versus noncooperative transitions, based on the coupling between interaction networks in proteins. The cooperativity inherent in a folding/unfolding reaction appears to be context dependent, and can be tuned via experimental conditions which change the stabilities of N and U. The evolution of cooperativity in protein folding transitions is linked closely to the evolution of function as well as the aggregation propensity of the protein. A large activation energy barrier in a fully cooperative transition can provide the kinetic control required to prevent the accumulation of partially unfolded forms, which may promote aggregation. Nevertheless, increasing evidence for barrier-less "downhill" folding, as well as for continuous "uphill" unfolding transitions, indicate that gradual non-cooperative processes may be ubiquitous features on the free energy landscape of protein folding.

Keywords: cooperativity; intermediates; one-state; population distributions; downhill folding; uphill unfolding

Introduction

The simplest possible way of describing the transitions between the native (N) and unfolded (U) states of a protein is the two-state U↔N model.[1] Many proteins appear to undergo completely cooperative folding and unfolding, as their folding reactions are found to meet both the kinetic and the thermodynamic criteria for two-state folding.[1–3] This deceptively simple depiction of protein folding has had great practical value. The two-state model allows an easy determination of the effects of mutations on the stabilities of the transition state (TS) and the N state, with respect to the U state, and enables indirect inferences to be made about the structure of the TS and its possible role as a nucleus in protein

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*Correspondence to: Jayant B. Udgaonkar, National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bengaluru 560065, India. E-mail: jayant@ncbs.res.in

folding reactions.[4,5] The criteria for two-state folding are, however, also met by a multi-state folder whose folding reaction is populated by intermediates that are too unstable to be detected experimentally.[6,7] In many cases, high energy intermediates can be stabilized by appropriate experimental manipulation,[8–10] and thereby become detectable. Nevertheless, the utility of two-state models is so great, that even when the folding kinetics may be more correctly describable as multi-state, the two-state model is invariably extended to account for the kinetics.

Multi-step folding is undeniable for many proteins because their folding intermediates are stable enough to accumulate and be easily detectable.[8,11–34] In such cases, structural characterization of intermediates provides a wealth of information on folding pathways, and allows different folding pathways to be distinguished from each other.[29,35,36] A common class of intermediates which provide evidence for the reduced cooperativity of protein folding/unfolding reactions is that of the molten globular (MG) forms. The detection of dry molten globules,[37–42] as well as wet molten globules,[43] demonstrates that changes in secondary and tertiary interactions, as well as changes in the solvation of the protein core, may occur in multiple stages.[19,44–47] When folding occurs in multiple steps via productive intermediates, the activation free energy to folding gets distributed between multiple barriers. This results in faster folding than when the activation free energy is concentrated in one barrier in "two-state" folding. But intermediates could also be transient structures trapped in local energy minima because they possess non-native interactions that have to be broken before folding can proceed.[48,49]

The dynamical coupling between the interactions which stabilize a well-packed N state determines the cooperativity of the protein folding or unfolding reaction. Strong coupling between stabilizing interactions would lead to an entirely cooperative two-state transition while a complete lack of coupling would result in a gradual/continuous noncooperative transition. Gradual folding[45,50–53] as well as unfolding[54–62] structural transitions have now been shown for several proteins. In fact, it has even been demonstrated that individual amino acid residues in a protein may have different equilibrium unfolding transitions,[63–68] providing strong evidence for non-cooperativity in protein folding reactions. Barrier-limited two-state transitions, and barrierless gradual transitions are at opposite ends of the cooperativity spectrum, with the former being entirely cooperative, and the latter being entirely noncooperative. Multi-step transitions with discrete intermediates illustrate reduced/limited cooperativity in protein folding reactions: individual steps may be fully cooperative but the overall reaction is noncooperative.

This review examines the extent of cooperativity inherent to folding and unfolding transitions, including downhill folding and uphill unfolding. The tunable nature as well as the structural basis of cooperativity on the free energy landscape of protein folding has been discussed. Early folding transitions that occur prior to the major structure-forming reactions,[69] as well as insights gained from theoretical and computational approaches[70,71] are not discussed here, as they have been reviewed elsewhere.

Experimental Determination of Cooperativity

The delineation of multiple steps, let alone gradual transitions, is experimentally challenging. "Two-state" folding transitions have been proposed for many proteins, on the basis of studies that utilized just a single experimental probe, which can, however, be misleading.[72] The hidden complexity of an apparently simple "two-state" transition is often revealed only by the use of multiple experimental probes[40,73,74] as well as different experimental conditions.[9,10] This is exemplified by studies on the protein monellin, using multiple methodologies (Fig. 1). Significant dispersion in the folding/unfolding rates of different parts of a protein structure, demonstrated by monitoring multiple sites in the protein, have provided direct evidence for asynchronous structural changes which occur in multiple steps for many proteins.[75–81] Several advances in the experimental techniques used to probe folding cooperativity[82–86] have yielded a quantitative understanding of thermodynamic cooperativity, which pertains to the number of states populated during a transition, as well as kinetic cooperativity, which pertains to the number of steps and free energy barriers between the initial and final states of a reaction.

Probing thermodynamic cooperativity

The classical way of determining the thermodynamic cooperativity of a folding reaction is to determine the ratio of the calorimetric enthalpy to the van't Hoff enthalpy, which is expected to be 1 for a two-state process.[87,88] A spectroscopic isobestic/isoemissive/isodichroic point will always be observed for equilibrium two-state unfolding, but it is not always remembered that the observation of such a single point where all the spectra for different stages of the transition superimpose, need not necessarily mean that the transition is two-state. Similarly, while multiple probes should yield coincident transition curves for two-state unfolding, the observation of coincident transition curves need not necessarily imply two-state unfolding.

Analysis of equilibrium unfolding transitions, induced by denaturants or temperature, can yield other useful insights into the cooperative nature of the unfolding reaction. For a true two-state process, the N and U protein baselines are expected to be

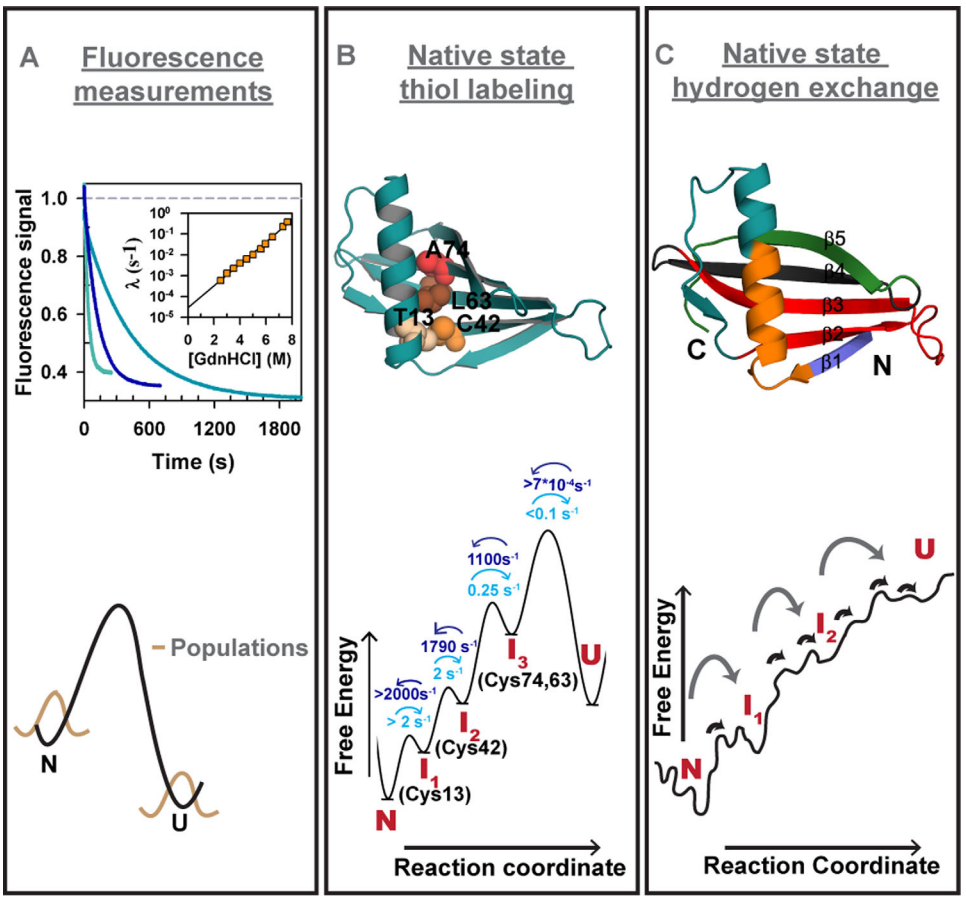

Figure 1. Experimental determination of cooperativity. A: Single exponential kinetics of the unfolding of monellin probed by fluorescence (upper panel; kinetic traces of unfolding in increasing denaturant concentration are shown from right to left) suggested a "two-state" cooperative transition. B: Site-specific unfolding/opening of four cysteine side chains (upper panel), probed by thiol labeling (SX) under native conditions, detected the presence of at least four discrete energy barriers.[217] This, along with steady state and TR FRET measurements[39,60] indicated limited cooperativity in the unfolding transition of monellin. C: Increasing structural resolution, by using hydrogen exchange (HX) which probes the backbone hydrogen bonding network in multiple sequence segments of the protein (upper panel), revealed a multitude of small barriers, of the order of thermal energy, resulting in a gradual uphill unfolding transition.[62,81]

independent of the change in equilibrium conditions, and the spectroscopic signal would be a linear sum of the N and U signals. On the other hand, large dependences of the N and U spectroscopic signals on denaturant concentration or temperature, which manifest as steep nonzero baselines in equilibrium unfolding curves, are likely to be indicative of gradual shifts of the N and U energy wells along the reaction coordinate, with a change in conditions.[51,89] Noncoincidence of equilibrium unfolding transitions measured by multiple probes is further indication of the presence of structurally distinct equilibrium intermediate(s).[17,64,67,68,90–92] For several proteins, such as the bovine prion protein (Fig. 2), even though multiple optical probes showed identical equilibrium unfolding curves, indicative of a "two-state" process, dispersed midpoints of denaturant or temperature-induced equilibrium unfolding transitions, measured for individual residues by NMR, suggested that the unfolding process was indeed noncooperative.[63–67] Moreover, the delineation of noncooperative cold denaturation of a protein which undergoes cooperative thermal unfolding,[93] showed that the thermodynamic cooperativity of a folding/unfolding transition is largely dictated by the nature of the interactions which undergo a change during the transition.

Probing kinetic cooperativity

Studies which aim to investigate the kinetic cooperativity of protein folding reactions have benefitted immensely from the advancement of relaxation methods,[94–96] including rapid mixing techniques.[53,97–101] However, interpretations drawn on the cooperativity of folding/unfolding transitions from these experiments rely heavily on the exponential nature of the observed kinetics. Although a barrier-limited two-state transition is expected to display single exponential kinetics, a barrier-less transition may be describable by either exponential or nonexponential kinetics.[102] Hence, cooperativity cannot be

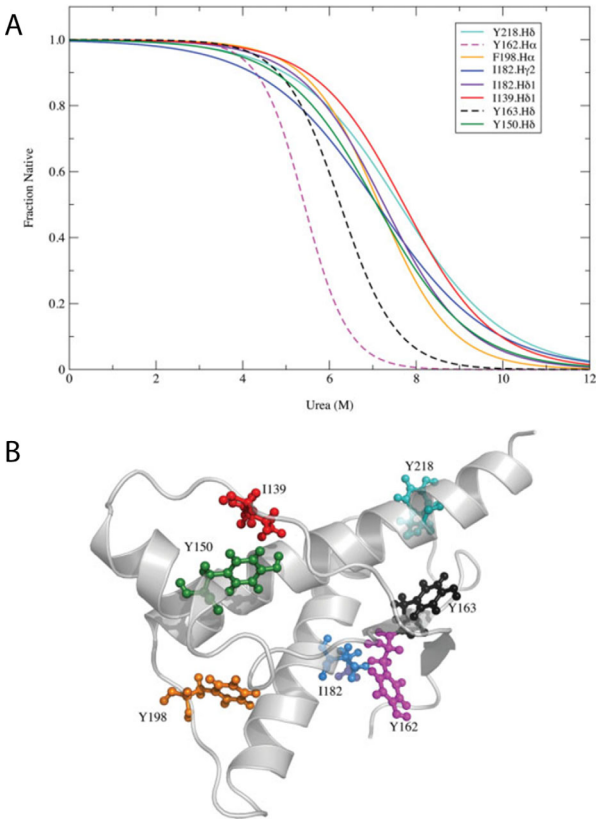

Figure 2. Equilibrium unfolding transitions at single amino acid resolution. Urea-induced unfolding transitions were measured for individual residues of bovine $PrP_{121–230}$ by NMR. A: The unfolding transition measured for each amino acid residue, shown on the protein structure in (B) was fit to a two-state N↔U model. The observed dispersion in the thermodynamic parameters obtained from the fits, which reflect the residue-specific sensitivity to urea denaturation, demonstrated the lack of thermodynamic cooperativity in the unfolding of the prion protein. Reprinted, with permission, from reference 66.

inferred reliably from the observation of exponential kinetics alone.[103] Nevertheless, relaxation experiments have provided the temporal resolution required to delineate the lack of kinetic cooperativity in early folding events[52,104,105] as well as in downhill folding transitions, which occur on fast timescales in the absence of activation free energy barriers.[50,106]

Reduced cooperativity in protein folding and unfolding reactions often manifests itself in the form of multi-exponential kinetics,[92] or in the presence of a lag phase in the kinetic curve, when one probe is used;[107–110] and in probe-dependent kinetics when multiple probes are used.[40,44,74,111,112] A lack of kinetic cooperativity in protein folding reactions is also evident in real time NMR experiments which have revealed differences in residue-specific folding and unfolding rates.[76,113,114] Moreover, the coupling of NMR measurements to fast mixing devices has provided detailed structural information on transient intermediate states populated in multi-step transitions, and thus delineated the hierarchy of conformational transitions for several proteins.[37,115,116] Advances in CPMG NMR relaxation experiments have further provided a wealth of insight into high energy intermediate states, whose populations can be as low as 0.5%.[117,118]

Monitoring population distributions

The most direct way to delineate the cooperativity of a protein folding reaction is to monitor the population distributions of structurally distinct species, as a function of reaction conditions (thermodynamic cooperativity) or reaction time (kinetic cooperativity). Time resolved (TR) fluorescence-based methodologies, which construct distributions of fluorescence lifetimes and, consequently, donor-acceptor distances in proteins, have demonstrated multiple steps as well as gradual transitions in apparently two-state folding proteins in both equilibrium[56,119] and kinetic[44,60,120–122] experiments. A TR-FRET study of the unfolding of monellin,[60] under highly denaturing conditions (Fig. 3), provided the first direct kinetic evidence for the gradual loss of structure at multiple sites during the unfolding of any protein. Moreover, denaturant-induced expansion of the N state and U states, which is often not detected by other ensemble optical measurements, have been directly delineated by TR-FRET measurements.[56,119]

Single molecule (sm) fluorescence-based methods,[123,124] which can also monitor population distributions, are especially advantageous as they can detect rare transitions even in the presence of a large activation free energy barrier. Although bimodal distributions corresponding to the N and U states have been observed for some proteins,[125,126] and are indicative of a cooperative transition, gradual shifts of the U state distribution[125,127,128] have revealed more subtle deviations from a true two-state process. Moreover, it remains unclear if the gradual shift corresponds to the inter conversion between two conformations of the U state or between U and a MG intermediate. Similarly, when exchange between two states is observed in measurements on tethered[129] or encapsulated[130] single protein molecules, it is not always clear whether the transitions are between U and N, or between a MG intermediate and N. Combining sm fluorescence methods with Markov state analysis has been very effective in providing a description of the complex network of conformationally distinct states in protein folding reactions.[31] The unimodal distributions observed in sm studies of downhill folding proteins[131] have provided further direct evidence of "one state" folding transitions.

Sm force spectroscopy, which measures the extension of a protein molecule as it folds and unfolds upon mechanical pulling, provides a detailed description of the free energy landscape of protein folding by determining the positions of different states along the reaction coordinate, the heights of

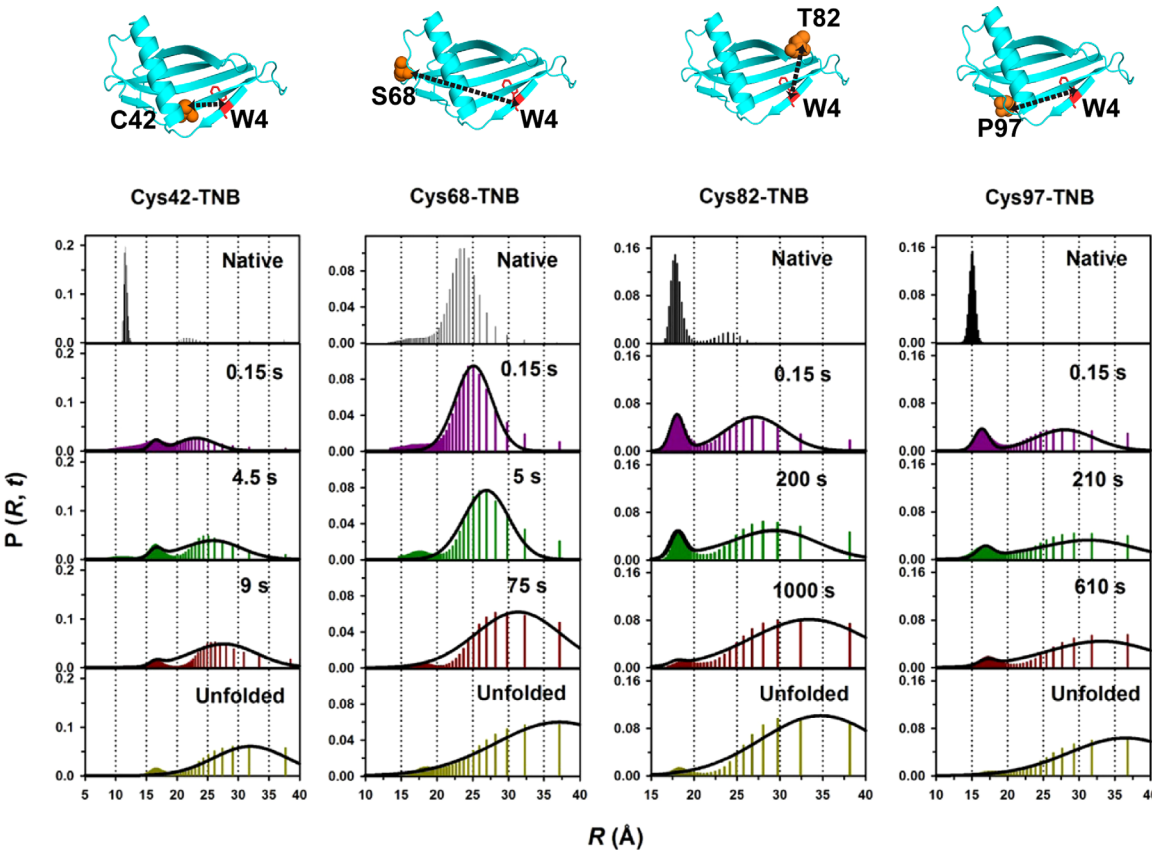

Figure 3. Gradual evolution of distance distributions in a kinetic unfolding experiment. The distributions of four distances in monellin, indicated on the protein structure in the top most panels, were monitored by multisite TR-FRET, during unfolding in the presence of 4 *M* GdnHCl. Unimodal distributions for one of the distances (Cys68-TNB) provided strong evidence of a continuous noncooperative expansion during unfolding. The bimodal distributions in the remaining three cases were centered at distances intermediate between those of the N and U states, suggesting the presence of intermediate ensembles. Continuous shifts in the distance distributions of the intermediate states provided further evidence for gradual noncooperative structural change. The data could be accounted for by a model in which structure was lost continuously during the gradual swelling of the protein during unfolding, which was describable by the Rouse model of polymer physics. Reproduced from Ref. 60.

free energy barriers as well as unfolding/refolding rates.[132] Sm pulling experiments have delineated the presence of intermediates and multiple steps in the unfolding of single domain,[133,134] multi-domain[135–138] as well as repeat proteins.[139,140] More importantly, while most experimental methods which detect intermediates cannot distinguish between on-pathway and off pathway species, folding and unfolding trajectories obtained from sm force spectroscopy measurements have directly delineated the kinetic connectivity between the N, U and intermediate states.[133,137] Mechanical pulling along different geometries, in circular permutants of a multi-domain protein, has further revealed that discontinuous domain topology promotes cooperativity, thereby establishing chain topology as an important determinant of folding cooperativity.[136]

Hydrogen Exchange (HX)

HX probes the formation and dissolution of secondary structure in a protein, by monitoring the exchange of backbone amide hydrogens (protiums) with a heavier isotope (deuterium) in solution, using either mass spectrometry (HX-MS) or NMR (HX-NMR).[141,142] Although HX-NMR affords a higher structural resolution, HX-MS experiments are very effective in determining folding cooperativity because they monitor population distributions, which cannot be probed by HX-NMR. Moreover, tuning the exchange regime between the EX2 and the EX1 limits[143] allows a delineation of both thermodynamic cooperativity (in the EX2 limit) as well as kinetic cooperativity (in the EX1 limit).

Exchange in the EX2 regime

Site-specific stabilities determined in the EX2 limit for many proteins, when mapped on to the protein structure, showed a clustering of residues with similar ΔG_{op} (free energy change associated with a structure-opening event) values delineating cooperative units of structure called "foldons".[23,96,112,141,144–149] The backbone amide sites within a foldon opened in a concerted manner, and the unfolding of each structural unit led to the formation of partially unfolded forms (PUFs)

which differed in stability from the N and U states. Folding of the protein appeared to occur via "sequential stabilization" of multiple foldons, thus resulting in a modular assembly of the native structure.[150,151] The delineation of PUFs for many proteins[23,96,147–149,152,153] was an example of the reduced thermodynamic cooperativity in protein unfolding reactions. On the other hand, for some proteins, such as T4 lysozyme,[154] a continuous dispersion of the ΔG_{op} values measured in the EX2 limit of exchange, provided evidence for gradual unfolding. It should, however, be noted that measurements in the EX2 limit cannot define the temporal order of structural transitions, and can, therefore, probe only the thermodynamic cooperativity of protein folding reactions.

Exchange in the EX1 regime

Exchange in the EX1 limit allows a direct determination of site-specific opening rates and, therefore, of the sequence of structure-opening events.[61,62,81,155–161] For cytochrome *c*[159] and RNase H,[162] residues with similar rates of opening were found to be localized to the same structural element, which was in agreement with the limited cooperativity associated with the presence of foldons in these proteins. Structural dispersion of residues with similar opening rates results in a diffuse loss of structure, and indicates a lack of discrete cooperative units and modular architecture.[81,1113,163] Moreover, a wide dispersion in opening rates leads to an asynchronous loss of structure with different backbone amide sites opening on different timescales. A determination of the sequence of structural transitions, from the opening rates measured in the EX1 limit, has shown that both modular as well as diffuse disassembly of structure can occur in a hierarchical manner, through a defined sequence of events (described in this article).

A further advantage of HX-MS experiments is that the measured mass distributions can distinguish directly between correlated (cooperative) and uncorrelated (noncooperative) structure-opening events, in the EX1 regime of exchange.[62,160] For the few proteins known to exchange in the EX1 limit under normal physiological conditions,[61,62,163] a large number of backbone amide sites located outside the structural core were found to undergo uncorrelated structural transitions, indicating a gradual loss of structure.[61,163]

HX-MS studies with monellin provide a very good example of the delineation of extreme noncooperativity in folding/unfolding transitions.[62,81] The observation of exchange in the EX1 limit allowed a direct demonstration of correlated versus uncorrelated openings in the protein. Nearly all the observed backbone amide hydrogens, including the slow exchanging core, opened in an uncorrelated manner under native conditions (Fig. 4), thereby providing evidence for an entirely noncooperative (gradual) unfolding transition which occurs in an 'uphill' manner, in the presence of a large free energy difference between the N and U states. The uncorrelated openings, as well as the dispersion in the opening rates, indicated several independent structure-opening events occurring in parallel. The gradual unfolding transition of MNEI was, nevertheless, associated with distinct exponential kinetic phases. These studies[62,81] showed that even when only one backbone amide site becomes labeled at a time, significant surface of the protein might become exposed to solvent, resulting in structural intermediates and even global unfolding.

Downhill Folding

Downhill folding transitions provide an example of the extreme noncooperativity observed in some protein folding reactions. Sufficient energetic bias towards either the N or the U state may result in a free energy landscape with only one energy minimum, giving rise to a downhill process, which is not limited by an activation free energy barrier.[103,164] In contrast, a cooperative two-state transition would be associated with bimodal population distributions, corresponding to the N and U energy minima. Cooperative transitions, limited by barriers, appear to be describable adequately by transition state theory, according to which the TS at the top of the barrier serves as the kinetic bottleneck of the reaction. However, in addition to the free energy barrier, a protein folding reaction is also limited by diffusion on the free energy landscape, which is largely a function of friction within the molecule (internal friction) as well as with the solvent (viscosity). The effective diffusion coefficient changes along the reaction coordinate of folding,[165,166] with parts of the free energy landscape with the lowest diffusion coefficient corresponding to the kinetic bottleneck in barrier-less downhill transitions.

The earliest experimental evidence for downhill folding, in the thermodynamic context, came from a study of the small 40-residue protein, BBL.[51] Probe-dependent kinetics and large, nonzero N and U baselines in equilibrium unfolding transitions were interpreted as hallmarks of a global downhill folding process, in which the free energy surface has a single minimum under all experimental conditions, which shifts continuously along the reaction coordinate. Statistical mechanical modelling further confirmed the presence of an ensemble of structures, which populated in a "one-state" manner, as the N state shifted continuously to the U state. However, subsequent kinetic studies measured a single 10 μs refolding phase for BBL which seemed to suggest that the refolding was barrier-limited.[167] Experiments on different BBL variants by multiple groups sparked a long-standing debate on the downhill versus two-state nature of BBL folding,[168–170] with current experimental and simulation studies appearing

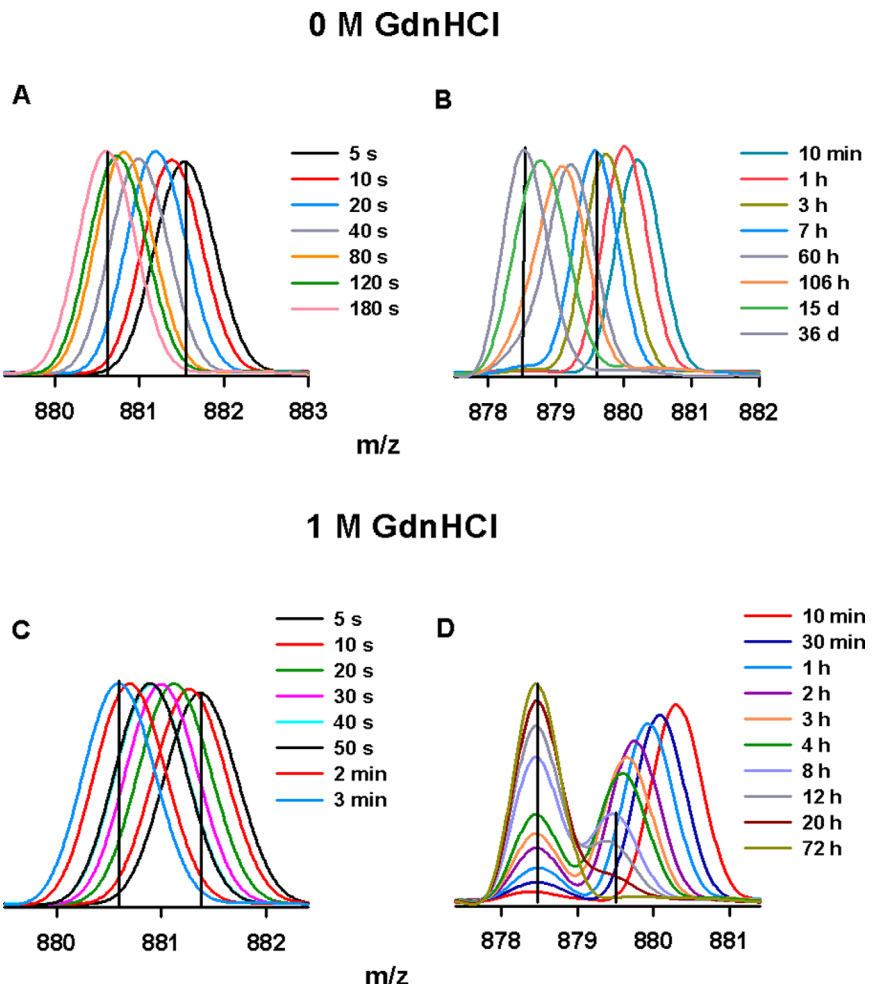

Figure 4. Delineation of cooperativity from population distributions. Mass distributions measured by HX-MS in the EX1 regime could directly differentiate between a cooperative and a gradual transition in monellin. Unimodal mass distributions at all time points of exchange, in 0 *M* GdnHCl (A and B) were indicative of a one-state gradual transition under native conditions, involving the opening of one backbone amide site at a time. The solid vertical lines in each panel indicate the mass distributions corresponding to the N state (at 5 s), U state (at the final time point of exchange) and at the end of each kinetic phase of exchange. The observation of bimodality upon the addition of 1 *M* GdnHCl (C and D) indicated a switch to a cooperative transition, which involved the all-or-none opening of a subset of 14 backbone amide sites in the protein core, during the slow global unfolding step. Reprinted, with permission, from Ref. 62.

to favor the downhill folding scenario.[132,171,172] BBL, and other proteins such as gpW,[89] which are rare examples of "natural" downhill folding proteins, provide an opportunity to understand the evolutionary factors which result in barrier-less versus barrier-limited processes.[106]

Kinetic studies on downhill folding proteins, which fold on a very rapid time scale, have received a huge impetus from advances made in rapid relaxation techniques. Due to the discrepancies associated with the interpretation of nonexponential kinetics,[102] downhill folding is best identified in kinetic experiments by tuning the reaction from a two-state to a barrier-less process via mutations,[173] or by changes in temperature[174] or solvent conditions.[175] Under experimental regimes in which the transition was two-state, a single "activated" kinetic phase which corresponded to the crossing of the activation free energy barrier was observed.[103,176] A shift to the downhill folding regime resulted in a rapid "molecular" phase which corresponded to the relaxation of the population of molecules from the top of the barrier into the native well. Tuning of the protein folding energy landscape allowed the observation of both time scales, providing an estimate of the waiting time or the dwell time in each of the energy minima (activated time scale) as well as the transition path time (molecular time scale).[176]

Tuning the Cooperativity of Protein Folding and Unfolding Transitions

Tuning between a two-state and a three-state transition

The mechanism by which a protein achieves its native structure is much more sensitive to

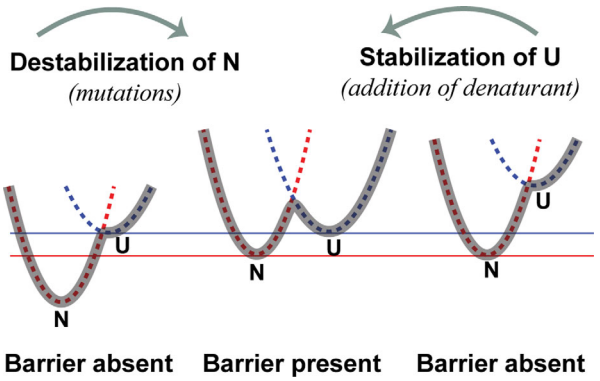

Figure 5. Tuning the cooperativity of protein folding/unfolding reactions. Changes in the stabilities of the N and U states, via changes in solvent conditions[62] or mutations of the protein sequence, can switch a barrier-limited cooperative process to a barrier-less transition. The point of intersection of the N and U state energy wells corresponds to the position of the TS along the reaction coordinate. The resultant free energy landscape is shown in grey. Reprinted, with permission, from Ref. 62.

environmental tuning than the final native fold itself. Changes in solvent conditions[9,10,32,92] as well as mutations[20,91,177,178] may cause the folding mechanism to switch between a two-state and a three-state transition, via destabilization or stabilization of an intermediate. Alternatively, subtle changes in folding conditions may cause a switch from a pathway on which only the N and U states are populated, to a pathway on which folding occurs via the accumulation of intermediates.[179,180] Multiple folding routes, each characterized by a different sequence of structural events, were conceptualized initially by comparing a protein folding transition to the assembly of a jigsaw puzzle,[181] and later envisaged by the energy landscape theory.[164,182]

Tuning between a two-state and a gradual transition

The folding and unfolding transitions of proteins may also be tuned from an entirely cooperative (two-state) reaction to an entirely noncooperative (gradual) transition. This drastic tuning of cooperativity on the protein folding energy landscape is possible as a result of a change in the stabilities of the N and U states,[62,103] as shown in Figure 5. The TS, defined by the point of intersection of the N and U energy wells, is higher in energy than the N or U states in a barrier-limited reaction. Stabilization of the N state, or destabilization of the U state, reduces the energy difference between the U state and the kinetic bottleneck of folding (i.e., the TS), thus resulting in a barrier-less process. Changes in experimental conditions such as temperature[50,174] and solvent conditions,[62,175] as well as mutational changes[173,183] (Malhotra and Udgaonkar, personal communication), alter N and U state stabilities, and can therefore switch a cooperative process to a gradual transition and vice versa.

Recent experiments with monellin[62] have shown how the cooperativity of a slow unfolding reaction may be tuned by changing experimental conditions. Under native conditions, the backbone amide sites in monellin opened up to HX, one at a time. The "uphill" unfolding transition therefore occurred gradually over many small barriers. The addition of denaturant, which preferentially stabilizes the U state,[62] or mutations which destabilize the N state (Malhotra and Udgaonkar, personal communication), caused a subset of backbone amide sites to open in a concerted manner (Fig. 4), over a single free energy barrier. Kinetic barriers on the free energy landscape of protein folding can, therefore, be tuned by altering the thermodynamic stabilities of the N and U states.

Ruggedness on the Free Energy Landscape of Protein Folding

Local kinetic traps and barriers, which confer ruggedness to the protein folding energy landscape, result in reduced folding cooperativity. The observation of wavelength-dependent folding kinetics, measured for $\lambda_{6\text{-}85}$,[165] revealed that the folding rate decreased with an increase in the degree of structural compaction, indicating that the protein folding energy landscape was rougher on the native than on the unfolded side of the activation energy barrier. The molecular time scale measured in the case of barrier-less downhill folding, which is limited by diffusion over the free energy landscape, provides a measure of the landscape ruggedness.[176] Another measure of ruggedness is provided by the transition path time, obtained from folding/unfolding trajectories in sm fluorescence experiments.[130,184,185] The observation that the transition path times, which are a measure of the actual time taken to cross the top of an activation energy barrier, were comparable while the folding rates, associated with the waiting times in the energy minima, varied considerably between different proteins,[130] suggested that the free energy landscapes of structurally diverse proteins may be similarly rugged. Roughness has also been quantified by determining internal friction,[186–191] or more directly by analysing the complexity of the kinetic phases associated with the unfolding reaction.[81] It has further been suggested that in addition to perturbing the stabilities of the N and U states, denaturants smoothen local kinetic traps and small intervening barriers, thereby increasing folding cooperativity[81,166] (Fig. 6). These observations highlight the limitations of drawing interpretations of cooperativity from experiments which use denaturant to induce folding or unfolding. The cooperativity observed in such studies may simply be an effect of the changes in solvent conditions

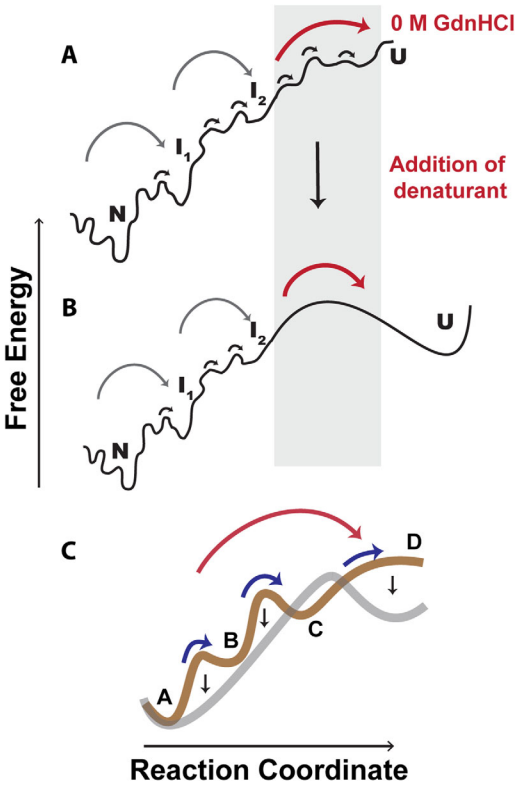

Figure 6. Ruggedness on the protein folding energy landscape. A subset of backbone amide hydrogens in monellin, which unfold gradually in native conditions (A), were induced to unfold cooperatively upon the addition of GdnHCl (B),[62] suggesting that denaturant smoothens the roughness of the protein folding energy landscape. This was further confirmed by the observation that the unfolding kinetics of a sequence segment spanning two β strands in the protein, simplifies from being a triple exponential in native conditions to a single exponential reaction in the presence of GdnHCl (C).[81] Denaturant smoothens the free energy landscape of protein folding (in gray), by reducing the intervening energetic barriers and kinetic traps present on a rough landscape (in brown). Reprinted, with permission, from Refs. 62 and 81.

on the stabilities of the TS and U state, and on the inherent roughness of the protein folding energy landscape.

Structural Basis of Cooperativity

Structural correlates to cooperativity in "two-state" folding

The folding of a protein involves the formation of a multitude of weak interactions, which individually may contribute very little to the energetics of the process, but which cumulatively result in a well-packed native structure. Although general principles are difficult to define, theoretical and experimental studies have elucidated certain structural correlates to the cooperativity and mechanism of protein folding.[192,193] For example, chain length has been suggested to be one of the determinants of folding cooperativity.[2] The packing requirements of every amino acid residue in a covalently linked polypeptide chain cannot be satisfied while meeting the structural and energetic requirements of the entire protein. This results in unfavorable interactions which manifest themselves as local kinetic traps and 'frustration' on the free energy landscape of protein folding.[194] The likelihood of unfavorable interactions is expected to increase with the chain length, given the fixed amino acid repertoire available to proteins. It has therefore been suggested that shorter chain lengths may facilitate cooperative "two-state" folding. However, a decrease in chain length may also compromise packing efficiency as there are now fewer residues available to satisfy the packing requirements of each amino acid residue in the protein. Short sequences may, therefore, also increase frustration, and thereby noncooperativity, as suggested by the observation that downhill folders are all small-sized globular proteins.[85]

Topological organization, which is the order of secondary structural elements along the amino acid sequence, has been experimentally shown to be another important determinant of folding cooperativity.[137] It has been suggested that multiple topological parameters, including contact order[195] as well as the distribution of local and nonlocal interactions,[196] dictate the complexity of the folding mechanism. Theoretical modelling of folding reactions has further suggested that the cooperativity observed in protein folding reactions, compared to noncooperative helix-coil transitions observed in peptides,[197,198] can be explained by the stabilizing effect of long-range tertiary interactions in a protein.[199,200]

Denatured states of proteins

A dynamical network of native-like interactions, already present in the denatured states of proteins, can generate a natural bias towards the native conformation, thereby reducing the conformational space sampled by a given sequence.[201–205] However, nonnative interactions found in the denatured states of proteins, which persist during folding, can result in the accumulation of intermediates in kinetic traps on the protein folding energy landscape.[48,49,105,206] The structural rearrangements required for breaking nonnative interactions may thus result in reduced folding cooperativity.

Models of protein folding

Three conceptual models have been proposed to describe the formation of structure during protein folding: the nucleation-condensation model, the framework model and the hydrophobic collapse model. These models provide qualitative descriptions of the stages at which secondary and tertiary interactions are formed, and thereby yield insights into the structural basis of folding cooperativity.

The nucleation-condensation model[7,207] posits that all interactions are formed concomitantly over a single activation free energy barrier via a unique, structurally diffuse TS which is conceived to be an expanded form of the native structure. The fundamental tenets of this model emerged from elegant protein engineering studies which delineated the structure of the TS in terms of the solvent exposure (Tanford β value),[5] or the extent of formation of interactions (Φ value).[4] For "two-state" folding proteins, Φ value analyses revealed diffuse TSs, in which most residues displayed intermediate Φ values.[208] It was thus suggested that the formation of the nucleus (nucleation), defined by the residues which had the highest Φ value, and the formation of the remaining structure (condensation) are coupled processes. While it provides a qualitative description of "two-state" transitions,[1] the nucleation-condensation model does not invoke the presence of folding intermediates, and cannot, therefore, explain the reduced cooperativity observed in the folding/unfolding transitions of many proteins.

The framework model,[14,15,209] posits that local secondary structural elements form first, and are subsequently stabilized by tertiary interactions. According to the hydrophobic collapse model,[74] folding is driven by the initial formation of a collapsed intermediate in which hydrophobic residues are sequestered away from the surrounding solvent. Subsequent formation of specific secondary and tertiary interactions results in the fully folded N state. The framework model and the hydrophobic collapse model are thus hierarchical folding models which envisage folding reactions to have limited cooperativity, with multiple steps and intermediate structures on the folding pathways.

For proteins which fold with limited cooperativity, via discrete intermediates, clusters of residues with very high (close to 1) or very low (close to 0) Φ values were observed.[210,211] The bimodal distributions of Φ values appeared to be a manifestation of polarized nuclei, which corresponded to the TS of each step in the folding reaction. Strong coupling within the nuclei, and weak coupling between them, results in a modular architecture of the protein, comprised of "foldons" which fold and unfold cooperatively. However, structural overlap and coupling between multiple nuclei may promote global cooperativity, as suggested in the case of the ribosomal protein S6.[212,213] Understanding the extent of dynamical coupling within the interaction networks in proteins is, therefore, the key to determining the structural and physical basis of folding cooperativity.

Structural changes in gradual transitions

In a gradual/continuous transition, which occurs over several small barriers of less than 2-3 k_BT, coupling between intramolecular interactions is very weak. The loss or gain of structure in a gradual transition is therefore expected to occur in a diffuse and asynchronous manner. HX-MS experiments on monellin have provided strong evidence for the absence of structural patterning and modular structural transitions in a slow unfolding reaction.[62,81] The uncorrelated opening of backbone amide sites, to HX, one at a time, was indicative of an entirely gradual unfolding transition. Mapping of segment-specific opening rates to the protein structure demonstrated a lack of modular architecture, and revealed structurally diffuse opening transitions during unfolding (Fig. 7). Moreover, multiple unfolding rates associated with a given secondary structural element provided rare evidence for an α helix or a β strand evolving noncooperatively during unfolding. An NMR study of the folding of a four-helix bundle protein has also demonstrated gradual structural changes which occur in a multitude of steps involving one or two amino acid side chains at a time.[214] Noncooperative gradual transitions, associated with structurally diffuse conformational changes, have been elucidated for other proteins as well.[68,113,154,163] In fact, the coupling of interaction networks has been quantified for a downhill folder, to provide a further understanding of the structural basis of noncooperativity in protein folding.[64] It is important to clarify here that the diffuse transitions that occur in gradual processes are different from the diffuse TSs envisaged for "two-state" folding proteins. The former are associated with multiple small barriers and result from a complete lack of dynamical coupling between interactions, while the latter are a consequence of strongly coupled interactions which form concomitantly over a single barrier.

Interestingly, the unfolding reaction of monellin, despite being entirely gradual, was found to occur in three well-defined kinetic phases,[62] which are typically taken to correspond to discrete steps in a reaction. A comparison of native state HX data[81] with native state thiol labeling (SX) experiments,[215] which probe the solvent-exposure of cysteine side chains engineered at different sites in the protein, revealed the structural rationale for the observation of exponential kinetic phases in a continuous transition. The multiple kinetic pauses observed in the HX-MS experiments could be attributed to the waiting times associated with the dissolution of local tertiary packing interactions in the protein. The gradual unfolding reaction of monellin[81] is therefore a very good example of uncorrelated secondary structural changes, resulting in diffuse noncooperative loss of structure, as well as strong coupling between tertiary and secondary interactions, resulting in the observation of discrete kinetic phases.

Evolutionary Causes and Consequences of Cooperativity

The demonstration of downhill folding[50,51,85] as well as gradual unfolding[54–62] indicates that large

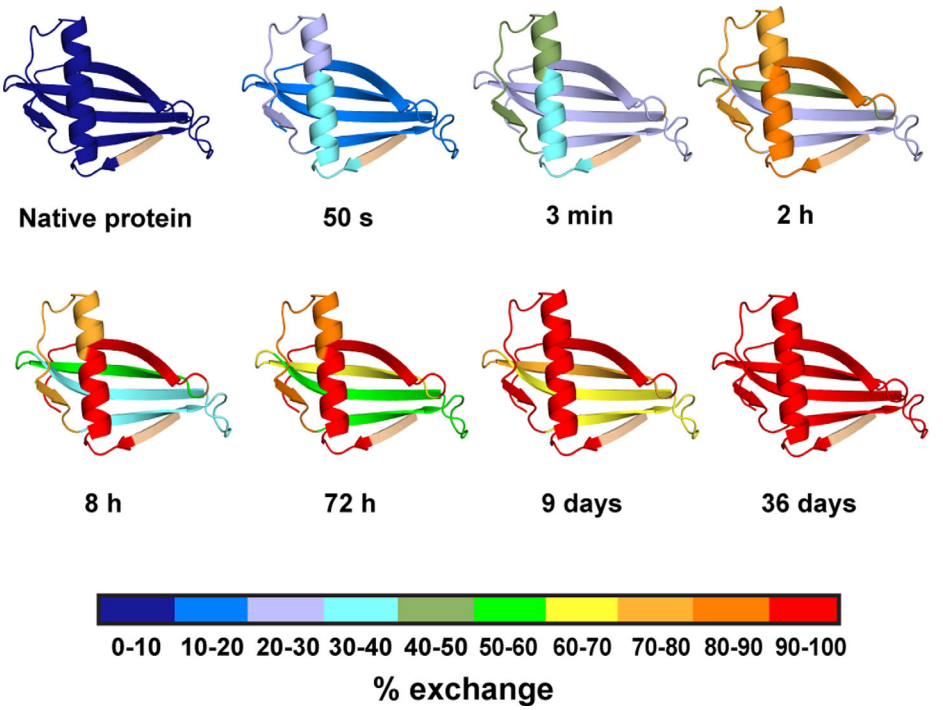

Figure 7. Structural transitions in a gradual unfolding reaction. HX-MS kinetics was measured for different sequence segments of monellin, generated by chemical fragmentation subsequent to exchange in 0*M* GdnHCl. Mapping the extents of exchange in individual sequence segments at different times of unfolding on to the protein structure revealed a diffuse and asynchronous dissolution of the backbone hydrogen bonding network in monellin. A dispersion in the extents of exchange within the α-helix, demonstrated the noncooperativity in the unfolding of individual secondary structural elements in the protein. A lack of modular architecture and distinct secondary structural units in monellin, evident in the above representation, provided a structural basis for the gradual unfolding of the protein under native conditions. Reprinted, with permission, from Ref. 81.

activation free energy barriers may not be obligatory features of folding and unfolding reactions. However, for many proteins, folding transitions are found to be slower than the expected timescales of barrierless reactions, because of the presence of an activation free energy barrier. It has, therefore, been suggested that barriers on the free energy landscape of protein folding may be by-products of the physical and evolutionary constraints on proteins.[106] The physical constraints arise from the availability of a limited set of amino acids from which a protein sequence can be constructed, which precludes perfect packing in the native states of proteins. Alternative packing arrangements, similar in energy to the N state, may incorporate non-native interactions, the breaking and rearrangement of which result in local kinetic traps, and thereby reduce folding cooperativity.

In addition to physical constraints, free energy barriers can also be affected by a folding-function trade-off[62,194]—a concept which has been experimentally[216] and theoretically[217,218] explored. Proteins have evolved to function, and the incorporation of functionality at structurally important residues may have affected protein folding cooperativity by either increasing[216] or decreasing[219] barriers on the free energy landscape of protein folding. A further evolutionary constraint arises from the competition between misfolding and aggregation. Marginal barriers, which result in faster folding, are also associated with faster unfolding reactions. Protein molecules will therefore shuttle between the N and U states more frequently than when the folding/unfolding reactions are barrier-limited.[220] An increase in the frequency of excursions to fully or partially unfolded states is expected to increase the aggregation propensity of proteins.[221] As shown in the case of $\lambda_{6\text{-}85}$,[176] mutant forms of the protein which folded in a two-state manner were less prone to aggregation than downhill folding variants. These observations further indicate that small changes in environmental conditions or sequence, which tune the cooperativity of folding, may also have dramatic effects on the aggregation of the protein.

The tunable nature of folding cooperativity suggests that changes in environmental conditions can be "sensed" by the effect they have on energetic barriers on the protein folding energy landscape. It has been proposed that downhill folding proteins which fold via a continuum of structures can serve as efficient molecular rheostats[51,86] in which, unlike switch on-switch off signaling molecules, the multitude of populated conformations may enable the protein to respond to subtle changes in environmental

conditions. Moreover, partial unfolding events, facilitated by the absence of a large free energy barrier to unfolding, increase the flexibility of the N state, which is a functional requirement for many enzymatic proteins.[222–224] Indeed, a theoretical model of ligand binding, called fly-casting,[225] posits that increased flexibility promotes binding by increasing the cooperativity of the binding process.[85,86]

Summary

The observation of gradual downhill folding and uphill unfolding transitions demonstrates that the inter-conversion of states on the free energy landscape of protein folding can occur in a "one-state" manner, with one species continuously shifting along the reaction coordinate and transitioning into another. High-resolution structural probes have demonstrated that the coupling between complex networks of interactions, determines the cooperativity of the folding/unfolding transition. A barrier-less transition can be switched to a barrier-limited process, via changes in the stabilities of the N and U states. Denaturants may have a further smoothening effect on the protein folding energy landscape, and the results of denaturant-induced unfolding reactions which aim to probe cooperativity should, therefore, be interpreted with caution. The tunable nature of folding cooperativity may provide the kinetic control required to prevent the formation of aggregation-competent species under some conditions, while allowing the protein to sample functionally relevant, partially unfolded states under other conditions. Moreover, the continuum of states populated in a barrier-less transition, may allow a protein to function as a molecular rheostat and respond to small changes in environmental conditions. In the future, the tunable nature of folding cooperativity may be exploited to tune the response of signaling molecules from being all-or-none to being gradual.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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