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Limited cooperativity in protein folding

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Theory and simulations predict that the structural concert of protein folding reactions is relatively low. Experimentally, folding cooperativity has been difficult to study, but in recent years we have witnessed major advances. New analytical procedures in terms of conformational ensembles rather than discrete states, experimental techniques with improved time, structural, or single-molecule resolution, and combined thermodynamic and kinetic analysis of fast folding have contributed to demonstrate a general scenario of limited cooperativity in folding. Gradual structural disorder is already apparent on the unfolded and native states of slow, two-state folding proteins, and it greatly increases in magnitude for fast folding domains. These results demonstrate a direct link between how fast a single-domain protein folds and unfolds, and how cooperative (or structurally diverse) is its equilibrium unfolding process. Reducing cooperativity also destabilizes the native structure because it affects unfolding more than folding. We can thus define a continuous cooperativity scale that goes from the 'pliable' two-state character of slow folders to the gradual unfolding of one-state downhill, and eventually to intrinsically disordered proteins. The connection between gradual unfolding and intrinsic disorder is appealing because it suggests a conformational rheostat mechanism to explain the allosteric effects of folding coupled to binding.

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Introduction

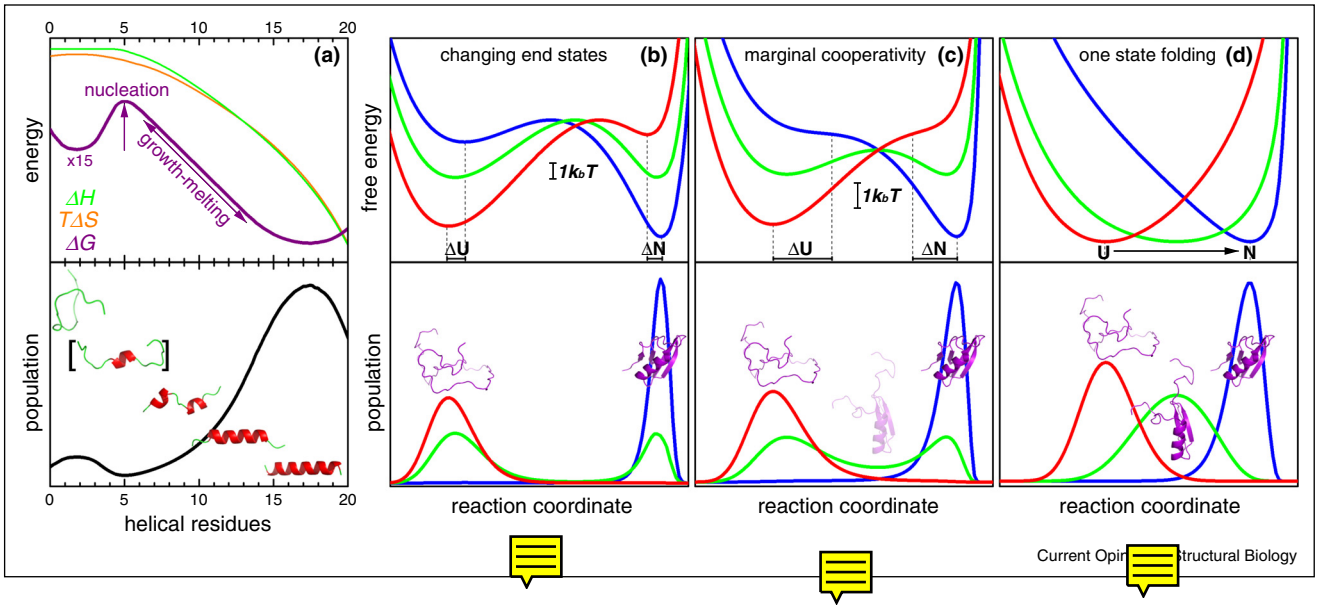
Protein folding requires the stabilization of the native 3D structure over all alternative conformations by formation of a network of weak interactions. The coordination of such interaction network is what we term folding cooperativity, a property that is at the heart of the folding mechanisms. The amount of cooperativity available to folding reactions is in principle restricted by the mesoscopic scale of proteins [1•]. It is further constrained by the need to fold within biologically reasonable times [2]. According to theory, the conflicting optimization for stability and foldability results in natural proteins with folding energy landscapes that are smooth and funneled towards the native state, and which thus have very limited cooperativity [3,4]. Theory in fact predicts that for some natural proteins folding can be an entirely gradual process (downhill) [3]. Simulations using coarse-grained protein folding models have stressed the difficulty of achieving cooperative folding from a design viewpoint [5,6]. Recent ultra-long atomistic molecular dynamics simulations on fast folding proteins have provided further support to the minimally cooperative nature of folding reactions [7••].

In contrast, the experimental analysis of folding cooperativity has been challenging, mostly because of the low resolution of conventional experiments and the lack of analytical procedures to assess cooperativity. However, there has been major progress in this front during the last years. The results obtained so far confirm the predictions from theory and simulations. Moreover, the new experimental procedures and techniques emerge as a powerful toolset that expands the realm of questions amenable to direct experimental testing, laying the ground for detailed comparison between experiments, theory and simulations. Here we discuss some of the key experimental observations that confirm the limited cooperativity of protein folding and how these results shed new light onto our general understanding of how proteins fold and function.

Folding scenarios under finite cooperativity

The experimental analysis of protein folding cooperativity requires guidelines for the interpretation of observations. As reference, it is illustrative to discuss a well-understood process with limited cooperativity such as the helix-coil transition (Figure 1a). Helix formation involves crossing a small nucleation barrier that is quickly followed by favorable multistep helix elongation [8]. The free energy surface has thus a low barrier and a shallow and broad native minimum (Figure 1a). There is significant conformational diversity (broad distribution of helix lengths), including significant populations of species at the barrier top (helix nuclei). A free energy perturbation induces the repopulation of all species, including the adjustment of the average helix length, the depopulation of helix nuclei, and the activated inter-conversion between the two minima. Thermodynamically, helix melting produces a sigmoidal curve with sharpness determined by helix cooperativity. Kinetically, the relaxation is biphasic with a faster diffusive

Figure 1



Scenarios of limited folding cooperativity. (a) Free energy surface and its enthalpic and entropic components (top) and the conformational distribution (bottom) associated with α-helix formation on short peptides as an illustration of the mechanistic implications of conformational transitions with limited cooperativity. **(b–d)** Representations of the free energy surface (top) and conformational distribution (bottom) expected for protein folding reactions under different levels of folding cooperativity. Blue curves represent native conditions, green curves the denaturation midpoint, and red curves unfolding conditions. (b) Folding scenario for the maximal cooperativity expected for single-domain proteins. The process is two-state like but its limited cooperativity makes the end states to change structurally in response to perturbation. (c) Scenario of marginal cooperativity generally expected for fast-folding protein domains. (d) Minimally cooperative, gradual unfolding process associated with one-state or global-downhill folding.

phase (adjustment amongst pre-formed helices) followed by global re-equilibration over the nucleation barrier [8].

The helix-coil transition provides simple guidelines for interpreting the experimental manifestations of folding with limited cooperativity. Using a one-dimensional representation of the folding free energy surface we can define three main scenarios. A maximally cooperative scenario includes slow folding reactions that involve crossing relatively high (several RT) free energy barriers (Figure 1b). In this case, only the unfolded and native states are ever populated, consistently with two-state thermodynamics. Nevertheless, perturbations of the free energy surface should also induce structural shifts of the end states, which would produce an additional, much faster kinetic phase. An intermediate scenario corresponds to fast folding domains. The free energy surface is shallow and the barrier at the denaturation midpoint is comparable to thermal energy (Figure 1c). The shallow surface produces a marginally cooperative unfolding process in which the end states experience large structural shifts upon perturbation and the barrier-top conformations are significantly populated (at both extreme conditions the surface becomes downhill). In the third scenario there is no barrier at any experimental condition, resulting in a free energy surface with a single minimum (one-state) that changes continuously upon perturbation (Figure 1d). The (un)folding process is then gradual and minimally cooperative, similarly to the helix-coil transition on pre-nucleated helices.

Conformational adaptation of unfolded and native states

Many slow-folding single-domain proteins seem to adhere to a two-state model. However, Figure 1b indicates that those proteins should experience structural changes of the end states. This problem has been investigated for the unfolded state using two experimental approaches. One approach relies on the analysis of missing amplitudes in chemical dilution kinetic experiments [9•]. Refolding kinetic experiments have persistently revealed a structural transition occurring within the stopped-flow dead time [10]. This burst phase involves chain compaction [11,12] and appears to be structurally gradual [13,14]. Moreover, recent experiments using faster continuous mixing methods have shown that this is a microsecond phase [15]. All these observations point to a non-cooperative structural change of the unfolded state. Another approach uses single-molecule FRET spectroscopy to resolve the structural properties of the unfolded and native states as a function of denaturant [16–18]. These experiments have also consistently shown the gradual compaction of the unfolded state for all two-state folding proteins analyzed so far [19•,20•].

Structural changes of the native ensemble might be of lesser magnitude since in terms of sensitivity to chemical denaturants the folding transition state is closer to the native than to the unfolded state [21]. Nevertheless, recent experimental works have reported structural changes in the native state of slow, two-state folding proteins. Several of these focus on SH3 domains, for which gradual native state disorder has been detected by single-molecule FRET [22••], hydrogen–deuterium exchange [23••], chemical shift analysis [22••], and from the observation of FRET-based burst phases during unfolding kinetic experiments [24]). A recent study of the U1A protein has also reported microsecond native state structural changes in response to laser T-jump perturbations [25]. The application of such high-resolution methods to other slow two-state folding proteins may reveal more examples of structurally changing native states.

Fast folding and marginally cooperative unfolding

Empirical estimates have set the bar for the fastest possible folding in the μs timescale [26,27••,28,29••]. Accordingly, proteins that fold in microseconds are expected to cross very small (if any) free energy barriers, and exhibit marginally cooperative unfolding (Figure 1c). The existence of a direct link between folding speed and unfolding cooperativity was established based on the analysis of mutants on several moderate (milliseconds) to fast (microseconds) folding proteins [30••]. The marginal cooperativity of fast folding has also been recently confirmed using long-timescale MD simulations on 10 different microsecond folding proteins [7••].

This scenario has interesting thermodynamic implications (Figure 1c). A marginally cooperative process should result in equilibrium unfolding that depends on the structural probe employed to monitor folding. Such behavior has been recently documented for several proteins that fold fast, but are no one-state downhill folders, like PDD [31], gpW [32] and $\lambda_{6\text{-}85}$ [33]. The thermodynamics of this scenario has also been investigated using differential scanning calorimetry, which detects broad ensembles and small populations of partially folded conformations [34]. DSC analyses of the fast-folding proteins gpW [32], villin [35], and PDD [36•] have confirmed their marginally cooperative unfolding. Interestingly, the minimal thermodynamic barriers (~1 RT) derived from DSC were consistent with independent barrier estimates from kinetic measurements. A comparative analysis has actually shown that DSC is sensitive enough to detect the minute populations at the top of barriers as high as 6 RT, which extends the applicability of the DSC approach to moderately fast, two-state folding proteins such as Csp [37]. The analysis of DSC protein unfolding data is normally model dependent [34]. However, this limitation has been recently removed by applying Bayesian analysis to the results obtained with multiple models [36•].

Different structural probes can also result in changes in kinetic experiments, as it has been shown for villin [38], En-HD [39] and $\lambda_{6\text{-}85}$ [33]. From a kinetic standpoint, a particularly intriguing aspect of marginally cooperative unfolding comes from the significant population of the species at the barrier top (Figure 1c). In this case, perturbations change the barrier top population, resulting in a minor ultrafast phase that corresponds to the downhill relaxation from the top (the molecular phase). A molecular phase of 1-2 μs has been observed in $\lambda_{6\text{-}85}$ [27••] and also in several WW domains [40]. This phase is equivalent to the folding transition path times obtained by single-molecule FRET methods [29••]. The fact that barrier-top conformations are populated (even if minimally) makes them amenable to atomic resolution structural analysis using NMR relaxation dispersion methods (RD-NMR), which detect perturbations on the native state chemical shifts arising from exchange with 'excited' states [41]. Although the time resolution of this approach is limited to about 100 μs, it has been recently possible to structurally characterize the barrier-top ensemble of the fast-folder gpW from RD-NMR measurements performed at very low temperature [42••].

One-state downhill folding

One-state folding corresponds to the lowest level of cooperativity that still results in formation of well-defined native structures [43]. This regime is an extreme case of the marginally cooperative scenario in which native and unfolded minima fully merge at the denaturation midpoint (Figure 1d). The thermodynamic properties are similar but more marked [30••]. Due to this reason, most thermodynamic analyses of folding cooperativity were originally developed to characterize one-state downhill and first applied to the one-state downhill folder BBL as benchmark [34,44••,45]. Interestingly, the kinetic consequences go in reverse because the diffusive re-equilibration induced by perturbation on a single welled surface is monophasic, mostly exponential and probe independent (for reasonably smooth surfaces) [46]. Probe dependence is still found in the kinetic amplitude as reflection of the differences in equilibrium unfolding [46].

Although initially met with skepticism [47], the case for one-state downhill folding is nowadays firmly established. Its equilibrium and kinetic properties have been analyzed theoretically in depth [43,48••]. The showcase protein BBL has been consistently shown to fold one-state on a battery of thermodynamic tests [34,44••,45], atomic-resolution structural analysis [49••], multi-probe ultra-fast kinetics [46] and microsecond-resolution single-molecule FRET experiments [50••]. One-state downhill folding has also been experimentally identified on other proteins, such as monellin [51••,52] and certain mutants of $\lambda_{6\text{-}85}$ [53••] and En-HD [54]. Several other fast folding domains have been catalogued as one-state folders on the basis

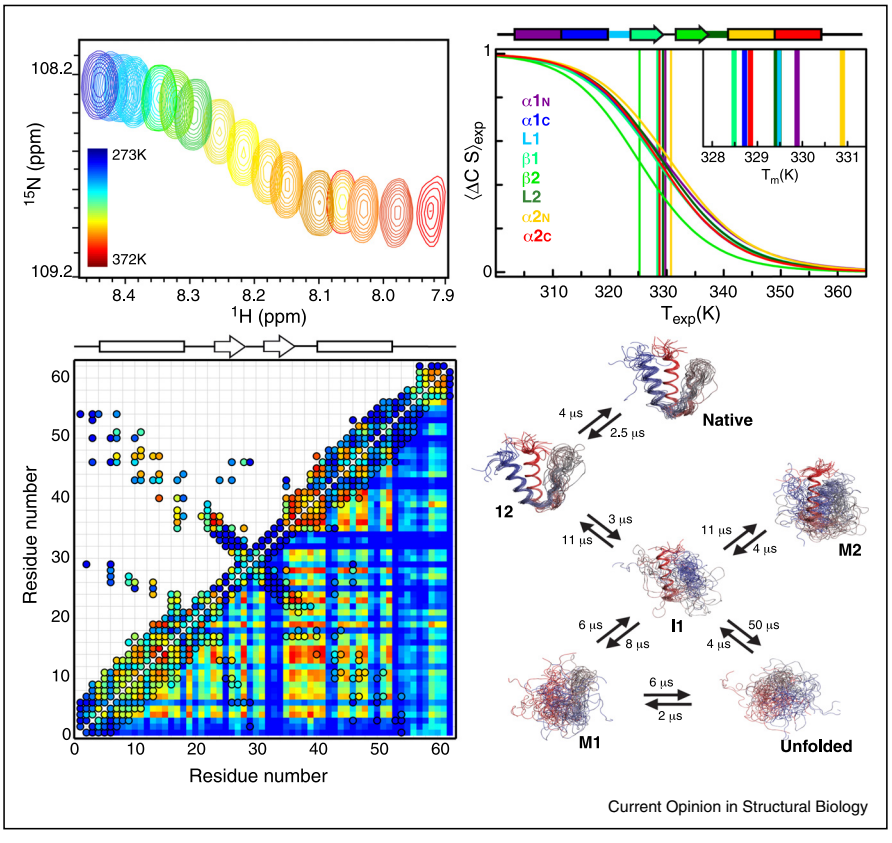
of the analysis of laser T-jump kinetic data [30••]. The one-state downhill scenario has also been studied in computer simulations with topology-based Go models [55], coarse-grained models [56•], and atomistic models [57,58]. Moreover, millisecond-long molecular dynamics (MD) simulations on fast-folding proteins have also confirmed the one-state downhill character of BBL and En-HD [7••].

High-resolution structural analysis of folding cooperativity

A scenario of limited cooperativity implies that it might be possible to resolve the structural heterogeneity of unfolding transitions from high-resolution thermodynamic data. This idea was proposed for identifying one-state downhill folding [43], but it is in principle extensible to any protein that exhibits probe dependent unfolding [59]. Practically, it has been pursued in two different forms.

The first one involves the global analysis of equilibrium unfolding data from multiple low-resolution structural probes (and DSC) with statistical mechanical models that include sufficiently detailed enumerations of the relevant partially folded conformations. This approach was pioneered on BBL [44••]. Subsequently, it has been applied to several marginally cooperative folding proteins such as villin [60], and more recently PDD [31], the de novo designed αtα [61], and P22 subdomain [62••]. Recent applications have utilized more detailed statistical mechanical models, sometimes in combination with kinetic data [60] or with select residue-specific infrared probes [61,62••]. Such studies confirmed that all these domains populate broad conformational ensembles and fold crossing minimal folding barriers, but they also revealed differences amongst them. For example, comparative analysis between structural homologues (BBL vs. PDD [63] and αtα vs. P22 [62••] provides important clues about their slight differences in cooperativity.

Figure 2



Structural analysis of folding cooperativity. This figure summarizes the structural analysis of folding cooperativity performed by NMR experiments and atomistic simulations on the fast folding protein gpW. Chemical shift (CS) thermal unfolding curves are measured experimentally from multidimensional NMR spectra of the fully assigned protein (top left) or by computing the average chemical shift from the atomic coordinates of simulated trajectories. CS curves (experimental or simulated) can be grouped to calculate the average unfolding behavior for each protein segment (top right). Pairwise cross-correlations between each CS curve of one residue with each CS curve of another residue lead to a matrix of residue-residue thermodynamic couplings in which red signifies the strongest and blue the weakest coupling (bottom left). The matrix also shows as circles the structural contacts obtained from the gpW 3D structure for reference. Experimental and simulation-generated matrices can be compared directly. In addition, structural and kinetic analyses of the simulated trajectories provide a mechanistic interpretation of the thermodynamic coupling matrix (bottom right).

The second approach attains atomic resolution by obtaining hundreds of NMR chemical shift unfolding curves that are cross-correlated to calculate the thermodynamic coupling between every possible pair of residues in the protein (Figure 2). These data provide a detailed structural mapping of folding cooperativity [64]. The NMR method was first tested on BBL, which showed an extremely broad distribution of atomic unfolding behaviors [49••]. Recently, the approach has been successfully extended to the fast-folder gpW, demonstrating its feasibility beyond the one-state downhill regime [65••]. The gpW data, which are not as heterogeneous, revealed a structurally delineated network of couplings between residues scattered throughout the sequence. In this case, long-timescale MD simulations were performed in parallel (Figure 2, bottom right). Simulations confirmed the conformationally heterogeneous and marginally cooperative unfolding process. One important feature is that matrices obtained from experiment and simulation can be compared directly, thus allowing the mechanistic interpretation of experiments and the benchmarking and refinement of simulation methods.

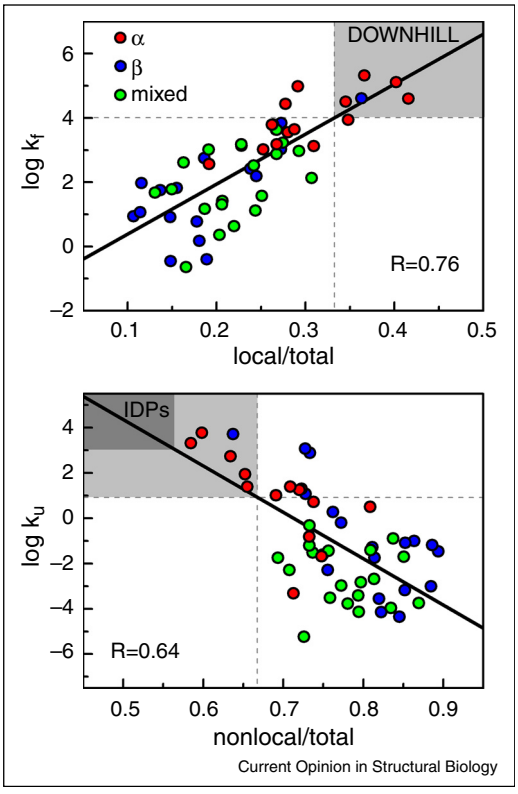
A folding cooperativity scale

The experimental results discussed in previous sections widely expand the catalogue of folding behaviors. The big question is whether it is possible to integrate all these observations to define a general scale of folding cooperativity.

The folding properties of any given protein are ultimately determined by its aminoacid sequence, but there are other factors worth considering. For example, protein domain size is key for determining folding rates [28], folding cooperativity [1] and even native state stability [66]. The empirical correlations between folding rates and coarse structural properties, such as the contact order [67] or its variants [68], indicate that the overall fold topology is another important determinant. The ratio between α-helix and β-sheet content also plays a role [69••]. In fact, a theoretical analysis of empirical data has concluded that size and secondary structure account for over 80% of the natural variability in folding and unfolding rates [69••].

From the viewpoint of folding cooperativity, a particularly interesting parameter defines the fractional contributions to native stability that come from local and from non-local interactions. This parameter was shown early on to play a pivotal role in folding cooperativity on lattice simulations [70]. To explore this relationship further we can take advantage of the recent theoretical analysis of a 52-protein database [69••] (Figure 3). Such analysis indicates that for natural domains the fraction of local interactions ranges from 0.1 up to 0.45. (0.9–0.55 for the non-local) and correlates with folding and unfolding rates (Figure 3). In other words, the larger the local fraction the faster and less cooperative is the (un)folding process. These correlations define a simple scale in which cooperativity is inversely proportional to the local fraction. The scale integrates all observed folding behaviors, highlighting that faster folding also implies faster unfolding, lower cooperativity and minimal stability. A local fraction of 0.33 delimits the transition between two-state-like and downhill folding. Further increases in local fraction lead to one-state downhill folding and ultimately to IDPs, which are thus one-state downhill folders that lack sufficient tertiary interactions to autonomously sustain a stable native structure. Another interesting realization is that the most cooperative natural domains are already close to the maximally attainable cooperativity (non-local fraction of 1).

Figure 3



Folding Cooperativity Scale. The fraction of the folding stabilization energy that comes from local and/or non-local interactions are closely related to the degree of folding cooperativity of the domain. Re-analysis of the empirical database of 52 single-domain proteins of De Sancho and Muñoz [69] shows that these parameters correlate with both the folding (top) and unfolding rates (bottom). These plots indicate that a local fraction higher than 0.33 results in the fast, marginally cooperative folding characteristic of the downhill folding regime (proteins that (un)fold barrier-less at some experimental conditions). Because the unfolding rate speeds up with an increasing local fraction at a faster rate than the folding rate does (the slope from the bottom plot is higher than from the top plot), the native state of fast-folding proteins is also marginally stable. This effect is further strengthened because at local fractions higher than 0.4 the folding rate levels off as the domains approach the folding speed limit. Therefore, local fractions higher than 0.45 (non-local below 0.55) produce intrinsically disordered domains (IDP regime).

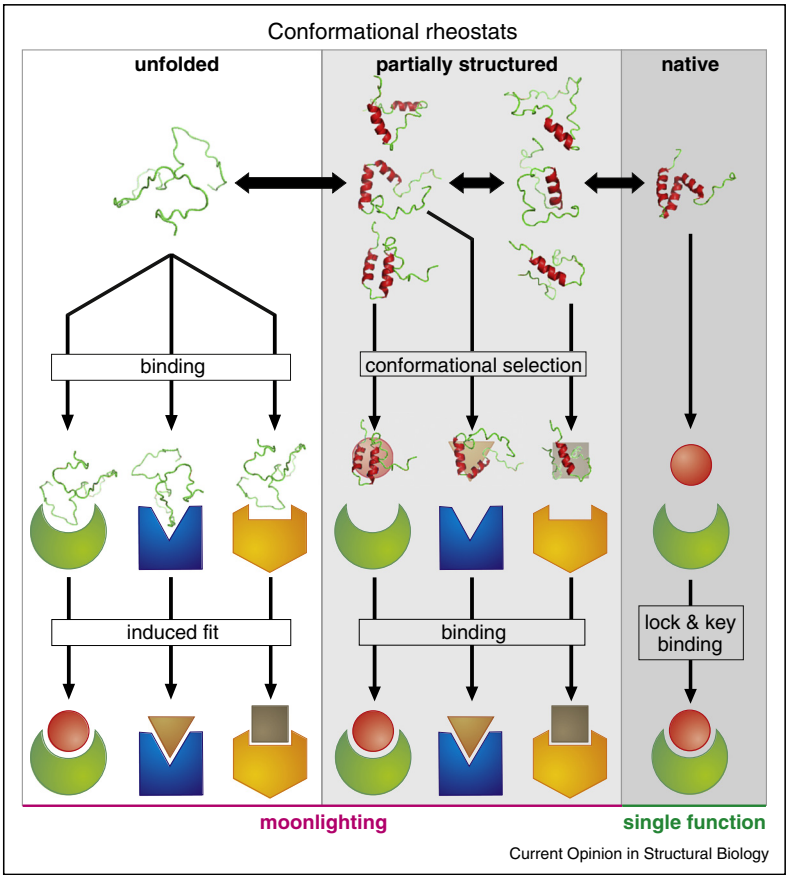
Functional possibilities of gradual (un)folding

The discovery of IDPs and the realization that they represent a large fraction of the proteome [71•] has spurted into a great deal of research activity on these domains [72]. The lack of a defined structure immediately inspired new functional modes emerging from the coupling between folding and binding [73]. Accordingly, experimental studies have reported disordered proteins that bind their partners by induced fit or conformational selection [74••], or bind to multiple, structurally diverse partners [75] resulting in moonlighting [76] and/or allosteric effects [77••]. In parallel, NMR structural analysis [78] and single-molecule fluorescence experiments [79] have shown that proteins classified as IDPs do have residual structure when unbound to their partners, and are able to form stable native structures under favorable conditions [80,81••].

Looking into those issues from a cooperativity viewpoint immediately suggests a mechanistic link between the functional modes associated to partial disorder and the marginally cooperative unfolding of downhill domains (Figure 4). Figure 4 shows how the coupling between binding and gradual folding explains in simple terms all the functional complexity and multiple binding modes reported for IDPs. The key is that modulation of the domain's stability affords an exquisite control of the properties of its conformational ensemble, which facilitates the implementation of different binding mechanisms, binding to structurally different partners, and complex allosteric effects. In other words, marginally cooperative downhill folding domains have a built-in mechanism to operate as conformational rheostats through folding coupled to binding [82].

There is amounting evidence that the complex binding modes commonly associated to IDPs do in fact involve the gradual conformational changes of molecular rheostats rather than discrete conformational transitions. For example, the nuclear coactivator binding domain (NCBD), which has been classified as an IDP [80,83], binds multiple partners

Figure 4



The gradual folding coupled to binding of conformational rheostats. The figure shows a schematic representation of how the gradual unfolding of one-state downhill folding domains (simplified here as a series of coupled conformational equilibria) can be coupled to the binding to structurally different partners through different binding mechanisms such as lock and key, conformational selection and induced fit. The affinity to different partners and the specific binding mechanism can be changed and controlled by simple modulation of the intrinsic native stability of the downhill domain (e.g. by changes in environmental conditions such as temperature, pH, partner concentration), thus resulting in protein moonlighting and positive or negative allosteric effects.

by folding into different conformers [80,84,85]. However, under stabilizing conditions, NCBD folds into a three helix bundle in a process that is gradual (one-state downhill) according to the multivariate analysis of experimental data and computer simulations [86]. It has also been noted that the denaturation temperature of homologous downhill folding domains from meso-thermophilic, thermo-thermophilic and hyper-thermophilic organisms coincides with the living temperature of the host organism, suggesting a functional need for populating partially folded conformations [82]. DNA binding domains are marginally cooperative fast folders [36•] that exhibit partial disorder when unbound [87]. Finally, it has been recently demonstrated that the coupling between proton binding and gradual (one-state) unfolding results in biosensors with ultra-wide dynamic range [88••].

As more folding coupled to binding processes are studied at high-resolution the mechanistic link between gradual (un)folding and disorder-mediated, complex binding may become increasingly apparent.

Conflict of interest statement

Nothing declared.

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