Article No. mb982118 J. Mol. Biol. (1998) 283, 669–678

JMB

A Pulse-Chase-Competition Experiment to Determine if a Folding Intermediate is On or Off-pathway: Application to Ribonuclease A

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A modified pulse-chase experiment is applied to determine if the native-like intermediate I_N of ribonuclease A is on or off-pathway. The ^1H label retained in the native protein is compared when separate samples of ^1H-labeled I_N and unfolded protein are allowed to fold to native in identical conditions. The solvent is ^2H_2O and the pH* is such that the unfolded protein rapidly exchanges its peptide NH protons with solvent, and I_N does not. If I_N is on-pathway, more ^1H-label will be retained in the test sample starting with I_N than in the control sample starting with unfolded protein. The results show that I_N is a productive (on-pathway) intermediate. Application of the modified pulse-chase experiment to the study of rapidly formed folding intermediates may be possible when a rapid mixing device is used.

Keywords: protein folding; on-pathway intermediate; pulse-chase experiment; ribonuclease A; hydrogen exchange

Introduction

Are protein folding intermediates on or off-pathway? This is an important question because if the intermediates are on-pathway, then the folding process can be elucidated by characterizing their structures. Conversely, if the intermediates are off-pathway, then the value of characterizing them is less certain.

Data supporting both the on-pathway and off-pathway nature of folding intermediates have been presented. Kinetic folding intermediates have been characterized and found to have secondary structure that corresponds to some fraction of the native protein structure, e.g. apomyoglobin (Hughson *et al.*, 1990; Jennings & Wright, 1993). These data support the conclusion that intermediates are on-pathway. Moreover, the molten globule intermediate of the α-lactalbumin alpha domain not only has a native secondary structure, but it also has an overall native tertiary fold (Peng *et al.*, 1995). A peptide fragment system resembles a folding intermediate and has native-like structure (Oas & Kim, 1988). The fractional change in free energy (transition state/native state) produced by a mutation (Φ) values found in the barnase folding intermediate show a similar pattern when plotted against residue number as those in the transition state (Matouschek *et al.*, 1992).

On the other hand, evidence indicates that some folding intermediates are off-pathway. Formation of incorrect disulfide pairing in bovine pancreatic trypsin inhibitor (BPTI; Creighton, 1975) and of ligation of heme by a non-native side-chain during the folding of cytochrome *c* have been well established (Sosnick *et al.*, 1994; Elöve *et al.*, 1994). Several very small proteins have been shown to fold extremely fast (1 ms or faster) without populated intermediates (Jackson & Fersht, 1991; Huang & Oas, 1995; Schindler *et al.*, 1995). New theoretical treatments of folding suggest that proteins might fold to unique structures on biologically relevant timescales without forming populated intermedi-

Abbreviations used: RNase A, ribonuclease A; I_N, native-like intermediate of RNase A; U_F, U_S, fast-folding and slow-folding forms, respectively, of unfolded RNase A; N, I, U, native, partly folded, intermediate and unfolded forms of a protein, respectively; pH*, pH meter reading in ^2H_2O; Φ, fractional change in free energy (transition state/native state) produced by a mutation; BPTI, bovine pancreatic trypsin inhibitor; 3D, three-dimensional; GdmCl, guanidinium chloride; k_{ex}, chemical exchange rate; *P*, protection factor; Mops, (*N*-morpholino) propanesulfonic acid.

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0022–2836/98/430669–10 $30.00/0

ates (Zwanzig *et al.*, 1992; Bryngelson *et al.*, 1995; Doyle *et al.*, 1997, and references cited therein).

The pulse-chase experiment is the traditional method of molecular biology to decide if an intermediate is on or off-pathway (see Lodish *et al.*, 1995). The pulse-chase procedure contains the following steps when applied to testing if ribosome-bound peptides are intermediates in protein synthesis. In the pulse step, an isotope-labeled amino acid is added to growing cells, where it equilibrates with a precursor pool and becomes incorporated into ribosome-bound peptides. In a corollary study, these peptides are isolated and characterized. In the chase step, cold amino acid is added to dilute out the label in the precursor pool, time is allowed to complete protein synthesis, and the amount of label chased into mature protein is determined. Questions of rate and yield are studied.

The pulse-chase experiment needs to be modified for use in studying folding intermediates where the 3D structures are formed chiefly by hydrogen bonds and hydrophobic interactions, and not by strong covalent bonds. Our adaptation includes a competition between folding and exchange with solvent of the ^{1}H labeled peptide NH protons. Step 1 (analogous to the pulse): the starting material is the unfolded protein (U) in 4 M guanidinium chloride (GdmCl) and the labeled folding intermediate (I) is formed by allowing the folding process to proceed part way in ordinary water. Thus, the peptide NH protons of I are ^{1}H-labeled. It is important to stop the folding process before a significant amount of labeled native protein (N) is formed. In a separate experiment, a control sample of ^{1}H-labeled U is also prepared. Step 2 (analogous to the chase): the H_2O is diluted out with ^2H_2O and folding continues until N is formed. The amount of ^{1}H-label retained in N is determined by 1D ^{1}H-NMR, by integrating the area under the peak envelope of peptide NH resonance lines. The control sample of U is allowed to fold under identical conditions as the test sample by starting folding and exchange simultaneously in 90% ^2H_2O, 10% H_2O, and its retained ^{1}H label is determined.

Provided the design of the experiment meets certain conditions, I is found to be a productive (on-pathway) intermediate when the label retained by N in the test sample is sufficiently larger than in the control. Two basic conditions must be satisfied. Firstly, the pH of the second step must be high enough so that the ^{1}H label exchanges out of U, before U forms I. The average exchange rate of NH protons in U can be computed from model compound data (Bai *et al.*, 1993; Connelly *et al.*, 1993) and the rate of the U $\rightarrow$ I reaction needs to be measured for comparison with the exchange rate in U. Secondly, the pH of the second step must not be high enough to cause the ^{1}H label in I to exchange out before I forms N. The average protection factor of the NH protons in I needs to be measured in order to compute the exchange rate in I. The rate of the I $\rightarrow$ N reaction also needs to be measured. The experiment is designed such that if I is off-pathway and must first unfold to U before forming N, then the label in I will be lost before N is formed, whereas the ^{1}H label will be retained in N if I is a productive intermediate.

This modified pulse-chase experiment is applied here to the native-like intermediate (I_N) of RNase A (Cook *et al.*, 1979). The advantages of I_N are: (1) it is kinetically stable for several hundred seconds at 0°C (Schmid, 1983); (2) the amounts of I_N and N can both be determined in a single unfolding assay (Schmid, 1983) in which I_N unfolds about ten times more rapidly than N; (3) although unfolded RNase A contains an entire set of unfolded species with different proline isomers (Houry & Scheraga, 1996, and references cited therein), I_N is the major folding intermediate and under some conditions accounts for 50% or more of the total sample; (4) I_N is formed relatively rapidly (five seconds) under the conditions studied by Schmid (1983); and (5) the exchange behavior of I_N has been studied by Brems & Baldwin (1985) in pulse labeling experiments as a function of pH. The *cis* $\rightarrow$ *trans* isomerization of both Pro93 and Pro114, which are *cis* in native RNase A, gives rise to significant amounts of slower folding species of unfolded RNase A, and I_N contains a *trans* isomer of at least Pro93 (Houry & Scheraga, 1996).

Results

Preparation of I_N

The preparation of test samples with major amounts of I_N was studied by Schmid (1983) and Brems & Baldwin (1985). Our conditions (20 seconds, at pH 4, 0°C, 0.8 M Na_2SO_4, 0.4 M GdmCl) are based on their results as well as on additional results using the unfolding assay of Schmid (1983). The 0.8 M Na_2SO_4, which is a protein stabilizer, increases both the maximum concentration of I_N and its rate of formation. It also compensates for the 0.4 M GdmCl remaining after dilution of the unfolded RNase A (see Materials and Methods).

The amount of I_N is determined by the unfolding assay (Schmid, 1983), which monitors the kinetics of unfolding of I_N and N by the change in tyrosine absorbance (287 nm); I_N and N have the same extinction coefficient within error. The GdmCl-induced unfolding curve of N, monitored by tyrosine absorbance, is shown in Figure 1: note the decrease in absorbance that occurs upon refolding. The data are fitted to a two-state unfolding reaction (N $\rightleftarrows$ U) and the baselines for N and U are fitted by the procedure of Santoro & Bolen (1988), which uses data inside as well as outside the transition zone to fix the baselines. The decrease in extinction coefficient upon unfolding (3080($\pm$150) cm^{-1} M^{-1} at 5 M GdmCl) is in fair agreement with the value at 4.6 M GdmCl (2800 cm^{-1} M^{-1}) given by Schmid (1983). The kin-

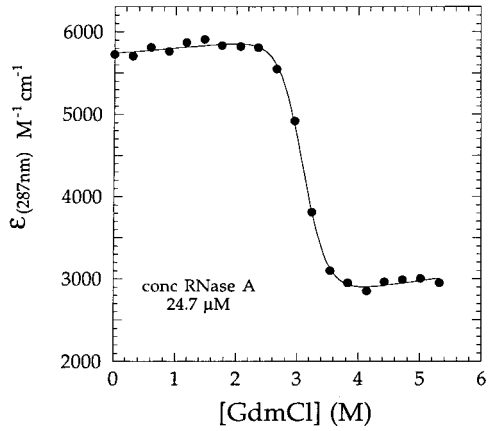


Figure 1. The equilibrium unfolding transition of RNase A induced by GdmCl, measured by the decrease in tyrosine absorbance at 287 nm. Conditions: 20 mM sodium acetate buffer (pH 4.3), 2.5°C, 24.7 μM RNase A.

etic unfolding assay (Figure 2) gives 50% I_N, 30% N and (by difference) 20% U. Under similar conditions, but after 15 seconds at pH 6, Schmid (1983) found 57% I_N, 26% N and 17% U; he showed that this composition is consistent with his measurements of the relaxation times for forming I_N (five seconds) and for converting I_N to N (130 seconds). The 20% U present after 20 seconds prefolding is chiefly in the form of one or more minor unfolded species containing different non-native proline isomers than the species forming I_N (see Schmid, 1983; Houry & Scheraga, 1996).

Conditions for step 2

In step 2, I_N is already formed and labeled and it has the choice of (1) unfolding to U and then folding to N (label is lost) or (2) folding directly to N (label is retained).

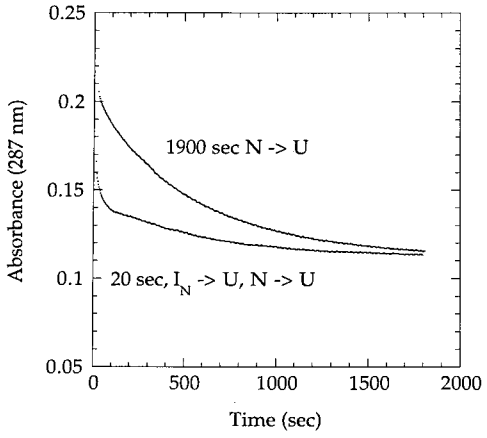


Figure 2. The unfolding assay (Schmid, 1983) used to measure the amounts of I_N and N at various times during refolding: times of 20 and 1900 seconds are shown here. Each kinetic curve is fitted to a sum of two exponentials; the total kinetic amplitude is the same in both curves. I_N unfolds approximately ten times more rapidly than N. Conditions: 5 M GdmCl (pH 6), 25°C.

The pH* (glass electrode reading without correction for the isotope effect) chosen for folding in step 2 represents a balance between two exchange processes: the pH* should be high enough so that ^{1}H label exchanges out of U before U forms I, but the pH* should be low enough so that the ^{1}H label does not exchange out of I before I forms N. The pH* used here (pH 7.4) is satisfactory, but a higher pH* such as pH 8.6 could have been used advantageously. From data given by Connelly *et al.* (1993), the exchange rate of an Ala-Ala peptide NH at pH* 7.4, ^2H_2O, 0°C, is 1.2 s^{-1}. Thus, exchange out of ^{1}H label from U is faster than formation of I_N from U (0.2 s^{-1}, Schmid, 1983). Note that this point is checked experimentally by measuring the loss of label resulting from exchange in the control.

The protection factors in I_N are known to be, on average, about tenfold lower than those in N (Brems & Baldwin, 1985), and it is easy to select conditions in which numerous protons are retained after I_N forms N. Many protected protons remain in I_N after ten seconds exchange at pH 10, which shows that these protons have large protection factors. We measured the time course of the $I_N \rightarrow N$ reaction by the unfolding assay for I_N and N (Figure 2) to determine when the reaction is over. Fitting the kinetic results (data not shown) to a single exponential indicates that the $I_N \rightarrow N$ reaction is complete in less than 500 seconds, in agreement with Schmid (1983). (The assay results suggest, however, that small amounts of partly folded protein remain at longer times.) Consequently, the pH* of 7.4 used in step 2 is well below the upper limit imposed by the condition that I_N not exchange out before it forms N, the protection factors of I_N, and the half time of the $I_N \rightarrow N$ reaction.

Measurements of protons retained

The results are reported here as the number of protons retained after folding, but this should be understood to mean the normalized area under the peptide NH envelope in a 1D NMR spectrum (see Materials and Methods for details). The precision of measuring this quantity is very likely better than the accuracy of measuring the number of retained protons.

Before performing the actual experiment, we measured the curve of ^{1}H retained *versus* time of folding in step 2, to check whether exchange-out of label occurs slowly in step 2, as expected. The results are shown in Figure 3. No matter how short step 2 is, little further exchange should occur after step 2 because the low pH* used for the quench (pH* 4) is adequate to slow down exchange while folding goes to completion. Figure 3 shows that some loss of label ($50 - 37 = 13$ protons) occurs slowly in step 2 between the starting time and 600 seconds, when the $I_N \rightarrow N$ reaction is complete. The protons which exchange-out at a

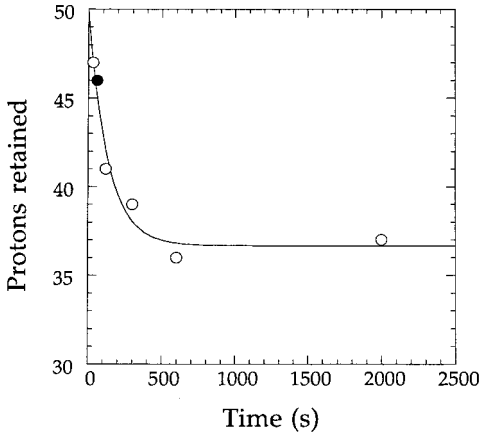


Figure 3. Decrease in the number of protons retained after folding with increasing duration of the competition between folding and exchange-out in step 2. Conditions: pH* 7.3, 27 mM Mops buffer, 90% ^2H_2O, 0°C (see Materials and Methods). The sample represented by the filled circle was allowed to fold for 40 seconds in step 1; the other samples folded for 20 seconds.

Table 1. Protons retained after folding

Initial label			
Row[a]	I_N[b]	U[c]	N[d]
1	36	22	60
2	41	24	53
3	41	25	51

[a] This Table shows the label retained when at the start of step 2 the label is present either in I_N, U or N. Three separate experiments were made: A and C and D. The data from A are shown in Figure 3, and samples A1 and A8 provide the values for N in rows 1 and 2, while sample A6 provides the value for I_N in row 1. Data from C provide values for U and I_N in row 2 and for U in row 1. Data from D provide values for U and I_N in row 3.

[b] Step 1: 20 seconds prefolding at pH 4, 0°C, to form ^1H-labeled I_N. Step 2: 600 seconds at pH* 7.4, 0°C, to allow I_N to form N. Peptide NH protons with low protection factors exchange with solvent (^2H_2O) in step 2, while NH protons with large protection factors are retained during the folding of I_N to N. The D samples used a lower concentration of Na_2SO_4 (0.4 M) in step 1.

[c] Step 1 omitted. Step 2 is the same as in [a], and the Na_2SO_4 solution usually added in step 1 is added to make the solution have the same composition as the test sample with I_N.

[d] ^1H-labeled N in ^2H–H_2O (pH*4). Sample D3, but not samples A1 and A8, exposed to step 2 for 600 seconds after being allowed to fold in step 1 for 3600 seconds.

measurable rate in Figure 3 probably come from I_N as well as from partly folded species formed from U. Labeled protons in U itself should exchange-out rapidly, but some partial folding of U may occur and slow the exchange. The protons in N with low protection factors, which might otherwise contribute to the results in Figure 3, are exchanged-out in the workup process which takes place in ^2H_2O after step 2.

Table 1 and Figure 3 summarize the results. The NMR assay for the number of retained protons is illustrated in Figure 4. The number of protons retained in the test sample at the end of the $I_N \rightarrow N$ reaction is 37 according to the data in Figure 3, which shows the time course of exchange-out in step 2. This number may be compared with values of 41 and 41, taken at a single time point (600 seconds) from two other experiments (see Table 1).

In the control samples from three experiments (Table 1), which initially contain U at the start of step 2, the number is 24 ± 2. Thus, using preformed I_N instead of U gives a significantly larger number of protons retained in step 2. An upper limit on the number of protons retained is provided by the three native protein samples: 55 ± 5.

Figure 3 shows that 13 (50–37) protons exchange out from the test sample with measurable kinetics while the $I_N \rightarrow N$ reaction takes place during step 2. They come from two sources: the 20% U present in the test sample at the start of step 2 (this accounts for at most 11 protons) and from protons in I_N whose protection

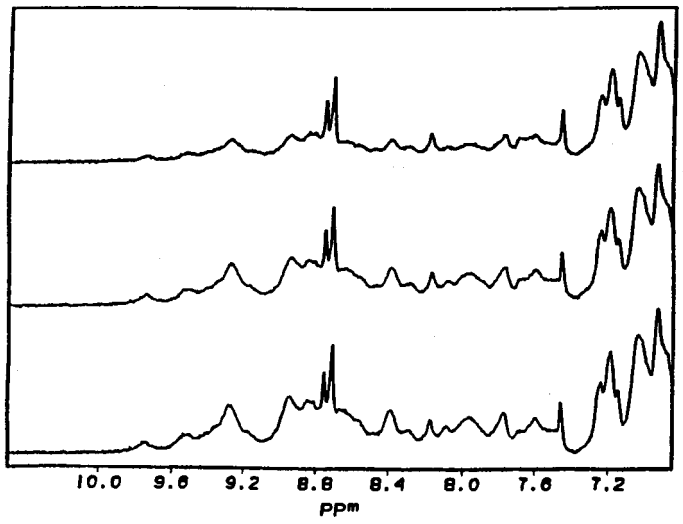


Figure 4. Illustrative 1D ^1H-NMR spectra of samples showing the number of retained protons after complete folding. From top to bottom, the samples are (see Table 1): D1 (25 protons), D2 (41 protons) and D3 (51 protons).

factors are low enough to cause exchange. A simulation of the results is given in Discussion for a simplified model.

The average number of retained protons in the control samples (24 ± 2) is not zero for two reasons. Firstly, U contains 20% fast-folding molecules which form N rapidly and account for 11 protons retained. At least part of the remaining 13 protons are undoubtedly retained because some U molecules rapidly undergo partial folding (see Udgaonkar & Baldwin 1990). An experiment was performed to check this point by increasing the pH* of step 2 to 8.6, to find out if part of the retained protons originate from molecules with small protection factors. Samples were taken after only a brief exposure (ten seconds) to pH* 8.6 in step 2: the number of retained protons in the control sample dropped from 24 to 16, or only five more than expected from the 20% fast-folding molecules. Thus, the number of retained protons in the control sample drops rapidly with increasing pH* even when the duration of step 2 is quite short, as expected if some of these protected protons originate from partially folded forms of U with low protection factors. The number of protons retained in the test sample with I_N present is 45, when the pH* of step 2 is 8.6, in good agreement with the exchange results at early times at pH* 7.4 (Figure 3).

We conclude that the factors which determine the different numbers of protons retained in the test samples, the control samples, and the samples of N, are understood satisfactorily. This knowledge, combined with the significant difference between the protons retained in the test samples (38 ± 3) and the control samples (24 ± 2), means that I_N is a productive (on-pathway) folding intermediate, i.e. I_N does not unfold to U before forming N.

Discussion

Criterion for an intermediate being on-pathway

The criterion used here for an on-pathway intermediate rests on the difference between the test sample and the control in the number of protons retained after folding. In most cases, some protons will be retained in the control sample and less than 100% of the protons will be retained in the test sample. We discuss above how large the difference must be between the test sample and the control in order for the difference to be significant: the difference must, of course, be well outside experimental error, but also the reasons must be understood for retention of protons in the control sample and for the difference between the native sample and the test sample.

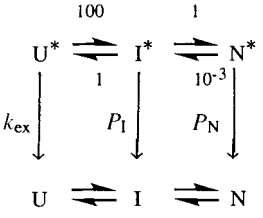
The number of protons retained in the control sample can be predicted for the competition between exchange and folding if the unfolded molecules present are fully unfolded (see Schmid & Baldwin, 1979). The prediction requires that the rate of the U → I reaction be known and that U remains fully unfolded before it forms I, so that the exchange rates of protons in U can be computed from model compound data. However, rapid folding of U to give a weak intermediate with detectable protection factors may also occur, and in this case the unfolded exchange rates are not applicable. It is then not possible to predict the value for the control sample and it is necessary to have an experimental control. In any case it is desirable to have direct experimental data on the competition between exchange and folding for the intermediate in the test sample. The experiment shown in Figure 3, measuring retained label *versus* time of folding in step 2, provides data on this competition.

The I_N intermediate of RNase A has long been thought to be on-pathway because isomerization of the critical proline residue(s) occurs 30 times faster in I_N than in unfolded RNase A (Cook *et al.*, 1979). It would be difficult to explain this behavior if I_N is off-pathway. A possible explanation for the increased isomerization rate was provided by Levitt (1981), who showed by a folding simulation how the difference in free energy between N and I_N may be used to increase the rate of proline isomerization in I_N.

Application of the pulse-chase-competition experiment to rapidly formed folding intermediates

At present it is controversial whether rapidly formed folding intermediates are on or off-pathway (see Introduction and Roder & Colón, 1997). The simulations shown below indicate that the pulse-chase-competition method should be applicable to this problem, provided the intermediate is formed with measurable kinetics in stopped-flow experiments so that the label can be placed in I in the test sample and in U in the control in step 1. The rapidly formed intermediate of apomyoglobin does have measurable kinetics under certain conditions (Jamin & Baldwin, 1996). In the following simulations of either an on-pathway or off-pathway model, the steady-state [I]/[U] ratio is 100 in both cases and the individual rate constants have been chosen so that the rate of forming N from the U ⇄ I steady-state mixture is approximately the same in both models. The exchange rates in I and N are k_{ex}/P, where P_I, P_N are the protection factors in I and N, respectively, and k_{ex} is the exchange rate in U.

(I) On-pathway model.



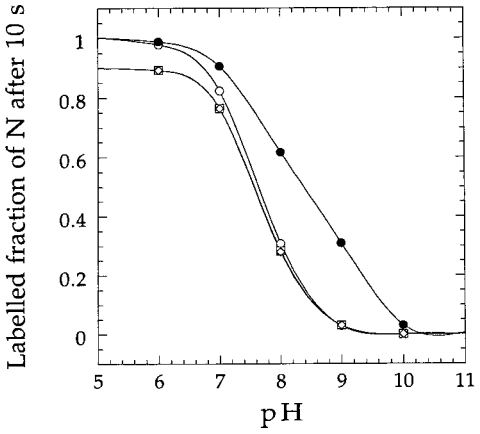
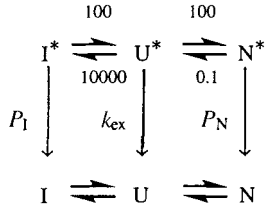


Figure 5. Simulation of the competition between exchange and folding for a hypothetical fast-folding protein. Details of the simulation are given in the text. Two cases are shown: in the first case the intermediate I is on-pathway (filled circles, label is present initially in I; open circles, label is present initially in U); in the second case I is off-pathway (open squares, label is present initially in I; open diamonds, label is present initially in U). Note that the label retained in case 1, but not in case 2, depends on whether the label is in I or U at the start of step 2.

(II) Off-pathway model.



The simulations were made for values of k_{ex} ranging from 1 to 10,000 s^{-1} and for times up to ten seconds; in Figure 5, k_{ex} is assumed to be 1 s^{-1} (pH 6.0) for exchange in H_2O and k_{ex} is assumed to be base-catalyzed. The values assigned to P_I and P_N are 10^3 and 10^6, respectively.

The results are shown in Figure 5 as protons retained in N *versus* pH after a folding time of ten seconds. For the on-pathway model, the retained label in N is much larger in the test sample than in the control for the pH range 7.5 to 9.5. In contrast, for the off-pathway model the results are the same when the initial label is in either I or U. Also, the off-pathway results are nearly the same as those in the on-pathway model for the case when the initial label is in U. Because some parameters of our on-pathway and off-pathway models are different, some of the retained label remains in I at pH values below 7 in the off-pathway model.

These simulations test a critical assumption in the design of the pulse-chase competition method. The assumption is that the results will be the same in the off-pathway model when the initial label is placed either in I or U. This assumption is made because I must unfold to form U before forming N in this model. The simulations indicate that this assumption is correct. The simulations also confirm that the pulse-chase-competition method can be extended to rapidly formed intermediates in certain cases. The method should be applicable when I is formed with kinetics that are slower than stopped-flow mixing, so that a test sample can be prepared that contains labeled I while the control contains labeled U, at the end of step 1. The two conditions discussed above for step 2 still apply: label must exchange out of U before I is formed in step 2, and prelabeled I must have large enough protection factors and must form N rapidly enough so that a measurable amount of label in I is retained in N at the end of step 2.

The simulations in Figure 5 show that a positive test for an on-pathway intermediate is expected in

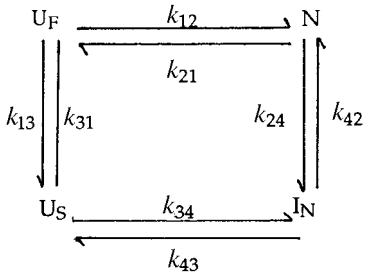
Table 2. Effects of varying k_{12} and k_{21} on the test for being on-pathway

A. *Varying* k_{12}	($k_{21} = k_{23} = 1$)		
k_{12}	k_{ex}	N*(+)	N*(−)
10^2	10^2	0.617	0.308
10^3	10^3	0.361	0.181
10^4	10^3	0.433	0.393
B. *Varying* k_{21}	($k_{12} = 10^2$, $k_{23} = 1$)		
k_{21}	k_{ex}	N*(+)	N*(−)
1	10^2	0.617	0.308
10	10	0.518	0.471
10^2	1	0.500	0.495
C. *Varying both* k_{12} *and* k_{21}	($k_{ex} = 10^2$, $k_{23} = 1$)		
k_{12}	k_{21}	N*(+)	N*(−)
10^2	1	0.617	0.308
10^3	10	0.491	0.446
10^4	10^2	0.472	0.467

The simulations for the on-pathway model (see Figure 5 and the text) were repeated for varying values of k_{12} and k_{21}. N*(+), N*(−) give the label present in N at ten seconds when initially the label is present either in I or U, respectively.

this example. *A priori*, the method should not distinguish between the on-pathway and off-pathway models if U and I equilibrate before I forms N and, therefore, it is important to investigate how the results depend on the values of k_{12} and k_{21}. Table 2 shows these results: the difference between the test sample and the control decreases as either k_{12} or k_{21} increases; it is particularly dependent on k_{21}. When the ratio k_{12}/k_{21} is held constant but both rate constants vary, the method ceases to distinguish between the off-pathway and on-pathway mechanism as the rate constants become large. Likewise, the experimental requirement that the kinetics of forming I from U must be measurable also puts an upper limit on the values of k_{12} and k_{21}. The data in Table 2 emphasize that it is important to simulate the predicted results to find out if a measurable difference between the test sample and the control is expected.

Simulation of the expected results for RNase A was made for the following model.



In this model, U_S is a slow-folding unfolded species with a non-native *trans* proline isomer and U_F is the corresponding fast-folding species with a native *cis* isomer. The treatment of the competing exchange reactions is shown below. Values assigned to the rate constants and protection factors are given in Materials and Methods. For simulation of the test sample, all label was placed initially in U_S; for the control sample, the label was placed in the equilibrium $U_F \rightarrow U_S$ mixture. The simulated results at 600 seconds are: test sample 0.983 label retained (0.940 in N, 0.043 in I_N) and control sample 0.301 label retained (0.289 in N, 0.012 in I_N).

The simulated results show that the on-pathway test is feasible and that the observed results are in the range expected. Note that the 20% U in the test sample at the start of step 2 does not correspond to U_S nor U_F, but to minor slow-folding species (Schmid, 1983; Houry & Scheraga, 1996) with other non-native proline isomers.

Future applications of the method

The method is intended for future use in determining if an observed rapidly formed folding intermediate is on or off-pathway. There are two general requirements for using the method. (1) The kinetics of forming the intermediate (I) must be measurable, otherwise it is not possible to begin step 2 with a test sample which has labeled I and a control sample which has labeled U. (2) Simulations of the folding and exchange kinetics in step 2 should be made to find out if the test for an on-pathway intermediate is feasible.

Note that the method is not intended for use in detecting an intermediate that has not yet been observed with conventional optical probes such as Trp fluorescence. Conceivably, the method might be adapted for this purpose, but then the limitations and requirements of the procedure would have to be spelled out.

An important property is that the method does not give a false-positive result. If a significant difference is found between the retained label in the test sample and the control, then I is on-pathway. Any ambiguity is limited to deciding what constitutes a significant difference in retained label. Note that the term on-pathway has a defined meaning here: if I must unfold to U before forming N, then I is off-pathway, but if I forms N without passing back through U, then I is on-pathway. Note also that a negative result may be ambiguous: it may mean either that I is off-pathway or that the conditions for using this method are not fulfilled. It should be possible to decide between these two alternatives by careful measurements of the rate constants for folding and exchange and by simulating the results.

Materials and Methods

Materials

Guanidinium chloride (GdmCl) was the ultra-pure grade from Gibco BRL. Sodium acetate and sodium sulfate were reagent grade from Baker. Mops (acid form) was from Sigma. RNase A was obtained from Sigma and was type XII A. For experiments C and D, Sigma RNase A R-5000 type II A was used. Deuterium oxide (99.9%) was from Isotech.

Methods

All solutions were prepared in double quartz-distilled water, or in $^2H-H_2O$ (99.9% ^2H) from Isotech. pH measurements were made at 0°C after calibration at 0°C with pH 2.00, 4.00 and 7.00 standards. pH* indicates the apparent pH reading in ^2H_2O solutions recorded without adjustment for the isotope effect. GdmCl concentrations were determined by refractive index (Pace *et al.*, 1989).

Equilibrium unfolding of RNase A (25 μM) by GdmCl was followed by ultraviolet (UV) absorbance (287 nm) in 20 mM sodium acetate buffer (pH 4.3) at 2.5°C. An extinction coefficient of 9800 M^{-1} cm^{-1} at 278 nm (Sela & Anfinsen, 1957) was used to determine RNase A concentration.

The unfolding assay of Schmid (1983) was used to measure the ratio of native, intermediate and unfolded RNase A at various times during refolding. Unfolded RNase A was allowed to refold, for varying periods of time, under identical conditions as step 1 of the pulse-

chase-competition experiment (0.8 M Na_2SO_4, 0.4 M GdmCl (pH 4), 0°C). These partially refolded solutions were then unfolded in 5 M GdmCl at pH 6 and 25°C where I_N and N unfold in two distinct kinetic phases. These kinetic phases were monitored by UV absorbance at 287 nm, and their amplitudes were compared to the total folding amplitude obtained from equilibrium unfolding to determine the fractional populations of I_N and N. The unfolded population (U) was then estimated as the difference between the total amplitude, and the sum of the amplitudes of N and I_N; U = total amplitude − (N + I_N).

The pulse-chase-competition experiment was performed as follows. RNase A (ca 20 mM) was first unfolded in 4 M GdmCl (pH 2) in double-distilled H_2O on ice for over one hour. Step 1 refolding was initiated by mixing a 70 μl aliquot of unfolded RNase A with 0.63 ml 0.88 M sodium sulfate, 22 mM sodium acetate. The resulting solution contained 2 mM RNase A, 20 mM sodium acetate, 0.8 M sodium sulfate, and 0.4 M GdmCl, and had a pH of 4.1. Under these conditions the fast-folding U_F molecules (20%) immediately form native RNase A, and a major fraction of the U_S molecules folds rapidly to I_N with rate of approximately 0.2 s^{-1} (Schmid, 1983). Formation of I_N was typically allowed to proceed for 20 seconds before initiating step 2 by adding the chase buffer (6.3 ml of ^2H_2O containing 30 mM Mops (pH* 7.4)). In control experiments, step 1 was omitted or extended to allow RNase A to refold completely before adding the ^2H_2O. The length of step 2 was varied to measure the effect on the extent of exchange. Finally, exchange was quenched by the addition of 3 ml of ^2H_2O containing 167 mM sodium acetate (pH* 3.8), which reduced the pH* to about 4.0. Under these conditions, native RNase A is stable and its protected amide protons exchange very slowly with solvent, allowing time for the samples to be concentrated without loss of the proton label. Surface amides can, however, exchange and come to equilibrium with the solvent (93% ^2H: 7% ^1H). The quenched samples were concentrated from 10 ml to about 0.8 ml using an Amicon apparatus with a YM-10 filter in a 4°C cold room to a final concentration of 0.8 mM.

To measure the extent of proton retention during folding, 1D ^1H NMR spectra of the samples were obtained using a General Electric GN-Omega spectrometer, operating at 500 MHz. Presaturation was used to reduce the ^1H_2O peak. The spectra were measured at 5°C. A recycling delay of 1.5 seconds was used. Typically, 512 scans (4096 real points, spectral width 7000 Hz) were recorded per spectrum. Trimethyl silyl propionate was the internal chemical shift reference.

NMR spectra were processed using Felix, version 2.30 from Biosym Technologies (San Diego). The FID was not premultiplied prior to Fourier transformation, so that peak areas would remain quantitative. Following phase and baseline correction with a zero-order polynomial function, the total area of amide peak region (10.00 to 7.35 ppm) was measured by integration. The total area of the nearby aromatic region (7.35 to 6.85 ppm) was also measured. The error in the peak area was about 10% (1σ, the reproducibility of the non-exchanging aromatic region). The area of the amide region retained after the ^2H_2O chase was compared to the amount retained by RNase A that had been allowed to refold completely before application of the ^2H_2O chase.

In addition, the area of the aromatic proton zone (7.35 to 6.85 ppm) was used to estimate the number of protons in the amide peak region using the complete peak assignments (Rico *et al.*, 1993; Robertson *et al.*, 1989). This aromatic zone contains 23 non-exchanging aromatic protons, three histidine protons, three slow exchanging amide protons, 14 fast exchanging side-chain protons, and seven fast exchanging amide protons. Assuming that the fast exchanging side-chains and amides come into equilibrium with solvent (93% ^2H, 7% ^1H) by the time the NMR spectrum was measured, these groups would contribute $(14 + 7) \times 0.07 = 1.5$ protons to the aromatic peak. Therefore, in total $23 + 3 + 3 + 1.5 = 31$ protons contribute to the intensity of the aromatic region. In the amide zone (10.00 to 7.35 ppm), the assignments show there are 37 slow-exchanging amide protons, five non-exchanging (or very slow) histidine side-chain protons, 41 fast-exchanging side-chain protons (from K, N, S, Y, R, and Q side-chains) and 72 fast-exchanging amide protons. When at equilibrium with solvent, the fast-exchanging groups are expected to contribute $(41 + 72) \times 0.07 = 8$ protons to the amide peak zone. To calculate the number of non-exchanged, slow amide protons remaining after the ^2H_2O chase, the area of the aromatic zone was first divided by the number of protons present (31 protons) to obtain an estimate of the area of one proton. Next, the area of the amide zone produced by slow amide protons was calculated by subtracting the contribution of the histidine and fast-exchanging protons (13 protons times the area for one proton determined from the aromatic region) from the total peak area. Finally, the area produced by slow-exchanging amide protons was divided by the estimated area of a single proton (estimated from the aromatic peak area) to give the number of amide protons which remain after the ^2H_2O chase. To calculate the percentage occupancy, the number of remaining protons was divided by the number of amide protons calculated to be present (50) under these conditions in the native state and then multiplied by 100. Since the conditions used here to record the 1D ^1H NMR spectra differed in temperature and salt concentration from those used by Rico *et al.* (1993) to assign the protein, it is certain that many of the resonances are altered in chemical shift. Therefore, the calculation of the number of retained protons given here should be considered a rough estimate only. We have tried to use 2D ^1H NMR spectra of RNase A in order to estimate accurately the number of protons remaining, but at these pH* values and temperatures, we could not interpret the spectra.

Simulations of the pulse-chase-competition experiments were performed using SIMFIT (Holzhütter & Colosimo, 1990). On and off-pathway models were simulated for up to ten minutes using a Runge-Kutta numerical integration routine. Values for the rate constants and protection factors used in simulating the RNase A results are as follows: k_{12}, 20 s^{-1}; k_{21}, 2×10^{-6} s^{-1}; k_{13}, 8×10^{-4} s^{-1}; k_{31}, 2×10^{-4} s^{-1}; k_{34}, 0.2 s^{-1}; k_{43}, 2×10^{-6} s^{-1}; k_{24}, 0.32×10^{-3} s^{-1}; k_{42}, 0.8×10^{-3} s^{-1}; k_{ex}, 1.2 s^{-1}; $P(I_N)$, 10^5; $P(N)$ 10^7.

The values for k_{34} and k_{42} are taken from Schmid (1983), for k_{13} and k_{31} from Schmid & Baldwin (1978), the estimate for the stability of N from Pace *et al.* (1990), the protection factors of I_N and the stability of I_N relative to N from Brems & Baldwin (1985), and the other rate constants are plausible values.

Acknowledgements

We thank Professor M. Rico for valuable discussion and an anonymous referee for a valuable suggestion. D.V.L. is a Fellow of the Leukemia Society of America. This research was supported by NIH grant GM19988.

References

Bai, Y., Milne, J. S., Mayne, L. & Englander, S. W. (1993). Primary structure effects on peptide group hydrogen exchange. *Proteins: Struct. Funct. Genet.* **17**, 75–86.

Brems, D. N. & Baldwin, R. L. (1985). Protection of amide protons in folding intermediates of ribonuclease A measured by pH-pulse exchange curves. *Biochemistry,* **24**, 1689–1693.

Bryngelson, J. D., Onuchic, J. N., Socci, N. D. & Wolynes, P. G. (1995). Funnels, pathways and the energy landscape of protein folding: A synthesis. *Proteins: Struct. Funct. Genet.* **31**, 167–195.

Connelly, G. P., Bai, Y., Jeng, M.-F. & Englander, S. W. (1993). Isotope effects in peptide group hydrogen exchange. *Proteins: Struct. Funct. Genet.* **17**, 87–92.

Cook, K. H., Schmid, F. X. & Baldwin, R. L. (1979). Role of proline isomerization in folding of ribonuclease A at low temperatures. *Proc. Natl Acad. Sci. USA,* **76**, 6157–6161.

Creighton, T. E. (1975). The two-disulphide intermediates and the folding pathway of reduced pancreatic trypsin inhibitor. *J. Mol. Biol.* **95**, 167–199.

Doyle, R., Simons, K., Qian, H. & Baker, D. (1997). Local interactions and the optimization of protein folding. *Proteins: Struct. Funct. Genet.* **29**, 282–291.

Elöve, G. A., Bhuyan, A. K. & Roder, H. (1994). Kinetic mechanism of cytochrome *c* folding: involvement of the heme and its ligands. *Biochemistry,* **33**, 6925–6935.

Holzhütter, H. G. & Colosimo, A. (1990). SIMFIT: a microcomputer software-toolkit for modelistic studies in biochemistry. *CABIOS,* **6**, 23–18.

Houry, W. A. & Scheraga, H. A. (1996). Nature of the unfolded state of ribonuclease A: effect of cis-trans X-Pro peptide bond isomerization. *Biochemistry,* **35**, 11719–11733.

Huang, G. S. & Oas, T. G. (1995). Submillisecond folding of monomeric λ repressor. *Proc. Natl Acad. Sci. USA,* **92**, 6878–6882.

Hughson, F. M., Wright, P. E. & Baldwin, R. L. (1990). Structural characterization of a partly folded apomyoglobin intermediate. *Science,* **249**, 1544–1548.

Jackson, S. E. & Fersht, A. R. (1991). Folding of chymotrypsin inhibitor 2.2. Influence of proline isomerization on the folding kinetics and thermodynamic characterization of the transition state of folding. *Biochemistry,* **30**, 10436–10443.

Jamin, M. & Baldwin, R. L. (1996). Refolding and unfolding kinetics of the equilibrium folding intermediate of apomyoglobin. *Nature Struct. Biol.* **3**, 613–618.

Jennings, P. A. & Wright, P. E. (1993). Formation of a molten globule intermediate early in the kinetic folding pathway of apomyoglobin. *Science,* **262**, 892–896.

Levitt, M. (1981). Effect of proline residues on protein folding. *J. Mol. Biol.* **145**, 251–263.

Lodish, H., Baltimore, D., Berk, A., Zipursky, S. L., Matsudaira, P. & Darnell, J. (1995). *Molecular Cell Biology*, 2nd edit., pp. 215–216, Freeman Co.

Matouschek, A., Serrano, L. & Fersht, A. R. (1992). The folding of an enzyme. IV. Structure of an intermediate in the refolding of barnase analysed by a protein engineering procedure. *J. Mol. Biol.* **224**, 819–835.

Oas, T. G. & Kim, P. S. (1988). A peptide model of a protein folding intermediate. *Nature,* **336**, 42–48.

Pace, C. N., Shirley, B. A. & Thomson, J. A. (1989). Measuring the conformational stability of a protein. In *Protein Structure: A Practical Approach*, vol. 316, IRL Press, New York.

Pace, C. N., Laurents, D. V. & Thomson, J. A. (1990). pH dependence of the urea and guanidine hydrochloride denaturation of ribonuclease A and ribonuclease T1. *Biochemistry,* **29**, 2564–2572.

Peng, Z., Wu, L. C. & Kim, P. C. (1995). Local structural preferences in the α-lactalbumin molten globule. *Biochemistry,* **34**, 3248–3252.

Rico, M., Santoro, J., Gonzalez, C., Bruix, M., Neira, J. L. & Nieto, J. L. (1993). Refined solution structure of bovine pancreatic ribonuclease A by ^{1}H NMR methods. Sidechain dynamics. *Appl. Magn. Reson.* **4**, 385–415.

Robertson, A. D., Purisima, E. O., Eastman, M. A. & Scheraga, H. A. (1989). Proton NMR assignments and regular backbone structure of bovine pancreatic ribonuclease A aqueous solution. *Biochemistry,* **28**, 5930–5938.

Roder, H. & Colon, W. (1997). Kinetic role of early intermediates in protein folding. *Curr. Opin. Struct. Biol.* **7**, 15–28.

Santoro, M. M. & Bolen, D. W. (1988). Unfolding free energy changes determined by the linear extrapolation method: 1. Unfolding of phenylmethanesulfonyl α-chymotrypsin using different denaturants. *Biochemistry,* **27**, 8063–8068.

Schindler, T., Herrier, M., Marahiel, M. A. & Schmid, F. X. (1995). Extremely rapid protein folding in the absence of intermediates. *Nature Struct. Biol.* **2**, 663–673.

Schmid, F. X. (1983). Mechanism of folding of ribonuclease A. Slow refolding is a sequential reaction via structural intermediates. *Biochemistry,* **22**, 4690–4696.

Schmid, F. X. & Baldwin, R. L. (1978). Acid catalysis of the formation of the slow-folding species of RNase A: evidence that the reaction is proline isomerization. *Proc. Natl Acad. Sci. USA,* **75**, 4764–4768.

Schmid, F. X. & Baldwin, R. L. (1979). Detection of an early intermediate in the folding of ribonuclease A by protection of amide protons against exchange. *J. Mol. Biol.* **135**, 199–215.

Schmid, F. X. & Blaschek, H. (1981). A native-like intermediate on the ribonuclease A folding pathway. 2. Comparison of its properties to native ribonuclease A. *Eur. J. Biochem.* **114**, 111–117.

Sela, M. & Anfinsen, C. F. (1957). Some spectrophotometric and polarimetric experiments with ribonuclease. *Biochim. Biophys. Acta,* **24**, 229–235.

Sosnick, T., Mayne, L., Hiller, R. & Englander, S. W. (1994). The barriers in protein folding. *Nature Struct. Biol.* **1**, 149–156.

Udgaonkar, J. B. & Baldwin, R. L. (1990). Early folding intermediate of ribonuclease A. *Proc. Natl Acad. Sci. USA,* **87**, 8197–8201.

Zwanzig, R., Szabo, A. & Bagchi, B. (1992). Levinthal's paradox. *Proc. Natl Acad. Sci. USA,* **89**, 20–22.

Edited by P. E. Wright

(Received 4 June 1998; received in revised form 27 July 1998; accepted 29 July 1998)