All roads lead to Rome? The multiple pathways of protein folding

Recent studies have found that protein folding reactions often proceed through two or more kinetically distinct pathways. In at least some cases, the observed folding intermediates act as kinetic traps, slowing the rate at which folding is completed. These findings have important implications for understanding how proteins fold *in vitro* and *in vivo*.

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Efforts to understand how proteins adopt their native three-dimensional structure have been strongly influenced by two observations. The first, due to Anfinsen and coworkers [1], is that proteins can fold spontaneously on a physiological timescale. The second, due to Levinthal [2], is that there are vastly too many conformations open to a protein for all of the possible states to be sampled. These observations seem, on the face of it, to be mutually exclusive. To resolve this paradox, it was proposed that proteins fold via specific pathways. Much of the experimental work on protein folding has concentrated on identifying folding intermediates and, in analogy to simple chemical reactions, ordering these intermediates into a discrete folding pathway [3].

Over twenty years of research has produced a wealth of data on the energetic and structural nature of protein-folding intermediates [4,5]. It is now clear that during the folding of most proteins, specific structural intermediates, often containing significant stable secondary structure, are formed rapidly. The kinetic role of the observed intermediates, however, is less well established. An implicit assumption in many folding studies is that the observed intermediates are critical in restricting the conformational space sampled by the polypeptide, thus allowing it to fold rapidly. In the simplest cases, the intermediates would form a direct linear pathway (Fig. 1a). Alternatively, the observed intermediates might assist folding but could be ordered in two or more distinct competing pathways (Fig. 1b). Recently, however, it has been found that the folding of several proteins can occur extremely rapidly (~10–50 msec) in an all-or-none manner that does not involve detectable folding intermediates (see [6] for discussion). These findings raise the issue of whether the observed intermediates generally assist folding, or whether they are the products of off-pathway reactions, having structures that are irrelevant or even detrimental to the formation of the final native state (Fig. 1c).

Experimental evidence for multiple folding pathways

One of the first protein-folding pathways to be studied, and still among the best characterized, is that of the oxidative folding of bovine pancreatic trypsin inhibitor (BPTI) [7,8]. Protein-folding intermediates are generally transient in nature, making them difficult to characterize. Proteins like BPTI, in which folding is thermodynamically linked to the formation of a set of native disulfide bonds, provide an exception, however. In such cases, it is possible to reversibly trap the disulfide intermediates present during the folding reaction and identify them. A particularly powerful aspect of these studies is that the trapped intermediates can be purified and allowed to re-enter the folding pathway. Thus, one can evaluate the kinetic role of a given species directly [9]. During the folding of BPTI at neutral pH, two species, each containing a single native disulfide bond, accumulate transiently (Fig. 2a). A second native disulfide bond in both of the well populated single disulfide species is formed rapidly, yielding two intermediates termed N' and N*. In contrast to the well populated single disulfide intermediates, which are in rapid equilibrium, the rate of interconversion between N' and N* is extremely slow. Moreover, the N' intermediate forms native BPTI ~30-fold more rapidly than does N*. Thus the folding of BPTI occurs via two distinct kinetic phases. Interestingly, these two phases are also observed in the presence of the enzyme protein disulfide isomerase, even though the overall rate of folding is increased dramatically [10].

A major source of heterogeneity in the folding reaction of a number of proteins, including ribonuclease A, ribonuclease T1 and staphylococcal nuclease, results from the slow rate of proline isomerization [11]. Prolines are unusual in that the peptide bond preceding these residues is often found in a *cis* configuration. Even in denatured proteins, the rate of isomerization of the *cis–trans* peptide bond is often slow compared to the rate of folding. As a consequence, those unfolded molecules that contain incorrect proline isomers frequently fold at a relatively slow rate. A number of tests have been developed to determine whether the presence of a slow-folding component in a folding reaction is due to proline isomerization. These tests include site-directed mutagenesis to remove proline residues that are suspected of causing the problem, addition of enzymes that catalyze proline isomerization to accelerate slow-folding steps, and elimination of the slow-folding phase in

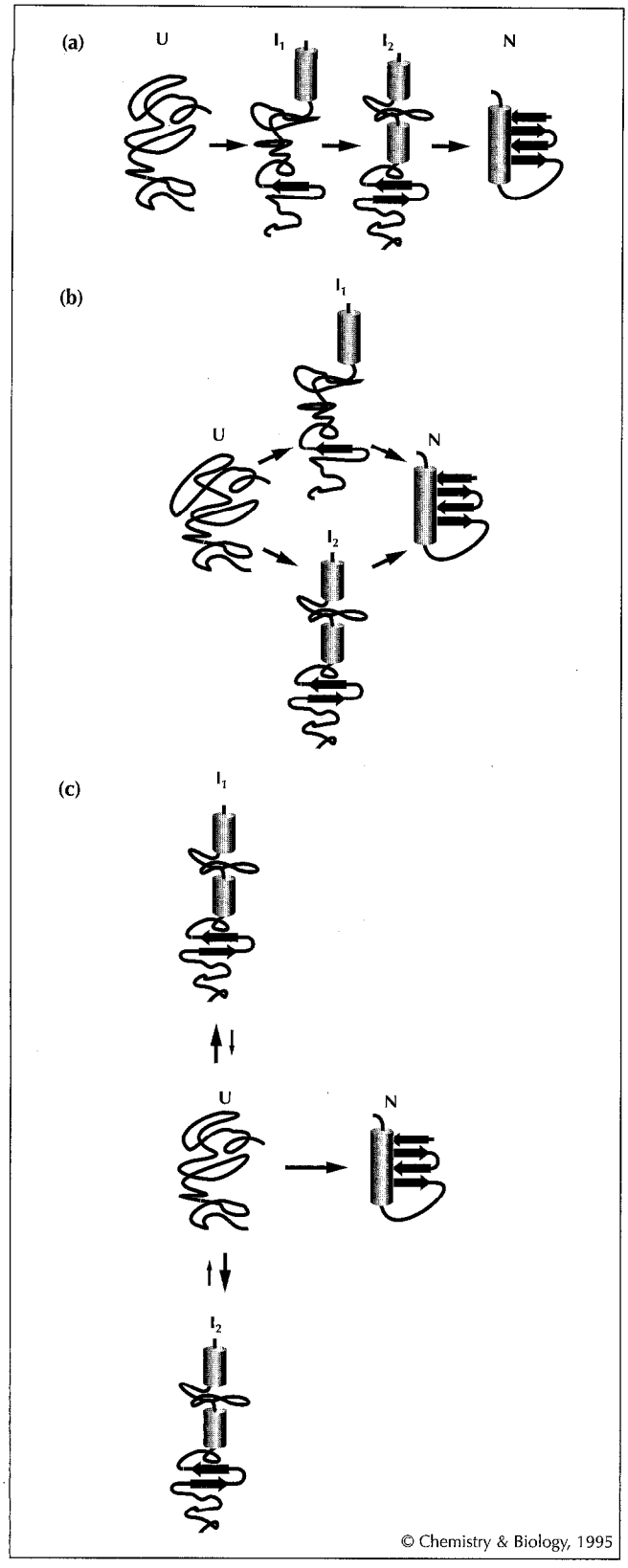

Fig. 1. Three schemes for the kinetic role of protein-folding intermediates. **(a)** A simple sequential pathway in which the observed intermediates are required to assist folding. **(b)** A set of parallel pathways in which the folding intermediates accelerate folding, but different competing parallel pathways exist. **(c)** A set of dead-end pathways in which the observed intermediates are kinetically trapped, dead-end species whose structure must be partly, or completely unfolded before folding is completed.

'double-jump' experiments, in which the protein is unfolded rapidly and a refolding reaction is initiated before the prolines can equilibrate.

A third example of a folding reaction involving complex kinetics is observed with cytochrome c (cyt c) [6]. In early studies, folding of cyt c was found to be relatively slow (taking several seconds) and to involve multiple distinct kinetic phases with well populated, structured intermediates. Recently, it has been found that a majority of the slow-folding phases result from inappropriate ligation of the attached heme group, by either of two His residues, to the coordination position occupied by Met80 in the native protein. When this ligation is prevented by lowering the pH, 50–70 % of the molecules fold rapidly (15 msec) with no observable intermediates. Under mildly denaturing conditions, cyt c forms a stable, compact intermediate which has many properties of the so-called molten globule state, such as a high degree of secondary structure with little stable tertiary structure [5]. Interestingly, folding of cyt c from the molten globule state occurs within 1 msec. This suggests that, contrary to common belief, the rate-limiting step in folding (at least for this protein) is the collapse to the molten globule state, not the transition of the molten globule to the native protein. The relatively rapid rate of folding of this molten globule intermediate also supports the notion that the structure of the cyt c molten globule assists the completion of folding.

Multiple kinetic phases observed in the absence of slow isomerization or ligation steps

In all of the above examples, the heterogeneity in folding rates was a consequence of intrinsically slow isomerization or ligation steps. Recently, however, examples of folding reactions involving multiple kinetic phases have been observed even in the absence of such slow steps. In a series of careful studies, it was found that dihydrofolate reductase (DHFR) refolds through four different kinetic channels (Fig. 2b) leading to four distinct, but related, native states [12]. The ratio of the four native states formed initially is under kinetic control, with equilibration to the thermodynamic minimum occurring only well after the initial folding reaction is completed. It was originally assumed that the four folding phases were due to proline isomerization. Double-jump experiments, however, showed that all four channels are populated within 20 s of unfolding at 15 °C. Thus, the different folding channels arise too rapidly to be due to proline isomerization. In addition, replacement of a cysteine residue, not located near the prolines in native DHFR, causes collapse of the four folding phases to two [13]. Since DHFR contains no disulfide bonds, replacement of this cysteine residue cannot be affecting the formation of different disulfide species.

Finally, folding of hen egg white lysozyme has also been observed to involve parallel pathways and distinct folding domains [14]. The native structure of lysozyme consists of two lobes separated by a cleft. The first lobe is

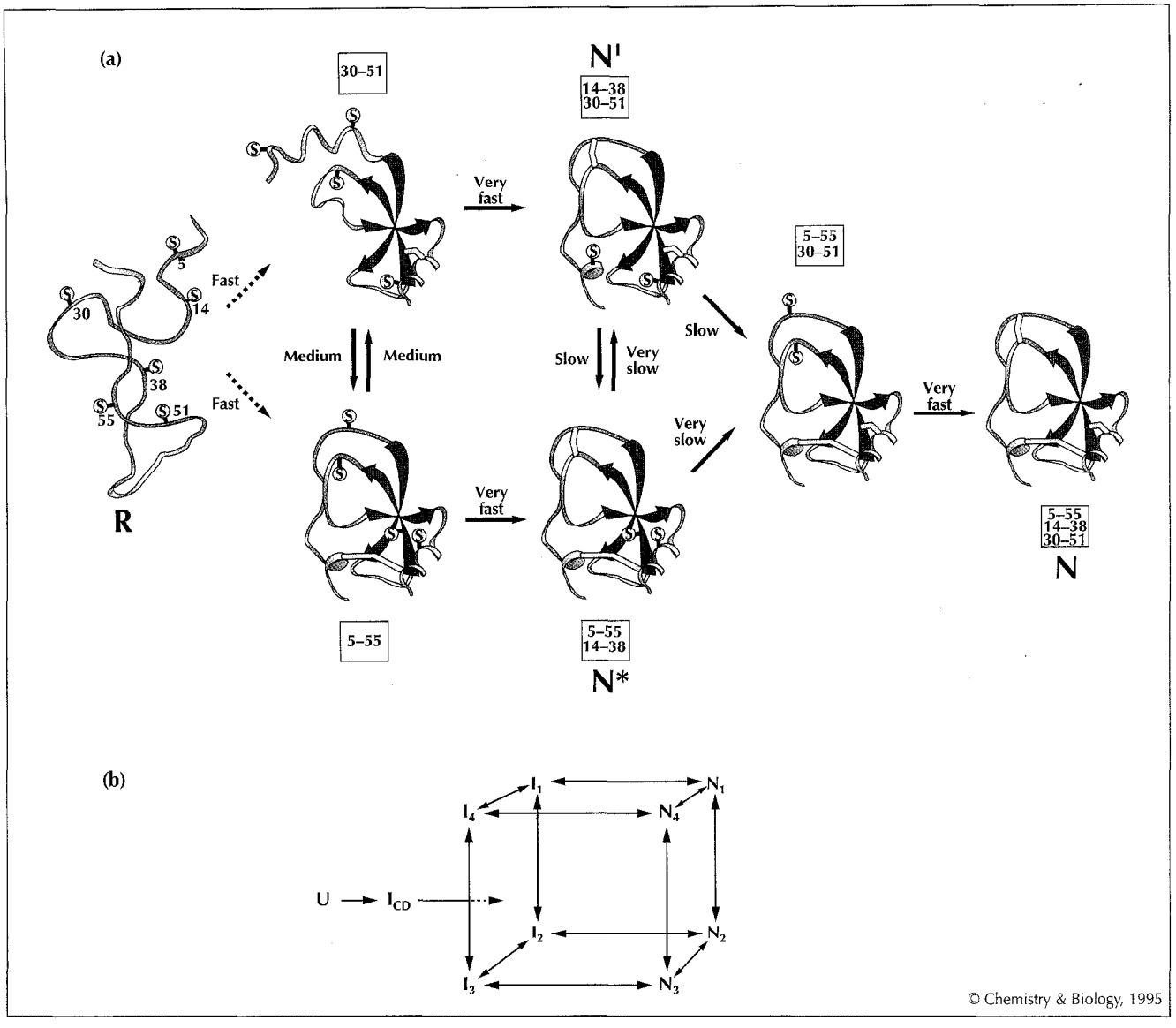

Fig. 2. Examples of folding reactions involving multiple distinct pathways. **(a)** Schematic representation of the kinetically preferred pathway for the folding of BPTI at pH 7.3, 25 °C [8]. All of the well populated intermediates contain only disulfide bonds found in native BPTI. **R** denotes the reduced protein. Qualitative descriptions of the relative rates of the intramolecular transitions associated with each step are indicated. The dotted arrows indicate that **R** is oxidized initially to a broad distribution of one-disulfide intermediates, which then rearrange rapidly to the intermediates containing disulfide bonds 30–51 and 5–55. A fork in the pathways is observed: the 30–51 intermediate yields a native-like species, termed N', which forms native BPTI on the hour timescale whereas the 5–55 species yields a kinetically trapped species, termed N*, that is stable for up to a week in these conditions. **(b)** Pathway for folding of *E. coli* DHFR [12]. Four distinct kinetic phases are observed leading to four native-like species. Only one of these species can bind the co-factor NADPH. Kinetic and mutagenesis studies indicate that these phases do not result from proline isomerization (see text and [12,13]).

predominantly α-helical and the second predominantly β-sheet. The folding of lysozyme has been studied extensively by a number of methods, including the use of pulsed-amide hydrogen–deuterium exchange. Amide hydrogens are readily exchanged with the solvent in unfolded proteins, but this exchange is often strongly inhibited when a protein folds. By measuring the rate of amide–hydrogen exchange as a function of folding time, it is possible, therefore, to monitor the formation of structure during the folding reaction. For the lysozyme studies, the rate of hydrogen exchange was examined by a combination of two-dimensional NMR, which measures the average rate at which individual amide protons exchange, and electrospray ionizing mass spectrometry, which distinguishes between different folding populations. At 20 msec, two major folding phases were detected: one in which the α-domain had achieved a high degree of protection while the β-domain contained no persistent structure, and a second in which no stable structure is observed in either the α- or β-domains. In addition, a third, less well populated pathway is observed in which both the α-domain and the β-domain are significantly protected. This heterogeneity is not due to residual structure in the unfolded state, as the ratio of the different populations does not depend on the denaturant used to unfold the protein [15].

In some cases, there may be only one major pathway

In contrast to the above results, recent studies from Fersht *et al.* [16] are consistent with the notion that folding of some proteins proceeds through a single major transition state. Examination of the effects of point mutations on the rate of folding and unfolding has proven to be a powerful tool for evaluating the nature of a folding reaction [17,18]. In particular, Fersht has introduced the use of a quantity, termed the Φ-value, determined by the relative effect of a mutation on the rate of unfolding versus the stability of the protein. The Φ-value is a useful quantity because it is a measure of the strength of an interaction involving the mutated residue in the protein-folding transition state. For example, a Φ-value of 0 or 1 indicates that an interaction is entirely absent or present, respectively (see [17]).

For both barnase and chymotrypsin inhibitor 2 (CI-2), a number of interaction sites were found to give fractional Φ-values. One possible explanation for a fractional Φ-value of, for example, 0.5, is that there is one or more transition states in which the interaction is present but weakened. Alternatively, there could be two parallel pathways of folding which are equally populated, one in which the interaction is entirely present, the other in which it is entirely absent. This latter model makes quantitative predictions about the effects that nearby mutations will have on the measured Φ-value. These predictions, however, are not borne out by the experimental measurements, ruling out the possibility that the fractional Φ-value represents an average between the values for intermediates in parallel pathways involving only fully formed and fully unfolded structures. Thus, although these experiments do not prove that there is a single folding pathway, they indicate that there are transition states in which the interaction is present, but weakened.

Intermediates that act as kinetic traps

The finding that the folding of a number of proteins can occur on the millisecond timescale without observable intermediates raises the question of whether the known stable folding intermediates, which often persist for hundreds of milliseconds or longer, are helping or inhibiting folding. The question of the kinetic importance of observed intermediates is particularly acute when a folding reaction proceeds through multiple phases, since (if unproductive intermediates exist) it is precisely those intermediates that persist at longer times which are most likely to be unproductive. A strong test of whether a folding intermediate is acting as a kinetic trap is to destabilize that intermediate, for example, by mutation or chemical denaturant. If the intermediate is acting as a trap, destabilization will increase the rate of folding. Negative results from such experiments are difficult to interpret, however, since denaturants and mutations may themselves slow the intrinsic folding rate. Nonetheless, a number of examples of folding intermediates that are acting as kinetic traps have been identified.

One of the earliest examples of a kinetic trap in folding was observed in the disulfide-linked folding of BPTI [7,8]. Here the protein accumulates as one of two native species, termed N' and N*, each containing two disulfide bonds (see above and Fig. 2a). Both N' and N* contain extensive native structure despite the lack of the final disulfide bond. The structure in these intermediates buries and constrains the remaining free cysteine residues, inhibiting disulfide formation and rearrangement. As a consequence, these species only slowly form the final native three-disulfide state. Disruption of the structure in both N' and N* by the addition of the chemical denaturant urea results in a substantial increase in the rate at which folding is completed. This counterintuitive result indicates that unfolding an intermediate that has some native structure can actually promote formation of the native state.

Native-like structure in a kinetic folding intermediate of RNase T1 also inhibits the formation of the final native state [19]. In this case, a specific tertiary interaction in a folding intermediate traps a proline residue in the incorrect isomer. Removing this contact, by replacement of a tryptophan residue with a tyrosine or addition of the denaturant urea, allows the proline isomerization step to occur unimpeded, increasing the rate of folding. It was also found that premature locking of a tertiary contact by the formation of the two native disulfide bonds slows the rate of folding, even though the disulfide bonds substantially increase the stability of the native state [20]. Interestingly, the presence of the disulfide bonds slows the rate of both the direct as well as the proline-limited folding reaction, suggesting that a high degree of flexibility is important even for folding reactions which do not involve proline isomerization.

Recent studies also suggest that structure in the observed folding intermediates of hen egg white lysozyme does little to help, and perhaps hinders, folding. In particular, it was found that reduction of one of lysozyme's four disulfide bonds prevented formation of the transient intermediate on the major refolding pathway containing persistent structure in the α-helical domain. Despite the loss of this intermediate, the overall rate of folding was not slowed [21]. In addition, it was found that the presence of low amounts of the organic solvent dimethylsulfoxide, which acts to destabilize proteins, increased the rate at which folding is completed. Intriguingly, even for CI-2 (which might fold through a single transition state; see above), mutagenesis studies indicate that breaking up a non-native hydrophobic cluster facilitates folding. This suggests that the rate-limiting step in the formation of the native state involves partial denaturation [22].

Implications for chaperone-mediated folding

The observation that protein folding often proceeds through multiple distinct pathways involving fast and slow phases provides insight into how folding might be assisted by cellular proteins. Although folding of small proteins can occur efficiently at low dilution *in vitro*,

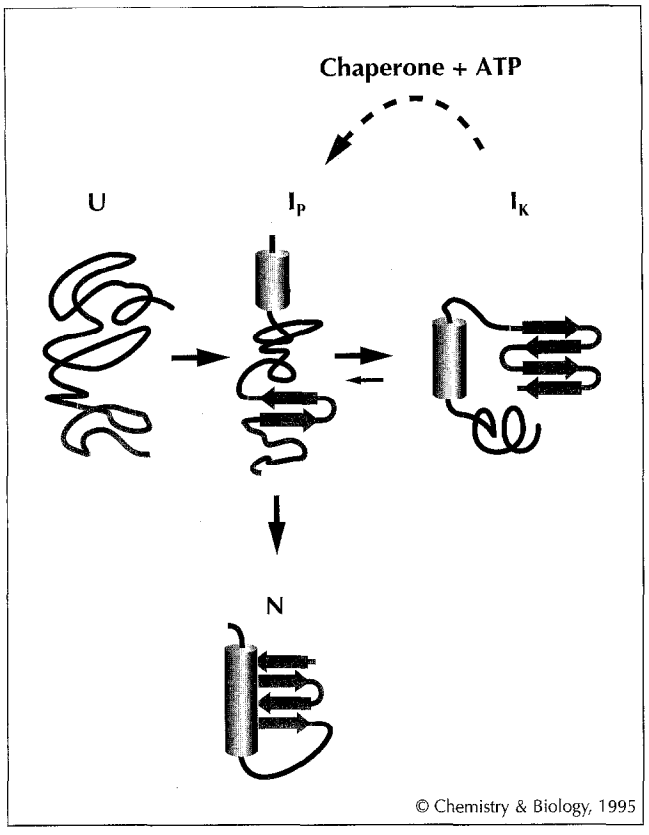

Fig. 3. Hypothetical model for one mechanism by which molecular chaperones could increase the efficiency of folding. **U** designates the fully unfolded state present under strongly denaturing conditions. I_p designates a productive protein-folding intermediate. I_k designates a kinetically trapped or perhaps mildly aggregated intermediate.

under non-ideal conditions, such as those in the cell, folding is often extremely inefficient due, in part, to off-pathway aggregation reactions. Recently, it has become clear that cells contain sophisticated machinery that assists protein folding, consisting of proteins termed molecular chaperones. Two major classes of molecular chaperones have been found: the Hsp70 family, of which the *Escherichia coli* protein DnaK is a member, and the chaperonin ring structures, including the *E. coli* protein GroEL. Despite major differences in function and structure between these two classes of molecular chaperones, both Hsp70 family members and chaperonins preferentially bind proteins that are in their denatured state. The affinity of this binding is modulated by the binding and hydrolysis of ATP. In addition, recent studies indicate that folding mediated by both Hsp70 [23] and chaperonins [24,25] occurs by multiple rounds of binding and release of non-native proteins, with a fraction of the molecules folding during each round. It has also been found that, as well as preventing off-pathway aggregation reactions, under some circumstances GroEL can increase the rate of folding by rescuing stable but unproductive intermediates in the folding of RuBisCO (ribulose 1,5-bisphosphate carboxylase-oxygenase) [25] and malate dehydrogenase [26].

The above considerations suggest that both Hsp70 and chaperonins might function in part by binding to kinetically trapped, slowly folding, or even mildly aggregated folding intermediates [8] (Fig. 3). This binding could facilitate the rearrangement of intermediates with incorrect proline isomers as well as the unfolding of kinetically trapped species. Finally, binding and/or hydrolysis of ATP would drive release of the unfolded protein. Folding could then be completed either in the bulk solution or perhaps while the protein remains associated with the chaperone. In this way, molecular chaperones could increase the fraction of the molecules that fold through rapid folding phases. This channeling of folding through inherently fast phases could lead to the observed rate enhancements sometimes seen in chaperone-assisted folding.

Conclusions

Folding of a protein is a vastly complicated reaction involving thousands of independent degrees of freedom. It is not surprising, therefore, that many folding reactions involve complex kinetics and multiple phases. Although the rapid rate of folding requires that the conformational space explored by a folding protein be highly restricted, it does not follow that the kinetic intermediates observed are involved in guiding a protein to its native states, nor is there any requirement that a protein fold by discrete pathways [27,28]. Much work needs to be done to establish the kinetic role of observed intermediates. These efforts should be aided by new statistical mechanical models [29,30] being developed which may provide a firm theoretical framework for understanding complex folding reactions.

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