Separation of the Nativelike Intermediate from Unfolded Forms during Refolding of Ribonuclease A†

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ABSTRACT: In an effort to determine structural properties of the nativelike intermediate (i.e., I_N) which forms during the refolding of RNase A, refolding samples were subjected to rapid HPLC gel filtration which allowed us to separate I_N from unfolded forms of RNase. The comparison of these samples, enriched in I_N and depleted of unfolded forms, with unseparated control samples at the same stage of refolding allowed certain conclusions to be drawn concerning the properties of I_N. First, the results show that the transition from I_N to native RNase occurs with only small changes in fluorescence. This means that the major fluorescence changes seen during normal refolding experiments must be associated with changes in proline isomerization of unfolded species and/or with the refolding step itself but not with the $I_N \rightarrow N$ step. Second, the fluorescence assay for isomerization of proline-93 shows that I_N exists with proline-93 in a state of isomerization identical with or very similar to native RNase; i.e., proline-93 is cis in I_N and not trans as suggested by others. All results are semiquantitatively consistent with our earlier refolding model and not nearly so consistent with alternative models which assume that most or all of the slow-refolding forms of RNase have proline-93 in the incorrect trans state.

Although more experimental information is available on the slow-refolding reaction of RNase A than for any other protein, there still is a lack of agreement on some important points of interpretation. Schmid (1981) has suggested that the fluorescence-detected refolding under strongly native conditions monitors the I_N (nativelike intermediate, or N_{CY} state in our terminology) to N reaction while the absorbance-detected refolding monitors the U_S (slow-refolding species) to I_N reaction. However, refolding data from our laboratory (Lin & Brandts, 1983b, 1984, 1987a,b) indicated that both fluorescence and absorbance monitor the same refolding processes (i.e., U_S to I_N and U_S to N) and that the I_N to N reaction is nearly silent for both methods of detection. More recently, it was proposed (Schmid, 1986; Schmid et al., 1986) that the double-jump fluorescence unfolding assay detects the I_N to N reaction and that the entire slow phase of RNase refolding (i.e., 80%) involves unfolded molecules with incorrect trans isomers of proline-93. On the other hand, our results from the fluoresence unfolding assay (Lin & Brandts, 1987a,b) as well as the results from isomer-specific proteolysis (Lin & Brandts, 1983a, 1987b) were consistent with the idea that only 25–30% of the unfolded molecules have the incorrect trans form of proline-93 and that these isomerize prior to refolding and not in the I_N to N step.

The best way to resolve these discrepancies is to separate the refolded RNase (i.e., native and nativelike intermediate) from unfolded RNase very quickly after refolding is started and then to directly examine their fluorescence properties individually in both refolding and unfolding buffers. If the fluorescence change during refolding is due exclusively to the I_N to N reaction, then removal of the unfolded RNase from I_N and N should have no effect on the fluorescence refolding amplitude when compared to a control sample with the same amount of I_N but without removal of the unfolded RNase. Likewise, any change in the amplitude from the fluorescence unfolding assay upon removal of the unfolded RNase from the sample should reveal whether that assay detects the I_N to N reaction or whether it detects isomerization of proline-93 primarily in the unfolded species.

Early in this study, we discovered that it is difficult to completely separate the unfolded RNase from I_N and N in a short time, particularly for samples treated with a prior $(NH_4)_2SO_4$ pulse to populate I_N. Various fractional precipitation and affinity column methods were tried in this study, and they failed to separate the unfolded and refolded RNase in the presence of $(NH_4)_2SO_4$. We finally settled on the use of HPLC gel filtration for the separation, performed at low temperature and in the presence of high urea concentration to slow down the I_N to N and refolding reactions during the separation. Although a complete separation of folded and unfolded forms was not possible, the method does remove nearly two-thirds of the unfolded RNase from refolded RNase in a short time and thereby permits one to differentiate the effect of the I_N to N reaction on fluorescence change from the effect of the unfolded RNase.

Materials and Methods

Materials. Bovine RNase (catalog no. R-5500), purchased from Sigma Chemical Co., was directly used without further purification. Cytidine cyclic 2′,3′-phosphate (cCMP, sodium salt; catalog no. C-9630) and cacodylic acid (sodium salt; catalog no. C-0250) were also obtained from Sigma Chemical Co. Urea (ultrapure; catalog no. 821527) and guanidine hydrochloride (Gdn-HCl, ultrapure; catalog no. 820539) were purchased from Schwarz/Mann. All other chemicals are of reagent grade, and their sources have been cited in previous papers (Lin & Brandts, 1987a,b).

Populating the Nativelike Intermediate and Separation of the Unfolded and Refolded RNases by HPLC Gel Filtration. The $(NH_4)_2SO_4$ pulse was carried out at 0 °C in ice. All pipets and syringes used for mixing and injection were also cooled to 0 °C. The detailed procedures for populating the nativelike intermediate are as follows: First, 20 μL of unfolded RNase (in 5.0 M urea, pH 2.0 at 0 °C) was mixed with 100 μL of

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1.2 M $(NH_4)_2SO_4$/0.2 M cacodylate buffer (pH 6.8 at 0 °C) for 22–24 s. At the end of the pulse, 120 μL of 7.1 M urea/0.07 M NaOAc solution (pH 6.5 at 0 °C) was quickly added to the RNase solution to slow down refolding and the $I_N \rightarrow N$ reaction. The solution was either further diluted (20 times) with 5 M urea/0.1 M NaOAc solution (pH 6.0) for the subsequent assays as a control run or immediately injected into the HPLC column for separation of the unfolded and refolded RNases.

An HPLC system consisting of a high-pressure metering pump (A-30-S, Rainin Instruments), a pressure gauge, a Valco sample injector [with a 100-μL sample loop and a Bio-Sil-TSK-125 gel filtration HPLC column (300 × 7.5 mm; Bio-Rad)], and a UV detector (V^4 absorbance detector, ISCO) was set up in a refrigerator thermostated at 2 °C. The column temperature was further controlled at 0 °C with a water jacket by circulating coolant from a circulator. The eluant (5 M urea/0.1 M NaOAc solution, pH 6.0) was in an ice bucket at 0 °C. The flow rate was set at 1.35 mL/min for all experiments. RNase eluated from the column was collected in a test tube (at 0 °C) for the following assays.

Assay for Unfolded and Refolded RNases. The amount of the unfolded and refolded RNases in a solution was determined by activity assay, using cCMP as substrate. The experimental procedures were similar to those previously described (Schmid & Blaschek, 1981; Lin & Brandts, 1983a). The details are as follows: RNase solution eluted either from the column or as a control sample without passing through the column (150 μL, at 0 °C) was pipetted into a thermostated cuvette (at 10 °C) containing 2.0 mL of cCMP (~0.3 mg/mL, in 4 M urea/0.1 M Tris, pH 7.2). After the solution was mixed for a few seconds with a spatula, the change in absorbance at 290 nm was followed with a Cary 14 spectrophotometer. The relative amounts of the unfolded and refolded RNases in a sample at the moment of assay were determined from the initial slope of each sample compared to that of the fully reactivated sample, whose activity was identical with native RNase.

Assay for the Native (N) and the Nativelike Intermediates (I_N). The Gdn-HCl unfolding assay at neutral pH, developed by Schmid (1983), was used to quantitate the relative amounts of the native and nativelike intermediates in solution. RNase solution (500 μL, at 0 °C) immediately after elution from the HPLC, or from a control sample, was pipetted into a thermostated cuvette (at 10 °C) containing 1.6 mL of 6 M Gdn-HCl/0.05 M cacodylate buffer (pH 6.0). After the solution was mixed for a few seconds with a spatula, the absorbance change at 287 nm was continuously followed until equilibrium. Two kinetic phases were seen if both the native and nativelike intermediates were present in the solution since the nativelike intermediate is unfolded faster than the native RNase in such buffers. The relative amounts of the native and nativelike intermediates in the solution were obtained from a semilog plot of the absorbance change vs time as described by Schmid (1983).

Fluorescence Refolding and Unfolding Assay. The instrumentation for fluorescence detection of RNase refolding and fluorescence unfolding experiments has been previously described (Lin & Brandts, 1983b, 1987a,b). For the fluorescence refolding assay, RNase solution (250 μL, at 0 °C) eluted from HPLC (or from a control sample) was pipetted into a thermostated cuvette (at 10 °C) containing 2.0 mL of 3 M urea/0.06 M NaOAc (pH 6.0) and mixed for a few seconds with the pipet tip. The change in fluorescence intensity was continuously monitored until equilibrium. For the fluorescence unfolding assay, the same procedures were followed except that 250 μL of RNase solution was mixed with 2.0 mL of 5 M Gdn-HCl/0.1 M glycine (pH 1.8).

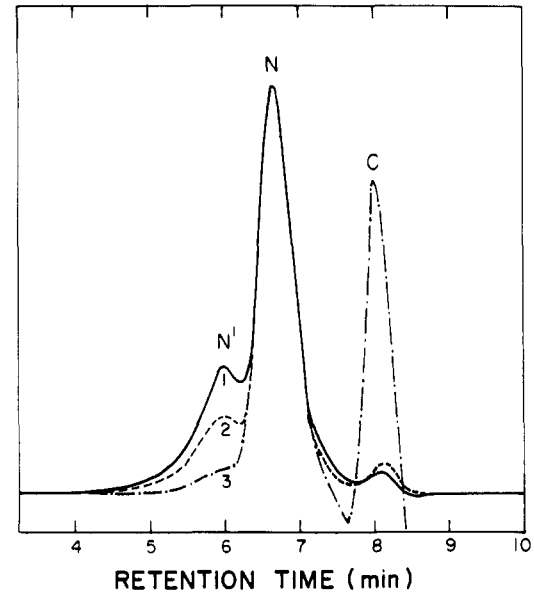

FIGURE 1: HPLC gel filtration of refolded RNase with different initial concentrations. The initial unfolded RNase concentrations were as follows: profile 1, 0.015 M; profilie 2, 0.006 M; profile 3, 0.0012 M. The height of the N peak has been normalized to the same magnitude to facilitate the comparison of N′. See the text for experimental details and the interpretation of peaks N and N′.

Results and Discussion

Formation of a Folded Aggregate during Refolding in $(NH_4)_2SO_4$. To carry out the experiments described later, the RNase solutions had to be strongly diluted at several points both before and after separation of the folded and unfolded forms on the HPLC column. To ensure that the final solutions contained adequate concentrations for spectroscopic monitoring, it was necessary to employ a fairly high RNase concentration for the initial precolumn refolding during the short $(NH_4)_2SO_4$ pulse. In carrying out control experiments, it was found that refolding at high RNase concentration in the presence of ammonium sulfate led to the formation of a minor contaminant in "fully-refolded" RNase, which migrated at a larger apparent molecules weight than the native form. This is illustrated in the HPLC profiles in Figure 1, obtained at three different RNase concentrations of 0.0012, 0.006, and 0.015 M. In these experiments, unfolded RNase at the indicated concentration was diluted 6-fold to become 1.0 M in ammonium sulfate (pH 6.5) and then allowed to fully refold (20 min) at either 0 °C or room temperature (results at the two temperatures were virtually identical). The samples were then placed on the HPLC column (0 °C) and eluted with 5 M urea, pH 6.0. As seen in Figure 1, the bulk of the protein emerges at the native peak position, labeled N, at 6.8 min but a small, concentration-dependent amount appears in the N′ peak at 5.9 min (the peak labeled C is due to cacodylate buffer). The maximum height of the N peak has been normalized to 1.0 in all cases to facilitate comparison, and it is seen that about 20% of the RNase is in the N′ peak at the highest concentration (profile 1), 8% at the intermediate concentration, (profile 2), and only a couple percent at the lowest concentration.

Samples of RNase from the left shoulder of the N′ peak were collected and characterized immediately. Although the N′ species was stable for long periods of time (at either low

temperature or room temperature) at the conditions under which formation occurred, rechromatography experiments showed that at lower concentrations (ca. 1.0×10^{-5} M RNase and room temperature) the HPLC migration characteristics of N′ changed slowly to that of the native form with a time constant of about 30 min. The enzymic activity of N′ was about 80% of native RNase and increased to 100% when N′ was converted to native protein by incubation at room temperature. Fluorescence unfolding experiments on N′ showed an amplitude which was 80% of that observed for the native form.

These experiments suggest that N′ consists of well-folded, active RNase in an aggregated state—perhaps mostly dimer. It could be similar to the active RNase dimer previously reported by Fruchter and Crestfield (1965). Further characterization was not attempted since it was irrelevant to the major concern in these studies.

Because the migration characteristics of the N′ peak are very close to those of unfolded RNase, it was impossible to chromatographically separate the two. In order to minimize the formation of N′ in the experiments which follow, the RNase concentration during the $(NH_4)_2SO_4$ pulse was kept as low as possible, corresponding to the intermediate concentration in Figure 1. The amount of N′ formed in the following experiments could be substantially less than in the above control experiments, since the duration of the $(NH_4)_2SO_4$ pulse was only 22–24 s, rather than the 20 min used in the controls. Even so, characterization of the peak, described below, does suggest some contamination by N′ so we prefer not to draw any conclusions based on the properties of the "unfolded peak". Fortunately, it is the folded peak which contains the nativelike intermediate in which we are interested, and this peak is not appreciably contaminated by N′.

Column Separation of Unfolded and Refolded RNases. In a typical refolding experiment, unfolded RNase was first pulsed in 1.0 M $(NH_4)_2SO_4$ (0.17 M cacodylate buffer, pH 6.5) for 22–24 s at 0 °C to populate the nativelike intermediate. Immediately after the pulse, the urea concentration of the sample was increased to 4 M to slow down refolding and the sample immediately injected onto the HPLC column (0 °C) to separate the folded and unfolded forms. A typical separation profile is shown in Figure 2 (profile 1), along with a control experiment (profile 2) carried out identically except that RNase was pulsed in 0.17 M cacodylate buffer with no $(NH_4)_2SO_4$. Two overlapping protein peaks are seen. The first at 5.8 min is primarily due to unfolded RNase while the second at 6.8 min is largely due to refolded RNase. However, because of incomplete peak separation and slow interconversion of forms occurring while the sample is on the column, the folded peak will be contaminated with some of the unfolded form and vice versa. Also, the unfolded peak will be contaminated by small amounts of the N′ species, discussed above, which forms during the short ammonium sulfate pulse and elutes at nearly the same position as the unfolded form. The precise composition of the peaks will be discussed in detail below. However, the important conclusion to be drawn immediately from comparison of curves 1 and 2 in Figure 2 is that the nativelike intermediate I_N, extensively populated under conditions of curve 1 which employed the $(NH_4)_2SO_4$ pulse, migrates in the second peak.

Quantitation of the Amounts of the Unfolded, Native, and Nativelike Intermediate Forms in Various Samples. To reduce cross-contamination of peaks, only samples from the left shoulder (5.5 min) of the first peak and the right shoulder (7.0 min) of the second peak (indicated by arrows in Figure 2) were collected for characterization and for carrying out unfolding and refolding assays. Our main interest is the second peak since it contains the I_N species. The first peak was characterized to some extent, however. Immediately after elution from the column, it was found to have ca. 70% unfolded RNase by its fluorescence refolding amplitude and to have ca. 20% enzymic activity which presumably is due to the presence of N′ species along with some contamination from the second peak.

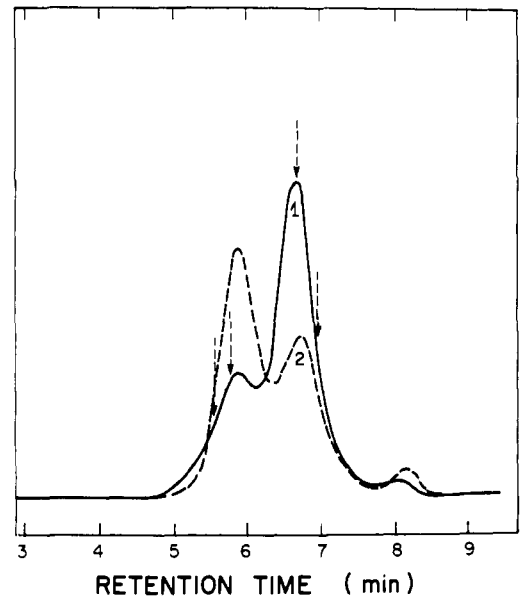

FIGURE 2: HPLC gel filtration of the unfolded and refolded RNases at 0 °C in 5 M urea/0.1 M NaOAc, pH 6.0. Unfolded RNase (0.006 M in 5 M urea, pH 2.0, at 0 °C) was first pulsed in 1.0 M $(NH_4)_2SO_4$, pH 6.5 (profile 1), or in 0.17 M cacodylate, pH 6.5 (profile 2), for 22–24 s at 0 °C. Immediately after the pulse, the urea concentration of the sample was increased to 4 M to slow down refolding and the I_N to N reaction, and the sample was immediately injected onto the column. Fractions of each peak were collected between the arrow indicators.

Immediately after collection from the column, the second peak assayed from 92% to 95% activity in three separate elutions, when compared to the same sample after being refolded for 1.5 h at room temperature (which has the same specific activity as native RNase). Assuming that the nativelike intermediate has the same activity as native RNase, this suggests that the second peak is still contaminated with ca. 7% of the unfolded RNase even after column separation. Control experiments showed that for samples treated identically but not separated on the column, there was 23% unfolded (i.e., inactive) RNase immediately after the pulse and 20% unfolded RNase at a time which corresponds to elution of the second peak. Thus, the column separation results in substantial enrichment of folded forms of RNase in the second peak.

The relative amounts of native RNase and nativelike intermediate in the second peak, immediately after elution, were determined by using the absorbance unfolding assay developed by Schmid (1983). As seen in Figure 3 (diamonds), these samples show both a fast and a slow phase, corresponding to the unfolding of the nativelike intermediate and native form, respectively. The relative amplitudes suggest that 35% *of the folded molecules* in the second peak correspond to the nativelike intermediate while 65% are native RNase. A control run (circles) on an aliquot allowed to sit at room temperature for 1.5 h after elution and before assay shows only the slow phase, as would be expected if all of the nativelike intermediate had converted to native RNase. Two other control experiments (not shown) were carried out. In the first, identically treated

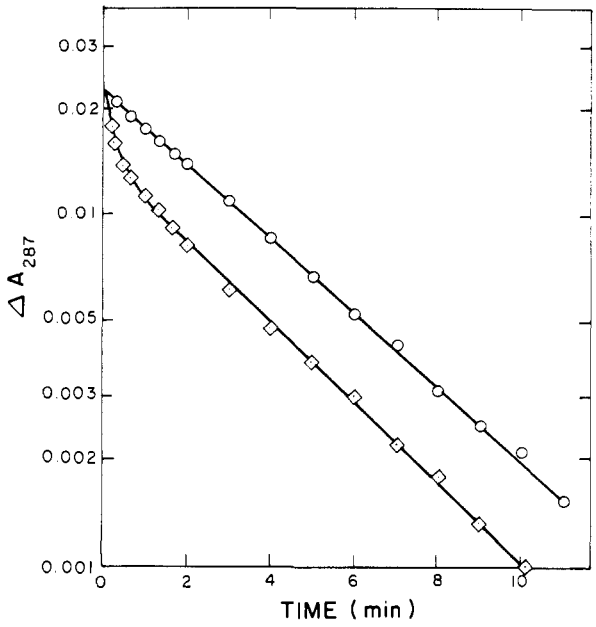

FIGURE 3: Neutral pH, Gdn-HCl unfolding assay for the native (N) and nativelike intermediate (I_N) of the second peak eluted from the HPLC column. Diamonds represent the data for the sample assayed immediately after elution, while circles represent the data for a sample (control run) which sat at room temperature for 1.5 h after elution and before assay.

samples were assayed immediately after the pulse. These contained about 65% *of folded forms* as the nativelike intermediate, showing that less than half of the intermediate is converted to native RNase while on the column. In the second, samples were not injected onto the column but were allowed to incubate at column conditions for an equivalent time. These samples also showed that about 35% *of the folded forms* were nativelike intermediate, indicating that the rate of the $I_N \rightarrow N$ reaction was unaffected by column separation.[1]

These results from activity and unfolding assays permit estimates of the amount of native, nativelike intermediate, and unfolded forms in three different refolding samples, i.e.: (I) immediately after pulse, unseparated, 27% N, 50% I_N, 23% U; (II) 7 min after pulse, unseparated, 52% N, 28% I_N, 20% U; (III) 7 min after pulse, folded peak, 60% N, 33% I_N, 7% U.

Fluorescence Refolding Studies. In view of the fact that the above three samples contain different amounts of the unfolded and intermediate forms of RNase, the refolding of each to fully native RNase is of interest. The data in Figure 4 show the direct fluorescence refolding for each of these samples. Also shown is a control experiment for refolding in the same final buffer, but omitting the $(NH_4)_2SO_4$ pulse. Relative to the control run, it is seen that each of the three pulsed samples exhibits a faster refolding phase in addition to the slow phase seen in the unpulsed control. This is expected and can be attributed to mass action effects produced by the pulse, as already discussed in detail (Lin & Brandts, 1987a). For present purposes, our major interest is in the refolding amplitudes for the three samples, which are very different as

[1] It should be mentioned that the relaxation times for I_N in these unfolding assays were the same for samples separated on the column as for unseparated samples, suggesting that the separation and sampling procedure produced no changes in the average properties of I_N. Also, relaxation times for both of these samples were identical with those seen for unseparated samples formed from refolding at much lower RNase concentration, showing that the small amount of aggregation, discussed earlier, was not accompanied by any obvious changes in the properties of monomeric I_N.

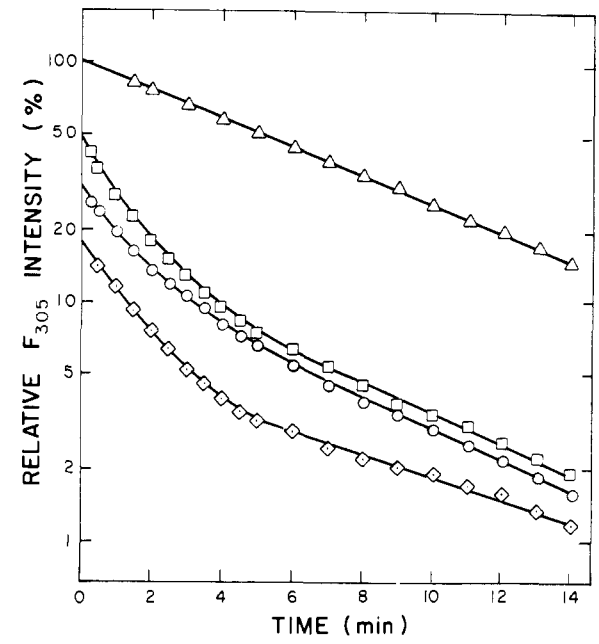

FIGURE 4: Direct fluorescence refolding assay for various RNase samples. (Squares) Sample I (i.e., assayed immediately after pulse, unseparated); (circles) sample II (i.e., assayed 7 min after pulse, unseparated); (diamonds) sample III (i.e., the second peak, 7 min after pulse, separated); (triangles) control sample (i.e., fully unfolded sample, no pulse, but assayed under the same final solution conditions). All data are normalized to the same final RNase concentration. The fluorescence change ($\Delta F_{305}/F_\infty$) of the control sample is assumed to be 100%.

Table I: Experimental and Predicted Amplitudes for Direct Fluorescence Refolding of Various RNase Solutions

sample	exptl amplitude (%)	calcd amplitudes (%) L–B model[a]	calcd amplitudes (%) alternative model[b]
unfolded control	100	100	100
I	47	48	91
II	30	33	60
III	17	12	50

[a] Average deviation = 3%. [b] Average deviation = 37%.

Table II: Experimental and Predicted Amplitudes for the Fluorescence Unfolding Assay for RNase Refolding

sample	exptl amplitude (%)	calcd amplitudes (%) L–B model[a]	calcd amplitudes (%) alternative model[b]
native control	100	100	100
I	19	15	9
II	56	53	40
III	78	82	50

[a] Average deviation = 4%. [b] Average deviation = 18%.

shown in Table I. Sample III, strongly depleted of unfolded RNase by column separation, shows the smallest extrapolated ΔF value of about 17% relative to the control. Sample II, treated identically with sample III but not depleted of unfolded forms on the column, shows a much larger refolding amplitude of 30%. Sample I, put into the final refolding buffer immediately after the pulse, has the largest refolding amplitude of 47%. It is seen immediately from these results, particularly by comparison of samples II and III, that the fluorescence refolding amplitude correlates very well with the amount of unfolded RNase in the sample and correlates very poorly with the amount of I_N.

Refolding Monitored by the Fluorescence Unfolding Assay. Since the fluorescence unfolding assay of Schmid et al. (1986)

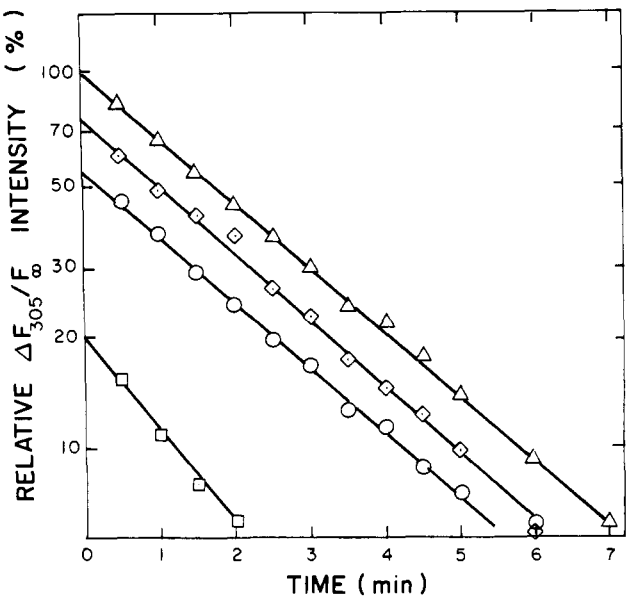

FIGURE 5: Fluorescence unfolding assay for various RNase samples. (Squares) Sample I; (circles) sample II; (diamonds) sample III; (triangles) control sample (i.e., fully refolded RNase). The $\Delta F_{305}/F_\infty$ values of the control sample are assumed to be 100%.

monitors exclusively the isomerization of proline-93 during refolding, it offers a more critical test of folding models than does the previous assay by direct fluorescence refolding. All three of the above samples were subjected to the fluorescence unfolding assay, along with the completely refolded control, and the results are shown in Figure 5 and Table II. The extrapolated normalized amplitude, $\Delta F/F_\infty$, for the fully refolded control is again adjusted to 100%.

Sample III, depleted of unfolded protein by column separation, shows an amplitude of 78% of the fully refolded control. This result shows that the folded forms in the second peak contain proline-93 in a state of isomerization very similar to that existing in the fully refolded protein, even though more than one-third of the folded forms are in the I_N state and not the native state. This argues strongly against all refolding models where I_N is assumed to contain the incorrect trans isomer for proline-93. Sample II, treated identically with sample III but not depleted of unfolded forms by column separation, shows a much smaller amplitude of 56% which reemphasizes that it is the unfolded forms and not I_N which contain incorrect isomers for proline-93. Sample I, as expected since very little time elapsed between the onset of refolding and the assay, showed the smallest normalized amplitude of 19%.

Comparison of Refolding Data with Model Predictions. The abbreviated folding model of Lin and Brandts (1984, 1987) is outlined in eq 1, The equilibrium concentrations of

$$\begin{array}{ccccc} N_{CX} & \rightarrow & D_{CX}\ (0.20) & \rightleftharpoons & D_{TX}\ (0.074) \\ \uparrow & & \uparrow\downarrow & & \uparrow\downarrow \\ N_{CY} & \rightarrow & D_{CY}\ (0.53) & \rightleftharpoons & D_{TY}\ (0.196) \end{array} \qquad (1)$$

the various unfolded denatured forms are given in parentheses. In this scheme, the CT process occurs horizontally among unfolded forms and corresponds to the cis–trans isomerization of proline-93 while the XY process occurs vertically and, although its exact nature is unknown, it may be due to a process other than proline isomerization (Lin & Brandts, 1984). The fast-refolding form D_{CX} quickly forms native RNase, N_{CX}, under all refolding conditions while, in the presence of $(NH_4)_2SO_4$, the nativelike intermediate N_{CY} (i.e., I_N) is formed rapidly from D_{CY}, and this is in turn converted slowly into the native form. Relative to a value of 0 for the native and nativelike intermediate forms, the two unfolded forms containing proline-93 in the cis isomer (i.e., D_{CX} and D_{CY}) were shown (Lin & Brandts, 1983) to have a fluorescent intensity of 1.0 while the two trans forms have an intensity of 2.5.

According to this model, application of the $(NH_4)_2SO_4$ pulse to the unfolded mixture leads to rapid refolding of D_{CX} to N_{CX} and of D_{CY} to the nativelike intermediate N_{CY}, so that immediately after the pulse nearly 100% of the still unfolded protein consists of the species D_{TX} and D_{TY}, which have proline-93 in the trans form. After dilution into high urea, the nativelike intermediate will no longer form, and both the XY and CT processes in the unfolded state will be slowed down considerably. At the time the second peak is collected off the column and assayed (i.e., 7 min after the end of the pulse), the unfolded RNase remaining still contains ca. 70% of the trans form of proline-93,[2] which is substantially higher than the equilibrium amount (ca. 27%; cf. eq 1).

Using the above information with no additional assumptions, we can use the model in eq 1 to calculate the expected amplitudes, for both direct fluorescent refolding and refolding monitored by the fluorescence unfolding assay, for any solution where the initial amounts of native, nativelike intermediate, and unfolded forms are known. Table I shows the calculated amplitudes for fluorescence refolding, based on the Lin–Brandts model, for solutions I–III. In all cases, the calculated amplitudes show excellent agreement with the experimental amplitudes with an average deviation of only 3% of the amplitude for total refolding. This is probably within experimental error.

The calculated amplitudes for the fluorescence unfolding assay for RNase refolding, which we assume monitors only the isomerization of proline-93, are shown in Table II for the Lin–Brandts model. In spite of the very large variations in these experimental amplitudes for solutions I–III from 19% to 78%, the calculated amplitudes agree very nicely with an average deviation of only 4%. This is especially noteworthy in the comparison between solutions II and III, which differ only in the fact that solution III has been depleted of unfolded forms by column separation.

We have also tried to reproduce both sets of experimental results with other models based on different assumptions. One alternative model of interest is based on assumptions along the lines of those proposed by Schmid, i.e.: (1) All slow-refolding forms (i.e., 80% of the total) have a trans isomer for proline-93. (2) The nativelike intermediate I_N still has a *trans*-proline-93 which converts to cis during the $I_N \rightarrow N$ step. (3) In direct fluorescence refolding (Table I), it is assumed that only the isomerization of proline-93 is monitored. In the fluorescence unfolding assay (Table II), it is assumed (as in the L–B model) that only the isomerization of proline-93 is monitored. With only these assumptions, amplitudes may be predicted from the amounts of N, I_N and U estimated to exist in solutions I–III from the direct measurements discussed earlier. The calculated amplitudes for this alternative model are also shown in Table I and II for direct fluorescence refolding and for the fluorescence unfolding assay for refolding, respectively. The predicted results from this alternative model

[2] Earlier studies (Lin & Brandts, 1983b) suggest a relaxation time of 220 s for the CT phase in 5 M urea at 10.5 °C and an E_a value of 17 000 cal/mol. Corrected to the present temperature of 0 °C, the relaxation time is ca. 700 s. Simulations from our model then show that if 100% of the unfolded forms have a *trans*-proline-93 at zero time, then 70% of the unfolded forms will have a *trans*-proline-93 after 7 min of refolding.

show exceedingly poor agreement with the experimental results with an average deviation of 37% for the direct refolding data in Table I and an average deviation of 18% for the fluorescence refolding assay results in Table II.

SUMMARY

In this study, rapid HPLC gel filtration has been used to separate the unfolded and refolded forms of RNase subsequent to a short $(NH_4)_2SO_4$ pulse, using column conditions which slow down the I_N to N reaction as well as the refolding steps. Using this technique, it was possible to obtain fractionated RNase samples which are largely depleted of unfolded forms (ca. 7%) but which still have I_N strongly populated (ca. 33%) and then to compare these samples with others which have not been depleted of unfolded forms. The results of these studies clearly lead to two qualitative conclusions: (1) Samples depleted of unfolded forms but containing large amounts of I_N show only a small amplitude for fluorescence refolding. Therefore, the major part of the fluorescence change during slow refolding occurs prior to the I_N to N step, i.e., either during isomerization in the unfolded forms or during the refolding step itself. (2) Samples containing large amounts of I_N but depleted of unfolded forms show a fluorescence unfolding amplitude very similar to native RNase. It follows that I_N must therefore have the same cis isomer for proline-93 as does native RNase if this unfolding assay truly measures only the isomerization of proline-93, as thought.

At the quantitative level, all of the results were shown to be consistent with our earlier model and inconsistent with an alternative model which assumes that the entire slow phase is due to isomerization of proline-93 and that I_N contains the incorrect isomer for proline-93.

Registry No. RNase, 9001-99-4.

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Experimental Charge Measurement at Leaving Oxygen in the Bovine Ribonuclease A Catalyzed Cyclization of Uridine 3′-Phosphate Aryl Esters†

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ABSTRACT: The title esters are demonstrated to be specific substrates of bovine pancreatic ribonuclease A (EC 3.1.27.5). The Brønsted dependence of k_{cat}/K_m at pH 7.50 for the enzyme-catalyzed cyclization versus the pK_a of the leaving phenol exhibits two regression lines of almost identical slope for respectively 2-chlorophenols and 2,6-unsubstituted phenols: $\log k_{cat}/K_m = -0.20pK_a^{ArOH} + 5.47$ ($n = 5$, $r = 0.957$); $\log k_{cat}/K_m = -0.17pK_a^{ArOH} + 5.79$ ($n = 4$, $r = 0.965$). Comparison of the Brønsted β_{lg}'s with that for the standard reaction where imidazole catalyzes the cyclization ($\beta_{lg} = -0.59$) indicates considerably less development of negative charge on the leaving oxygen in the enzyme case, providing experimental evidence for the hypothesis that electrophilic assistance is involved in catalysis. The existence of two essentially parallel Brønsted correlations is not reflected in the standard reaction of substrate with imidazole. Modeling studies indicate that the phenyl ring of the substrate can take up a range of positions away from the active site; the presence of ortho chloro substituents considerably restricts the motion of the phenyl leaving group.

Bovine pancreatic ribonuclease A (EC 3.1.27.5) has been studied intensively with regard to its structure and the structure of enzyme–effector complexes [see, for example, Howlin et al. (1987) and Richards and Wyckoff (1971)]. The currently accepted mechanism (Figure 1) (Fersht, 1985) involves formation and decomposition of the cyclic 2′,3′-nucleotide by closely similar processes. Formation of the intermediate is thought to involve histidine-119 has an acceptor acid for the leaving 5′-hydroxyl group of the adjacent nucleotide; histidine-12 could function as a base to remove the proton of the 2′-hydroxyl group, thus assisting its intramolecular attack on the phosphodiester. The mechanism involving acid–base catalysis was inferred from the pH dependence of the kinetics (Deavin et al., 1966).

We present here a study of the kinetics of the ribonuclease A catalyzed reaction of the cyclization of uridine 3′-(aryl phosphate) substrates (I). The sensitivity of k_{cat}/K_m to leaving group substituent gives rise to a Brønsted β_{lg} coefficient that may be used to determine the change in charge at the leaving oxygen atom from ground state to the transition state of the

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