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Intermediates in the Refolding of Ribonuclease at Subzero Temperatures. 2. Monitoring by Inhibitor Binding and Catalytic Activity†

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ABSTRACT: The kinetics of refolding of ribonuclease A were monitored by the return of catalytic activity and inhibitor binding at −15 °C in 35% methanol cryosolvent at pH* 3.0 and 6.0. Catalytic activity was measured with cytidine 2′,3′-cyclic monophosphate as substrate; inhibitor binding was determined with the competitive inhibitor cytidine 2′-monophosphate. Biphasic kinetics were observed at pH* 3.0 for both return of catalytic activity and inhibitor binding. At pH* 6.0 the rate of return of catalytic activity was monophasic, whereas that of inhibitor binding was biphasic. For both inhibitor binding and catalytic activity one of the observed rates was pH-dependent. Full return of catalytic activity was obtained at the completion of the refolding process. The observations are interpreted in terms of two parallel pathways of refolding for slow-refolding ribonuclease, with several native-like, partially folded intermediate states on the minor slow-refolding pathway. Of particular note is the presence of at least one such species that has inhibitor-binding capacity but not catalytic activity. This may be rationalized in terms of the known native structure. In addition, an intermediate is postulated which has the incorrect Pro-93 conformation and only partial catalytic activity (42% of the native). The slowest observed transient is attributed to the isomerization of this proline residue and return of full catalytic activity.

This paper is the second in a series in which the folding of ribonuclease A (RNase)[1] was studied in aqueous methanol cryosolvents and at subzero temperatures. These conditions were used in order to facilitate the detection of partially folded intermediate states in the folding process. Previous investigations have shown that the native state of RNase in 35% methanol is essentially the same as that in aqueous solution (Biringer & Fink, 1982a) and that the refolding process is multiphasic (Biringer & Fink, 1982b, 1988a,b). In the present research we have used the ability of the folded enzyme to bind competitive inhibitors and to catalyze the hydrolysis of substrate as a means of measuring the rate at which the native protein is formed. We assume that only a native-like state will possess a competent catalytic apparatus, although inhibitor binding could occur with only a partially formed active site.

Experimental Procedures

Materials

Ribonuclease A was purified as described previously (Biringer & Fink, 1982a, 1988a,b). 2′,3′-CMP and 2′-CMP were purchased from P-L Biochemicals and used without further purification. Methanol was HPLC grade, and buffer materials were AR grade. Cryosolvents were prepared as described previously (Biringer & Fink, 1988a).

Methods

Inhibitor Binding. Refolding kinetics were measured by using the binding of 2′-CMP to monitor the formation of the inhibitor binding site. The stoichiometry of the binding has been determined to be 1:1 in aqueous solution (Schmid & Blascheck, 1981) and is presumed to be the same in cryosolvent. The protein was unfolded by heating a stock solution (0.5 mM) in 35% methanol, pH* 3.0, at 70 °C for 10 min, which has been shown to fully unfold the protein as judged by proton NMR (Biringer & Fink, 1982a). The unfolded enzyme was injected into 0.8 mL of solution containing 100 μM 2′-CMP in 35% methanol (0.033 M sodium formate, pH* 3.0, or 0.033 M sodium acetate, pH* 6.0) at −15 °C. The reaction was monitored at 254 nm for the pH* 6.0 experiments and at 271.5 nm for the pH* 3.0 experiments. The isosbestic point for the enzyme folding when followed by UV absorbance is 254 nm and is independent of pH. At pH* 6.0 the binding reaction can be monitored without interference from the folding. At pH* 3.0 the *binding* reaction also exhibits an isosbestic point at 254 nm. At 271.5 nm a minimal change in absorbance due to protein (i.e., tyrosine burial) is observed whereas a large change due to 2′-CMP binding can be seen. The small contribution of the absorbance change due to protein

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[1] Abbreviations: RNase, ribonuclease A; 2′-CMP, cytidine 2′-monophosphate; 2′,3′-CMP, cytidine 2′,3′-cyclic phosphate; NMR, nuclear magnetic resonance; N, native state; U, unfolded state; pH*, apparent pH of aqueous–organic cryosolvent, as determined with glass electrode/pH meter; HPLC, high-performance liquid chromatography; AR, analytical reagent.

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Table I: Kinetics of Refolding of Ribonuclease A at −15 °C, in 35% Methanol[a]

	pH* 3.0				pH* 6.0			
signal	k_I(Amp) × 10^2	k_{II}(Amp) × 10^2	k_{III}(Amp) × 10^3	k_{IV}(Amp) × 10^4	k_I × 10^2	k_{II}(Amp) × 10^2	k_{III}(Amp) × 10^3	k_{IV}(Amp) × 10^4
ΔA_{286}	6.2 ± 0.9 (dec)	1.3 ± 0.4 (19)	1.7 ± 0.4 (19)	2.9 ± 1.0 (19)	5.4 ± 1.2	1.0 ± 0.2 (−8)	3.3 ± 0.9 (9)	7.3 ± 1.0 (8)
2′-CMP	–	2.1 ± 0.5 (9)	–	3.4 ± 0.8 (13)	–	1.8 ± 0.3 (19)	2.5 ± 0.3 (5)	–
2′,3′-CMP	ND	ND	3.2 (11)	2.4 (15)	–	ND	–	3.4 (23)

[a] The values in parentheses are the amplitudes of the given transient expressed as a percentage of the total amplitude change between U and N under the experimental conditions (see text). Rate constants are in units of s^{-1}, Amp = amplitude, ND means not determined, (–) means the reaction was not observed, and the errors shown are the standard deviations.

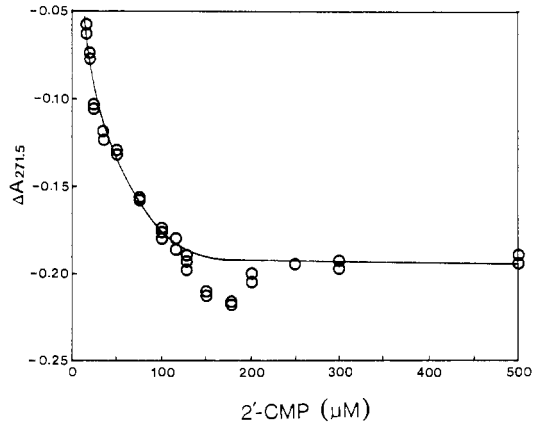

FIGURE 1: Absorbance change produced by binding of 2′-CMP to RNase A at −15 °C as a function of 2′-CMP concentration in 35% methanol, pH* 3.0 and 0.033 M sodium formate. The final enzyme concentration was 30 μM. The values given represent the change in absorbance produced after the addition of 50 μL of a 0.05 mM enzyme stock solution to 800 μL of a solution containing the given concentrations of 2′-CMP. In each case the absorbance change due to dilution and the absorbance of the enzyme was taken into account.

(tyrosine) folding was eliminated from each data set by subtraction of the average absorbance change from several folding experiments performed in the absence of 2′-CMP.

The concentration of inhibitor was chosen from the results of a series of experiments where native enzyme, at the concentration used in the assay, was added to different concentrations of inhibitor (Figure 1).

Catalytic Activity. The catalytic activity of RNase A was measured by its ability to hydrolyze cytidine 2′,3′-cyclic monophosphate. Under saturating conditions in aqueous solution, the reaction is zero order, the slope being directly proportional to enzyme concentration. In the presence of methanol, methanolysis is the preferred reaction. At −15 °C in 35% methanol conditions of saturation over extended time periods are beyond the solubility limits of the substrate. Under the conditions utilized here, the initial portion of the kinetic trace was linear. Experiments were performed for a series of enzyme (native) concentrations, and the initial slope was found to be directly proportional to the enzyme concentration.

In each experiment a 25-μL aliquot of stock RNase A solution consisting of 0.15 mM protein in 35% methanol, pH* 3.0, and 0.033 M sodium formate was heated in a glass syringe for 10 min at 70 °C. The sample was then injected into 225 μL of 35% methanol and pH* 3.0 (0.033 M) sodium formate, or pH* 6.0 (0.033 M) sodium acetate, precooled to −15 °C in a spectrophotometer. Control experiments showed that the maximum temperature reached in this process was −11 °C. The protein was allowed to refold for various time periods, and then 1.0 mL of precooled (−15 °C) 5 mM 2′,3′-CMP in 35% methanol, pH* 6.0, and 0.033 M sodium acetate was added. The hydrolysis reaction was monitored at 296 nm where the absorbance change due to protein folding is virtually nonexistent and the change observed for hydrolysis is quite large.

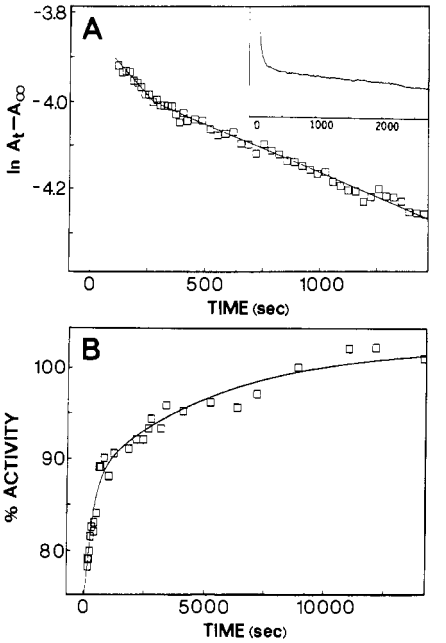

FIGURE 2: Time-dependent changes in the refolding of RNase A in 35% methanol, at −15 °C and pH* 3.0. (A) Monitored by the return of inhibitor (2′-CMP) binding. The data are displayed as a semilog plot in order to show the biphasic nature of the process. The inset shows the time-dependent changes in absorbance at 271.5 nm, where the binding was measured. Only the first part of the reaction is illustrated, in order to more clearly show its biphasic nature. (B) Rate of return of catalytic activity toward 2′,3′-CMP. The assays for catalytic activity were carried out as described in the text.

The activity is expressed as a percentage of that expected for the native enzyme under the assay conditions. The reported time value was taken as the time the protein was allowed to fold plus the time required to reach the center of the linear portion of the initial velocity curve. The standard error for each experimental point is ±3.0%.

Other procedures were as outlined in the preceding paper (Biringer & Fink, 1988a).

RESULTS

Catalytic Activity. The rate of return of catalytic activity was determined by using the substrate 2′,3′-CMP. Figures 2B and 3B show the data for the folding at pH* 3.0 and 6.0. Each square represents the results of a single assay, and the solid line gives the best fit to one (pH* 6) or two consecutive (pH* 3) first-order processes. Table I gives the rates and amplitudes. The amplitude data are given as a percentage of the catalytic activity obtained from native RNase at the same protein concentration. Due to technical difficulties it was not feasible to obtain accurate data during the first 50–70 s of the refolding reaction, and thus activity measurements in the fast-phase region (Biringer & Fink, 1988a,b) are not presented. The kinetics at pH* 3.0 are biphasic, and the rates correspond to the third and the fourth phases observed by other methods (Biringer & Fink, 1988a,b). The kinetics at pH* 6.0 are

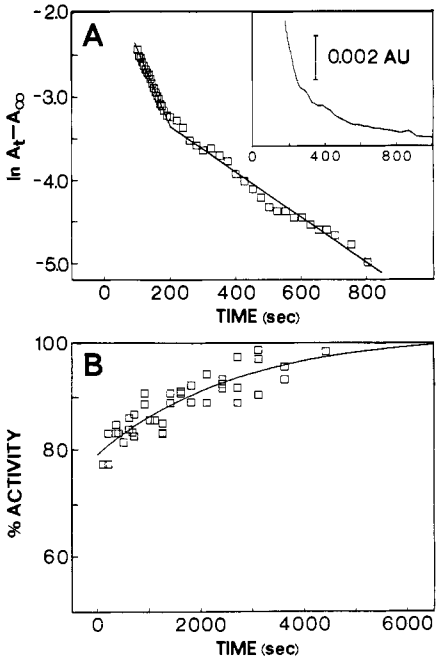

FIGURE 3: Time-dependent changes in the refolding of RNase A in 35% methanol, at −15 °C and pH* 6.0. (A) Monitored by the return of inhibitor (2′-CMP) binding. The data are displayed as a semilog plot in order to show the biphasic nature of the process. The inset shows the time-dependent changes in absorbance at 271.5 nm, where the binding was measured. (B) Rate of return of catalytic activity toward 2′,3′-CMP. The assays for catalytic activity were carried out as described in the text.

monophasic with a rate corresponding to the slowest phase observed by other methods (Biringer & Fink, 1988a,b). At both values of pH* the total amplitude corresponds to 23–26% of the total, the remaining portion having returned at a faster rate. Within experimental error, full catalytic activity was present upon completion of the slowest phase.

Inhibitor Binding. The refolding process was measured by the binding of the competitive inhibitor 2′-CMP. In order to determine the minimum concentration of inhibitor necessary to saturate the enzyme under the refolding conditions, a series of experiments was performed in which native enzyme was added to different concentrations of 2′-CMP (12.5–500 μM). The final enzyme concentration was 30 μM in each experiment. The results are given in Figure 1. The leveling off of the absorbance change at 100 μM 2′-CMP indicated that this concentration was the minimum necessary for saturation of the enzyme at pH* 3 and −15 °C. Since the inhibitor binding is much tighter in aqueous solution at pH 6 than at pH 3 it was assumed that a similar situation would exist in 35% methanol as well. In order for the change in absorbance of 2′-CMP to be a direct measure of the concentration of intact binding sites during folding, it must be linear with respect to enzyme concentration. A series of experiments was performed in which enzyme concentration was varied (25–33 μM RNase A) and the 2′-CMP concentration held constant. Three different inhibitor concentrations were tested (25, 50, and 100 μM 2′-CMP); otherwise, the experimental methodologies were identical with those for the saturation controls described above. The results showed that the absorbance change is linear with respect to enzyme concentration at each 2′-CMP concentration tested.

The results of typical refolding experiments are shown in Figures 2A and 3A for pH* 3.0 and 6.0, respectively. The kinetic data (Table I) are the average of several experiments. At both pH*'s the kinetics are biphasic. The rate of the fastest of the two phases is pH-independent. The slower phase is pH-dependent, with the rates differing by almost an order of magnitude between pH* 3 and 6. The amplitudes for each phase are pH-dependent, but the total amplitude appears constant at a value around 22–24% of the total expected. The contribution to the absorbance change at 271.5 nm from the changes in tyrosine exposure due to refolding was negligible, i.e., 7% at 60 s and 1% after 120 s.

Discussion

The results indicate that the rates of return of catalytic activity and inhibitor binding during refolding are multiphasic and noncoincident. The latter observation is consistent with different regions of the molecule becoming "native" at different rates. The observation that refolding is multiphasic with these probes means that the observed transients could represent either or both of two possibilities: a fraction of the molecules present could be in their native state, or all the molecules could be present in an intermediate state which has partial activity. In addition, there are two categories of models that can account for multiphasic kinetics of refolding: multiple intermediate states on a single folding pathway (sequential intermediates) or multiple (parallel) folding pathways from different unfolded species, or a combination of these. In fact, we assume that the early stages of refolding will involve concurrent parallel pathways (for a given unfolded configuration or proline conformation), which gradually converge to a single (or limited number of) pathway(s) for the later stages of folding.

The refolding of RNase A is complicated by the presence of different forms of the unfolded protein (Garel & Baldwin, 1973; Garel et al., 1976) which have been attributed to incorrect cis/trans isomers of proline (Brandts et al., 1975; Nall et al., 1978; Schmid & Baldwin, 1979; Schmid, 1983; Lin & Brandts, 1983a–c; Krebs et al., 1985; Schmid et al., 1985; Mui et al., 1985). Two major slow-refolding forms (U_S^I, U_S^{II}) of RNase have been recognized in investigations in aqueous solution. A similar situation exists in aqueous methanol (Biringer & Fink, 1988b). In particular, double-jump unfolding assays indicate two slow-folding, parallel paths with approximately 30% and 50% of the total protein. The remaining 20% of the protein refolds very rapidly, corresponding to U_F, the fast-refolding material.

Return of Catalytic Activity. The results show that 100% catalytic reactivity is recovered during refolding in 35% methanol at −15 °C at both pH* 3 and pH* 6. At both values of pH* a total of approximately 75% of the protein had already become catalytically active by 1–2 min. The fast-refolding material, U_F, accounts for 20% of this; the remaining 55% must be accounted for either by additional material that folded to the native state faster then our observation times or by an intermediate state (or states) that possesses (possess) partial catalytic activity but is (are) not fully native, or by a combination of these. It is possible that some refolding occurs during the cooling and mixing process to initiate the refolding. However, on the basis of comparison of thermally unfolded and guanidinium-unfolded protein which show similar amplitudes for refolding transients, plus the fact that control experiments show that the maximum temperature increase was ≤4 °C (i.e., to −11 °C) (Biringer & Fink, 1988a,b), we believe that the amount of refolding occurring during the mixing process is small. In addition, double-jump unfolding assays (Biringer & Fink, 1988b) show that at the earliest sampled times in refolding only 20% (from the fast-refolding state) of native protein is present.

At pH* 3 the kinetics of refolding as determined by catalytic activity were biphasic, the rates $(k_{III}, k_{IV})^2$ corresponding

closely to the two slowest processes observed at pH* 3 by absorbance (Biringer & Fink, 1988b), whereas at pH* 6 the kinetics were monophasic (k_{IV}) and corresponded to those of the slowest observed process. On the basis of the amplitudes of the reaction, it appears that the faster of the two observed processes at pH* 3 became slower at pH* 6. The rate of the slowest process was independent of pH, which would be expected if the process was the isomerization of proline. By comparison with observations in aqueous solution (Lin & Brandts, 1983a–c; Schmid, 1983; Krebs et al., 1985; Mui et al., 1985; Schmid et al., 1986), it is reasonable to attribute the slowest phase at both pH*'s to the isomerization of Pro-93 from its nonnative trans conformation to its native cis conformation. There are several lines of evidence that are consistent with this interpretation, and they are enumerated in the following paper (Biringer & Fink, 1988b). This interpretation does not rule out the possibility that the isomerization of Pro-114, which is also cis in the native state, is also involved in causing slow refolding; however, its isomerization should be manifested as a signal in the refolding of nitro-Tyr-115 (Biringer & Fink, 1988a).

Data from other studies of this system indicate that the slow-folding material corresponds to two different unfolded states, U_S^I and U_s^{II}, occurring as 30% and 50%, respectively, of the total unfolded protein (Biringer & Fink, 1988b) (Scheme I). On the basis of the amount of catalytic activity that returns in the observed transients (23–26%), we assign these kinetic phases to the minor slow-folding pathway. This means that the unfolded material on the major (U_S^{II}) pathway must have regained full catalytic activity in the first 2 min of refolding at −15 °C. We can therefore place a lower limit of around 2×10^{-2} s^{-1} on the rate at which U_S^{II} becomes catalytically active. This rate constant is consistent with rates measured by other techniques for one of the steps on the major folding pathway (Biringer & Fink, 1988b). The assignment of the observed transients at pH* 3 corresponding to the return of catalytic activity (k_{III}, k_{IV}) to steps k_2 and k_3 in the minor refolding pathway in Scheme I implies that I_2 has partial catalytic activity (42% of native, on the basis of the relative amplitudes of steps 2 and 3). We propose that this intermediate is essentially native-like, with the exception of the region around Pro-93.

Scheme I

$$U_F \rightarrow N \qquad (20\%)$$

$$U_S^I \xrightarrow{k_1} I_1 \xrightarrow{k_2} I_2 \xrightarrow{k_3} N \qquad (30\%)$$

$$U_S^{II} \xrightarrow{k'_1} I'_1 \xrightarrow{k'_2} N \qquad (50\%)$$

Inhibitor Binding. At both pH* 3 and 6 the binding of 2′-CMP was biphasic, with the rate of the faster observed phase being pH-independent and that of the slower phase increasing from pH* 3 to 6. The sum of the amplitudes for the two phases corresponded to approximately 25% of the inhibitor-binding capacity at both pH* values, just as in the case of the catalytic activity. Again, two interpretations are possible: the observed changes correspond to processes only on the minor slow-refolding pathway, or each of the observed phases corresponds to the formation of native (or native-like material) on different slow-folding pathways. On the basis of the data for the rate of return of catalytic activity, and other probes (Biringer & Fink, 1988a,b), we favor the former explanation, that is, that the major slow-folding material regained its inhibitor-binding capacity more rapidly than 2×10^{-2} s^{-1} and the observed changes reflect processes on the minor pathway.

Comparison of the rates of inhibitor binding and catalytic activity at pH* 6 demonstrates that an intermediate must be present that has inhibitor-binding capacity but that lacks full catalytic activity. This can be rationalized on the basis of the structure of RNase A. The imidazole of His-12 is essential for catalytic activity and resides on the N-terminal helix which is connected to the rest of the structure by a loop around residue 20. (It is this loop that is cleaved by subtilisin to give the S-protein and S-peptide molecules in RNase S.) One can thus conceive a folding intermediate in which the N-terminal 20 residues are not in the cleft they occupy in the native state (even though the helix may be present), and thus the enzyme is catalytically inactive. Such an intermediate could bind a competitive inhibitor, such as 2′-CMP, since the major source of binding of the pyrimidine is Thr-45, and the positive charges of His-119 and Lys-41 will be present to facilitate binding of the phosphate of 2′-CMP. The pyrimidine binding site includes two hydrogen bonds to Thr-45 and other interactions with the core of β-pleated sheet in this vicinity which is presumably formed early in the folding (Matheson & Scheraga, 1979). Thus I_4 (and perhaps I_3) may well be an intermediate in which the basic core of β-sheet structure of the native protein is present, but in which the N-terminal region, residues 1–20, has not yet moved into the cleft formed by the bent β-sheet.

In summary, the rate of return of inhibitor binding and catalytic activity in the *major* slow-folding pathway was too rapid to observe with the present experimental method at −15 °C. The implication is that either the native state or a native-like intermediate is formed in a process faster than 2×10^{-2} s^{-1} under these conditions. The return of catalytic and binding activity on the minor slow-folding pathway is much slower than on the major pathway and occurs in two phases. At both pH* 3 and pH* 6 the faster observed transient for inhibitor binding is at least an order of magnitude faster than the rate of return of catalytic activity, implicating at least one intermediate with inhibitor-binding activity but not catalytic activity. The slower transient for the return of catalytic activity is associated with the slowest step in the refolding, attributed to the isomerization of Pro-93. The data are best interpreted in terms of the kinetic mechanism shown in Scheme I which implies several relatively native-like species on the minor folding path. A more detailed discussion of the folding pathway is given in the following paper (Biringer & Fink, 1987b) using information from several different probes.

Registry No. 2′-CMP, 85-94-9; 2′,3′-CMP, 633-90-9; RNase, 9001-99-4.

[2] We will use the convention that roman numeral subscripts refer to *observed* rate constants, e.g., k_I, whereas arabic numerals, e.g., k_1, will be used for rate constants from model schemes.

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Intermediates in the Refolding of Ribonuclease at Subzero Temperatures. 3. Multiple Folding Pathways†

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ABSTRACT: The kinetics of refolding of ribonuclease A have been measured at −15 °C by monitoring the intrinsic fluorescence and absorbance signals from the six tyrosine residues. For each probe multiphasic kinetics were observed. The burial of tyrosine residues, as determined by the change in absorbance at 286 nm, revealed four phases, whereas the kinetics of refolding monitored by fluorescence revealed only two phases. The rates of the transients detected by fluorescence were independent of pH. One of the faster transients detected by ΔA_{286} involved a decrease in absorbance, which is consistent with solvent exposure, rather than burial, and suggests the possibility of an abortive partially folded intermediate in the earlier stages of folding. Double-jump unfolding assays were used to follow the buildup and decay of an intermediate in the refolding reaction at −15 °C. At both pH* 3.0 and pH* 6.0 the maximum concentration of the intermediate was 25–30% of the total protein. The existence of a second pathway of slow folding was inferred from the difference in rate of formation of native enzyme and breakdown of the observed intermediate, and by computer simulations. In addition, the unfolding assay demonstrated that 20% of the unfolded protein was converted to native at a much faster rate, consistent with observations in aqueous solution that 80% of unfolded ribonuclease A consists of slow-folding species. Kinetics and amplitude data from these and other refolding experiments with different probes were used to develop possible models for the pathway of refolding. The simplest system consistent with the results for the slow-refolding species involves two parallel pathways with multiple intermediates on each of them. Several independent lines of evidence indicate that about 30% of the unfolded state refolds by the minor pathway, in which the slowest observed phase is attributed to the isomerization of Pro-93. The major pathway involves 50% of the unfolded state; the reason why it refolds slowly is not apparent. A native-like intermediate is formed considerably more rapidly in the major slow-refolding pathway, compared to the minor pathway.

Preliminary experiments (Biringer & Fink, 1982a,b, 1988; Fink, 1986; Fink & Painter, 1987) have indicated that investigation of the folding of proteins in aqueous methanol cryosolvents at subzero temperatures may provide an important new method for determination of the pathway of protein folding. In particular, this approach facilitates the stabilization of partially folded states with exposed hydrophobic surfaces. By monitoring probes reflecting the state (native or solvent exposed) of different regions of the molecule, it is possible to map out the details of the folding pathway.

The work reported herein is a continuation of our studies on the folding of ribonuclease A. Complications exist since unfolded ribonuclease is a mixture of slow (U_S)[1] and fast (U_F) folding species (Garel & Baldwin, 1973). The fast-refolding molecules constitute 20% of the total in aqueous solution, and the remaining slow-folding molecules are of at least two distinct types (Schmid & Blascheck, 1981). It was first proposed by Brandts et al. (1975) that the slow-folding molecules differ from the fast-folding ones by virtue of proline cis/trans isomerization.

There have been several reports that an intermediate with properties very similar to native RNase is formed during the folding under native conditions (Kim & Baldwin, 1982; Cook

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[1] Abbreviations: RNase, ribonuclease A; proton NMR, proton nuclear magnetic resonance; pH*, apparent pH of mixed aqueous–organic solvent; U, unfolded state; N, native state; nitro-Tyr-115, derivative of ribonuclease A in which Tyr-115 has been nitrated; Gdn·HCl, guanidine hydrochloride; ΔF, change in fluorescence; IEF, isoelectric focusing.

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