On-pathway versus off-pathway folding intermediates

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Rapidly formed molten globule intermediates accumulate at the start of the folding reactions of several small proteins. Opinion is sharply divided as to whether they are on-pathway or off-pathway intermediates. I discuss recent experiments aimed at resolving this issue. Specific points include whether a 'rollover' in the plot of folding rate versus denaturant concentration implies that a folding intermediate is or is not on-pathway; whether the failure to observe folding intermediates for some small proteins implies a different folding mechanism or only that the intermediates are less stable; possible interpretation of 'fast-track' folding of hen lysozyme; and the significance of recent results in the search for unfolding intermediates.

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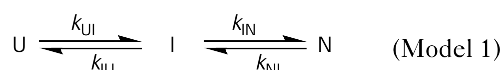
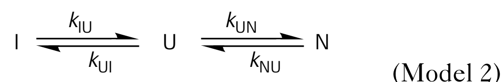
Introduction

Folding intermediates accumulate rapidly when refolding is initiated for a dozen or so small single-domain proteins (see [1–3] for reviews). The pressing issue raised by recent results is whether the intermediates are on-pathway:



(Model 1)

or off-pathway:



(Model 2)

In some refolding reactions [4,5], more than one kinetic intermediate is observed. The focus here is on the first observed intermediate, which equilibrates with the unfolded protein at the start of refolding.

Dependence of the folding rate on denaturant concentration

A logical starting point in the discussion of on-pathway versus off-pathway intermediates is consideration of the rate constants for folding and unfolding and how they depend on denaturant concentration. Although each of the two models above has two steps and four rate constants, often the overall rate constants for folding (k_f) and unfolding (k_u) are the only ones measured because the $U \rightleftharpoons I$ reaction occurs within the mixing time and only its amplitude can be measured.

(3)

In most cases, the rate constants for the $U \rightleftharpoons I$ step are found to be too fast to be measured by stopped-flow methods. The concentrations of U and I can be measured at the start of refolding, however, and the equilibrium constant K_{UI} (i.e. k_{UI}/k_{IU}) can then be determined.

Standard analysis of kinetic mechanisms shows that when equilibration between U and I precedes the step in which N is formed, the folding rate is proportional to f_I, the fractional concentration of I, in the on-pathway model:

$$k_f = f_I \, k_{IN} \qquad \text{(Model 1)} \qquad (4a)$$

whereas k_f is proportional to $(1–f_I)$ in the off-pathway model:

$$k_f = (1–f_I)k_{UN} \qquad \text{(Model 2)} \qquad (4b)$$

$$f_I = [I]/([I] + [U]) = K_{UI}/(1+K_{UI}) \qquad (4c)$$

Thus, accumulation of I has opposite effects in the two models: it speeds up the formation of N in the on-pathway model, but it slows down k_f in the off-pathway model.

For each single step in folding or unfolding, the logarithm of the rate constant depends linearly on the denaturant molarity C [6,7]:

$$\ln k_{ij} = \ln k_{ij}(H_2O) + (m^{\ddagger}_{ij}/RT)C \qquad (5)$$

The coefficient $m^{\ddagger}$, which describes the denaturant dependence, is approximately proportional to the change in buried surface area that occurs between the reactant and the transition state. The equilibrium constant for the step is given by equation 5a:

$$\ln K_{ij} = \ln K_{ij}(H_2O) + (m_{ij}/RT)C \qquad (5a)$$

The equilibrium coefficient m_{ij} equals the difference between the coefficients for the forwards and backwards reactions:

$$m_{ij} = m^{\ddagger}_{ij} – m^{\ddagger}_{ji} \qquad (5b)$$

Testing the equality in equation 5b provides a basic test of whether the reaction is a two-state reaction without any populated intermediates.

The case that f_I is too small to be measured directly is of particular interest because folding then appears to be a two-state reaction, although in reality the observed folding rate depends on the concentration of a real intermediate. In this case, $k_{IU} >> k_{UI}$ and k_f becomes proportional to K_{UI} in the on-pathway model.

$$k_f = K_{UI}\, k_{IN} \quad \text{(Model 1)} \quad (6)$$

$$\ln k_f = \ln k_f(H_2O) + (m^{\ddagger}_f/RT)C \quad (6a)$$

Combining equations 5, 5a, and 6 gives equation 6b:

$$m^{\ddagger}_f = m_{UI} + m^{\ddagger}_{IN} \quad (6b)$$

Consequently, for the on-pathway model, the linear dependence of ln k_f on C results from k_f being proportional both to f_I and to k_{IN}. If there is no intermediate, instead there being only the transition state, the linear dependence of ln k_f on C is still observed. When the plot of ln k_f against C bends over as a folding intermediate accumulates, this behavior is referred to as a 'rollover'.

According to the on-pathway model, the rollover occurs because k_f is proportional to $K_{UI}k_{IN}$ when f_I is small (equation 6) and to k_{IN} alone when f_I equals 1. According to the off-pathway model, the rollover occurs because k_f is proportional to k_{UN} when f_I is small and to $(1–f_I)k_{UN}$ as f_I approaches 1 (equation 4b).

Equations 4–6 are used below in discussing a number of topics. First, considering different possible interpretations of a rollover. Second, testing for two-state folding. Third, asking if the folding rate depends on stability. Fourth, detecting kinetic folding traps and avoiding them via 'fast track' folding. And fifth, probing for unfolding intermediates.

Interpretation of a rollover by the off-pathway model

In 1990, Fersht and co-workers [8] brought the problem of off-pathway intermediates into focus with the observation that the folding rate of barnase falls below the expected linear dependence of ln k_f on C at low urea concentrations, where a folding intermediate I accumulates at the start of folding. They also studied some less stable mutants of barnase and found a smaller accumulation of I when the mutants refold; they found that the rollover then begins at lower urea concentrations, coincident with the accumulation of I. They concluded that the slowing down of the folding rate, compared to expectation, was caused by the accumulation of the intermediate or by a change in the reaction mechanism to give a different rate-limiting step.

Two-state folding reactions

A natural next step is to ask how fast are the folding reactions of small proteins that show no detectable intermediates. There are now several examples; for a recent list, see [9]. In 1991, Jackson and Fersht [10] argued that the fast folding behavior of chymotrypsin inhibitor 2 (CI2) can be attributed to the absence of detectable folding intermediates (see also Creighton [11]). Schmid and co-workers [9] made the same argument for the small cold-shock protein from *Bacillus subtilis* (CspB), after subjecting it to a battery of tests that show the absence of folding intermediates. They suggest that the intermediates found in the folding reactions of other small proteins are rapidly formed secondary structures that are not on the folding pathway. In considering the fast folding reactions (ms) of these small proteins that do not show folding intermediates, one should remember that the folding rates of proteins cannot be predicted from their structures and the property of being fast folding cannot be unambiguously correlated with the presence or absence of folding intermediates.

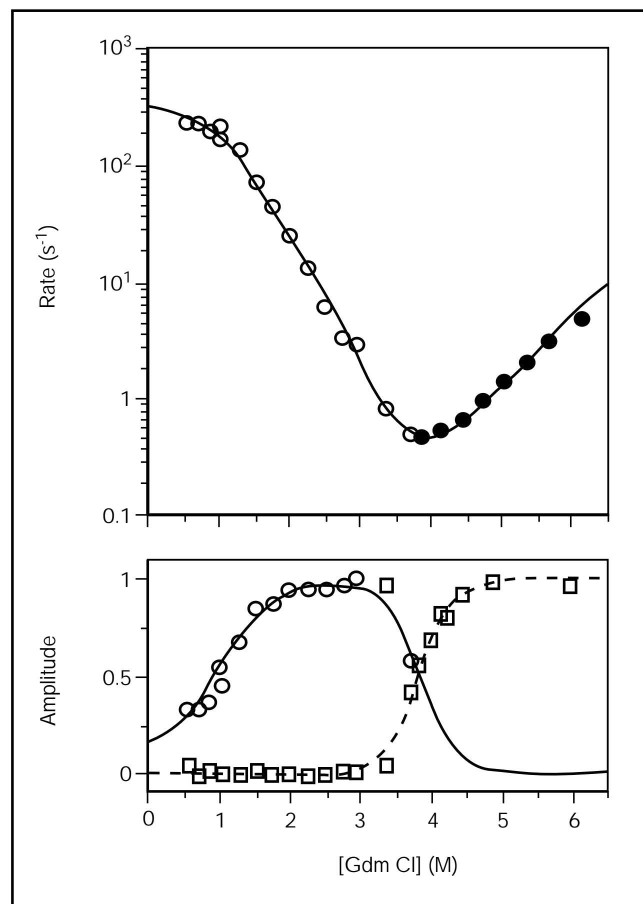
Interpretation of a rollover by the on-pathway model

A different view of the problem of on-pathway versus off-pathway intermediates has been provided by Roder and co-workers [12]. They made mutants of ubiquitin at a single residue position and then measured their folding kinetics as a function of guanidinium chloride (GdmCl) concentration. The results are fitted to the three-state on-pathway model (Model 1), but the authors point out that, because the kinetics of the $U \rightleftharpoons I$ step are too fast to be measured in stopped-flow experiments, their results do not distinguish between the on-pathway and off-pathway models. The results make sense, however, when interpreted by the on-pathway model.

Ubiquitin is a small protein (76 residues) with a β-sheet and a short helix. It folds rapidly (ms). In the 'pseudo-wild-type' protein, Phe45 has been replaced by Trp to allow the folding kinetics to be monitored by Trp fluorescence. The residue chosen for mutation, Val26, is in the hydrophobic core between the helix and the β-sheet. It has been replaced both by larger (Leu or Ile) and smaller (Ala or Gly) non-polar residues. Ubiquitin has three *trans* proline residues that produce minor amounts of slow-folding species; these are taken into account in the analysis and are not discussed here. When the folding kinetics are fitted to a three-state model, the kinetic results give values of ΔG_{NU}, the stability of the native protein, that are in good agreement with the equilibrium values measured from unfolding transition curves.

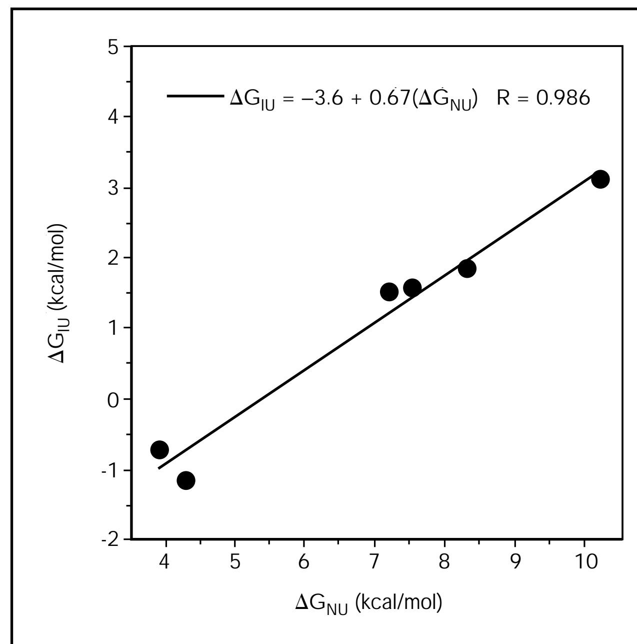
Four of their results are particularly relevant to the problem of distinguishing between the on-pathway and off-pathway models. Firstly, there is a rollover in the plot of ln k_f versus C (GdmCl) when I accumulates at the start of folding (Fig. 1). Roder and co-workers do not, however,

Figure 1



Unfolding and refolding kinetics of ubiquitin mutant Phe45→Trp (adapted from Fig. 6a of [12], prepared by S Khorasanizadeh and H Roder). The upper panel shows the observed rate constant for the major refolding reaction (open circles) and unfolding reaction (filled circles). The solid line is fitted to equation 4a. The lower panel shows the unfolding transition of the native conformation (squares) and of the intermediate (open circles) measured by tryptophan fluorescence. The solid and dashed lines are the kinetic amplitudes and equilibrium transition predicted by the three-state model (Model 1).

Figure 2



The stability to unfolding of the intermediate (ΔG_{IU}) plotted against the stability of the native species (ΔG_{NU}) for a set of ubiquitin mutants, including data taken in the presence of the stabilizing salt 0.4 M Na_2SO_4. (The data are from Tables 2 and 3 of [12]: ΔG_{IU} is $RT\ln K_{UI}$ measured in kinetic folding experiments and ΔG_{NU} is measured from equilibrium unfolding curves.)

interpret the rollover to mean that k_f is proportional to ($1-f_I$) as suggested by the off-pathway model. Instead, they use the on-pathway model in which k_f is proportional to f_I, and they point out that the rollover occurs as $f_I \rightarrow 1$, where the effect of the intermediate on the folding rate is approaching saturation. Secondly, they find that less stable mutants of ubiquitin show a reduced tendency to accumulate I at the start of folding (compare results for barnase [8]). Thirdly, a less stable mutant of ubiquitin, Val26→Ala, which shows apparent two-state folding kinetics, can be made to show three-state kinetics, with the accumulation of I at the start of folding, by adding the stabilizing salt 0.4 M Na_2SO_4. This salt increases the stability of the wild-type protein by 3.0 kcal mol^{-1}. Thus, other small proteins, such as CI2 [10] and CspB [9], may fail to show folding intermediates only because the intermediates are not stable enough. Fourthly, when the factors affecting k_f (see equation 4a) are compared for the different mutant proteins, k_f is found to depend solely on f_I (or K_{UI}) to a good first approximation. The values of k_{IN} differ by only a factor of 2 among the mutants, whereas the values of K_{UI} and of k_f differ by a factor of 10^3.

A linear correlation is seen between ΔG_{IU} and ΔG_{NU} (Fig. 2). The fact that the stability of the folding intermediate correlates directly with the stability of the native conformation supports the suggestion that I is on the folding pathway because there is a direct relation between the effects of mutations on the stability of I and of N: compare the kinetic analysis of the effects of mutations in barnase on the stabilities of I, N and the transition state for I→N [8]. Similar results were found for cytochrome *c* (cyt *c*) [13] when the stability of the pH 2 equilibrium intermediate was compared to that of the native species.

Although these experiments by Roder and co-workers [12] do not prove that I is on the folding pathway of ubiquitin, they do show that it has the properties expected for an on-pathway intermediate. They also show that the arguments for off-pathway intermediates in the folding reactions of

other small proteins could be used to argue instead for on-pathway intermediates.

Scheraga and co-workers [14] use the on-pathway mechanism to represent the folding rate of the very fast folding species of unfolded RNase A. They do not measure the concentration of the intermediate by an independent method.

Folding rates and stability

Sauer and co-workers [15] mutated most of the residues of Arc repressor to Ala in single mutants and determined the folding and unfolding rates in conditions where the folding reaction is two-state, without detectable intermediates. Arc repressor is small (53 residues) and forms a symmetrical dimer. The refolding reaction follows bimolecular kinetics and the transition state for folding occurs after dimerization.

The authors find a striking correlation of ln k_f and ln k_u with ΔG_{NU}, the stability of the native species. The effects of mutations on the unfolding rate are much larger than the effects on the refolding rate. It is well known that the unfolding and refolding rates of small proteins depend strongly on denaturant concentration, and the question is sometimes asked whether this behavior implies a general dependence of these rate constants on the stability of the native conformation. The results of Sauer and co-workers [15] indicate that the answer is yes.

In the ubiquitin system [12] discussed above, ln k_f also depends on ΔG_{NU} but, more particularly, ln k_f depends on ΔG_{IU} through equation 6a and the dependence of k_f on K_{UI}. In the case of Arc repressor, the folding kinetics are two-state and a folding intermediate has not yet been demonstrated, unlike the intermediates shown by ubiquitin, barnase and several other small proteins. The refolding kinetics of wild-type Arc repressor deviate from being second order at high repressor concentrations [16], which suggests that such an intermediate may be populated in these conditions.

Theoretical models of the folding process

Theories and Monte Carlo simulations of the folding process (see [17] and references therein) suggest that non-native contact interactions between sidechains act as kinetic traps during folding. The simulations indicate that folding follows several alternative pathways simultaneously [17]. Because alternative folding pathways can make use of different transition states, these theoretical models suggest there may be no unique rate-limiting step in folding. Molecules which escape the kinetic traps fold on a 'fast track'.

Kinetic traps in the folding of cyt *c* and other proteins

An example of a non-native contact that causes a folding intermediate to accumulate was found by Sosnick, Englander and co-workers [18] (see also [19]). When cyt *c* is unfolded by GdmCl at pH 6, the unfolded protein still contains heme ligands provided by protein sidechains; a non-native ligand (probably an unprotonated histidine sidechain) replaces the Met80 ligand found in native cyt *c*. The non-native ligand appears to be responsible both for slow refolding at pH 6 and for the failure of the early folding intermediate to include the middle helix of cyt *c*, although it contains both the amino-terminal and carboxy-terminal helices. At pH 2, a somewhat different folding intermediate is stable at equilibrium: it contains all three major helices [20]. When the pH 2 intermediate is used as the starting material for refolding at pH 4.9, the folding reaction is fast (ms) as compared to the slow folding reaction (s) observed when the GdmCl-unfolded protein at pH 6 refolds at pH 6 [18]. When overall folding is measured at a series of pH values, the amplitude of the slow-folding component decreases with pH as expected if titration of a histidine residue releases the non-native ligand from the heme [19]. When the non-native contact breaks during folding at pH 6, the kinetic intermediate containing the amino-terminal and carboxy-terminal helices probably does not need to unfold entirely in order to complete the folding process. Thus, kinetic mechanisms more complex than Model 2 are likely to be required when kinetic traps are present during folding.

Because the non-native heme ligand is present in GdmCl-unfolded cyt *c* before the folding reaction is initiated at pH 6, this example resembles the case in which two unfolded species, each with its own refolding pathway, are produced by proline isomerization after unfolding [21]. On the other hand, in contrast to the case provided by proline isomerization, the cyt *c* example makes the point that a non-native contact can produce a kinetic trap in folding [18]. The type of non-native contact commonly envisaged as producing kinetic traps in folding is the hydrophobic contact (see [17] and references therein), but specific examples of this kind have not yet been demonstrated. One wants to see proof that a non-native contact slows down folding by the test that folding speeds up when the contact is eliminated by mutation.

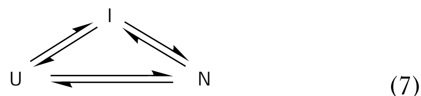
An example of a non-covalent folding reaction that speeds up when a contact is removed by mutation has been found [22]. The mutation Trp59→Tyr in RNase T1 accelerates the refolding reaction of a slow-folding unfolded species more than 10-fold. The slow-folding species is produced by the *cis*→*trans* isomerization of Pro39 after unfolding. There is a tight contact between Pro39 and Trp59 in the structure of RNase T1, and the likely explanation [22] is that the Trp59→Tyr mutation relieves a tight contact when *trans*→*cis* isomerization of Pro39 occurs during refolding.

When folding is coupled to the reoxidation of disulfide bonds, the presence of native structure can inhibit

disulfide bond formation for steric reasons [23,24]. This is a clear example of a kinetic trap in folding, but it pertains to the formation of disulfide bonds which are covalent bonds.

Alternative folding pathways: fast-track folding of hen lysozyme

By using a specific and sensitive assay for the native species N, Kiefhaber [25] recently discovered fast-track folding of hen lysozyme. His results fit the parallel pathway model:



(7)

where I is an intermediate on the major (86%) slow pathway and 14% of the molecules fold directly ($U \rightleftharpoons N$) on a fast track. I is formed with a relaxation time of 30 ms, which suggests a lag in the formation of N on the major pathway if I is on-pathway. The specific assay for N is based on unfolding kinetics: the native species unfolds slowly, and its concentration can be measured in conditions where folding intermediates unfold rapidly and do not interfere with this assay.

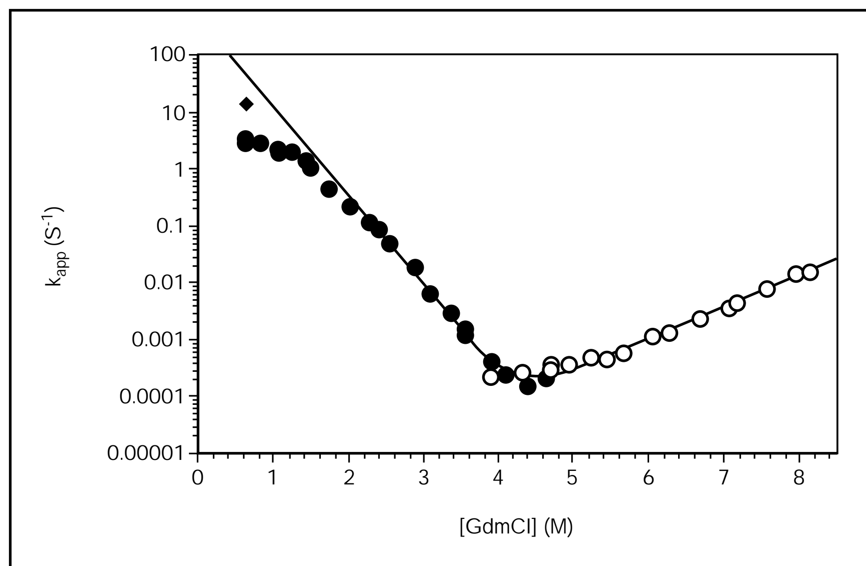
The folding rate observed for fast-track folding is close to the extrapolated rate (in the plot of ln k_f versus C) on the slow track at this value of C (0.6 M GdmCl). There is a rollover on the major pathway (Fig. 3) as I accumulates. Consequently, a possible explanation for the two pathways is a kinetic trap at low GdmCl concentrations on the major pathway, which causes I to accumulate and the folding rate to slow down. Some molecules escape this kinetic trap and fold on a fast track.

The folding kinetics of hen lysozyme have been studied in detail, using several different probes of folding, by Dobson *et al.* [3]. This system represents the best documented case of complex folding kinetics shown by a fairly small (albeit two-domain) protein. Curiously, despite the complex folding kinetics shown by probes of folding intermediates, other probes that are specific for the native conformation apparently show simple folding kinetics [3]. A special assay was required to resolve folding on a fast track by a minor species [25]. The problem of understanding why the native conformation is formed with relatively simple kinetics, compared to the complex kinetics found for folding intermediates by the NMR-hydrogen exchange method, appears to be a general problem [21].

Alternative folding pathways may exist for other reasons besides kinetic traps. The case of dihydrofolate reductase (DHFR) has been studied in detail by Matthews and co-workers [26]. There are two alternative conformations of the native (i.e. fully folded) species, one of which can bind the ligand NADPH; the other form cannot. Isomerization between the two forms is slow (~40 s). There are separate folding pathways for the two folded conformations [26], and there are also separate unfolding pathways (see below).

Alternative folded forms of human insulin have been found by Weiss and co-workers [27]. They are formed in the coupled processes of folding and disulfide bond formation, and the variant folded form differs from the standard form both in folding and in the pairing between cysteine residues in two of the three disulfide bonds. The possible significance of alternative folded forms for understanding folding pathways has been discussed recently by Dobson [28].

Figure 3



Observed rates of unfolding (open circles) and refolding (filled circles, filled diamond) of hen lysozyme in 0.6 M GdmCl, pH 5.2, 20°C (reprinted from [25] with permission of T Kiefhaber). The diamond shows the folding rate of the minor (14%), faster folding reaction.

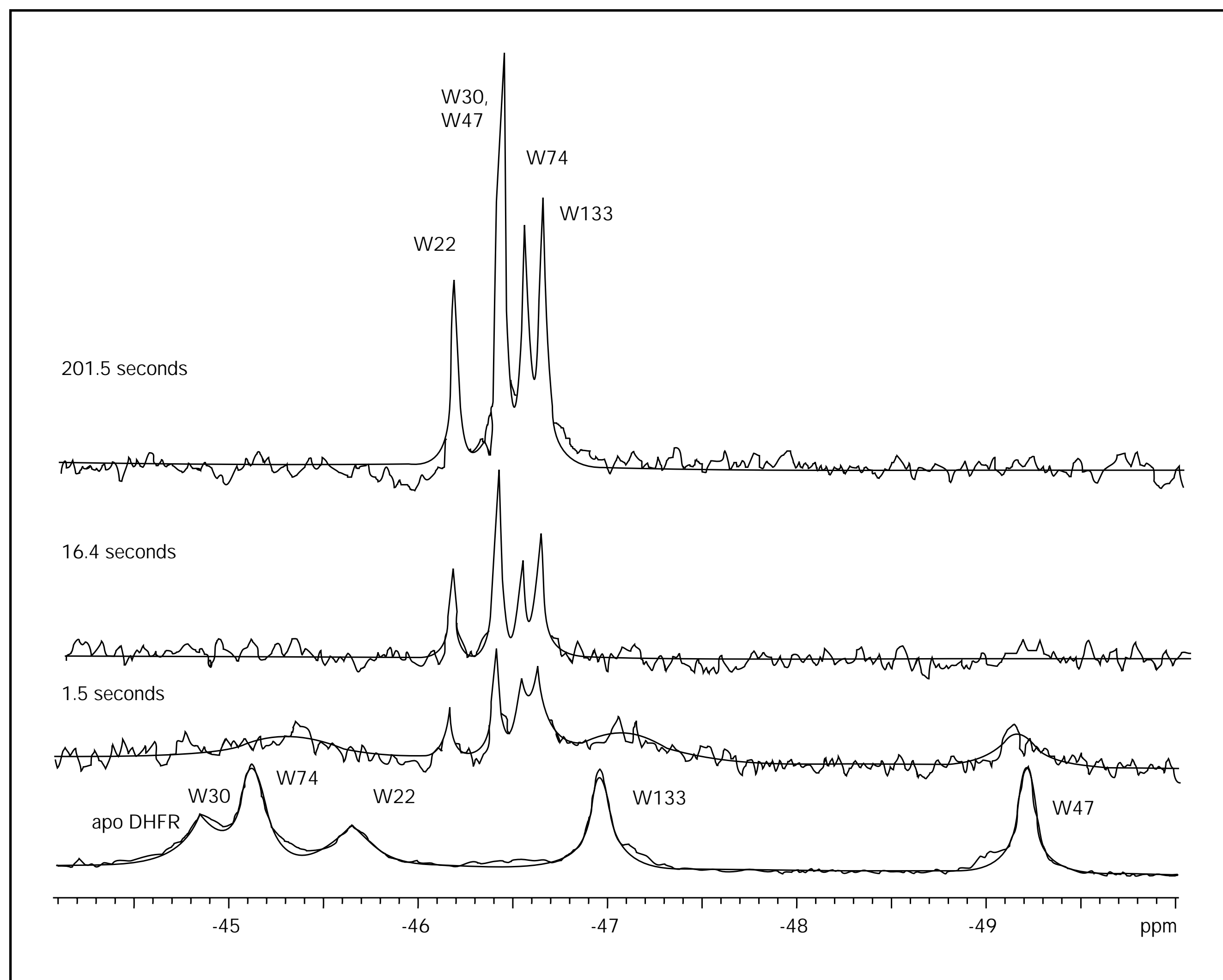
Unfolding intermediates and the rate-limiting step in unfolding

Finding the rate-limiting step in refolding has been stymied by uncertainties about the role of populated intermediates in refolding and about the causes of alternative folding pathways. Probably these problems are linked to each other: it seems likely that the nature of folding pathways will not be understood until the nature of the rate-limiting step in folding and unfolding is understood.

There are good reasons for trying to uncover the nature of the rate-limiting step by studying the unfolding pathway and by searching for unfolding intermediates. One reason is that kinetic traps for intermediates are less likely to be operative at high denaturant concentrations, where unfolding is studied, than at low denaturant concentrations, where refolding is studied. Another reason is that the transition state for unfolding is generally judged to be closer to the native state than to the unfolded state, according to measurements of surface area exposed to solvent [29,30], so that studying unfolding intermediates should be extremely helpful in learning whether there is a unique transition state and, if so, in determining its properties.

The basic test used in the past to probe for unfolding intermediates [31,32] was to compare the kinetics of unfolding monitored by various probes, especially probes of both secondary and tertiary structure, to ask if unfolding follows a single exponential curve, if the unfolding curves monitored by different probes are superimposable, and if the amplitude of each one accounts for the entire change expected from equilibrium data. These tests have

Figure 4



One-dimensional ^{19}F-NMR spectra of dihydrofolate reductase (DHFR) taken at various times after initiating unfolding by stopped-flow mixing to give 5 M urea, pH 7.2, at 22°C. (Reprinted from [35] with permission of C Frieden.) The five tryptophan residues are labeled with ^{19}F at position 6.

failed to demonstrate intermediates in the unfolding reactions of typical small proteins [31,32]. The reason usually given for refolding intermediates being easy to observe, whereas unfolding intermediates are not detectable, is that refolding intermediates should occur after the rate-limiting step in unfolding.

Nevertheless, evidence for an unfolding intermediate was obtained recently [33] using one-dimensional proton NMR to monitor the GdmCl-induced unfolding of RNase A at a low temperature (10°C, pH 8.0, 4.5 M GdmCl), where unfolding is slow enough (relaxation time ~6 min) to be monitored by taking successive one-dimensional spectra. The change in intensity with time of the resolved, upfield-shifted methyl resonance of Val63 was monitored. The results indicate that an intermediate is formed rapidly in which this resonance no longer has the chemical shift seen in the spectrum of the native protein. The intermediate is not detected by CD at wavelengths used to monitor the disappearance either of α-helices (222 nm) or of optically active tyrosine sidechains (275 nm). In the same unfolding conditions, NMR-hydrogen exchange was used to monitor the unfolding of 49 individual peptide NH protons [34]. The aim was to find out whether any partly unfolded intermediates are formed that have some hydrogen bonds broken before the rate-limiting step in unfolding is reached. The conclusion was that all 49 NH protons undergo rapid hydrogen exchange only upon global unfolding. Putting these experiments together led to the suggestion [33] that sidechains acquire some motional freedom before the rate-limiting step in unfolding, which occurs when water enters the hydrophobic core and causes rapid hydrogen exchange.

An informative study of a similar unfolding intermediate of DHFR was made by Hoeltzli and Frieden [35], who use ^{19}F NMR and stopped-flow mixing to monitor an unfolding reaction with a relaxation time of 70 s. The five Trp residues of DHFR have been replaced by 6-^{19}F-Trp. These five resonances are well resolved in the native spectrum and are moderately resolved in the unfolded spectrum (Fig. 4). At 1.5 s after the start of unfolding (5 M urea, 22°C, pH 7.2), the native resonances have almost entirely disappeared and the intensities of the unfolded resonances correspond to the amount expected (20%) for rapid unfolding of the minor native form of DHFR (see above). Unfolding of the major native form (80%) can be monitored by CD and by Trp fluorescence and also by the appearance of the unfolded resonances in the one-dimensional spectrum, with closely similar kinetics being found by each method. The unfolding intermediate has resonances that are too broad to be observed, either because of conformational heterogeneity in the population of molecules or because exchange occurs between alternative conformations on an intermediate time scale, neither fast nor slow. The ^{19}F stopped-flow method should be applicable to the study of unfolding intermediates in many different protein systems.

A different method of probing for unfolding intermediates has recently been introduced by Bai, Englander and co-workers [36] who applied it to cyt *c*. The method probes for partly unfolded intermediates which have some peptide NH protons able to undergo rapid hydrogen exchange, in equilibrium with the native protein. These are equilibrium species, and their relation to intermediates that appear in the kinetic process of unfolding, beyond the transition zone, remains to be determined. A program of systematic study of unfolding intermediates is beginning.

Conclusions

New experiments on the role of intermediates in folding pathways are coming forth to challenge older, accepted ideas. Experiments with ubiquitin show that a rollover (a slowing down of the increase in folding rate with decreasing denaturant concentration) can be caused by the transient accumulation of a folding intermediate even though the intermediate is on the direct folding pathway. The ubiquitin studies also show that whether two-state or three-state folding kinetics is observed may depend solely on the stability of a folding intermediate, with the folding rate being proportional to the concentration of this intermediate in either case. Discovery of an alternate pathway (a fast track) for forming the native conformation of hen lysozyme shows that the subject of folding pathways is more complex than was realized earlier. The introduction of ^{19}F NMR for monitoring unfolding kinetics in real time demonstrates that DHFR, like RNase A, shows an unfolding intermediate with altered sidechain chemical shifts; this method provides a widely applicable procedure of probing for unfolding intermediates in other protein systems. A search for partly unfolded intermediates, with some peptide hydrogen bonds broken, in the unfolding kinetics of RNase A was made using the sensitive method of NMR-hydrogen exchange; the results showed that rapid hydrogen exchange occurs only upon global unfolding. These different methods of searching for unfolding intermediates suggest that sidechains acquire some motional freedom before the rate-limiting step of unfolding, which occurs when water enters the hydrophobic core.

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