

Structural Intermediates Trapped during the Folding of Ribonuclease A by Amide Proton Exchange[†]

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ABSTRACT: In the folding reaction of the slow-folding species (U_S) of ribonuclease A (RNase A), the slow isomerization of wrong proline isomers provides a suitable trap for kinetic folding intermediates at low temperatures (0–10 °C). Partly folded intermediates are known to accumulate before proline isomerization takes place, after which native RNase A is formed. We have been able to measure the protection from amide proton exchange which is provided by structure in the intermediates at different times along the folding pathway. Previous work has shown that, by labeling the amide protons of the unfolded protein before initiating refolding, an early

folding intermediate can be detected. The new pulse-labeling method presented here can be used to label later folding intermediates. Our results indicate that, in conditions which strongly favor the native protein, intermediates are formed which provide protection against exchange. However, when folding is initiated in 2.5 M Gdn-HCl, 10 °C, pH 7.5, conditions in which folding goes to completion but there are no spectroscopically detectable intermediates, then no intermediates are detected by our method. Alternate minimal mechanisms for the folding of U_S are presented.

Previous work has shown that proline isomerization can be used as a kinetic trap for intermediates in the folding of RNase A.¹ The results can be summarized as follows. (1) There are two classes of unfolded RNase A: a fast-folding class, U_F , and a major (80%) slow-folding class, U_S (Garel & Baldwin, 1973, 1975a,b; Brandts et al., 1975; Garel et al., 1976; Hagerman & Baldwin, 1976). (2) The $U_F \rightleftharpoons U_S$ interconversion reaction in unfolded RNase A has been shown to be acid catalyzed and to have other specific properties of proline isomerization or of peptide bond isomerization (Schmid & Baldwin, 1978, 1979a), and it is probable that the slow-folding molecules of RNase A have one or more wrong proline isomers, as first proposed by Brandts et al. (1975). (3) There is recent evidence that, in the folding of RNase A at low temperatures, structural intermediates accumulate before

proline isomerization takes place. An assay has been devised for wrong proline isomers which can be used during folding (Cook et al., 1979), and the results show that, at 0–10 °C, the folding reaction monitored by tyrosine absorbance occurs well in advance of proline isomerization. Schmid (1980) has found that folding monitored by tyrosine fluorescence follows the same kinetic progress curve as the assay for wrong proline isomers, demonstrating that the proline isomerization reaction can also be observed by a spectral technique. (4) Specific structure is formed in the next to last folding reaction monitored by tyrosine absorbance because a binding site for the specific inhibitor 2'-CMP is formed in this reaction (Cook et al., 1979; Schmid & Blaschek, 1980). Consequently, before proline isomerization takes place, a quasi-native intermediate, I_N , is formed in the folding reaction monitored either by tyrosine absorbance or by 2'-CMP binding. (5) A different intermediate, formed very early in folding, has been observed

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¹ Abbreviations used: RNase A, bovine pancreatic ribonuclease A with disulfide bonds intact; RNase S, a derivative of RNase A cleaved at the peptide bond between residues 20 and 21; U_S and U_F , slow- and fast-folding species, respectively, of RNase A; Gdn-HCl, guanidinium chloride; Mops, morpholinopropanesulfonic acid; BPTI, bovine pancreatic trypsin inhibitor; 2'-CMP, cytidine 2'-phosphate.

by first ^3H labeling the amide protons of unfolded (U_S) RNase A and then initiating refolding in conditions where complete exchange-out of the ^3H label will occur unless a folding intermediate is formed rapidly (Schmid & Baldwin, 1979b).

For RNase A, there are known to be at least two U_S species. When refolding occurs at 0–10 °C, a minor slow-folding species (U_S^I , 20%) can be distinguished from a major species (U_S^{II} , 80%) which folds more rapidly (Cook et al., 1979; Schmid & Blaschek, 1980). The evidence that the two folding reactions monitored by tyrosine absorbance arise from separate U_S species is based on stoichiometry: each folding reaction produces a natively-like species able to bind the specific inhibitor 2'-CMP (Cook et al., 1979; Schmid & Blaschek, 1980). The different U_S species probably differ in the isomeric conformations of their proline residues.

RNase A contains four proline residues. The following factors determine the relative concentration of a U_S species with a wrong isomer of a particular proline residue. In the X-ray structure of RNase S (Wyckoff et al., 1970) two prolines are *cis* (Pro-93, Pro-114) and two are *trans* (Pro-42, Pro-117). Isomerization after unfolding is expected to give a mixture of *cis* and *trans* isomers for each proline residue, with typical *cis:trans* ratios of 20:80 (Brandts et al., 1975) or 10:90 [cf. Grathwohl & Wüthrich (1976)]. Consequently, among the different possible U_S species, the unfolded one with a wrong *trans* isomer (i.e., Pro-93 or Pro-114) is expected to occur at a higher relative concentration (e.g., 4–9-fold) than the one with a wrong *cis* isomer (i.e., Pro-43 or Pro-117). From this consideration, the U_S species with wrong *trans* isomers of Pro-93 and Pro-114 are expected to dominate the refolding kinetics. However, there is another consideration, in addition to a possible sequence dependence of the *cis:trans* ratio. Certain proline residues are likely to be accommodated in the native (or natively-like) protein structure with almost equal ease in either the *cis* or *trans* conformation (Levitt, 1980). These residues have been termed "nonessential" because the correct isomeric conformation is not essential for natively-like folding (Schmid & Baldwin, 1978) or "type I" (Levitt, 1980). Wyckoff [quoted by Schmid & Baldwin (1978)] has suggested that Pro-114 of RNase may be type I or nonessential. Any proline residue whose correct isomeric conformation is not essential for natively-like folding is not likely to interfere with rapid folding when the wrong isomer is present. Consequently, not all proline residues of RNase A may give rise to U_S species. Our best guess is that the major species (U_S^{II}) has a wrong *trans* isomer of Pro-93. However, the residues responsible for producing the different U_S species have not yet been identified.

Proline isomerization provides a suitable trap for intermediates in folding since (a) proline isomerization is the final (or nearly final) and slowest step of the folding reaction, (b) partial folding does occur before proline isomerization takes place, and (c) the kinetic trap is applicable to the major form of the unfolded protein (U_S^{II}). By using this kinetic trap, we have studied the folding reactions of the U_S species of RNase A. This has been done by radioactively labeling exchangeable amide protons and making use of the fact that folding reactions (especially H bonding) greatly retard the rate of amide proton exchange (Englander et al., 1972). The factors which slow down exchange in native proteins (exclusion of H_2O and H bonding) retard exchange in bovine pancreatic trypsin inhibitor (BPTI) by as much as 10^9 (Molday et al., 1972; Karplus et al., 1973) and in RNase A by as much as 10^6 (Molday et al., 1972; Schmid & Baldwin, 1979b).

In the method of Schmid & Baldwin (1979b), amide proton exchange can be used to label protons protected by folding only

if a structural intermediate is formed rapidly after the start of folding. The trapped protons which have been studied are those which are stable to exchange-out in native RNase A for at least 6 h in the folding conditions (50 out of 119 protons at pH 6, 10 °C). The results show that over 20 of a possible 50 protons can be stably trapped when folding takes place at pH 6, 10 °C, in strongly native folding conditions. It is also possible to choose marginally native folding conditions where almost no protons are trapped in the competition between exchange-out and folding, probably because the folding intermediates are not stable enough to be well populated.

Here we introduce a new pulse-labeling method for detecting structural intermediates during folding. There are three aims. (1) The first is to find if the evidence for an early folding intermediate (Schmid & Baldwin, 1979b) can be confirmed by a different technique. (2) The second is to circumvent the requirement for knowing the exchange rates of the different classes of amide protons in the unfolded protein. (3) The third is to ^3H label the folding intermediates which are formed later in the folding process.

The experimental design is as follows. (a) Refolding is initiated in the absence of radioactivity. (b) After folding is allowed to occur for a variable period of time, ^3H is added. Those protons whose exchange rates are retarded by the formation of folding intermediates will be protected from labeling. (c) After 10 s of exposure to ^3H , the exchange reaction is quenched by lowering the pH to pH 2.7. (d) Folding is allowed to go to completion and the ^3H is separated from the partially labeled protein on a Sephadex G-25 column. (e) Exchange-out is allowed to occur for 20 h at 10 °C, pH 6, so that only the stably protected protons remain. (f) The number of stably trapped protons is determined by using a filter assay (Schreier, 1977).

The pulse-labeling method introduced here differs from the previously used competition method (Schmid & Baldwin, 1979b) in two ways, in addition to providing a method of labeling intermediates at any time during folding. (a) Whereas the competition method measures the trapping of protons by a folding intermediate, the pulse-labeling method measures the exclusion of label by folded structure. (b) By varying the duration of the pulse, it is possible in principle to label differentially structural units with different stability constants. This has not yet been tested in practice.

Experimental Section

Materials

RNase A (Sigma type XII A, lot no. 55C8250 and 17C8098) was used after further purification by chromatography on CM-Sephadex 50 (Garel, 1976). Guanidinium chloride (Gdn-HCl) was of ultrapure grade from Schwarz/Mann. Ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$ and morpholino-propanesulfonic acid buffer (Mops) were from Sigma Chemical Co. All other reagents were of analytical grade. Concentrations of RNase A were determined spectrophotometrically at 278 nm by using a molar absorbance of $9.8 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ (Sela & Anfinsen, 1957). ^{14}C Formaldehyde and ^3H water were obtained from New England Nuclear. Phosphocellulose paper, No. P81, was made by Whatman. ^{14}C -Labeled RNase A was prepared by sodium borohydride reduction of RNase A treated with ^{14}C formaldehyde (Rice & Means, 1971).

Methods

(a) *Unfolding of RNase A.* RNase A is unfolded in 3.0 M Gdn-HCl and 40 mM glycine, pH 2, at room temperature for 20 min to ensure that the unfolding reaction has reached its

final equilibrium ratio of fast- and slow-folding species, which is essentially independent of temperature (Garel & Baldwin, 1975a). The unfolded protein is then cooled down to 10 °C.

(b) *Refolding of RNase A.* Refolding of the unfolded protein is initiated in either (1) strongly native conditions [0.25 M Gdn-HCl; 0.20 M (NH₄)₂SO₄; 50 mM Mops; pH 7.5; 10 °C] or (2) in conditions in which no intermediates have been detected but folding does go to completion [2.5 M Gdn-HCl; 50 mM Mops; pH 7.5; 10 °C] [Cook et al., 1979; cf. Tsong & Baldwin (1978) and Nall et al. (1978)]. These will be referred to as "strongly native folding conditions" and "marginally native folding conditions", respectively.

(c) *³H Labeling of Proteins (General Aspects).* The probe that we have used in these experiments is the labeling of the amide protons of the polypeptide backbone via exchange with the solvent containing [³H]H₂O. Model compound studies have shown that, if a proton is H bonded, the H bond must be broken before exchange can occur (Englander et al., 1972). The reaction is both acid and base catalyzed with a "pH_{min}" around 3 (Englander & Englander, 1978). We have taken advantage of this fact by exchanging label into the protein at pH 7.5, where exchange occurs in less than 1 s. This ensures that all accessible, exchangeable protons will be labeled immediately upon addition of the [³H]H₂O (i.e., before additional folding becomes significant). After a 10 s exposure ("pulse") to [³H]H₂O, the exchange reaction is quenched by adding formic acid to pH 2.7 (the pH_{min} for RNase A). In all experiments, the [³H]H₂O concentration during the labeling pulse is 20 mCi/mL.

(d) *³H Labeling at pH 2.7 (Control to Check the Quench).* This experiment was done to check the effectiveness of the pH 2.7 quench. Refolding of the unfolded RNase A is initiated by diluting the Gdn-HCl to 0.25 M and adjusting the pH to 2.7 (i.e., the pH used as a quench). Five seconds after initiating refolding, [³H]H₂O is added. During the 5 s of refolding, the fast-folding species (U_F) refolds completely while the slow-folding species (U_S) does not fold significantly. Refolding of U_S is allowed to continue in the presence of the [³H]H₂O for 20 min at 3 °C, pH 2.7. These conditions correspond to those used in the separation of the labeled protein as described under section h.

(e) *Pulse ³H Labeling of Native RNase A (Control).* As a blank, native RNase A is diluted to the same pH and [Gdn-HCl] as the corresponding final refolding conditions used with the unfolded proteins. [³H]H₂O is then added and the exchange reaction is quenched 10 s later.

(f) *Pulse ³H Labeling of Unfolded RNase A (Control for Complete Labeling).* [³H]H₂O is added to the unfolded RNase A. Five seconds later, refolding is initiated by diluting the Gdn-HCl and raising the pH to 7.5. The refolding buffer contains additional [³H]H₂O to keep the tritium concentration constant at 20 mCi/mL. Five seconds after the addition of refolding buffer, the exchange reaction is quenched.

(g) *Pulse ³H Labeling of Partially Folded RNase A (Labeling of Intermediates).* Refolding of the unfolded RNase A is initiated by diluting the Gdn-HCl and raising the pH to 7.5. After folding is allowed to occur for a variable period of time, [³H]H₂O is added. Those protons whose exchange rates are retarded by the formation of folding intermediates will be protected from labeling. Ten seconds after the addition of [³H]H₂O, the exchange reaction is quenched.

(h) *Separation of Labeled Protein.* In all cases the [³H]-H₂O is separated from the tritiated protein by passing the solution over a Sephadex G-25 column (Englander & Englander, 1972) equilibrated with 50 mM sodium formate, pH

2.7. The column is run at 2–4 °C and the separation takes ~20 min. During this time, the refolding reaction goes to completion.

(i) *Assay for Stably Trapped Protons.* After separation, the tritiated protein fraction is adjusted to pH 6.0 at 10 °C to initiate exchange-out of the protein. The exchange-out curve of native RNase A has been given by Schmid & Baldwin (1979b). After 20 h of exchange, the number of protons stably trapped in the pulse labeling is determined by using a filter assay described by Schreier (1977). Trace amounts of [¹⁴C]RNase A are used as an internal concentration standard to compute the number of stable protons trapped per molecule of RNase A. The term "stable protons" in this paper means stable to exchange-out for 20 h at pH 6, 10 °C. All values have been corrected for an ³H-¹H equilibrium isotope effect of 1.2 (Englander & Poulsen, 1969) and for a [U_S]/[U_F] ratio of 80:20.

(j) *Spectroscopic Methods.* Tyrosine fluorescence quenching is monitored with a thermostated Turner Model 430 spectrofluorometer. Fluorescence is monitored at 305 nm with excitation at 268 nm. The emission monochromator bandwidth is 15 nm. Tyrosine absorbance is measured with a Cary 118 spectrophotometer with jacketed cell holders at 287 nm.

Results

(a) *Labeling of RNase A at the Quenching pH.* In RNase A the average amide proton exchange rate is 3×10^4 faster at pH 7.5 than at pH 2.7 according to the data given by Molday et al. (1972). However, some protons, especially the side chain primary amide protons of asparagine and glutamine residues, exchange with significant rates at pH 2.7 (Molday et al., 1972). Exchange-out of these will therefore result in the loss of radioactive label from the protein during the separation procedure at pH 2.7. To check the magnitude of this effect, we measured the exchange into the unfolded protein (U_S) under conditions similar to those used for the separation. Exchange-in rather than exchange-out of the protein is measured since the former has a much lower background and is therefore more sensitive. A total of 3.2 protons were stably labeled in this experiment (pH 2.7, 3 °C). This value represents the maximum effect of the side chain amide protons on our measurements since the unfolded protein (U_S) is used here whereas the pulse-labeling experiments are made on partly folded protein, with some protected amide protons.

(b) *Pulse Labeling of Native RNase A.* Proteins are known to participate in reversible conformational equilibria ("breathing" reactions) which permit amide proton exchange (Englander, 1975). These breathing reactions allow some of the amide protons of a protein to become stably labeled even if, on a time average, the protein is completely folded. In our experiments, labeling of the protein via breathing reactions is reduced by allowing the exchange to occur for only 10 s. When native RNase A in strongly native folding conditions is exposed to a 10 s pulse of labeling, only 0.1 proton per molecule of RNase A is stably trapped. When native RNase A in marginally native folding conditions is used, 1.5 protons per molecule of RNase A are stably trapped in the 10 s pulse. Under both conditions, the breathing reactions do not make a large contribution to the ³H trapping. These values are subtracted from the corresponding values obtained with unfolded proteins.

(c) *Pulse Labeling of Unfolded RNase A.* In these experiments [³H]H₂O is added to the unfolded protein before refolding is initiated. In this way all exchangeable protons are labeled. The number of protons per RNase A molecule

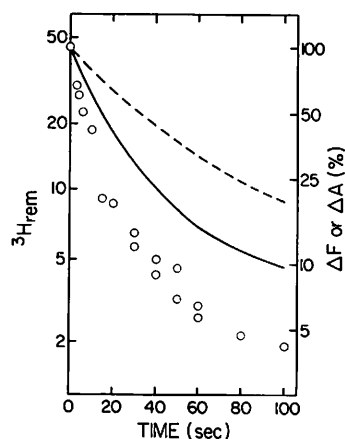


FIGURE 1: Refolding of RNase A in strongly native folding conditions [0.25 M Gdn-HCl; 0.20 M $(\text{NH}_4)_2\text{SO}_4$; 50 mM Mops; pH 7.5; 10 °C]. (---) Fluorescence changes (ΔF) observed at 305 nm (excitation at 268 nm). (—) Absorbance changes (ΔA) observed at 287 nm. (O) Number of protons trapped per RNase A molecule (${}^3H_{\text{rem}}$) after 20 h exchange-out at pH 6, 10 °C, vs. the time of refolding before the 10 s labeling pulse was applied. The data are plotted so that the ${}^3H_{\text{rem}}$ value at 0 s (i.e., complete labeling) and the 100% values for fluorescence and absorbance coincide.

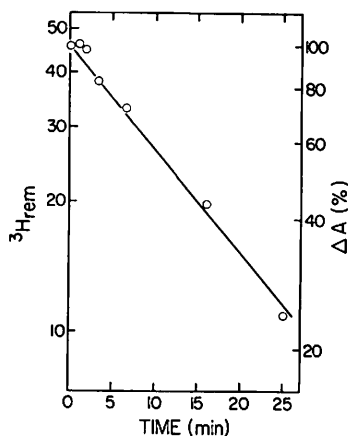


FIGURE 2: Refolding of RNase A in marginally native folding conditions [2.5 M Gdn-HCl; 50 mM Mops; pH 7.5; 10 °C]. (—) Absorbance changes (ΔA) observed at 287 nm. (O) Number of protons stably trapped, expressed in the same manner as in Figure 1.

stably trapped in this manner is 45 for the strongly native folding conditions and 46 for the marginally native folding conditions. These values are in close agreement with the value of 48 protons for the number of stable protons per molecule in RNase A at pH 6 (Schmid & Baldwin, 1979b).

(d) *Pulse Labeling after Varying Times of Folding.* The dashed line in Figure 1 presents the kinetic changes in fluorescence observed in strongly native folding conditions. As mentioned previously, the slow change in fluorescence during folding monitors the formation of native RNase A (Schmid, 1980). Also shown in Figure 1 is the absorbance (A_{287}) change that occurs during folding. Under these conditions, the tyrosine absorbance monitors chiefly the formation of the quasi-native intermediate I_N (Cook et al., 1979; Schmid, 1980). The data points in Figure 1 are a plot of the number of protons stably trapped per RNase A molecule (${}^3H_{\text{rem}}$) vs. the time of refolding before addition of $[{}^3\text{H}]\text{H}_2\text{O}$. The data are plotted so that the ${}^3H_{\text{rem}}$ value at 0 s (i.e., complete labeling) and the 100% values for fluorescence and absorbance coincide. It is clear that the pulse-labeling kinetics are distinct from both the kinetics monitored by tyrosine absorbance and those monitored by tyrosine fluorescence.

Figure 2 shows the corresponding plot, in marginally native folding conditions, of the number of protons stably trapped per RNase A molecule (${}^3H_{\text{rem}}$) vs. the time of refolding before the addition of $[{}^3\text{H}]\text{H}_2\text{O}$. The addition of 2.0 M Gdn-HCl has only a small effect on the exchange kinetics of the stable amide protons in native RNase A (Schmid & Baldwin, 1979b). Also shown in Figure 2 is the change in absorbance (A_{287}) that occurs during folding. At Gdn-HCl concentrations above 2.0 M, where folding intermediates are substantially destabilized, both fluorescence and absorbance monitor the formation of native RNase A (Schmid, 1980).

Discussion

(a) *Tests of the Pulse-Labeling Method.* The new method presented here was developed to enable us to label folding intermediates at any time point in the folding reaction. A short labeling pulse was used to minimize the effects of "breathing" reactions on our measurements (Englander, 1975). Our control experiments indicate that the breathing reactions do not make a significant contribution to the ${}^3\text{H}$ labeling. The

major problem with our pulse-labeling method is that the pH quench does not stop exchange of a few protons. This introduces an error of less than 10% in our measurements as determined both by our control for complete labeling (i.e., pulse labeling of unfolded RNase A) and by our direct check on the quench.

However, these protons are not a serious problem in our measurements as is demonstrated in Figure 2. In these experiments, refolding was initiated in marginally native folding conditions. Under these conditions, the percent of protons trapped by the pulse-labeling method should equal the percent of unfolded protein present since (1) the native protein does not trap a significant number of protons during the labeling pulse and (2) there are no significant levels of intermediates (i.e., spectroscopically detectable ones) present during folding. Figure 2 shows that the percent of protons trapped does equal the percent of unfolded protein present, as measured by absorbance, at any time of folding. We conclude that the errors introduced by loss of radioactive label during the separation of the labeled protein are relative minor.

(b) *Effect of Two or More Unfolded Species.* It might be supposed that, if there are two or more U_S species which refold at different rates, then the folding kinetics measured by different probes (as in Figure 1) need not be superimposable even if there are no populated structural intermediates in folding. We show here that this is not correct. Consider a folding experiment in which the protein is unfolded (U) at $t = 0$ and native (N) at $t = \infty$ and the folding kinetics are monitored by two probes, for example, tyrosine absorbance and tritium pulse labeling. Previously it has been shown (Labhardt & Baldwin, 1979) that, if the normalized kinetic progress curves obtained with two probes are not superimposable (i.e., the kinetic ratio test is positive), then a populated folding intermediate is detected. The kinetic ratio test is valid when the following conditions are satisfied: (1) the reaction must be monomolecular (e.g., the folding of RNase A) and (2) different initial species (i.e., unfolded proteins) must be indistinguishable by the probes used.

RNase A unfolded by Gdn-HCl appears to be completely unfolded [see review by Tanford (1968)]. The different U_S species of RNase A are believed to differ from one another only in the cis-trans conformation of their proline residues (Schmid & Baldwin, 1978). Since the U_S species initially present are completely unfolded, they should have the same fluorescence quantum yield, absorbance extinction coefficient, and number of exposed amide protons. Figure 3 illustrates

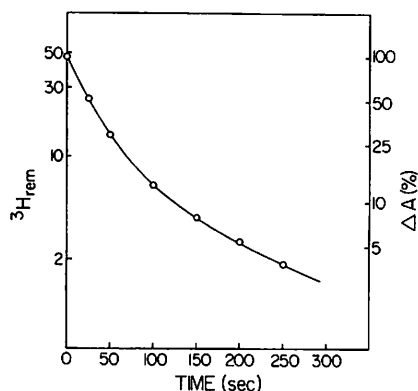


FIGURE 3: Computed kinetic progress curve for a hypothetical folding reaction without intermediates but with two unfolded forms which refold at different rates. The two unfolded species are present in a 20:80 ratio with time constants for folding of $\tau = 150$ s and $\tau = 30$ s, respectively. Folding is measured by tyrosine absorbance and by protection of amide protons against exchange. Both unfolded species are assumed to have the same extinction coefficient in measurements of tyrosine absorbance. (—) The normalized curve calculated for absorbance changes (ΔA). (O) Calculated number of protons stably trapped per RNase A molecule (${}^3H_{\text{rem}}$). The calculations show that when there are multiple unfolded species present initially, which have different rates of folding, then the kinetic progress curves become complex but the normalized folding kinetics are still superimposable [see the analysis of the kinetic ratio test by Labhardt & Baldwin (1979)].

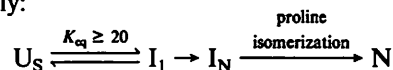
the point that, if two completely unfolded species are present which have different rates of refolding, the kinetic ratio test is still negative if there are no populated structural intermediates during folding. The proof has been given by Labhardt & Baldwin (1979). Figure 3 shows a hypothetical folding reaction in which there are no populated intermediates and initially two different unfolded species are present, with different rate constants for folding. The kinetics for this hypothetical folding reaction are complex but, as Figure 3 shows, there is still exact correlation between the folding kinetics measured by amide proton protection and spectroscopy. The lack of correlation between folding kinetics measured by different probes (Figure 1) requires the presence of at least one kinetic folding intermediate in addition to the quasi-native intermediate I_N , whose formation is monitored by tyrosine absorbance (Cook et al., 1979).

(c) *Intermediates in the Folding of RNase A.* The results in Figure 1 show that, under strongly native folding conditions, the folding kinetics measured by protection against 3H labeling precede both of the spectroscopically measured curves for folding. This shows that, before I_N is formed, at least one additional intermediate is formed which provides protection against exchange. However, when folding is initiated under conditions in which there are no spectroscopically detectable intermediates, the pulse-labeling results fall on the same line as folding measured by absorbance (Figure 2). This last result indicates that, when later intermediates are not detectable by comparison of spectral probes, early intermediates are also not detectable by 3H labeling. The experimental conditions used here (10 s pulse at pH 7.5, 10 °C) will not detect intermediates with a stability constant of less than ~ 50 because, at pH 7.5, 10 °C, the average time constant for proton exchange in unfolded RNase A is ~ 0.2 sec. Therefore, a 10 s pulse is long enough to label amide protons whose exchange rates are slowed down by a factor of 50 or less.

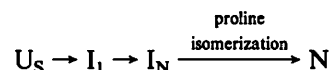
(d) *Confirmation of the Existence of an Early Folding Intermediate.* It has already been shown that an intermediate I_1 , which traps ~ 20 amide protons, is formed very early (< 3 s) in the folding reaction under native conditions (Schmid &

Baldwin, 1979b). The earliest time points that we obtained with the pulse-labeling technique were 3–4 s after the initiation of refolding in strongly native folding conditions (Figure 1). For these time points, $\sim 60\%$ of the stable amide protons were labeled. In other words, ~ 20 protons were protected from the label within 4 s after folding is initiated. This confirms the previous observation of the early folding intermediate I_1 (Schmid & Baldwin, 1979b). The present pulse-labeling method does not rely on knowing the exchange rate constants for amide proton exchange in the unfolded protein.

(e) *Alternative Minimal Mechanisms for the Folding of U_S .* A minimal mechanism for the folding of RNase A (U_S) under strongly native conditions that is consistent with our pulse-labeling data is the sequential mechanism in which I_1 is formed immediately:



An alternative minimal mechanism which is also consistent with our data is one in which K_{eq} is very large, so that the $U_S \rightarrow I_1$ reaction is irreversible, and in which I_1 is formed at a finite rate:



In addition to being consistent with the pulse-labeling data in Figure 1, both of the alternative mechanisms are consistent with the competition experiments between exchange-out and refolding performed by Schmid & Baldwin (1979b).

(f) *Possible Applications of the Pulse-Labeling Technique.* In contrast to the method used previously, pulse labeling can be used to label folding intermediates at any time in the folding reaction. However, the results presented here lack the specificity needed to detect intermediates other than I_1 and I_N . It is probable that additional intermediates besides I_1 and I_N are formed, but fitting of our results to either of the two mechanisms given above (P. S. Kim, unpublished data) indicates that it is not necessary to include additional intermediates in order to explain the pulse-labeling data. In future work it should be possible to characterize further I_1 and I_N (and to detect additional intermediates on the pathway between I_1 and I_N) either by using 1H - 2H exchange and analysis by proton NMR [note the assignment of the slowly exchanging amide protons in BPTI by Dubs et al. (1979)] or by 1H - 3H exchange and analysis by rapid protease digestion and separation of peptides (Rosa & Richards, 1979).

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