Protection of Amide Protons in Folding Intermediates of Ribonuclease A Measured by pH–Pulse Exchange Curves†

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ABSTRACT: pH–pulse exchange curves have been measured for samples taken during the folding of ribonuclease A. The curve gives the number of protected amide protons remaining after a 10-s pulse of exchange at pHs from 6.0 to 9.5, at 10 °C. Amide proton exchange is base catalyzed, and the rate of exchange increases 3000-fold between pH 6.0 and pH 9.5. The pH at which exchange occurs depends on the degree of protection against exchange provided by structure. Pulse exchange curves have been measured for samples taken at three times during folding, and these are compared to the pulse exchange curves of N, the native protein, of U, the unfolded protein in 4 M guanidinium chloride, and of I_N, the native-like intermediate obtained by the prefolding method of Schmid. The results are used to determine whether folding intermediates are present that can be distinguished from N and U and to measure the average degree of protection of the protected protons in folding intermediates. The amide (peptide NH) protons of unfolded ribonuclease A were prelabeled with ^3H by a previous procedure that labels only the slow-folding species. Folding was initiated at pH 4.0, 10 °C, where amide proton exchange is slower than the folding of the slow-folding species. Samples were taken at 0-, 10-, and 20-s folding, and their pH–pulse exchange curves were measured. After the pulse of exchange-out was quenched at pH 3.0, the sample was allowed to refold completely (15 min) and was subjected to a cutoff exchange step, designed to remove ^3H from the labile amide protons of the refolded protein, before the remaining ^3H was measured. The results are as follows. (1) The pH–pulse exchange curve of N is broad compared to that of U because N contains several classes of protected protons with widely separated exchange rates. The initial part of the curve for N is separated from the curve for U by 5 pH units. This corresponds to a 10^5-fold degree of protection for the least protected class of protons in N remaining after the cutoff exchange step. (2) The pH–pulse exchange curve of the sample taken at zero time of folding is separated from the curve for U by 1.4 ± 0.3 pH units. This indicates that an early folding intermediate (I_1) is formed rapidly, whose protected protons show an average 25-fold degree of protection. (3) The pH–pulse exchange curve of I_N was obtained after correction for known amounts of N and U in the prefolding sample of I_N. The curve for I_N is separated from that of N by 0.6 pH unit when the initial portions of the two exchange curves are compared. This corresponds to a 4-fold lower degree of protection in I_N than in N. (4) The samples taken at 10- and 20-s folding show pH–pulse exchange curves that can be represented by mixtures of U, I_1, I_N, and N. Other folding intermediates may be present but are not resolved by this technique.

Amide proton exchange has been used to trap protected protons in folding intermediates of RNase A[1] (Schmid & Baldwin, 1979; Kim & Baldwin, 1980) and of RNase S (Brems & Baldwin, 1984). The results show that numerous peptide NH protons are protected from exchange even at the earliest time of folding of the slow-folding species at 0–10 °C. Because the results indicate that extensive H-bonded structure is present early in folding, they support the framework model for folding in which α-helices and β-sheets are formed early in folding and interact with each other to make a scaffold for the tertiary structure. The application of amide proton exchange to protein folding experiments has been reviewed by Kim (1985). Intermediates in the folding of pepsinogen (McPhie, 1982) and of the α subunit of tryptophan synthase (Beasty & Matthews, 1985) have also been studied by amide proton exchange. By using a pulse of exchange with D_2O followed by analysis of the trapped protons in ^1H NMR, it is possible to resolve and identify individual protons that have been trapped in folding intermediates (Kuwajima et al., 1983), but the application of this approach to RNase A is waiting for resonance assignments to be made for the amide protons in RNase A.

There are certain problems in the methodology of pulse labeling that remain to be worked out. Here we introduce a pulse-labeling method for measuring the average stability of the protected protons in folding intermediates. An earlier pulse-labeling method (Kim & Baldwin, 1980) measures exchange-in during folding by giving a short pulse of $[^3H]H_2O$ at different times in folding. The earlier method labels the unprotected protons whereas the protected protons are the ones of interest. When the protected protons are ^3H labeled, the label can be used to measure the degree of protection against exchange. The degree of protection of an amide proton (for example, in a folding intermediate) is the reciprocal of its observed exchange rate times its exchange rate in the unfolded protein fully exposed to solvent at the same pH and temperature. The ^3H label might be used to find the structural locations of protected protons [cf. Rosa & Richards (1979) and Brems & Baldwin (1984)].

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A with S–S bonds intact; U_S and U_F, slow- and fast-folding species of unfolded RNase A; GdmCl, guanidinium chloride; I_1, early folding intermediate; I_N, native-like folding intermediate; U, unfolded; N, native.

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In the present method, which measures exchange-out, the slow-folding species of the unfolded protein are selectively ^3H labeled by the procedure of Schmid & Baldwin (1979). Folding takes place at pH 4.0, 10 °C, where exchange is slower than folding. Exchange-out is measured in a 10-s pulse at different times of folding; the pH of the pulse is varied from 6.0 to 9.5, giving a 3000-fold range over which the exchange rate of each amide proton is varied. The result is the overall pH–pulse exchange curve of the species present at a given time in folding. This curve can be used to find out if folding intermediates are present and to measure the average degree of protection of the protected protons. The pulse is quenched at pH 3.0, where folding is allowed to go to completion. Afterward, a cutoff exchange step (1000 s, pH 7.0, 10 °C) removes the labile protons, in order to avoid artifacts that may result when the labile protons are studied [cf. Schmid & Baldwin (1979)].

One of the objectives is to measure the pH–pulse exchange curve of I_N, the native-like intermediate that is formed late in folding (Cook et al., 1979; Schmid, 1981; Schmid & Blaschek, 1981; Schmid, 1983). A prefolding procedure has been given by Schmid (1983) for obtaining a sample that contains chiefly I_N by folding in strongly native conditions. He has determined the composition of the species present in the prefolding I_N sample (59% I_N, 13% U, 28% N) on the basis of his unfolding assay for measuring I_N in the presence of N. We have developed an independent method based on amide proton exchange for determining the proportions of these species, and we confirm his analysis. On the basis of the results, we have been able to determine the pH–pulse exchange curve of I_N. The pH–pulse exchange curves of U, N, and I_N are compared with those of samples taken at 0-, 10-, and 20-s folding, pH 4.0, 10 °C, after prelabeling with ^3H the slow-folding species of RNase A.

Materials and Methods

Materials

RNase A was purchased from Sigma (grade XIIA, lot no. 82F-8120) and purified chromatographically according to Garel (1976). Concentrations were determined by absorbance at 278 nm with an extinction coefficient of 9.8×10^3 cm^{-1} M^{-1} (Sela & Anfinsen, 1957). Guanidinium chloride (GdmCl) was ultrapure grade from Schwarz/Mann. $(NH_4)_2SO_4$ was enzyme grade from Sigma. [^{14}C]HCHO and [^{3}H]H_2O were from New England Nuclear.

Methods

^3H Exchange. ^3H was determined by scintillation counting after the ^3H-labeled RNase was adsorbed at pH 3.0 on phosphocellulose filters, as described by Schreier & Baldwin (1976) and Schreier (1977). Protein concentrations were determined by ^{14}C, with a trace amount of added ^{14}C-labeled RNase as an internal concentration standard (Schreier & Baldwin, 1976). ^{14}C-Labeled RNase was prepared by Na$CNBH_3$ reduction of the protein in the presence of [^{14}C]-formaldehyde, as described by Jentoft & Dearborn (1979).

Pulse Labeling of Folding Intermediates. The slow-folding species of unfolded RNase A were selectively labeled by exchange with [^{3}H]H_2O as described by Schmid & Baldwin (1979). The unfolded protein (0.15 mM) was kept in 4 M GdmCl and 50 mM glycine, pH 2.0. To initiate refolding at pH 4.0, 10 °C, the unfolded protein was diluted 10–20-fold into refolding buffer (0.1 M sodium cacodylate, 0.1 M glycylglycine, pH 4.1) to give a final pH of 4.0. For the pulse of exchange-out at higher pHs, the pH was fixed by adding a small, predetermined volume of NaOH. The pulse was quenched by adding a small volume of concentrated formate, pH 2.8, bringing the pH to 3.0. After 15 min, to complete folding, the sample was subjected to an exchange-out step to remove ^3H from labile amide protons in N; the conditions were 1000 s, pH 7.0, and 10 °C.

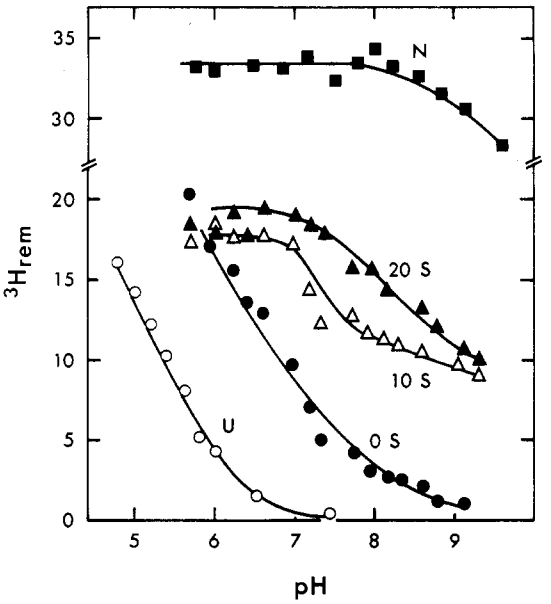

FIGURE 1: pH–pulse exchange curves for N (native RNase A, obtained after 30-min folding, ■), for U (unfolded in 4 M GdmCl, ○), and for samples taken at 0- (refolding initiated simultaneously with exchange-out, ●), 10- (△), and 20-s (▲) folding, pH 4.0, 10 °C. The slow-folding species of unfolded RNase A have been selectively labeled with ^3H. Exchange-out is measured in a 10-s pulse. After the pulse was quenched at pH 3.0 and folding was allowed to go to completion, ^3H has been removed from the labile amide protons of N by a cutoff exchange step (1000 s, pH 7.0, 10 °C).

Pulse Labeling of I_N. A sample containing a high proportion (59%) of the quasi-native intermediate I_N was prepared according to Schmid (1983): the sample was obtained after 15-s folding at pH 6.0, 0 °C, in 0.8 M $(NH_4)_2SO_4$ and 0.4 M GdmCl. For measurements of pulse exchange, [^{3}H]H_2O was added together with the NaOH used to fix the pH of the pulse. The pulse was quenched, and the sample was processed as described above, except that the excess [^{3}H]H_2O was removed by gel filtration (Sephadex G-25) and the conditions of the final exchange-out step were 12 h, pH 6.0, and 10 °C.

Results

pH–Pulse Exchange Curves of N and U. The pH–pulse exchange curves of N and U are separated by at least 5 pH units (Figure 1) for the set of protons remaining after the cutoff exchange step. Only the beginning of the curve for N is shown; the complete curve would extend over several pH units. Since the exchange rates in U are base catalyzed, this set of amide protons exchanges 10^5 times faster in U than in N. Folding intermediates whose protected amide protons have stabilities less than 10^5 can be characterized by measuring their pH–pulse exchange curves in this pH range.

The pH range in which protected amide protons can be identified as belonging to folding intermediates is more narrow. We can define this pH range by specifying that, in order for a folding intermediate to be measurable, it must contain more than one protected proton. Then the pH range extends from pH 6.7, where one out of 33.5 protons in U remains unexchanged, to pH 8.5, where one out of 33.5 protons in N exchanges out. The pH–pulse exchange curve for U has been measured in 4 M GdmCl, which introduces some uncertainty into the exchange rates. The exchange rates in S-peptide at

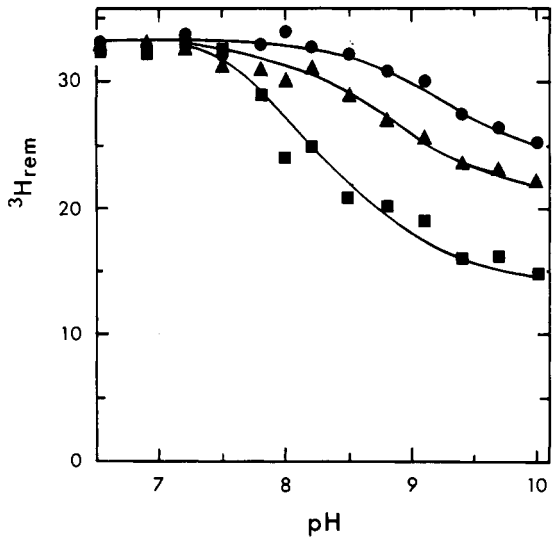

FIGURE 2: pH–pulse exchange curves for N in which pulse length is varied: ●, 10 s; ▲, 100 s; ■, 1000 s. The samples of N have been labeled with ^3H, and labile ^3H-labeled protons have been removed by a cutoff exchange step, as described in Figure 1.

pH 4.8 are 3-fold slower in 6 M GdmCl than in H_2O (Schmid & Baldwin, 1979). We suppose that 4 M GdmCl introduces an uncertainty of ±0.3 pH unit in the position of the pH–pulse exchange curve for U. If a sample from a refolding experiment has more than one [^3H]amide proton stable to exchange at pH 6.7 but exchanging out at pH 8.5, these amide protons must belong to folding intermediates and not to N or U.

To find out whether only a few or many protons of N, remaining after the cutoff exchange step, undergo significant exchange in this pH range, the pH–pulse exchange curve of N was measured for pulse lengths of 100 and 1000 s (Figure 2). For each pulse length the pH–pulse exchange curve is S-shaped, and about half of the core protons in N exchange in a 1000-s pulse at pH 10.0.

The number of protected protons in N is defined both by the conditions of the cutoff exchange step (1000 s, pH 7.0, 10 °C) and by the fact that all samples, including N and U, have been selectively ^3H labeled in conditions that label only the slow-folding species (see Methods). No correction has been made for this effect since there are losses of ^3H in selectively labeling the slow-folding species. The sample of N was obtained by allowing the prelabeled, unfolded protein to refold for 30 min at pH 4.0, 10 °C; the sample of U was the starting material in 4 M GdmCl, used without initiating refolding.

pH–Pulse Exchange Curves of Samples Taken at 0-, 10-, and 20-s Folding. The pH–pulse exchange curve for the zero-time sample, in which exchange was started simultaneously with refolding, is shown in Figure 1. Similar competition experiments were done by Schmid & Baldwin (1979), but folding was allowed to go to completion at the ambient pH. Their results indicate that an early intermediate, labeled I_1 (Nall et al., 1978; Schmid, 1983), is formed rapidly when refolding is initiated. Our experiments confirm this conclusion and show that the pH–pulse exchange curve of I_1 is similar in shape to that of U and is displaced from U by 1.4 ± 0.3 pH units.

The pH–pulse exchange curves of the samples taken at 10- and 20-s folding have different shapes than the curves for I_1 and U. They are broader and also displaced to progressively higher pHs, indicating that the average stability of the protected protons increases with time of folding. The 10- and 20-s samples contain several protected protons that can be identified as belonging neither to U nor to N: they are stable to exchange at pH 6.7 but undergo exchange at pH 8.5. To analyze further the pH–pulse exchange curves of these samples, it is necessary to examine the pH–pulse exchange curve of I_N and to determine the amounts of I_N present at each time.

pH–Pulse Exchange Curve of I_N. Figure 3 shows the pH–pulse exchange curve for the species present in the prefolding I_N sample, taken at 15 s in the folding conditions of Schmid (1983). Exchange-in is measured, in contrast to the data in Figures 1 and 2, and the exchange-in curves of U and N are given for comparison. The total number of ^3H-labeled protons in U is larger than the number in N in Figures 1 and 2 because the ^3H label is not restricted to the slow-folding species and because the conditions of the final exchange-out step are different (see Methods). By using specific unfolding assays for I_N and N, Schmid (1983) found the composition of the prefolding I_N sample to be 59% I_N, 28% N, and 13% U, where U was defined as everything other than I_N + N. We have measured the composition of the 15-s sample by a different method (see below) and find good agreement with Schmid's results.

The pH–pulse exchange curve for I_N (Figure 3) is obtained by subtracting the ^3H incorporation expected for 13% U and 28% N and then normalizing to 100% I_N. The early folding intermediates are included with U in this calculation; since the sample contains only 13% U, this is not too serious.

There are clear differences between the pH–pulse exchange curves of I_N and N. Exchange of I_N is significant at pH 8.0 whereas exchange into N begins only above pH 8.5. At pH 9.5 twice the number of ^3H-labeled protons exchange into I_N as into N.

Composition of Prefolding Sample of I_N Determined by Amide Proton Exchange. In order to check the composition of the prefolding sample of I_N, we developed the following method based on amide proton exchange. The sample is assumed to consist of three species: U, I_N, and N. The early folding intermediate I_1 is included with U.

Part A. The prefolding sample is allowed to finish folding in conditions where the amide protons of U are exchangeable but the amide protons of I_N and N are stable to exchange. [^3H]H_2O is present during folding (pH 5.9, 0 °C; see footnotes to Table I), and a cutoff exchange step after folding removes ^3H from the labile protons of the fully folded protein. The results of part A distinguish U from I_N + N.

Part B. The sample is first unfolded rapidly in conditions worked out by Cook et al. (1979) to give a mixture of U_F + U_S, and the mixture is analyzed by ^3H incorporation during folding. At pH 5.9, 0 °C, U_F refolds rapidly without incorporating much ^3H (Table I) whereas U_S refolds slowly and incorporates ^3H. The unfolded sample is allowed to refold in the same conditions as part A. In the unfolding step N unfolds to give U_F, I_N unfolds to give U_S (Cook et al., 1979; Schmid, 1983; Schmid et al., 1984), and U is U_S before and after the unfolding step. The results of part B distinguish N from U + I_N.

Details of the procedure, and analysis of the results, are given in the footnotes to Table I. Samples of N and U are treated in the same manner (see Table I) as the prefolding sample of I_N. The results give 13% U, 58% I_N, and 29% N, in good agreement with the unfolding analysis of Schmid (1983).

Folding at pH 4.0 Measured by Other Probes. To interpret the pH–pulse exchange curves of the folding intermediates (Figure 1), it is important to have assays for I_N and N at different times in folding. We have used the unfolding assay of Schmid (1983) to measure N. Folding measured by tyrosine

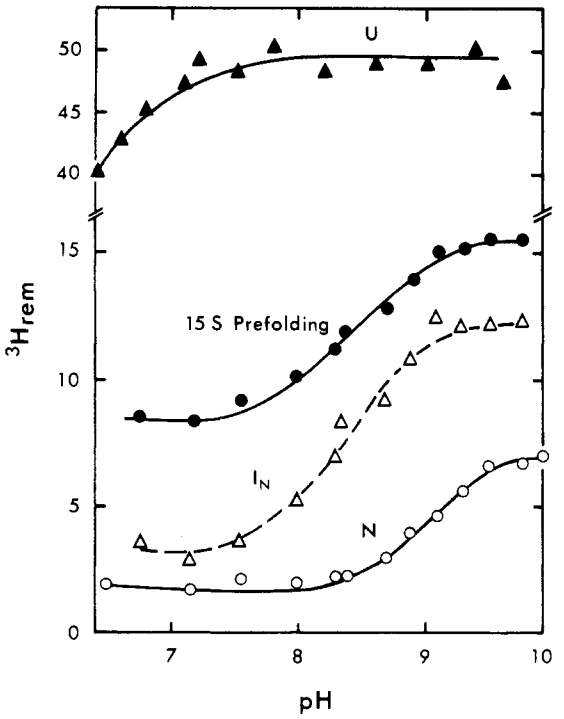

FIGURE 3: pH–pulse exchange curves in which exchange-in is measured in a 10-s pulse. The purpose is to obtain the exchange curve for I_N (△) from the curve for the prefolding I_N sample (●) and from the composition of this sample given by Schmid (1983) (13% U, 28% N, 59% I_N; see also the analysis in Table I). The pH–pulse exchange-in curves of U (in 4 M GdmCl, ▲) and of N (○) are also given. The samples are allowed to complete folding (15 min) at pH 3.0 after the pulse. ^3H is removed from the labile amide protons of N in a cutoff exchange step (12 h, pH 6.0, 10 °C) that differs from the one used for Figures 1 and 2. The pH–pulse exchange curve for I_N was obtained by solving at each pH for y_I in the equation $y_{mix} = y_I f_I + y_U f_U + y_N f_N$, where y is $^3H_{rem}$, f is the fraction of each species, I is I_N, and y_{mix} is $^3H_{rem}$ in the prefolding I_N sample. The values of f_I, f_U, and f_N are given above, and the values of y_U and y_N are shown in the figure.

Table I: Analysis[a] of Prefolding I_N Sample[b] by Amide Proton Exchange

sample	[^{3}H]amide protons incorporated during folding[c] in [^{3}H]H_2O: part A (untreated)	part B (unfolded rapidly)[d]
N	1.5	2.8
U[e]	43	43
prefolding I_N sample (I_N, N + U)	7.1	31

[a] [^{3}H]H_2O is added when refolding is initiated. After folding is complete (25 min), [^{3}H]H_2O is removed by gel filtration and each sample is processed as described under Methods for pulse labeling of I_N, including the cutoff exchange step after folding. Part A: The prefolding sample of I_N is allowed to complete refolding in [^{3}H]H_2O in conditions described below (see footnote *c*). Part B: The prefolding sample of I_N is first unfolded rapidly (see footnote *d*) and then allowed to complete refolding in the same conditions (see footnote *c*) as for part A. Analysis for U in the 15-s I_N sample: (7.1 − 1.5)/(43 − 1.5) = 13% U; U + I_N = (31 − 1.5)/(43 − 1.5) = 71%; I_N = 71 − 13 = 58%; N = 100 − 71 = 29%. [b] The I_N sample is obtained by 15-s folding at 0 °C, 0.8 M $(NH_4)_2SO_4$, 0.4 M GdmCl, and pH 4.0 (Schmid, 1983). In the analysis, the 15-s sample is assumed to have three species: N, I_N, and U = U_S (U_F folds rapidly to N). [c] The folding conditions are chosen so that ^3H is incorporated during the folding of U_S but not of U_F. The conditions are 25 min, pH 5.9, 0 °C, 0.72 M GdmCl, 0.44 M $(NH_4)_2SO_4$, and 0.1 M sodium cacodylate. [d] The conditions of the rapid-unfolding step (Cook et al., 1979; Schmid et al., 1984) are chosen to minimize the $U_F \rightleftharpoons U_S$ interconversion during unfolding. The conditions are 55 s, 5 M GdmCl, pH 2.0, and 0 °C. [e] U is unfolded initially in 4 M GdmCl, pH 2.0.

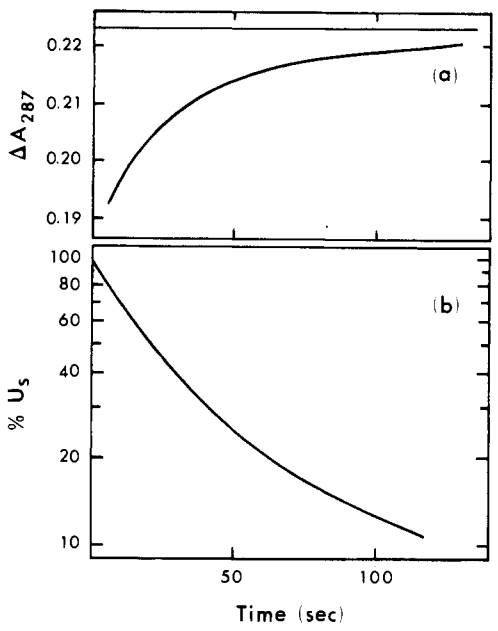

FIGURE 4: Folding of U_S species of RNase A at pH 4.0, 10 °C, and 34 μM RNase A, measured by tyrosine absorbance: (a) absorbance at 287 nm vs. time; (b) percent unfolded U_S molecules remaining, as measured by tyrosine absorbance. The fast-folding species U_F refolds within the time of manual mixing. The intercept at $t = 0$ in (a) was found by extrapolation of the plot of ln ΔA vs. t to $t = 0$.

absorbance gives the sum of I_N and N, according to the data of Schmid & Blaschek (1981), who found that the regain of enzymatic activity during folding of the U_S species is proportional to the change in tyrosine absorbance. The semilog plot of folding measured by tyrosine absorbance is nonlinear (Figure 4) because there are minor U_S species in addition to the major species [cf. Schmid & Baldwin (1979) and Lin & Brandts (1983a,b, 1984)] and, in strongly native folding conditions, the different U_S species have distinguishable folding kinetics (Schmid & Blaschek, 1981; Lin & Brandts, 1983a; Schmid et al., 1984). The proportion of I_N + N found from Figure 4 is 30% at 10-s folding and 51% at 20-s folding. The amount of N at each time, as measured by the unfolding assay of Schmid (1983), is 6% at 10-s folding and 13% at 20-s folding.

Discussion

pH–Pulse Exchange Curves of Folding Intermediates. These results define the pH–pulse exchange curves of two folding intermediates: the late intermediate I_N (Figure 3) and the early intermediate I_1, which is formed rapidly at the start of refolding (Figure 1). The curves for I_1 and I_N are clearly resolved from each other and from the curves for U and N. The results give information about the stabilities of the protected amide protons in I_1 and I_N.

The pH–pulse exchange curve for I_1 is close to the curve given by Schmid & Baldwin (1979; see their Figure 6) for ^3H trapped during folding as a function of pH when the unfolded protein is ^3H labeled and there is a competition between exchange-out and folding. They measured completion of folding by tyrosine absorbance ($t_{1/2}$ = 25 s, pH 6.0) and assumed that N is formed in this reaction; I_N had not yet been discovered. They give a calculated curve for the competition between exchange and folding when there are no folding intermediates and when folding occurs with the kinetics given by the change in tyrosine absorbance. The calculated curve of Schmid & Baldwin (1979) may be taken as the curve for the case that there are no folding intermediates except I_N and the amide protons are protected once I_N is formed.

As judged by the stability of protected amide protons, I_1 is closer to U than to N and I_N is quite close to N. The folding intermediates present at 10- and 20-s folding show pH–pulse exchange curves (Figure 1) that are intermediate between those of I_1 and I_N. One would like to know whether they represent mixtures of I_1 and I_N or whether additional intermediates can be detected. Since I_1 shows almost complete exchange at pH 9.2 (Figure 1), we can ask whether the protected protons remaining at pH 9.2 in the 10- and 20-s samples can be accounted for by the known amounts of N and I_N. At 10-s folding there are 7.8 protected protons expected vs. 9.5 observed, and at 20-s folding there are 13.1 expected vs. 10.5 observed. These numbers are based on 6% N and 24% I_N at 10 s and 13% N and 38% I_N at 20 s (see Folding at pH 4.0 Measured by Other Probes under Results) with 25 protected protons in I_N and 29.5 in N (estimated from Figure 3 and corrected to the smaller set of protons studied in Figure 1). Only rough agreement is found, but the comparison indicates that more sophisticated experiments will be required to resolve additional intermediates.

Properties of I_N That Distinguish I_N from N. The folding kinetics of the slow-folding (U_S) species of RNase A are complex in strongly native folding conditions both because intermediates are populated when the major U_S species refolds and because the minor U_S species are not negligible. Because of the latter source of complexity, it is important to document those properties of intermediates that clearly distinguish an intermediate both from N and from U. The native-like intermediate I_N is close to N both in tyrosine absorbance and the ability to bind cytidine 2′-monophosphate (2′CMP) (Cook et al., 1979; Schmid & Blaschek, 1981) and in enzymatic activity (Schmid & Blaschek, 1981). Lin & Brandts (1983b) have proposed that the folding kinetics of the slow-folding species might be interpreted without invoking I_N, but they have withdrawn this proposal (Lin & Brandts, 1984) following the demonstration by Schmid (1983) that I_N can be clearly distinguished from N by its 15-fold faster rate of unfolding in specified conditions. A second property by which I_N can be clearly distinguished from N is that I_N unfolds to give U_S whereas N unfolds to give U_F, and U_S is easily distinguished from U_F in refolding assays (Cook et al., 1979; Schmid et al., 1984). We show here that I_N can be clearly distinguished from N by a third property, namely, the displacement to lower pHs of its pH–pulse exchange curve (Figure 3).

Limitations of This Pulse-Labeling Method. It is desirable but difficult to label with ^3H the protected amide protons rather than the ones that are accessible to exchange during folding. To accomplish this, it is necessary to make two compromises that introduce some uncertainty into the data; see also Kim (1985). The first is the choice of pH 4.0 for folding. At this pH $t_{1/2}$ for the exchange of fully exposed amide protons is in the range 30–300 s [cf. Englander et al. (1972) and Figure 3] whereas $t_{1/2}$ for folding measured by tyrosine absorbance is 22 s (Figure 4). Thus, exchange-out during folding is not negligible. The consequence is a slow decrease with time of folding of the number of [^3H]amide protons. The choice of pH 4.0 for folding, rather than pH 3.0 where loss of ^3H by exchange would be reduced, is dictated by the fact that folding intermediates become less stable as the pH is reduced below 4, as indicated by a decreased folding rate below pH 4.0 when folding is measured at 25 °C (Nall et al., 1978).

The second factor is the need to label selectively the U_S species of RNase A. If the U_F species is ^3H labeled, then U_F folds rapidly compared to exchange at pH 4.0 and the amide protons in U_F become protected against exchange. In pH–pulse exchange curves of the type shown in Figure 1 the protected protons appear as N, stable to exchange over most of the pH range. Since the U_F:U_S ratio is known accurately to be 18:82 (Schmid, 1982), one could ^3H label both U_F and U_S before refolding and then subtract the amount of ^3H incorporated that corresponds to [^3H]U_F → [^3H]N, assuming 18% U_F. But labeling U_F with ^3H makes a large contribution to the total ^3H incorporation, and this procedure gives an undesirable scatter of the data (Schmid & Baldwin, 1979). In unfolded RNase A, there is a constant, slow equilibration between U_F and U_S: $t_{1/2}$ is about 10 min at 0 °C (Schmid & Baldwin, 1978). Equilibration between U_F and U_S occurs on the column used to separate ^3H-labeled protein from excess [^3H]H_2O and occurs also as the unfolded protein stands before refolding is initiated. This results in some ^3H incorporation from [^3H]U_F → [^3H]N. An upper limit on this effect is provided by the pH–pulse exchange curve for the zero-time sample, where the total amount of N present is less than or equal to that computed from ^3H remaining at pH 9.0 (see Figure 1).

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Registry No. RNase A, 9001-99-4; H_2, 1333-74-0.

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