Exploiting the downhill folding regime via experiment

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Traditionally, folding experiments have been directed at determining equilibrium and relaxation rate constants of proteins that fold with two-state-like kinetics. More recently, the combination of free energy surface approaches inspired by theory with the discovery of proteins that fold in the downhill regime has greatly widened the battlefield for experimentalists. Downhill folding proteins cross very small or no free energy barrier at all so that all relevant partially folded conformations become experimentally accessible. From these combined efforts we now have tools to estimate the height of thermodynamic and kinetic folding barriers. Procedures to measure with atomic resolution the structural heterogeneity of conformational ensembles at varying unfolding degrees are also available. Moreover, determining the dynamic modes driving folding and how they change as folding proceeds is finally at our fingertips. These developments allow us to address via experiment fundamental questions such as the origin of folding cooperativity, the relationship between structure and stability, or how to engineer folding barriers. Moreover, the level of detail attained in this new breed of experiments should provide powerful benchmarks for computer simulations of folding and force-field refinement. [DOI: 10.2976/1.2988030]

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From a chemical standpoint natural proteins are intriguing objects that share a distinctly polymeric character with the uniqueness of their biologically determined composition ("the aminoacidic sequence"). It is through their unique aminoacid sequence that proteins can defeat the large conformational entropy inherent to any polymer and fold into specific three-dimensional structures stabilized only by weak noncovalent interactions. The combination of large numbers of degrees of freedom and intricate networks of weak interactions should make protein folding a highly complex and inefficient process. Nevertheless, we know experimentally that the basic folding elements (protein domains) fold spontaneously, efficiently, and, often, with macroscopic features that resemble first-order transitions or two-state chemical reactions. Solving this conundrum has often been considered the protein folding problem.

One of the difficulties in studying folding has been obtaining a common conceptual framework between theoreticians and experimentalists. Such framework in which theory is used to develop tools for analysis and interpretation of experiments and to make fundamental predictions amenable to experimental testing has finally emerged from efforts started in the late 1980s and 1990s (Bryngelson *et al.*, 1995; Oliveberg and Wolynes, 2005). According to these ideas, protein folding is viewed as diffusion on a hyperdimensional corrugated energy landscape with a bias towards the native structure (the folding funnel)

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(Bryngelson *et al.*, 1995). In addition to explaining why folding is efficient and reasonably fast, the energy landscape approach promotes the use of low dimensional free energy projections as powerful means to analyze folding experiments. From projecting the energy landscape onto one or a few order parameters comes the realization that folding barriers arise from an early decrease in conformational entropy that is not entirely compensated by the formation of stabilizing interactions (Onuchic *et al.*, 1997). Interestingly, the principle of an entropy-energy mismatch as source of folding barriers comes with a corollary: the prediction of barrierless (downhill) folding (Bryngelson *et al.*, 1995). In the downhill folding regime proteins cross very small barriers of less than 3 *RT* at the denaturation midpoint and fold without barriers in some favorable conditions (Gruebele, 2008). Downhill folding is more than a curiosity. It can have tremendous practical implications because, in contrast to two-state folding, downhill folding could lead to direct experimental exploration of the entire folding process, both kinetically (Eaton, 1999) and thermodynamically (Muñoz, 2002).

In the last years downhill folding has become an experimental reality (Naganathan *et al.*, 2006). The application of ultrafast folding techniques has introduced 1 μs as an empirical estimate for the folding speed limit (Hagen *et al.*, 1996; Kubelka *et al.*, 2004) and has resulted in the experimental identification of many microsecond-folding proteins (Muñoz, 2007), which accordingly are in or near the downhill folding regime (Naganathan *et al.*, 2007). The cumulative introduction of mutations that speed folding up has been used to approach the downhill scenario kinetically (Liu *et al.*, 2008; Yang and Gruebele, 2003). Recently, the downhill folding arena has been extended to include natural protein domains of mid-size and $\alpha+\beta$ topology (Fung *et al.*, 2008). In parallel, the identification of global downhill or one-state folding, in which there is no barrier at any degree of denaturational stress (Garcia-Mira *et al.*, 2002; Naganathan *et al.*, 2005a), has been exploited to investigate the thermodynamic implications of the downhill regime. From this work we have now tools to estimate the height of thermodynamic (Muñoz and Sanchez-Ruiz, 2004; Naganathan *et al.*, 2005b) and kinetic (Naganathan *et al.*, 2007) folding barriers, approaches to determine the structural heterogeneity of equilibrium unfolding (Naganathan and Muñoz, 2008; Sadqi *et al.*, 2006), and the opportunity to measure conformational dynamics as a function of the degree of folding.

In this perspective we review some of these recent developments making emphasis on the open questions and discussing novel experimental approaches that could lead to addressing some of these questions in the near future.

PROTEIN FOLDING BARRIERS

The observation of two-state-like folding implies the presence of a free energy barrier separating the unfolded and native state ensembles. In principle such two-state barrier must be sufficiently high as to make the population at its top negligible. Practically, this definition implies that the free energy at the barrier top must be over 3 *RT* higher than the free energy of the ground state (native or unfolded depending on denaturational stress) (Gruebele, 2008). Of course, the implication is that even proteins that behave strictly as two-state experimentally could have quite low barriers, which is in fact what current estimates of folding speed limits suggest (Naganathan *et al.*, 2006). This realization reconciles two-state and global downhill folding as two extremes of a continuum of folding barriers where the height limit might be determined in the end by requirement of folding within biologically relevant times. These ideas come naturally from the application of a free energy surface approach to protein folding and suggest that some important aspects of the folding process are overlooked when experiments are interpreted as elementary chemical reactions (e.g., the widely used scheme $U \underset{k_u}{\overset{k_f}{\rightleftarrows}} N$). The argument can be turned around to say that the free energy surface approach makes some general predictions about protein folding reactions that would be difficult to rationalize within conventional chemical views. Some of these properties emerge in the downhill folding regime as deviations from two-state behavior and, therefore, can be used as diagnostic of this regime (Naganathan *et al.*, 2007). Other non-two-state features predicted by a free energy surface approach should be observable even in folding reactions over large barriers. The latter are particularly interesting as tests of the free energy approach and suggest experimental strategies to address important questions about the origin of folding barriers.

Interesting predictions of the free energy surface approach for two-state—like folding

In a free energy approach the multidimensional folding energy landscape is projected onto one or a few order parameters resulting in a low (or even one) dimensional free energy surface that describes the entire thermodynamic properties of the folding process.

When the free energy projection produces a good reaction coordinate then the folding kinetics are described as diffusion on that free energy surface. A general consequence of free energy approaches is that any thermodynamic quantity of the system is a continuous function of the order parameter(s) rather than just a discrete value. Figure 1 illustrates this point with an example of projection onto a single order parameter that represents the degree of native structure in terms of local conformation. The figure shows the projection of the two opposing forces in folding: conformational entropy (in energy units) and stabilization free energy. The latter includes contributions from specific interactions (e.g., hydrogen bonds, van der Waals, electrostatics) and solvation free energy, and it is only weakly temperature dependent (Akmal and Muñoz, 2004). The two opposing forces change

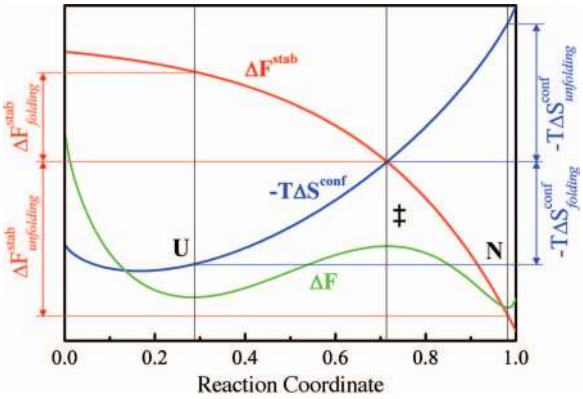

Figure 1. Representation of the balance between the two opposing forces in protein folding in a one-dimensional free energy surface. The stabilization free energy is shown in red, the cost in conformational entropy in blue, and the resulting free energy surface in green. The partitioning of stabilization free energy (ΔF^{stab}) and conformational entropy (ΔS^{conf}) on both sides of the barrier top is shown on the sides of the figure.

continuously with the degree of folding and, according to both theoretical arguments and computer simulations, with increasing curvature towards the fully native state (see red and blue curves in Fig. 1). Depending on the difference in curvature of the two functions the total projected free energy develops a barrier separating two minima at low and high degrees of order (green curve in Fig. 1), or is downhill.

The barrier top arises at the order parameter value in which the misbalance between the decreases in conformational entropy (i.e., increases in $-T\Delta S_{conf}$) and stabilization free energy (i.e., ΔF_{stab}) is maximal. An important prediction is then that the height of the barrier is a fraction of the equilibrium cost in conformational entropy upon folding, which in turn would explain the scaling of experimental folding rates with protein size (Li *et al.*, 2004; Naganathan and Muñoz, 2005; Thirumalai, 1995). Structure and sequence can further modulate the barrier height by changing the curvature of these functions. For example, local interactions are already present at low values of the order parameter because they form by just ordering a few contiguous residues. Thus, increasing the contribution of local interactions to the stabilization energy would decrease its curvature and that of the conformational entropy as well, resulting in smaller barriers. When the barrier decreases below the 3 *RT* threshold the two minima start moving closer together. This is manifested in changes in typical macroscopic observables that have been recently used to diagnose downhill folding from kinetic experiments (Naganathan *et al.*, 2007). Because the barrier is always a small fraction of the total entropy cost, very slight curvature changes in the stabilization energy can result in large differences in barrier heights, pointing to a high tunability range at the disposal of natural selection. The misbalance between conformational entropy and stabilization free energy as origin of folding barriers has been tested empirically in several two-state proteins (Akmal and Muñoz, 2004). In the future it would be interesting to carry out similar experiments and analysis in other two-state-like proteins to further test this result.

Another interesting consequence of the free energy surface approach to protein folding is that the fractions of conformational entropy (β_{conf}) and stabilization free energy (β_{stab}) realized at the top of the barrier are expected to change drastically as a function of denaturational stress. This is in stark contrast with the chemical analysis in which the top of the barrier is another state (i.e., transition state) with fixed structural and thermodynamic properties (Fersht *et al.*, 1992). In free energy surface approaches, when the stabilization energy changes in magnitude the intersect between the two opposing functions (stabilization energy and conformational entropy in energy units) necessarily moves. As a consequence the top of the barrier always shifts along the reaction coordinate following Hammond's behavior [see Fig. 2(A)]. This effect is independent of the type of denaturation procedure (e.g., thermal, chemical). The shift of the barrier top along the reaction coordinate is small, especially for high barriers, and linear. However, these slight shifts in the barrier top position correspond to large changes in β_{conf} and β_{stab}, which greatly decrease as protein stability increases [Fig. 2(C)]. Here it is important to emphasize that such changes in thermodynamic properties of the barrier top are intrinsic to the free energy surface approach and thus could be considered a prediction of universal behavior in protein folding.

Therefore, a key question is whether these large changes in β_{conf} and β_{stab} are compatible with the wide observation of linear limbs on plots of the logarithm of the folding relaxation rate as a function of denaturational stress (chevron plots). V-shaped chevron plots have been traditionally considered strong evidence of invariant folding transition states (Huang *et al.*, 2007; Meisner and Sosnick, 2004). However, it is easy to demonstrate that, in spite of the large intrinsic changes in β_{conf} and β_{stab}, the free energy surface approach produces chevron plots with essentially linear limbs that are perfectly compatible with experimental results [Fig. 2(B)]. The critical difference is that the barrier top has no fixed thermodynamic properties, but moves monotonically from close to the unfolded state in native conditions toward the native state at increasing denaturational stress.

There are currently empirical estimates of β_{conf} and β_{stab} in native conditions for six two-state proteins (Akmal and Muñoz, 2004). These estimates, which were obtained from the temperature dependence of the two-state folding and unfolding rates, indicate a barrier top with roughly 1/3 of the total change in conformational entropy, 1/4 of the change in stabilization free energy, and little variation among the six proteins (Akmal and Muñoz, 2004). Both the small values in stabilizing conditions and the similarity among proteins are in close agreement with predictions from the free energy sur-

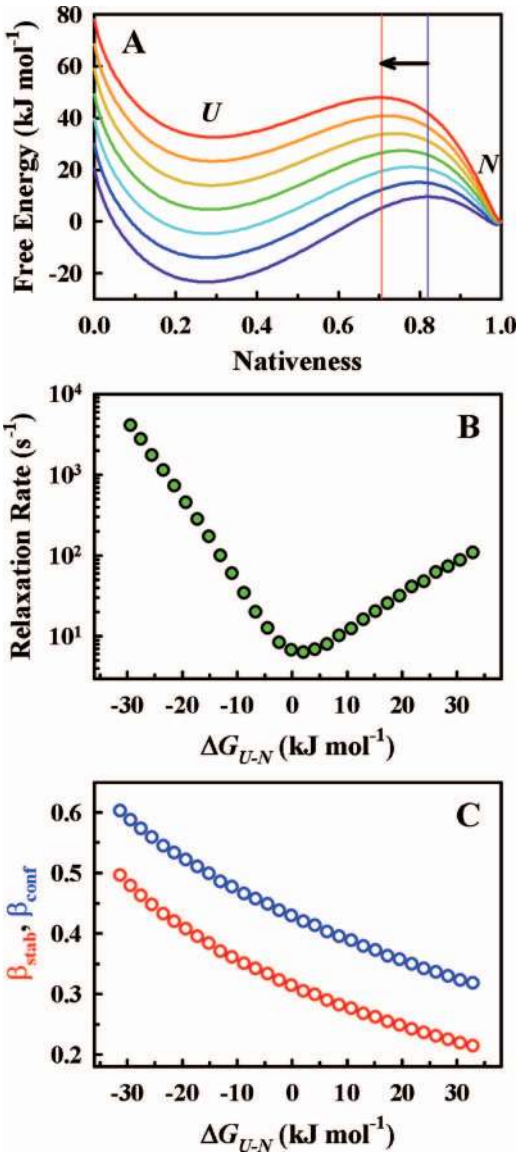

Figure 2. Predictions of the free energy approach for the top of the folding barrier. Panel (A) shows that the barrier top is expected to shift linearly towards the unfolded state when the denaturational stress decreases (or protein stability increases). In this figure the color code represents denaturational stress as proportional to the energy in the spectrum of light (e.g., purple corresponding to the strongest denaturational stress). Panel (B) shows that in spite of the movement of the barrier top the logarithm of the folding relaxation rate as a function of denaturational stress still exhibits the characteristic V-shape. Panel (C) shows an example according to the free energy approach of the large changes with denaturational stress that are expected for the fractions of conformational entropy (β_{conf}) and stabilization free energy (β_{stab}) realized at the barrier top.

face approach. Along the same lines, Brönsted analysis of over 800 mutations on 26 proteins obtained from the literature produces an average phi value of 0.24 (AN Naganathan and V Muñoz, unpublished results). Phi values report on the fraction of the destabilization energy introduced by site-directed mutations that is realized on the top of the folding barrier (Fersht *et al.*, 1992). Therefore, the average phi-value could be seen as an estimate of β_{stab} (ignoring changes in conformational entropy induced by mutation) for all those proteins and mutants. The agreement between this average phi value and the β_{stab} estimate for six proteins obtained with a completely independent method is tantalizing. Nevertheless, the important evidence still missing is whether β_{conf} and β_{stab} do increase as proteins become less stable. This issue could perhaps be investigated more deeply taking advantage of the wealth of mutational data already available.

Thermodynamic versus kinetic folding barriers

Free energy projections should reproduce protein folding thermodynamics exactly. Protein folding kinetics and dynamics are more complex because the combination of order parameters onto which the energy landscape is projected must also represent the time progress of the folding process. The inherent difference is that the folding time depends on fluxes rather than populations. This aspect is highlighted in Fig. 3, which shows a simplified two-dimensional surface with two minima and rugged microtopography. The two-dimensional surface represents a two-state system separated by a barrier. As expected, projecting the surface onto one of the two order parameters results in a typical two-state profile (red curve in Fig. 3) in which a large fraction of the intrinsic surface roughness is smoothed out. The free energy at each value corresponds exactly to the population of the complete slice in the projection (green curve in Fig. 3 for the slice at the barrier top). Therefore, it is possible to define a thermodynamic folding barrier as the ratio between the populations

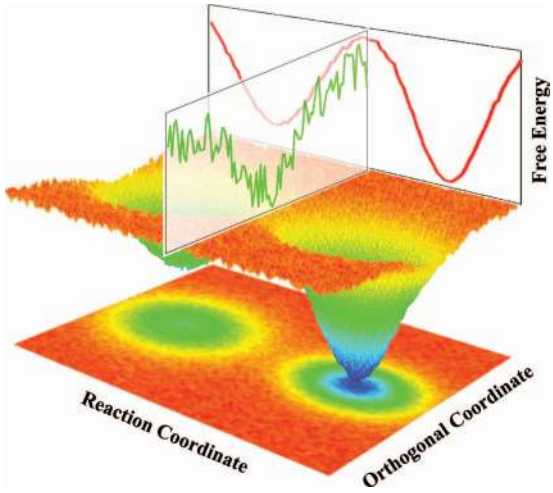

Figure 3. Multidimensional folding landscapes and free energy projections. The figure depicts as an example a simple two-dimensional free energy surface with two global minima and rough topography. The projection of the free energy surface onto one order parameter (e.g., the reaction coordinate) produces the smoother two-state free energy profile shown in red. The top of the free energy profile corresponds to the sum of the populations of the orthogonal slice shown in green.

on the bottom of one well and the area on the top in energy units. This barrier is exact in terms of populations in equilibrium and independent of the roughness. The surface roughness, however, does have two important effects on the kinetics. The smoothed out roughness along the reaction coordinate slows down the transit time. It is directly accounted for in the effective diffusion coefficient for the projected surface (Socci *et al.*, 1996) and, therefore, it does not affect the magnitude of the kinetic folding barrier. A second effect arises from landscape roughness orthogonal to the reaction coordinate, which can result in local bottlenecks and thus in a nonequilibrium distribution of net fluxes over the many local valleys at the barrier top (green curve in Fig. 3). This situation would result in effective kinetic barriers that are higher (even using the correct effective diffusion coefficient) than the thermodynamic barrier. Therefore, comparison between thermodynamic and kinetic estimates of folding barriers can give us important clues about the basic topography and degree of overall roughness on the folding landscapes of natural proteins.

How to estimate thermodynamic folding barriers? A method based on the quantitative analysis of differential scanning calorimetry (DSC) thermograms has been developed recently (Muñoz and Sanchez-Ruiz, 2004). This method requires fitting the thermogram obtained in absolute heat capacity units to an idealized one-dimensional free energy surface. In its first applications the method showed that it could clearly distinguish between global downhill folding and two-state-like folding over large barriers (Muñoz and Sanchez-Ruiz, 2004). Later on the analysis of 15 proteins with available DSC and folding kinetic data showed a very good correlation between the thermodynamic barrier at the midpoint temperature obtained from DSC and the folding time at 298 K (Naganathan *et al.*, 2005b). This result was important for two reasons. First, it indicates that the folding landscape of these proteins, which all exhibit simple folding kinetics, is reasonably smooth. Second, it provided a good test of the performance of the method.

However, there still are many open questions. Since the DSC method relies on detecting enthalpy fluctuations, it is only quantitative when the barriers are not too large. A crude estimate based on the analysis of the 15 proteins mentioned above suggests that ~ 6 *RT* might be a safe upper limit for the quantitative sensitivity of the method. Here is where proteins that fold in or near the downhill folding regime become important. Very recently, the method has been applied to estimate the thermodynamic barrier of two ultrafast folding proteins: the villin headpiece domain (Godoy-Ruiz *et al.*, 2008) and gpW (Fung *et al.*, 2008). The thermodynamic barriers at the midpoint from DSC were both positive, but small [~ 2 *RT* for villin (Godoy-Ruiz *et al.*, 2008) and below *RT* for gpW (Fung *et al.*, 2008)], consistently with their microsecond folding times. The most interesting result, however, is that such barriers were compared with empirical estimates of kinetic folding barriers. In the villin headpiece, a kinetic barrier was estimated by taking the 70 ns fast phase observed in laser temperature-jump experiments as diffusion coefficient for the slower microsecond phase (Godoy-Ruiz *et al.*, 2008). The kinetic barrier so obtained was also small, although ~ 1 *RT* higher than the best estimate of the thermodynamic barrier. This difference could reflect a higher kinetic barrier (i.e., orthogonal roughness), and/or an overestimated diffusion coefficient at the barrier top. The latter would be the case if landscape roughness does increase along the reaction coordinate and the 70 ns fast phase corresponds to an unfolded state relaxation. The kinetic barrier for gpW was not obtained using an estimated diffusion coefficient, but from the temperature dependence of the folding relaxation rate analyzed with a free energy surface approach (Fung *et al.*, 2008). In this case thermodynamic and kinetic barriers were smaller and in closer agreement, although the kinetic estimate was again a bit higher. Therefore, the results in these two proteins suggest folding landscapes with some residual roughness. But overall, the agreement is encouraging and supports the feasibility of using low-dimensional free energy projections to describe the folding kinetics of protein domains. In the future, we should try to improve the procedures for estimating both thermodynamic and kinetic barriers and to apply them to other proteins that exhibit simple folding kinetics.

Another interesting result has come from the DSC analysis of the two structural homologues hen egg-white lysozyme and bovine α-lactoalbumin. These two are much larger proteins (>120 residues) with the same structure, but very different biological roles (Halskau *et al.*, 2005). In spite of having the same size and structure, the thermodynamic barriers for these two proteins are very different. According to the DSC method the thermodynamic barrier of hen egg-white lysozyme is very high (>14 *RT*), whereas bovine α-lactoalbumin has a marginal barrier below 2 *RT* both in the apo- and holoforms (Halskau *et al.*, 2008). This observation provides an explanation to the different properties that these two proteins have in equilibrium and suggests a biological role for the modulation of the thermodynamic folding barrier (Halskau *et al.*, 2008). However, in this case thermodynamic barriers do not correlate at all with folding kinetics, which are slow and multiphasic in both cases (Rothwarf and Scheraga, 1996; Troullier *et al.*, 2000).

Therefore, it seems that in larger proteins with chemical crosslinks (both hen egg-white lysozyme and bovine α-lactoalbumin have several disulphide bonds in the native structure) the folding landscape is much more rugged and the kinetics mostly controlled by escape from local traps. The question is whether the decoupling between thermodynamic folding barriers and kinetics is due to the presence of chemical crosslinks or to the increasing structural complexity of these proteins. To address this question it would be interesting to study other proteins with chemical complexity that,

ideally, is controllable experimentally. One such candidate could be cytochrome c in which there is a possibility of histidine-heme misligation (Sosnick *et al.*, 1994) together with submillisecond folding when misligation is impeded by addition of large quantities of imidazole (Chan *et al.*, 1997).

Engineering folding barriers: from two-state to downhill and back

If folding barriers arise from the difference in curvature between stabilization energy and conformational entropy it should be possible to manipulate the barrier height by changing either of the two through mutation. Several ideas of how to achieve this are emerging from recent experiments in the downhill regime. Putting these ideas into practice may give us the opportunity to decrease the folding barriers of two-state proteins down to the downhill folding limit, and the other way around. This ability to engineer folding barriers will allow testing experimentally possible biological roles for both two-state and downhill folding.

One obvious approach is to change the ratio between local and nonlocal contributions to the stabilization energy. As discussed above, a high content of stabilizing local interactions should decrease the barrier because these interactions are realized early in the folding process. Gruebele and coworkers have exploited this idea and created superfast mutants of the λ repressor by combining several mutations that enhance the helical propensity of its native α helices (Yang and Gruebele, 2003; 2004). Moreover, the two natural proteins that have been identified as downhill folders, BBL (Garcia-Mira *et al.*, 2002) and gpW (Fung *et al.*, 2008), are α helical and have very high intrinsic helical propensity according to the helix algorithm AGADIR (Muñoz and Serrano, 1994). The counter example is given by the villin headpiece sub-domain, which is α helical and folds as fast, but appears to have a barrier near the 3 *RT* threshold (Godoy-Ruiz *et al.*, 2008). Based on AGADIR, the intrinsic helical propensity of the villin sequence is very low, suggesting that its barrier results from little contribution from local interactions to its stability.

Another factor is the presence of a tightly packed core, which is also related to the presence of buried aromatic residues. A tightly packed core will involve the formation of complex tertiary interaction networks (Chan *et al.*, 2004) and possibly the simultaneous expelling of several water molecules (Liu and Chan, 2005). In other words, a tightly packed core solidified with aromatic residues is likely to result in highly curved stabilization energy (see Fig. 1), and thus high folding barriers. Here again, downhill folders BBL and gpW share a structurally loose hydrophobic core with very low aromatic content. Besides, Gruebele and coworkers appear to have succeeded in producing a near global downhill folding version of λ repressor by weakening its hydrophobic core (Liu and Gruebele, 2007). In parallel, Fersht and co-workers have introduced tryptophan residues in the core of BBL and other structural homologues such as PDD and POB (Ferguson *et al.*, 2004; 2005), all belonging to a protein family in which the absence of tryptophan is strictly conserved. The introduction of core tryptophan residues appears to enhance an incipient thermodynamic barrier in PDD (Naganathan *et al.*, 2005b). Moreover, the tryptophan mutants of some of these proteins (not BBL) do show slightly steeper chevron plots (Ferguson *et al.*, 2004; 2005), suggesting the presence of a midpoint barrier in the 2–3 *RT* range (Naganathan *et al.*, 2007).

Electrostatic interactions are a third factor suggested by structural comparison of the naturally downhill folders BBL and gpW (Fung *et al.*, 2008). Electrostatic interactions act on long distances and thus could be used to engineer the relative stability of partially folded conformations that belong to the thermodynamic barrier. In fact, the large differences in thermodynamic barrier between hen egg-white lysozyme and bovine α-lactoalbumin have been linked to their distinct surface charge distributions (Halskau *et al.*, 2008). The charge distribution of these two proteins with almost identical three-dimensional (3D) structure is very different, and so is their pattern of pK values with large deviations from model compound values for hen egg-white lysozyme and no significant deviations for bovine α lactoalbumin (Halskau *et al.*, 2008). A simplistic theoretical analysis of the electrostatic interactions in their conformational ensembles does in fact suggest that the surface charges in bovine α-lactoalbumin are distributed to preferentially stabilize partially folded conformations. Of course the most striking result when comparing the two proteins is that the barrier modulation is in this case a remarkable ~12 *RT*.

The combination of the three factors provides a powerful toolbox for engineering down or up folding barriers. Local interactions can be easily manipulated, at least in α-helical proteins (Muñoz and Serrano, 1996). There also is a history of successes in engineering surface charge distributions to increase protein stability (Sanchez-Ruiz and Makhatadze, 2001) that could be adapted to tune folding barriers. Finally, Fersht and co-workers have demonstrated great heuristic ability in successfully introducing aromatic residues in the loose cores of small ultrafast folding proteins (Ferguson *et al.*, 2004; 2005).

ATOM-BY-ATOM ANALYSIS OF PROTEIN FOLDING

One of the most attractive features of the downhill folding regime is that all partially folded conformations are susceptible to populate at significant levels by tuning the degree of denaturational stress (Muñoz, 2002). The implication is that the entire folding process can be resolved with high resolution equilibrium experiments. This has been recently demonstrated analyzing the equilibrium thermal unfolding process of the global downhill folder BBL at the atomic level (Sadqi *et al.*, 2006). From these experiments we have seen that unfolding-induced changes in the electronic environ-

ment of individual atoms in the protein are extremely complex. Such structural complexity is expected for a global downhill folding protein, and to a lesser extent for proteins with small folding barriers (Muñoz, 2007). Although experimentally challenging and arduous, characterizing the structural features of equilibrium protein unfolding atom by atom provides a unique opportunity to investigate the microscopic origin of common macroscopic observations as well as their link with the native 3D structure.

Microscopic interpretation of equilibrium protein unfolding

Equilibrium unfolding experiments of protein domains in which the integrity of the native structure is monitored with a low-resolution spectroscopic technique produce what is often called a sigmoidal curve. Sigmoidal unfolding curves are typically interpreted as indicative of the ratio between fully folded and fully unfolded molecules (1 at the midpoint) (Aune and Tanford, 1969). Likewise, a single peak in DSC thermograms is simply seen as reflecting the maximum in $p_N(1-p_N)$, where p_N is the probability of the protein being in the native state as a function of temperature (Freire and Biltonen, 1978). This interpretation is convenient because it simplifies the analysis of unfolding experiments. However, it produces an important conceptual dead end: how to explain microscopically the large and varying broadness observed in equilibrium unfolding experiments.

Atom by atom equilibrium unfolding experiments in the downhill folding regime have provided a solution to this problem. In downhill proteins the global unfolding transition is very broad, and the unfolding process is expected to be structurally heterogeneous (Garcia-Mira *et al.*, 2002). Nevertheless, the large heterogeneity in atomic unfolding curves does not imply that the unfolding process is completely uncorrelated. In fact, the first moment of all the atomic unfolding curves of the downhill protein BBL is a simple sigmoidal that superimposes with the unfolding curve monitored with a low-resolution technique. Moreover, the structural heterogeneity [measured as the standard deviation in p_N for all atomic probes (Sadqi *et al.*, 2006)] as a function of temperature follows exactly the shape of a DSC thermogram (Fig. 4). In other words, the unfolding heterogeneity at the atomic level is the origin of the two basic macroscopic observations: the sigmoidal unfolding curve and the peak in enthalpic fluctuations at the midpoint. Besides, these results indicate that there is a close connection between the broadness of the global unfolding curve and the structural heterogeneity at the atomic level. This relation is exactly what would be expected from statistical mechanical arguments (Kouza *et al.*, 2006). In principle, the same argument can be taken beyond downhill folding to proteins with larger barriers (Klimov and Thirumalai, 2002). The idea is that it should be possible to estimate the degree of structural heterogeneity of any protein unfolding process simply from the broadness of the global equilibrium curve or DSC thermogram, and, by exten-

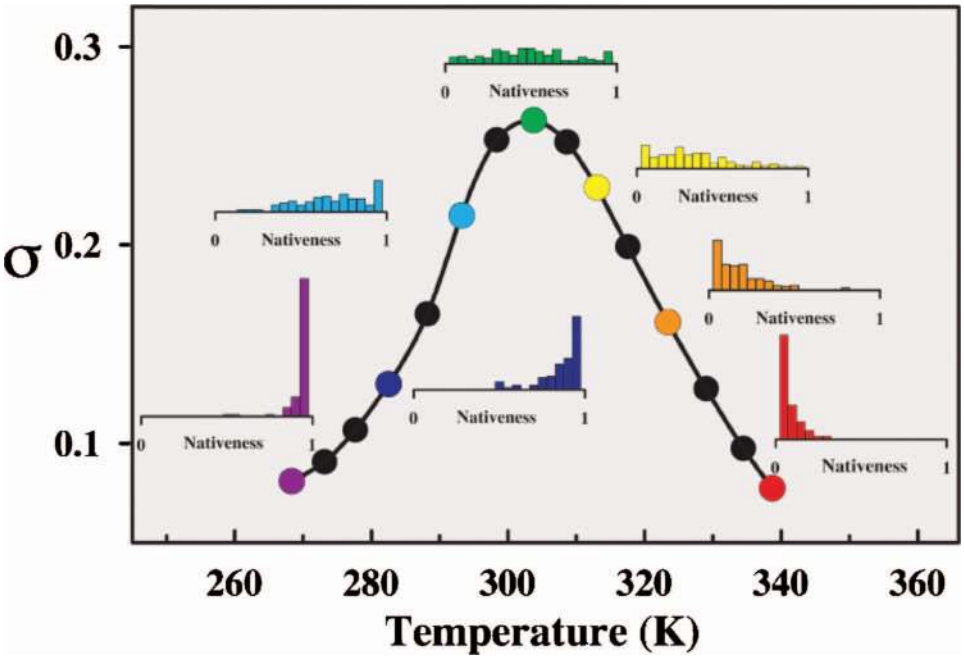

Figure 4. Microscopic connection between structural heterogeneity at the atomic level and the broadness of the global unfolding curve. The histograms show the variability in atomic unfolding behaviors for BBL as determined by Sadqi *et al.* from NMR experiments (Sadqi *et al.*, 2006). The peaked curve corresponds to the standard deviation of the histograms, which coincides with a typical single-peaked DSC thermogram.

sion, estimate the magnitude of the thermodynamic folding barrier. This important hypothesis, which extends a bridge between traditional folding experiments and statistical mechanics, should be confirmed in other experimental systems. Proteins with small barriers might be ideal candidates to further test this important point.

Interaction networks and the origin of folding cooperativity

The basic idea behind multiprobe equilibrium unfolding experiments with atomic resolution is that the energetic coupling between different atoms in a protein is in general small. For global downhill folding the average coupling is only slightly higher than *RT* (Sadqi *et al.*, 2006), and thus the protein unfolds gradually. But even two-state-like folding proteins should exhibit limited energetic coupling because stabilizing interactions are weak and not too many. Besides, the approach can be taken much further and map the specific interaction networks that stabilize proteins in their native structures. To achieve this one could look for correlations in the atomic unfolding behaviors of each pair of residues in the protein (Sadqi *et al.*, 2006). Such pairwise residue couplings should reflect how protein stabilization energy is distributed through the sequence. This is an important piece of information not accessible by any other experimental means. With the coupling matrices at hand it becomes possible to look for chemical, structural, and sequence patterns, and thus unravel the energetic factors that determine protein stability and folding.

Currently the only protein that has been analyzed in this way is BBL (Sadqi *et al.*, 2006; 2007). This single dataset, however, is sufficient to illustrate the potential of the approach. By grouping the atomic probes of BBL according to chemical properties it was possible to find out that most tertiary interactions break at temperatures close to the global midpoint whereas the backbone local conformation melts at higher temperatures. An interesting follow up question would be whether this is a universal property or depends of the ratio between local and nonlocal interactions in the protein. Another interesting result was the identification of a small single cluster of residues with strong couplings. The residues in this cluster belong to the hydrophobic core of the protein and correspond with the critical network of interactions holding the native structure together. In the BBL matrix there also are many couplings between residues not in spatial contact, representing propagation through more than one contact. Such propagation of energetic coupling can be seen as the source of protein folding cooperativity. Accordingly, the amount of folding cooperativity in a protein would be directly related to how dense the critical interaction network is. In the downhill folder BBL the critical network indeed appears to be rather sparse (Sadqi *et al.*, 2006), whereas in proteins with larger folding barriers a denser network should be expected. This concept is analogous to the critical packing density of Akmal and Muñoz (2004). Interestingly, it suggests a very specific role for aromatic residues in the protein core. The bulky and semirigid character of aromatic sidechains will allow them to act as very efficient propagators of energetic coupling, and thus as enhancers of folding cooperativity.

In the future it is critical for this kind of analysis to be extended to other proteins. Here we should emphasize that conceptually the method does not need to be restricted to downhill folding proteins. The advantage of downhill folding proteins is that experimental resolution is maximal since the global unfolding process is broader and more heterogeneous. But, the limitations in applicability are mostly technical. The first one is related to line broadening effects in the nuclear magnetic resonance (NMR) spectrum. Proteins with folding times within the NMR chemical exchange time scale ($\sim$0.1–5 ms) will be very hard to study due to the difficulty of resolving severely broadened NMR crosspeaks. For proteins with high cooperativity (i.e., with a high folding barrier and sharp equilibrium unfolding) this problem disappears again because folding times in the slow chemical-exchange regime will result in two sets of crosspeaks (folded and unfolded), which can be assigned independently in $^{15}N-^{13}C$ multidimensional NMR experiments (Farrow *et al.*, 1997). However, for highly cooperative unfolding processes the difficulty lies in resolving small differences in atomic behaviors that could very well be within the accuracy limit of the analysis (Sadqi *et al.*, 2007).

Connections between 3D structure and folding

Another useful practical application of atomic-resolution multiprobe equilibrium unfolding experiments is their use as benchmarks for computer simulations. The wealth of structural unfolding information originating from these experiments provides challenging quality controls for the performance of all atom computer simulations. In particular, two different groups have recently performed replica exchange molecular dynamics simulations in explicit solvent of BBL unfolding (Pitera *et al.*, 2008; Zhang *et al.*, 2008). These simulations are remarkably good in reproducing the overall unfolding behavior of BBL and the large structural heterogeneity observed experimentally. However, the specific atomic details are still quite different from experiment. The lack of agreement at the atomic level might not be too surprising given the approximate nature of the force fields, but it does suggest that this type of experimental data could be extremely useful for testing and further refining atomic-resolution force fields.

Simulations with simplified protein models can also benefit significantly from these experiments. Simulations on simplified models often use Go-like potentials, which only consider native interactions. Go-potentials are parameterized from a matrix of native contacts that is obtained from the structural coordinate pdb file of the protein. These potentials

assume almost perfectly smooth funneled landscapes for natural proteins, an assumption that has been supported by their success in predicting folding rates (Muñoz and Eaton, 1999). A second important assumption is that the geometric contacts determined from a 3D structure are a good representation of the critical interaction matrix of the protein. However, comparison between the contact map of BBL and the residue-residue coupling matrix reveals very little overlap [Fig. 5(A)]. The majority of the structure-derived contacts do not coincide with experimentally resolved couplings [blue dots in Fig. 5(A)]. Interestingly, the few contacts that also produce strong couplings [red dots in Fig. 5(A)] tend to involve highly conserved core residues in the BBL family (I20 and V34 for instance). The implication is that only a few geometric contacts are involved in stabilizing the native structure whereas the rest are thermodynamically adventitious.

The question then becomes how to identify those geometric contacts that are more likely to stabilize the structure. Sequence conservation in the protein family may offer some clues. Structures obtained by NMR offer another possibility. NOEs are the basic experimental information used to calculate NMR structures. The observation of a NOE between two residues guarantees that there is a contact in the native structure, but calculated structures tend to have many more contacts than NOEs are observed. This is easily appreciated comparing the NOE map of BBL [Fig. 5(B)] with the geometric contact map [Fig. 5(A)]. Not only the NOE map is sparser, it also agrees much better with the coupling matrix, especially for the long distance NOEs that are critical to define the 3D structure. Nevertheless, the overlap is still not perfect, and there are some long-range NOEs that do not coincide with experimentally resolved couplings. Intriguingly, these NOEs are often adjacent to residue pairs involved in strong noncontacting couplings.

An additional advantage of NOE maps is that they do not depend on the structure calculation procedure. For example, recent simulations have highlighted the large differences between the contact maps of two versions of BBL that differ on the unstructured tails and experimental conditions (Cho *et al.*, 2008). The many more long-range geometric contacts of the BBL variant with longer tails predict a small folding barrier at midpoint (i.e., $\sim 4\ RT$) that is not present in the simulations on the shorter variant (Cho *et al.*, 2008). However, the NOE maps of the two BBL variants are very similar. If anything, the shorter variant has a few more long-range NOEs. In fact, the main reasons for the largely increased long-range geometric contacts on the longer variant seem to be a single weak NOE between the δHs of L2 and βHs of A17 together and slight differences in the structure calculation protocol. It would be interesting to perform similar computer simulations using NOE maps. But in general, all these considerations suggest that Go-like potentials should probably be parameterized with NOE maps when available.

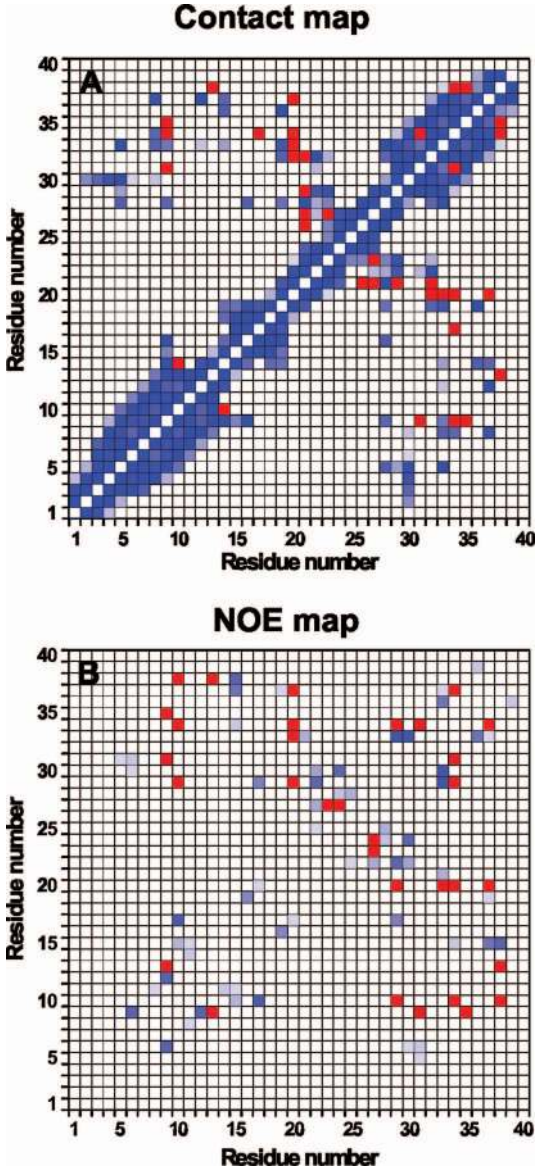

Figure 5. Comparison between structure and residue-residue thermodynamic coupling matrix. Panel (A) shows in blue the contact map obtained from the structural coordinate file of BBL (2CYU.PDB). The stronger the shade of blue the stronger the contact (either shorter distances or more atomic contacts). Dots in red correspond to geometric contacts that coincide with strong thermodynamic couplings. Panel (B) shows a similar plot using the experimental NOE map of BBL (obtained from the list of experimental restraints in the 2CYU.PDB entry). The color code is as in (A).

FOLDING KINETICS VERSUS CONFORMATIONAL DYNAMICS

Another exciting area of the experimental studies on the downhill regime is the possibility of probing conformational

dynamics during protein folding by performing time-resolved relaxation experiments (Eaton, 1999). The relaxation of barrier crossing processes depends on the probability of populating the top of the barrier and the diffusion coefficient for crossing it (Hanggi *et al.*, 1990). In other words, the relaxation rate is largely determined by the energetics. On downhill processes, however, the relaxation depends entirely on the diffusive path through the free energy surface: curvature, reequilibration distance, and diffusion coefficient, which can then be measured experimentally. To take full advantage of this feature requires thinking outside of classical chemical kinetics.

What are the trademarks of diffusive downhill folding dynamics?

The following brings us to the issue of how to interpret folding relaxation decays and identify specific trademarks to distinguish between activated folding kinetics and diffusive folding dynamics. The features of two-state kinetics are very well known. The relaxation decay should be a single exponential with amplitude that depends on the initial and final conditions according to the equilibrium unfolding. The two-state relaxation rate should be independent of the magnitude of the perturbation and the structural probe used to measure it.

There are more subtleties regarding downhill folding relaxations. This is so because downhill relaxation decays depend critically on fine-grained properties of the folding energy landscape. The roughness that is smoothed out by the free energy projection (see Fig. 3) is embedded onto the diffusion coefficient, which will now depend on the position on the reaction coordinate (Socci *et al.*, 1996). Furthermore, free energy projections could conserve residual roughness at long length scales relative to the reaction coordinate, resulting in rugged surfaces (Gruebele, 2008). Depending on the height of the peaks and troughs relative to thermal energy, the latter could be seen as folding via multiple intermediates. If the position dependence of the diffusion coefficient and/or the residual ruggedness on the projected surface is large, one should expect downhill relaxations resembling stretched exponential decays. Likewise, the observed relaxation time could become highly dependent on the structural probe employed, or on the diffusive path. This could be the regime observed by Gruebele and co-workers on ultrafast folding variants of λ repressor (Ma and Gruebele, 2005; Yang and Gruebele, 2004), and Tokmakoff and co-workers in ubiquitin (Chung and Tokmakoff, 2008).

On the other hand, a relaxation on a smooth harmonic well produces single exponential relaxation decays with relaxation times that just depend on the curvature of the well and the diffusion coefficient. Therefore, downhill folding free energy surfaces will result in single exponential, probe independent decays when the projected surface is smooth and the diffusion coefficient has little position dependence. The relaxation time should also be probe independent and exhibit a rather flat, still V-shaped chevron plot. In this case, the minimum in the chevron plot arises when the surface is broadest (lowest curvature), which typically corresponds to the global denaturation midpoint. This is the regime that has been recently found to apply to laser T-jump experiments on the natural protein BBL (P Li, FY Oliva, AN Naganathan, and V Muñoz, in preparation).

The experiments on BBL also addressed how to distinguish between this downhill scenario and activated kinetics. Here, most clear criteria come from the kinetic amplitude information. Like for any other reequilibration relaxation, the amplitude of downhill folding relaxations should follow the equilibrium unfolding behavior displayed by the probe. Therefore, experiments using probes that produce very different equilibrium unfolding curves should result in equally probe dependent amplitudes with a single relaxation time. This observation is very specific because probe dependent amplitudes rule out two-state folding kinetics, whereas the concomitant disconnect between relaxation rates and apparent stabilities cannot be explained with other activated-kinetics schemes. In multistate activated kinetics the different probes could be detecting different intermediates, but then the observed rates are always proportional to the equilibrium stabilities. Unfortunately, in most kinetic experiments in protein folding the amplitudes are not reported. Given their obvious importance, an extra effort should be made to measure both relaxation times and amplitudes in fast-folding experiments.

Probing folding diffusion coefficients experimentally

Once it has been established that a folding relaxation is downhill, the issue becomes how to take advantage of its special features to obtain critical dynamic information about protein folding reactions. Particularly, measuring diffusion coefficients along the reaction coordinate would answer several important questions. First, diffusion coefficients measured at barrier top values of the reaction coordinate will provide empirical estimates of the true preexponential factor to employ for two-state-like folding. This has been recently done at 333 K for BBL, obtaining a preexponential of $\sim 10^6$ s^{-1} (P Li, FY Oliva, AN, Naganathan, and V Muñoz, in preparation). Second, the changes in diffusion coefficient as folding progresses report on the topography of the underlying energy landscape, and should help in understanding the different conformational modes involved at the various stages of folding.

Another important issue refers to the temperature dependence of the diffusion coefficient. Current estimates suggest that folding diffusion coefficients slow down by an order of magnitude from typical midpoint temperatures (i.e., 330–340 K) to room temperature (Naganathan *et al.*, 2007), but it is important to test this estimate with direct experimental information. Moreover, if the diffusion coefficient is

indeed very temperature dependent (both from landscape roughness and solvent coupling) it may also exhibit strong size dependence (Naganathan *et al.*, 2007). This would be of significance to further understand the temperature dependence of folding rates. However, because the diffusion coefficient is expected to depend on both temperature and reaction coordinate, it is difficult to separate the two contributions with standard nanosecond temperature jump experiments. One possibility is to perform T-jumps of different size to the same final temperature, which given the current performance of laser T-jump instruments would only resolve cases with highly position dependent diffusion coefficients. Combining temperature with chemical denaturants is not a perfect alternative either, because it will be difficult to extricate changes in diffusion coefficient from possible changes in surface curvature induced by chemical denaturant. The ideal experiment in this case would consist on measuring relaxation decays on a globally downhill folding protein at fixed temperatures starting from clearly different initial conditions (ideally in both folding and unfolding directions). The free energy minimum will shift with temperature tracking the reaction coordinate, whereas relaxations from different initial conditions will report on the diffusion coefficient through the visited paths at each fixed temperature.

CONCLUSIONS

Protein folding theory has advocated for the use of low-dimensional free energy projections as an analytical tool to tackle the inherent microscopic complexity of protein folding reactions. This idea was quickly applied with great success to the analysis and interpretation of computer simulations of protein folding, which produce a wealth of otherwise hard to handle information. In contrast, protein folding experiments tend to produce simple results that have been traditionally interpreted in analogy to elementary chemical reactions. The recent discovery of downhill protein folding is changing this state of affairs. Downhill folding cannot be explained with chemical models, which require discrete states interconnected by activated kinetics, and confirms critical theoretical predictions. Furthermore, the downhill folding regime can be exploited to access all those intermediate folding stages that hold the key to the folding mechanisms and which had been deemed intrinsically inaccessible to experiment. Following on these ideas, the combination of detailed experiments on downhill folding with their analysis with free energy surface approaches is crystallizing into a set of approaches that offer the opportunity to address experimentally many of the fundamental questions in protein folding. These questions range from the microscopic interpretation of classical equilibrium unfolding experiments and their connection to folding cooperativity, the thermodynamic and kinetic origin of folding barriers, to the timescales of the conformational motions leading proteins in their search for the native structure. Last, but not least, the closer connection of these new experimental data with theory and their high level of detail provide direct benchmarks for computer simulation methods and even for force-field refinement.

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