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The nature of protein folding pathways: The classical versus the new view

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Summary

Pulsed hydrogen exchange and other studies of the kinetic refolding pathways of several small proteins have established that folding intermediates with native-like secondary structures are well populated, but these studies have also shown that the folding kinetics are not well synchronized. Older studies of the kinetics of formation of the native protein, monitored by optical probes, indicate that the folding kinetics should be synchronized. The model commonly used in these studies is the simple sequential model, which postulates a unique folding pathway with defined and sequential intermediates. Theories of the folding process and Monte Carlo simulations of folding suggest that neither the folding pathway nor the set of folding intermediates is unique, and that folding intermediates accumulate because of kinetic traps caused by partial misfolding. Recent experiments with cytochrome *c* lend support to this 'new view' of folding pathways. These different views of the folding process are discussed. Misfolding and consequent slowing down of the folding process as a result of cis–trans isomerization about prolyl peptide bonds in the unfolded protein are well known; isomerization occurs before refolding is initiated. The occurrence of equilibrium intermediates on the kinetic folding pathways of some proteins, such as α-lactalbumin and apomyoglobin, argues that these intermediates are not caused by kinetic traps but rather are stable intermediates under certain conditions, and this conclusion is consistent with a sequential model of folding. Folding reactions with successive kinetic intermediates, in which late intermediates are more highly folded than early intermediates, indicate that folding is hierarchical. New experiments that test the predictions of the classical and the new views are needed.

Introduction

Studies of protein folding intermediates matured rapidly after 1988 and NMR played a key role in this transformation. Earlier, most studies of intermediates in the kinetic process of folding consisted of efforts to prove (or disprove) the existence of structural intermediates. The NMR hydrogen exchange method of pulse labeling folding intermediates (Roder et al., 1988; Udgaonkar and Baldwin, 1988) changed this situation. Studies of several small proteins showed that structural intermediates are easily demonstrated by this method and the results gave some information about their structures (for reviews, see Baldwin, 1993; Dobson et al., 1994; Evans and Radford, 1994; Woodward, 1994).

Moreover, the full range of NMR structural methods can be used to analyze equilibrium molten globule intermediates and the significance of these intermediates for the mechanism of folding began to be understood in this period (for reviews, see Kuwajima, 1989; Ptitsyn, 1992). Although these partly folded forms are found at equilibrium under nonphysiological conditions where the native conformation is unstable, typically at acidic pH, sometimes the same partly folded forms appear as kinetic intermediates in the folding process under physiological conditions (Ikeguchi et al., 1986; Jennings and Wright, 1993) and this provides a link between these stable equilibrium species and the kinetic process of folding. Molten globule intermediates present severe technical difficulties, because they aggregate at low concentrations and their

resonances in the NMR spectrum are poorly resolved. Nevertheless, determination of the 3D structure of the protein backbone has been achieved in some cases (Feng et al., 1994; Redfield et al., 1994). Thus, NMR studies of folding intermediates have come of age. Models of intermediates on the coupled pathways of disulfide bond formation and folding have been made by peptide synthesis (Oas and Kim, 1988; Staley and Kim, 1990) and NMR is the basic tool used to analyze their structures.

The question discussed here is the nature of protein folding pathways: is there a well-defined pathway with sequential intermediates, as in an ordinary chemical reaction, or does folding follow multiple pathways without passing through a unique transition state? Experimentalists began by assuming that folding follows a simple sequential mechanism with defined intermediates, by analogy to an ordinary chemical reaction (Kim and Baldwin, 1982; Schmid, 1983). This is the 'classical view'. The 'new view' was put forward first by theorists, based on statistical mechanical theories of folding and on Monte Carlo simulations of folding (Bryngelson and Wolynes,

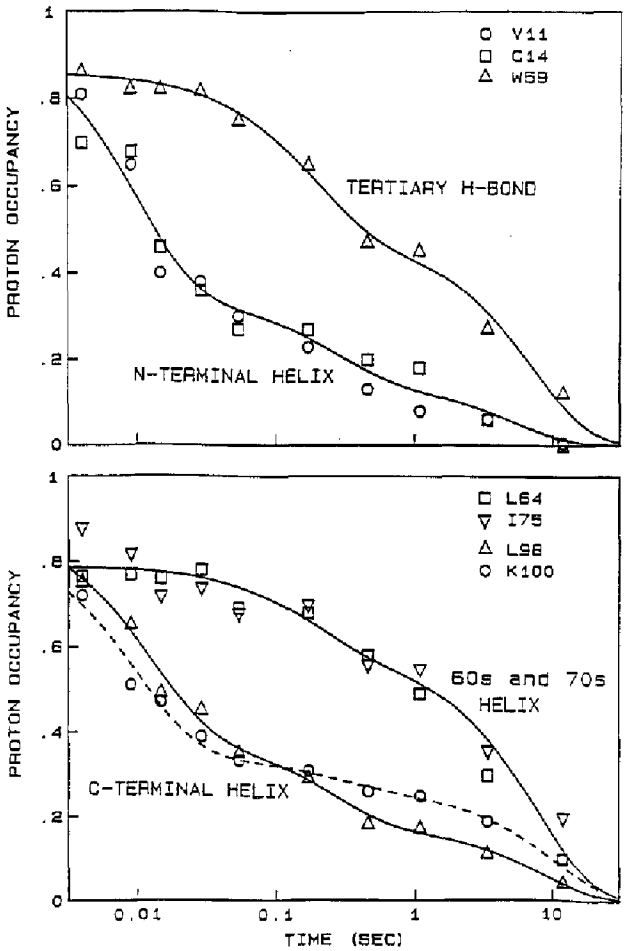

Fig. 1. At least three successive kinetic phases of folding of oxidized cytochrome *c* at pH 6.0, 10 °C, are shown, measured by protection against exchange of peptide NH protons in the N- and C-terminal helices. Results for other protons are also shown. Reproduced with permission from Roder et al. (1988).

1987,1989; Shakhnovich et al., 1991; Guo et al., 1992; Leopold et al., 1992; Camacho and Thirumalai, 1993; Dill et al., 1993; Shakhnovich and Gutin, 1993; Abkevich et al., 1994a,b; Chan and Dill, 1994; Sali et al., 1994a,b; Shakhnovich, 1994). These studies focused on the nature of the 'energy landscape' for folding, and on special properties of proteins that might explain their ability to fold rapidly. Experimentalists were slow to grasp the point that, according to these theories and simulations, folding intermediates are the result of kinetic traps; moreover, intermediates are predicted to be partly misfolded.

Independently, Sosnick et al. (1994) performed experiments on the folding of cytochrome *c* that led to a model with key features in common with the new view of the theorists. They found that a major kinetic folding intermediate is produced by an identifiable kinetic trap, the presence of a nonnative heme ligand in the starting material for folding (see also Elöve et al., 1994), and also that, by using an equilibrium molten globule species as the starting material, folding can be made to occur very rapidly. Thus, they suggest that in general folding intermediates, which are populated transiently, are formed by kinetic traps in folding, so that the accumulation of folding intermediates causes the folding process to slow down. Their work has been discussed by Creighton (1994). A corollary of this new view is that molecules which escape folding traps can fold on a 'fast track'. Dobson and coworkers found some suggestive evidence for fast-track folding of hen lysozyme (see below and Radford et al., 1992; Dobson et al., 1994; Itzhaki et al., 1994).

Lack of synchrony in folding kinetics studied by pulsed hydrogen exchange

Pulsed hydrogen exchange experiments give the locations of protected amide (peptide NH) protons during the kinetics of protein refolding. This information allows comparison of the predicted secondary structure of a folding intermediate to the known secondary structure of the native protein (for reviews, see Baldwin and Roder, 1991; Englander and Mayne, 1992). Moreover, by varying the pH of the pulse, the stability of a folding intermediate can be determined, or a lower limit can be set when the protection factors exceed this practical limit (about 1000) (Udgaonkar and Baldwin, 1990; Elöve and Roder, 1991). Experiments monitoring the kinetics of folding by pulsed hydrogen exchange have been performed for several small proteins and in most cases the predicted secondary structure of the folding intermediate comprises either the entire secondary structure of the native protein or else a large part of it. Thus, these results indicate that in many cases native-like secondary structures are formed early in folding and are sufficiently stabilized to provide protection against H-exchange. A different result was found for the β-sheet protein interleukin-1β (Varley et al., 1993): al-

though circular dichroism (CD) measurements indicate that β-structure is formed within 25 ms, protection against H-exchange begins only after 1 s. Miranker et al. (1991) demonstrated that the α-helix and β-sheet domains of hen lysozyme become protected against exchange in separate stages of folding, using a competition method based originally on ^{3}H labeling (Schmid and Baldwin, 1979) that was adapted for NMR analysis by Roder and Wüthrich (1986).

A disturbing feature of experiments in which refolding intermediates are characterized by pulsed H-exchange is the fact that the folding kinetics are not synchronized, in contrast to studies of the formation of the native protein made using optical probes. In other words, a reaction that is expected to be a single-step reaction, such as the protection of a specific peptide NH proton in a folding intermediate, actually occurs in two or even several kinetic steps. Formation of the native protein usually follows a single exponential curve, provided that refolding of a particular unfolded species is studied (for a review, see Schmid, 1992; and see below). If folding follows a simple sequential model, then the acquisition of protection against H-exchange for any amide proton is expected to follow a single exponential curve under most conditions (Udgaonkar and Baldwin, 1990), but instead complex kinetics, with two or more successive phases, are commonly observed. For ribonuclease A, only one half of the expected amount of unfolded protein forms the initial major folding intermediate that is monitored by H-exchange, and the remaining unfolded protein folds only gradually, with poorly defined kinetics (Udgaonkar and Baldwin, 1990). For cytochrome, at least three separate kinetic phases are observed for the acquisition of protection by amide protons in the N- and C-terminal helices (Roder et al., 1988; see Fig. 1). Related results, demonstrating kinetic complexity in the acquisition of protection against H-exchange, have been observed for barnase (Bycroft et al., 1990), hen lysozyme (Radford et al., 1992) and ribonuclease T1 (Mullins et al., 1993), and parallel pathways of folding have been found by using other probes of folding for dihydrofolate reductase (Touchette et al., 1986) and for a fatty acid binding protein (Ropson et al., 1990).

The lack of synchrony in the acquisition of protection against H-exchange during the folding of cytochrome *c* is shown in Fig. 1, reproduced from Roder et al. (1988). A conspicuous feature is that the middle helix (the 60s and 70s helix) is not stabilized in the major early folding intermediate, although the N- and C-terminal helices are both stabilized with the same folding kinetics. On the other hand, all three helices are stabilized in the equilibrium molten globule intermediate at pH 2 (Jeng et al., 1990). The reason for the failure of the early kinetic intermediate to form the middle helix is that a kinetic trap prevents its formation, i.e., a trap caused by a nonnative heme ligand

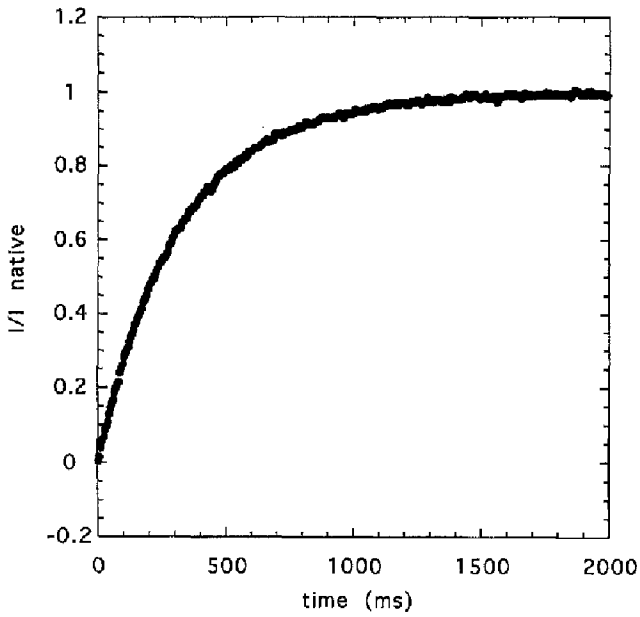

Fig. 2. Kinetics of formation of native hen lysozyme at pH 5.2, 0.54 M GdmCl, 20 °C, monitored by the change in fluorescence upon binding of a specific inhibitor, 4-methylumbelliferyl-*N,N'*-diacetyl-β-chitobiose (see Kato et al., 1982). Redrawn with permission from Itzhaki et al. (1994).

(probably a histidine side chain) in place of Met^{80} (Elöve et al., 1994; Sosnick et al., 1994). Cytochrome *c* provides a specific example of how a partly misfolded intermediate can accumulate during the kinetic process of folding. Similar examples can be taken from the older literature on the effects of cis–trans isomerization of proline residues in producing fast- and slow-folding forms of an unfolded protein (see below and the review by Schmid, 1992).

The complexity of the folding kinetics of hen lysozyme has been analyzed in detail by Dobson and co-workers (Radford et al., 1992; Dobson et al., 1994; Itzhaki et al., 1994). The kinetics of acquisition of protection are complex for each of the amide protons, and sometimes show more than two kinetic phases. Folding of some molecules on a fast track is suggested by the kinetics of acquiring protection against exchange, but specific probes for the formation of native protein (near-UV CD and inhibitor binding) do not show any native protein formed in a fast phase (Itzhaki et al., 1994). Instead, the kinetics of formation of native protein follow a single exponential curve (see Fig. 2), without a measurable lag, as in earlier studies of folding kinetics based on optical probes. The puzzle posed by the results of Itzhaki et al. (1994) is that the rate-limiting step in the formation of native protein does not appear to depend on the prior extent of folding measured by H-exchange. It should be remembered that amide proton protection is limited to rather early events in folding: the largest protection factors that can be measured by pulse labeling during the kinetics of folding are about 10^3, whereas native proteins commonly show protection

factors of 10^8 or larger. Docking of the separately folded α-helix and β-sheet domains (see Dobson et al., 1994) is likely to be the rate-limiting step in the formation of the native structure. The slowness of this step relative to the folding of the α-helix and β-sheet domains may explain why formation of the native structure follows a single exponential curve (Denton et al., 1994).

Relation between synchrony of folding and conformation of the unfolded state

Certain slow reactions in the unfolded protein, such as cis–trans isomerization about prolyl peptide bonds, are known to cause initial misfolding, slow down the folding process, and to have a directing influence on the folding pathway. This behavior has been studied thoroughly for proline isomerization. Figure 3 shows an example, taken from a study of porcine ribonuclease (Grafl et al., 1986; for a review see also Schmid, 1992). The unfolding/refolding kinetics of this protein, together with the X-ray structure of ribonuclease A, indicate that the *cis*-proline residues Pro^{93}, Pro^{114} and Pro^{115} are responsible for the different slow-folding species shown in Fig. 3. The neighboring proline residues Pro^{114} and Pro^{115} apparently isomerize in unison and, when they are trans, a particularly slow-folding species is formed. Proline isomerization is sufficiently slow that assays of the refolding behavior as a function of time after the initial unfolding (see Fig. 3) provide a simple test for the slow formation of different unfolded species that are distinguished by their refolding behavior.

Other slow reactions in the unfolded state are known that can produce heterogeneity in refolding behavior. Figure 1 illustrates heterogeneity in the refolding kinetics of cytochrome *c*, and it is known that the interchange of heme ligands in unfolded cytochrome *c* involves slow steps (Tsong, 1977). It has been found recently that a cis non-prolyl peptide bond gives rise to a slow-folding species by cis–trans isomerization after unfolding (Odefey et al., 1994). This finding may be of general significance for understanding heterogeneity in refolding kinetics measured by pulsed H-exchange, because for all proteins there is a small but finite probability that in the unfolded state any peptide bond will be cis (see Lin and Brandts, 1978; Udgaonkar and Baldwin, 1990).

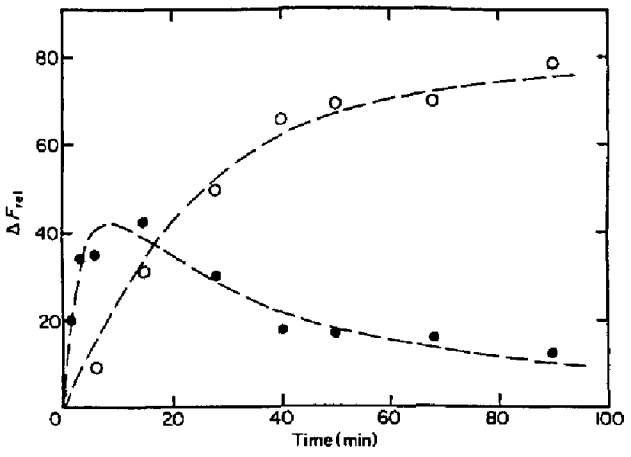

Fig. 3. Formation of the two slow-refolding species of porcine ribonuclease at pH 2.0, 5.0 M GdmCl, 10 °C, by slow cis–trans isomerization of prolyl peptide bonds. The fast-refolding species is formed directly as unfolding occurs and accounts for 100% of the unfolded protein at t = 0. The amounts of the fast- and slow-refolding species at various times after unfolding are measured by a refolding assay. Reproduced with permission from Grafl et al. (1986).

These examples illustrate the point that, in searching for a case where folding occurs on a 'fast track', it is essential to find out whether the fast-folding molecules arise from a particular species of the unfolded protein. Recently a minor (7%), very fast folding form of the well-studied protein ribonuclease A was found (Houry et al., 1994). These authors suggest that it has correct cis isomers of the two *cis*-proline residues, Pro^{93} and Pro^{114}, and that these residues produce slow-folding species when either proline residue is trans. Such examples suggest that lack of synchrony in refolding kinetics will often be the result of conformational heterogeneity in the unfolded protein. Because the unfolded protein exists in a wide range of conformations, conformational heterogeneity is present when refolding is initiated. If conformational heterogeneity is the correct explanation for the lack of synchrony in the refolding kinetics monitored by H-exchange, then the unfolding kinetics should be synchronized because unfolding starts from a unique conformation, the native protein. Indeed, a study of the unfolding kinetics of ribonuclease A indicates that unfolding monitored by H-exchange occurs with synchronized kinetics: the process follows a single exponential time course and the entire protein sample follows these kinetics (Kiefhaber and Baldwin, 1995).

Equilibrium molten globule forms as early intermediates on kinetic pathways of folding

The existence of equilibrium molten globule forms of proteins such as apomyoglobin and α-lactalbumin on kinetic pathways of folding argues against the accumulation of folding intermediates by kinetic traps. These forms are equilibrium intermediates and their unfolding/refolding behavior is freely reversible, starting either from the unfolded protein, the intermediate, or the native protein. The fact that these equilibrium intermediates also occur as intermediates on kinetic pathways of folding strongly suggests that they are not caused by misfolding in kinetic traps and instead are on-pathway, sequential folding intermediates. In general, when a kinetic intermediate accumulates transiently on a reaction pathway, there is a barrier preventing the rapid formation of the next species on the pathway. In the case of protein folding, the barrier may represent partial misfolding that must be undone

before the next species on the pathway can be formed. What separates the new view from the classical view of folding is the issue of whether folding is a sequential process with obligatory intermediates: can some molecules escape these barriers and fold on a fast track? The occurrence of parallel pathways in the folding pathways of some proteins complicates the argument.

With regard to the relation between an equilibrium folding intermediate and the corresponding kinetic intermediate, the most detailed structural comparison has been made for apomyoglobin. The existence of an equilibrium folding intermediate (I_1) near pH 4 was shown, using CD measurements of helix content, by Griko et al. (1988). Earlier fluorescence measurements (Kirby and Steiner, 1970; Irace et al., 1981) also indicated the presence of an intermediate. Three of the helices of native myoglobin, the A-, G- and H-helices, are stabilized in I_1, according to the locations of protected amide protons (Hughson et al., 1990). During the kinetic process of refolding at pH 6, intermediate I_1 is formed rapidly, within the stopped-flow mixing time (Jennings and Wright, 1993); it is identified by an exact correspondence between the locations of the protected amide protons in the early kinetic intermediate and in the equilibrium intermediate at pH 4. At least two kinetic folding intermediates can be resolved; I_1 corresponds to the equilibrium intermediate at pH 4.

$$U \leftrightarrow I_1 \rightarrow I_2 \rightarrow \cdots N \qquad (1)$$

The B-helix is stabilized in addition to the A-, G- and H-helices in the second intermediate, I_2. Both I_2 and the native protein (N) are formed slowly, i.e., on the seconds time scale. Recently, conditions have been found in which a second intermediate (the same as or closely similar to I_2) is also a stable equilibrium intermediate: its identity was established by NMR hydrogen exchange (Loh et al., manuscript in preparation).

The conditions needed for the appearance of an equilibrium folding intermediate I on the kinetic pathway of folding are well understood (see Kuwajima, 1977; Barrick and Baldwin, 1993; Sanz and Fersht, 1993). (1) I should be stable relative to U under refolding conditions; (2) I should be formed rapidly from U; and (3) folding should follow the simple sequential model. These conditions are met for apomyoglobin (Barrick and Baldwin, 1993; Jennings and Wright, 1993; Loh et al., manuscript in preparation). The sequential character of the folding reaction of apomyoglobin is suggested by the facts that I_2 contains additional structure (the B-helix) compared to I_1, and I_2 is more stable than I_1. These results strongly support the classical view of folding pathways. The interactions that stabilize I_1 and I_2 have not yet been identified. A site-directed mutagenesis study (Hughson et al., 1991) indicated that mutations at helix pairing sites have only marginal effects on the stability of I_1, in contrast to native apomyoglobin where close-packed interactions between side chains contribute strongly to stability.

Matthews and co-workers have provided extensive evidence that the folding pathways of tryptophan synthase α subunit and dihydrofolate reductase are hierarchical (for a review, see Matthews, 1993), that folding proceeds through stages of increasing complexity, and that folding intermediates accumulate at these stages. In order to reconcile the extensive evidence for sequential folding with the new view of folding pathways, it seems necessary to postulate a series of kinetic traps that occur at successive stages in folding. An example of such a specific trap is provided by the work of Kiefhaber et al. (1992). They found that replacement of Trp^{59} by tyrosine in ribonuclease T1 markedly speeds up a refolding step in the proline-limited pathway that is present when Pro^{39} (which is cis in the native protein) is trans in the unfolded protein. Apparently, there is a tight contact involving Trp^{59} when Pro^{39} isomerizes from trans to cis during refolding, and the replacement of Trp^{59} by tyrosine facilitates the proline isomerization.

If this compromise between the new and the classical view of folding pathways is adopted, namely that folding follows sequential pathways (sometimes more than one pathway) but that folding intermediates accumulate because of traps in folding, then the critical question is: can some molecules escape these traps and fold on a fast track? And, if folding on a fast track can be demonstrated, do the fast-folding molecules originate in a particular conformation in the unfolded state?

Some other small proteins have not yet been found to form equilibrium molten globule intermediates, but nevertheless show early kinetic folding intermediates whose properties resemble those of molten globule species (for a review, see Baldwin, 1993). One reason why these proteins do not exhibit equilibrium folding intermediates is because the native species is too stable. It has been shown by Sanz and Fersht (1993) that mutants of barnase that are strongly destabilized relative to wild type do show equilibrium intermediates in urea-induced unfolding, although wild-type barnase shows only kinetic folding intermediates (Matouschek et al., 1992). Cytochrome *c* is an exception to the general scheme discussed above, because the equilibrium molten globule intermediate differs in its structure from the early kinetic folding intermediate. As discussed above, all three major helices of the native protein are stable in the equilibrium intermediate at pH 2 (Jeng et al., 1990), whereas only the N- and C-terminal helices are stable in the early kinetic intermediate during folding at pH 6.0.

If refolding intermediates accumulate transiently because partial misfolding blocks their progress, then it may be possible to detect this misfolding by pulsed hydrogen-exchange experiments. When an intermediate unfolds partially in order to form a different structure, this un-

folding event can be detected by using a long labeling pulse and a high pH of labeling (Baldwin, 1991). Peptide NH protons that are labeled by virtue of nonnative structure, present in a folding intermediate at the time when the exchange pulse is first applied, will lose their label when transient unfolding occurs. When the labeling pulse is made at pH 10, as in initial experiments on the folding of ribonuclease A (Udgaonkar and Baldwin, 1988), the protein needs to be unfolded for only 0.1 ms in order to exchange out its label. Systematic experiments of this kind, aimed at detecting partial misfolding, have not yet been reported but are clearly feasible.

Conclusions

The complex refolding behavior predicted by theories and simulations of the folding process (see above) almost seems to follow directly from the models used, which are closely similar in spirit to the jigsaw puzzle model of Harrison and Durbin (1985), in which folding can start anywhere and the folding pathway is determined by chance events as well as by the energetics of folding. There are two major *caveats* in drawing this conclusion. First, secondary structures are often ignored in simulations of folding, because of practical difficulties in representing them with a lattice model. Most experimentalists see the formation of secondary structure as a major factor determining the folding pathway. As Richards (1992) pointed out, pattern recognition is an important part of solving a jigsaw puzzle and the pattern imposed by the cooperative formation of α-helices and β-sheets is likely to be an important factor in determining the folding pathway. Second, folding pathways and folding intermediates may have been selected through evolution so as to have a rapid and robust pathway for arriving at the native structure, see Abkevich et al. (1994a) and Sali et al. (1994a,b). The random misfolding seen in the simulated folding reactions of random sequences need not carry over to the folding behavior of real proteins.

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Note added in proof

A new pulsed H/D exchange folding study has just been published that is relevant to the discussion presented here (Kuszewoki, J., Clore, G.M. and Gronenborn, A.M. (1994) *Protein Sci.*, **3**, 1945–1952).

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