Protein Folding: Matching Theory and Experiment

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ABSTRACT The impact of folding funnels and folding simulations on the way experimentalists interpret results is examined. The image of the transition state has changed from a unique species that has a strained configuration, with a correspondingly high free energy, to a more ordinary folding intermediate, whose balance between limited conformational entropy and stabilizing contacts places it at the top of the free energy barrier. Evidence for a broad transition barrier comes from studies showing that mutations can change the position of the barrier. The main controversial issue now is whether populated folding intermediates are productive on-pathway intermediates or dead-end traps. Direct experimental evidence is needed. Theories suggesting that populated intermediates are trapped in a glasslike state are usually based on mechanisms which imply that trapping would only be extremely short-lived (e.g., nanoseconds) in water at 25°C. There seems to be little experimental evidence for long-lived trapping in monomers, if folding aggregates are excluded. On the other hand, there is good evidence for kinetic trapping in dimers. α-Helix formation is currently the fastest known process in protein folding, and incipient helices are present at the start of folding. Fast helix formation has the effect of narrowing drastically the choice of folding routes. Thus helix formation can direct folding. It changes the folding metaphor from pouring liquid down a folding funnel to a train leaving a switchyard with only a few choices of exit tracks.

INTRODUCTION

A central focus in protein folding studies now is matching theory and experiment: finding out how to represent a protein molecule during the process of folding so that simulations catch the essential features of the process, and then learning from the simulations how to interpret experimental data in the most realistic manner.

In the 1960s and early 1970s, before sufficient data were available to convince the biophysics community of the existence of kinetic folding intermediates, it was common practice to assume that the initiation of folding is nucleation limited, to explain why folding intermediates are not observed. Nucleation was assumed to be difficult, and succeeding steps in folding were assumed to be fast enough that refolding intermediates would not be detectable, although a nucleation model of this type predicts that intermediates should be detectable in the unfolding kinetics (see Tsong et al., 1972). Good experimental data on the kinetics of folding were available from Pohl (1969), but only for the kinetics measured in the seconds time range. The kinetic behavior in faster time ranges was not examined, and the kinetic data in the seconds time range were found to fit the two-state model.

In 1971, two papers reported the discovery of kinetic intermediates in faster time ranges: Ikai and Tanford (1971) found biphasic unfolding and refolding kinetics of cytochrome *c* (cyt *c*) in a stopped-flow study of the GdmCl-induced unfolding transition, and Tsong et al. (1971) found biphasic unfolding kinetics of RNase A (ribonuclease A) in a temperature jump study of its thermal unfolding. Soon afterward, chymotrypsinogen A was shown to have complex unfolding kinetics when examined by the stopped-flow or the temperature jump method (Tsong and Baldwin, 1972), and so two of the proteins classified by Pohl (1969) as showing two-state folding in fact showed kinetic intermediates when studied by fast-reaction methods.

The folding kinetics of RNase A were found by Garel and Baldwin (1973) to be largely dominated by behavior resulting from two unfolded states, a minor ($\cong$ 20%), fast-folding U_F state and a major ($\cong$ 80%), slow-folding U_S state. This finding led to the proposal by Brandts et al. (1975) that the $U_F \rightleftharpoons .U_S$ reaction of unfolded RNaseA is *cis-trans* isomerization about proline peptide bonds, and later to the demonstration by Schmid and Baldwin (1978) that the $U_F \rightleftharpoons .U_S$ reaction is in fact acid-catalyzed, as expected for proline isomerization. Confirmation of the proline model led to the study of the folding kinetics of U_S at 10°C (Cook et al., 1979), where proline isomerization is extremely slow (time range 10^3 s). The question was: How far does folding proceed before it is stopped by the barrier of a wrong proline isomer?

The results showed that U_S folds almost completely: it forms a native-like folding intermediate I_N, as measured by binding of the specific inhibitor 2′CMP. There are two *cis* proline residues in RNase A, Pro^{93} and Pro^{114}, and they account for the high proportion of the slow-folding U_S species. Pro^{93} is believed to isomerize in the $I_N \rightarrow N$ step (see Houry et al., 1994; Houry and Scheraga, 1996). I_N has an important property: it behaves as a discrete species when tested by unfolding kinetics. It unfolds with a single unfolding rate, which is ~10 times faster than the unfolding of N (Schmid, 1983) (see Fig. 1). These results provided the basis for representing folding as an ordinary chemical reaction

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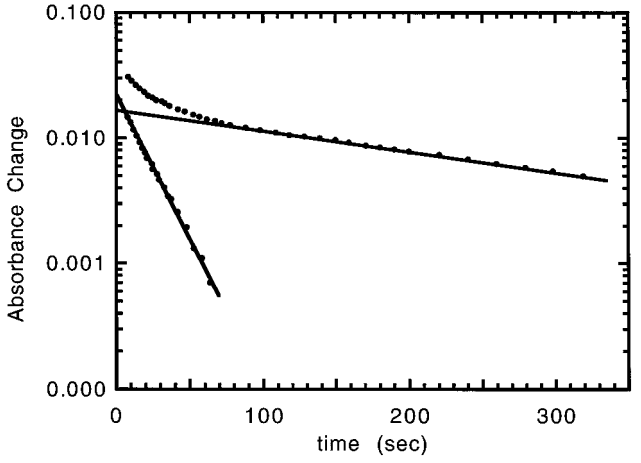

FIGURE 1 The unfolding kinetics of the native-like intermediate (I_N) of ribonuclease A, compared with the unfolding kinetics of the native protein (N), in a mixture of both species in 4.6 M GdmCl, 0.04 M $(NH_4)_2SO_4$, pH 6, 10°C (redrawn from Schmid, 1983). Both I_N and N unfold with single exponential kinetics; the unfolding rate of I_N is ~20 times faster than that of N.

with discrete intermediates—the "simple sequential model" of Kim and Baldwin (1982) and Schmid (1983):

$$U \rightleftharpoons I_1 \rightleftharpoons I_2 \cdots I_n \rightleftharpoons N \quad (1)$$

In this model, folding is analogous to an ordinary chemical reaction or to a metabolic pathway: there are successive steps, each one marked by the formation of a specific intermediate, and one step is usually rate-limiting; the transition state $I^{\#}$ for folding is assigned to this step. Experimentally, it is often difficult to populate and observe the intermediates I_1, I_2. . . , but the procedure for working out the pathway is proposed to be the same as for a metabolic pathway: the procedure is to isolate and characterize the intermediates. Populating the intermediates often requires particular conditions ("strongly native" conditions) for folding: low temperature, low denaturant concentration, and the presence of a stabilizer such as Na_2SO_4. In less stabilizing conditions, intermediates may not be observed. Some proteins may have intermediates that are too unstable to be observed; nevertheless, the simple sequential model formally represents these cases.

Harrison and Durbin (1985) argued that the simple sequential model is physically unrealistic for protein folding: the folding process should resemble instead the assembly of a jigsaw puzzle, with many possible pathways, a random assortment of intermediates, and no unique transition state. Most later simulations and theories of protein folding proved to have the properties they proposed (see reviews by Wolynes et al., 1995; Eaton et al., 1996; and Dill and Chan, 1997). Simulations of the folding process led to a new folding metaphor of pouring liquid into a "folding funnel" with partially rough sides (Leopold et al., 1992; Wolynes et al., 1995).

Folding simulations and recent experiments give a new view of the transition barrier

In a chemical reaction in which bonds are made and broken, the transition state intermediate is highly unstable and is never detectably populated. This is a consequence of strain and the large bond energies involved. In protein folding, the only bonds made and broken are hydrogen bonds with comparatively small bond energies, if folding reactions involving disulfide bonds or other strong bonds are excluded. Nevertheless, it has often been assumed in folding studies that the transition state intermediate in folding should be a highly unstable species that never reaches a detectably large population, and that it should have a highly specific structure.

Recent experiments indicate that this is the wrong picture, and that the transition barrier is broad and not especially high. Most simulations and theories of the folding process show this behavior (for reviews, see Karplus and Sâli, 1995; Eaton et al., 1996, 1997; and Klimov and Thirumalai, 1996). It should not be surprising that the transition barrier is broad, because individual steps in folding involve small changes in free energy. The most likely mechanism for producing a sharply peaked barrier is one in which the process that forms the transition state is highly cooperative (Shakhnovich et al., 1996).

The experimental test, which has been carried out, is to determine whether the position of the barrier is fixed, as expected if it is sharply peaked, or whether the barrier peak can be changed by mutation, as expected if the barrier is broad. The barrier peak position is determined experimentally from the change in solvent-accessible surface area between the unfolded protein and the transition state species, as compared to the change for complete folding to the native state. This ratio is estimated from (m_f/m), where m_f and m are found from the denaturant dependences of the refolding rate and the folding equilibrium constant, respectively (Tanford, 1970; Chen et al., 1989).

The nature of the transition barrier has been analyzed by mutational studies of three proteins: barnase (Matouschek and Fersht, 1993), Arc repressor (Milla et al., 1995), and the N-terminal domain of λ- repressor (Burton et al., 1997). Matouschek and Fersht find that not only does the peak position of the transition barrier change with mutation, but the height of the barrier ($\Delta G^{\#}$) is also directly correlated with its position for two sets of structurally connected mutants. This behavior is expected if the species at the barrier peak are closely similar to nearby folding intermediates. In a mutational analysis of the transition barrier in Arc repressor by Sauer and co-workers, 44 of the 53 residue positions were replaced by alanine. Milla et al. (1995) then determined the mutant unfolding and refolding rates and their dependences on denaturant (urea) concentration, and they found that some mutations cause significant changes in the solvent accessibility of the species at the barrier peak (see Fig. 2), although most mutants have values that cluster around the wild-type value. These studies lend strong support to a funnel type of folding mechanism in which the

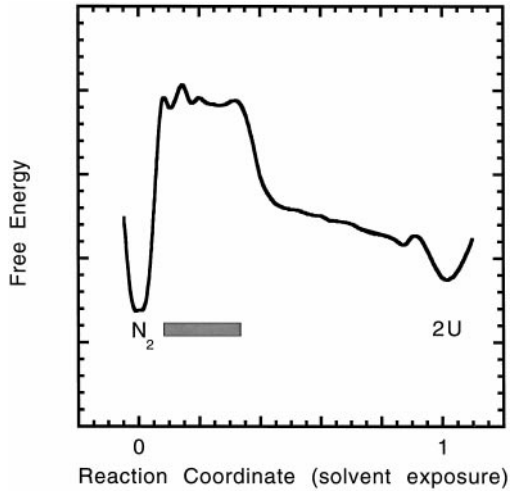

FIGURE 2 A possible diagram of the transition state barrier in the unfolding/refolding kinetics of Arc repressor (redrawn from Milla et al., 1995). The diagram is based on the results for 44 mutant proteins of unfolding and refolding rates measured as a function of urea concentration. The results can be explained if the barrier is low and broad: the fractional solvent accessibility of the species at the top of the barrier is found to vary among the mutants studied from 0.08 to 0.31 (shown by the *solid bar*). N_2 is the native dimer and 2U is the unfolded monomer (two molecules).

transition state species are closely related in their structure and properties to other nearby folding intermediates. A different approach to the search for movement of the barrier peak position is to look for curvature in the plot of ln k versus denaturant concentration, where k is the unfolding or refolding rate constant. Curvature is expected if the peak position moves with denaturant concentration, and curvature has been reported for a two-state folding reaction by Silow and Oliveberg (1997). A simple quantitative funnel model has been introduced by Zwanzig (1995, 1997). It makes the point that folding can occur rapidly when there is no ordered pathway of folding. In this model, every step of the folding process is equally likely and has the same rate and energetics as every other step until the last step, when an energy bonus is given for forming the final native structure. Folding proceeds through a continuum of intermediates. This model is the antithesis of the simple sequential model: there is no defined pathway of folding and no specific transition state species. A common experimental view of the transition state barrier is that the barrier peak is reached by making a few favorable contacts at a considerable cost in conformational entropy. Once these contacts have been made, additional stabilizing contacts can be made with little penalty in conformational entropy, and folding goes downhill. Zwanzig's 1995 model shows, however, that even if the stabilizing contacts are spread evenly throughout the folding process, except for the last step, there is still a free energy barrier preceding the last step, and it arises from conformational entropy. Zwanzig's model has been modified by Doyle et al. (1997) to include the cooperativity of folding as a variable parameter, as well as the relative strengths of local versus nonlocal interactions. The height of the transition barrier increases with the cooperativity of folding, as might be expected. Zwanzig's model has the virtues of extreme simplicity and of allowing the kinetics to be calculated explicitly from the parameters assigned to the model. Thus it should prove useful in helping to ferret out universal properties of real folding reactions.

Fast formation of α-helices

Recent measurements of the kinetics of α-helix unfolding, in both a peptide (Williams et al., 1996) and a protein (native apomyoglobin; Gilmanshin et al., 1997), indicate that solvent-exposed helix unfolding and refolding occur at least as fast as the 10^{-7}-s time range, in agreement with older results for synthetic polypeptides (Schwarz, 1965; Hammes and Roberts, 1969; Gruenewald et al., 1979). Schwarz's (1965) theoretical treatment, which adapts the Zimm-Bragg (1959) helix-coil theory to the kinetics of helix unfolding and refolding, indicates that helix nucleation occurs in the 10^{-7}-s time range, and helix propagation is $\sim 10^3$ times faster; his theory was illustrated with ultrasound absorption data for the helix-coil transition. The newer results give measured rates of helix unfolding, because they are obtained from laser-induced temperature jump measurements of helix unfolding, and the elementary rate constants for helix nucleation and propagation remain to be determined in these systems. Thus far the data have been analyzed by the two-state model, which gives an apparent rate constant for refolding as well as unfolding. Helix refolding should occur in the same time range as unfolding when refolding and unfolding kinetics are compared in fixed final conditions inside the transition zone and only small perturbations of the initial equilibrium are made.

The rate of helix nucleation, 10^7 s^{-1}, can be compared with the rate of closing small (6–10 residue) loops, estimated by Hagen et al. (1996) to be 10^6 s^{-1}. They suggest that closure of small loops can be regarded as the elementary step in forming the tertiary structures of proteins. Eaton et al. (1997) suggest that formation of a tight reverse turn should occur in the same time range as helix nucleation at a unique site, ~ 0.5 μs. Nucleation of a helix at random sites is faster (0.1 μs) because it can occur at any of several sites. They also consider estimated rates of other elementary steps in folding, such as β-hairpin formation.

Because helix nucleation is 10 times faster than the fastest loop closure reaction, it follows that incipient helices are already present when formation of the tertiary structure begins. The helices formed by peptides from helix-containing segments of proteins are only marginally stable (Muñoz and Serrano, 1994). If helices in proteins can be stabilized rapidly, on a time scale close to that of helix nucleation, then helix formation is expected to dominate the folding kinetics of helical proteins. Recently Oas and co-workers

reported that a thermostable mutant of the N-terminal domain of λ repressor, an all-helix protein, can fold in only 20 μs (Burton et al., 1997). This is the fastest folding reaction of a native protein yet reported, although the folding time of 20 μs at 0 M urea was obtained by extrapolation.

The mechanism of stabilization and the folding kinetics of intermediates formed by helical and partly helical proteins are only beginning to be studied, chiefly by making use of systems in which the same intermediate is found at equilibrium in nonnative conditions (e.g., at acid pH) and as a transient intermediate in the refolding kinetics of the native protein. NMR-hydrogen exchange has been used to examine the similarity between the structures of equilibrium and kinetic intermediates for apomyoglobin (Hughson et al., 1990; Jennings and Wright, 1993) and ribonuclease H (Dabora et al., 1996; Raschke and Marqusee, 1997). It is generally thought that loose hydrophobic interactions between helices stabilize these intermediates, but there is also evidence for partial close packing in the acid forms of cytochrome *c* (Marmorino and Pielak, 1995) and apomyoglobin (Kay and Baldwin, 1996).

A model for the stabilization of helices by close packing interactions is the coiled-coil structure, in which two helices lie parallel to each other by means of gentle supercoiling. Formation of the dimeric coiled-coil structure by peptides (~30 residues) is extremely fast, almost diffusion-controlled (Sosnick et al., 1996a; Wendt et al., 1997). Helix formation occurs as a two-state reaction, and it is not yet possible to separate helix nucleation and growth in this system. In molten globule intermediates, the contacts between helices are looser than in coiled coils, and stabilization of helices is likely to occur on an equally fast or faster time scale, but few experimental results are available. The formation of a partly helical folding intermediate of apomyoglobin occurs in a few milliseconds in the urea-induced unfolding/refolding transition of this intermediate at pH 4.2 (Jamin and Baldwin, 1996), but the folding reaction has not yet been monitored by a direct probe of helix formation. The introduction of laser T-jump thermal unfolding monitored by Fourier transform infrared spectroscopy (Gilmanshin et al., 1997) should soon give data on the folding/unfolding kinetics of helices in molten globule intermediates.

Fast helix formation means that helices must be explicitly included in folding simulations of helical proteins. Until now, secondary structures have usually been omitted in simulations (see, however, Dinner et al., 1996), and so has the formation of peptide H-bonds. Honig and Cohen (1996) point out that omission of peptide H-bonds in folding simulations is a major factor limiting the physical reality of the results. Often the lattice models used for simulating folding do a poor job of representing helices. The problem has been explored by Levitt and co-workers (see Park and Levitt, 1995). They conclude that off-lattice models are significantly better than lattice models, and they show that a simple off-lattice model can be computationally efficient.

Populated folding intermediates: productive on-pathway intermediates or folding traps?

Simulations and theories of folding often predict that populated intermediates are folding traps (see reviews: Dill and Chan, 1997; Eaton et al., 1996). Intermediates accumulate because they hit barriers; simulations suggest that folding can go around these barriers. As pointed out above, folding may instead proceed on fixed tracks, and the alternatives may be either to tunnel through the barrier or to back up until a switch point is reached.

If the folding process does not encounter serious barriers, folding of helical proteins can be extremely fast, in the submillisecond time range (see Burton et al., 1997; also Robinson and Sauer, 1996). Nevertheless, the fact that these proteins still show normal (i.e., single exponential) folding kinetics means that the barrier is greater than $5kT$ (Eaton et al., 1997). Some small proteins that are largely nonhelical can also fold rapidly (milliseconds): chymotrypsin inhibitor 2 (Jackson and Fersht, 1991), cold shock protein B (Schindler and Schmid, 1996), ubiquitin (Khorasanizadeh et al., 1996), and protein L (Scalley et al., 1997). Consequently, slower folding reactions are regarded today with the suspicion that folding has hit a barrier that is not intrinsic to the folding process. This point was driven home by finding that the folding of oxidized cytochrome *c* occurs in a slow time range (0.1–1 s) chiefly because wrong heme ligands obstruct the folding process (Sosnick et al., 1994, 1996b; Elöve et al., 1994). Monitoring the ligand exchange reactions during folding by resonance Raman spectroscopy (Takahashi et al., 1997) makes it possible to determine the roles of ligand exchange reactions as barriers to folding. Some examples are known of proteins with huge kinetic barriers to refolding: for example, in the serpin family (Wang et al., 1996; Tani et al., 1997), and in some secreted bacterial proteases, such as subtilisin (Bryan et al., 1995).

The folding traps predicted by Wolynes, Onuchic, and co-workers have been compared to being caught in a glass transition (Wolynes et al., 1995). An experimental analogy is provided by the conformational substates of myoglobin that interconvert slowly compared to the geminate rebinding of CO after it is released by flash photolysis from myoglobin (Frauenfelder et al., 1988). The dynamic behavior of these substates has been discussed in relation to the behavior of spin glasses (Frauenfelder et al., 1991). Experimental data have been taken at subzero temperatures in glycerol media of very high viscosity, and the high solvent viscosity plays a major role in damping down the interconversion between substates (Ansari et al., 1992). When the data on substate dynamics are extrapolated to water at 25°C, substate interconversion appears to be extremely fast (microseconds to nanoseconds or faster) compared to most folding reactions. Thus trapping by this mechanism might be expected to affect the kinetics of only the fastest folding reactions, in the submillisecond time range. However, Klimov and Thirumalai (1996) have also predicted kinetic trapping during folding by a somewhat different mecha-

nism, and experimentalists are watching for possible examples of kinetic trapping as molten globule intermediates. These intermediates are known to readily form aggregates (Silow and Oliveberg, 1997) and dimers (Eliezer et al., 1993).

There is evidence for conformational substates and kinetic trapping in much slower time ranges when the behavior of dimers and higher oligomers is studied (see Weber 1986; Subramaniam et al., 1996; Rietveld and Ferreira, 1996; Sinclair et al., 1994; and references therein). The β-subunit of a bacterial luciferase, which is an $\alpha\beta$ heterodimer, can be kinetically trapped as an inactive β_2 homodimer (Sinclair et al., 1994). The β_2 homodimer is not formed by domain swapping (for a review, see Bennett et al., 1995) or by loop interchange. Its x-ray structure has been determined (Thoden et al., 1997), and the structure of the β_2 dimer interface is remarkably similar to the corresponding surface structure of the β-subunit in the $\alpha\beta$ heterodimer. It is important to understanding the folding mechanism to learn why kinetic trapping is readily observed with dimers and oligomers, but remains a question mark with small monomeric proteins. Folding reactions that involve disulfide bond formation are another matter: kinetic trapping in monomers is readily demonstrated, as shown initially by Creighton (1975).

The denatured state of a protein consists of a vast ensemble of structures, and it is plausible that folding can start simultaneously on alternative pathways. This has been demonstrated to occur in dihydrofolate reductase (Jennings et al., 1993) and in hen lysozyme (Wildegger and Kiefhaber, 1997). In these cases, the alternative folding pathways both lead to native protein, but the folding rates on alternative pathways are different. In the case of hen lysozyme, the presence of a populated intermediate on one pathway slows down the folding rate on that pathway.

Direct experimental methods are needed for determining if a folding intermediate is on-pathway. In molecular biology, there is a standard experiment for this purpose: the pulse-chase experiment. The intermediate is first labeled, then isolated, and afterward the reaction is started up again with a "cold chase," and it is determined whether the label appears in product at the rate expected if the reaction must go through this intermediate. There are serious technical problems in adapting the pulse-chase experiment to the study of folding intermediates, but it is hoped that these difficulties will be overcome. The most direct approach to determining the structures of folding intermediates, as well as the interactions that stabilize them, is to study populated intermediates. Otherwise, only indirect approaches can be used. Consequently, it is of prime importance to determine whether populated kinetic intermediates are on-pathway.

This paper is dedicated to Gregorio Weber on his 80th birthday.

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