Evidence for the Existence of Three or More Slow Phases in the Refolding of Ribonuclease A and Some Characteristics of the Phases†

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ABSTRACT: The slow refolding kinetics of RNase A have been analyzed, by using a nonlinear least-squares program for deconvoluting the kinetic phases and applying statistical tests for quality of fit. It is found that a minimum of three slow phases are required to fit the kinetic data properly, and this is true whether the method of detection is absorbance or fluorescence. Since the number of phases and the relaxation times for each phase are independent of the method of detection, it is concluded that the same three rate-limiting processes are seen by absorbance and fluorescence. These phases correspond to the XY, CT, and ct phases described in our earlier studies. The fact that fluorescence-detected kinetics are somewhat slower than absorbance-detected kinetics is a trivial effect due not to differences in relaxation times but to the fact that the amplitude of the CT phase is enhanced in fluorescence measurements, at the expense of the faster XY phase, because of intrinsic fluorescence changes associated with the isomerization of proline-93. By use of a new double-jump technique [Schmid, F. X., Grafl, R., Wrba, A., & Beintema, J. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 872], it is shown that proline-93 isomerizes as the rate-limiting step in only one of the three phases, the CT phase, and that this phase involves only 25–30% of the RNase molecules. There is still no indication as to the molecular events that occur in the large, ammonium sulfate dependent XY phase, which is the pathway for formation of the nativelike intermediate.

The slow refolding reaction of RNase A, which is thought to involve 80% of the unfolded molecules, is known to be a complex kinetic process when monitored by either absorbance or fluorescence changes at low temperature (Cook et al., 1979; Schmid, 1981; Lin & Brandts, 1983b, 1984; Mui et al., 1985; Schmid et al., 1986). Because of this complexity, considerable differences still exist in the literature concerning not only the interpretation of results at the molecular level but even concerning the number of slow phases that exist as well as their relative amplitudes and relaxation times. Refolding data from our laboratory (Lin & Brandts, 1983b, 1984) have been interpreted in terms of three slow phases (i.e., the XY, CT, and ct phases) with respective amplitudes of ca. 50:25:5 in absorbance refolding. The same three phases were found in fluorescence refolding and had experimentally identical relaxation times, although the relative amplitude of the CT phase was much larger at the expense of the XY phase. Parallel studies using isomer-specific proteolysis suggested that the CT phase was rate-limited by isomerization of ca. 25–30% of the unfolded molecules that had proline-93 in the incorrect trans configuration and that the small ct phase was rate-limited by the isomerization of ca. 5% of the molecules that contained proline-114 in the incorrect trans configuration. The structural events involved in the XY phase were not assigned, although it was suggested that they may not involve proline isomerization. The large differences in relative phase amplitudes in fluorescence and absorbance refolding were attributed to intrinsic changes in fluorescence associated with the isomerization of Tyr-92–Pro-93, which is silent in absorbance. It was also concluded that I_N, the nativelike intermediate suggested by others (Cook et al., 1979; Schmid, 1983), was formed during the XY phase[1] when refolding took place under strongly native conditions. Formation of I_N is apparently responsible for the strong increase in the rate of the XY phase, seen in both absorbance and fluorescence, as ammonium sulfate is added to the refolding buffer, while the CT and ct phases are not significantly affected. All of these results are consistent with the idea that the nativelike intermediate does not contain incorrect isomers for either proline-93 or proline-114.

On the other hand, Schmid and co-workers (Schmid, 1981; Schmid et al., 1986) resolve the slow refolding reaction into only two kinetic phases: a major phase (65–70% of the amplitude) resulting from the refolding of U_s^{II} and a minor phase (10–15%) from the refolding of U_s^{I}. In contrast to our observations, Schmid (1981) found that the relative amplitudes of the two phases are identical in absorbance- and fluorescence-detected refolding but that the difference lies in the rate constants: fluorescence-detected refolding is much slower than absorbance-detected refolding and ammonium sulfate acts to accelerate only the absorbance-detected kinetics. Schmid (1981) concluded that absorbance-detected refolding monitors the $U_s \rightarrow I_N$ reaction under strongly native conditions and that the fluorescence-detected refolding monitors the $I_N \rightarrow N$ reaction. More recently, Schmid et al. (1986) suggest that both U_s^{I} and U_s^{II} have the incorrect trans isomer of proline-93 and that, under strongly native conditions, refolding to the fully active, nativelike intermediate proceeds for both of these species before the correct isomers are formed. Thus, the entire slow phase is assumed to involve unfolded molecules with incorrect isomers of proline-93, in contrast with the results from isomer-specific proteolysis, where only 30% of the unfolded molecules were found to have a trans proline-93.

Nearly all of the kinetic results available on RNase refolding in the past have been analyzed by the method of peeling-off exponentials. There is considerable uncertainty involved in this method, due to the much larger errors that are propagated into the semilogarithmic plots close to the equilibrium point

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[1] Consistent with our previous use (Lin & Brandts, 1983b, 1984) of the term "XY phase", it will be used here to denote the kinetic process by which major refolding occurs spectroscopically for those unfolded molecules that contain an incorrect Y configuration. Under marginally stable conditions, this XY process as defined here results in formation of native RNase while, under very stable conditions, it results in formation of the nativelike intermediate.

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for each of the phases. This factor is very difficult to handle visually. In this paper, refolding data are analyzed by a nonlinear least-squares procedure with automatic weighting of errors in order to avoid this difficulty. Fluorescence and absorbance data are subjected to both two-phase and three-phase analysis under different conditions in order to try to understand the discrepancies in the literature. Finally, the isomerization of proline-93 is reinvestigated by using isomer-specific proteolysis in both the presence and absence of an enzyme (i.e., peptidylprolyl cis–trans isomerase) known to catalyze proline isomerization in an attempt to uncover artifacts that might have been present in the original study (Lin & Brandts, 1983a).

Materials and Methods

Materials. Bovine RNase (catalog no. R-5500, lot no. 25F-80501), trypsin (from porcine pancreas, catalog no. T-0134, log no. 44F-0150), and soybean trypsin inhibitor (catalog No. T-9003, lot no. 103F-8110), purchased from Sigma Chemical Co., were used without further purification. Pepsin (from porcine stomach mucosa, catalog no. P-7012, lot no. 60F-8057), also obtained from Sigma Chemical Co., was passed through a Sephadex G-25 column to remove autolysis products (Rose & Richard, 1979) and then lyophilized. Aminopeptidase P (APP) was purified from *Escherichia coli B* as described previously (Yaron & Berger, 1970; Lin & Brandts, 1979b). Ammonium sulfate (reagent grade; Fischer) was passed through Chelex-100 (Bio-Rad) to remove impurities. All other chemicals are reagent grade, and their sources have been cited in previous papers Lin & Brandts, 1979a,b, 1983a–c, 1984, 1985).

Purification of Peptidylprolyl Cis–Trans Isomerase (PPI). The enzyme was isolated from pig kidney obtained from a local slaughterhouse. The purification procedures are very similar to those of Fischer et al. (1984), with the following modifications: (1) The step of chromatographing with Bio-gel DEAE-A50 was omitted. (2) The enzyme solution, after having passed through Sephadex G-200, was concentrated and further purified on a Sephadex G-75 column. (3) A different substrate, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (Sigma Chemical Co.; catalog no. S-4511, lot no. 104F-0588), instead of L-glutanyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide, was used to assay for activity during purification. The final enzyme solution was about 70% pure as indicated by sodium dodecyl sulfate gel electrophoresis and had an activity of ca. 150 units/mg. Here, one unit is defined as the turnover rate of 1 μmol/min of the cis form of SU-Ala-Ala-Pro-Phe-pNa under the conditions described by Fisher et al. (1984).

Absorbance Measurement of RNase Refolding. A Cary 14 spectrophotometer equipped with a thermostated cell holder was employed to follow the absorbance change (at 287 nm) as a function of time during RNase refolding. All experiments were carried out at 10 °C. The unfolded RNase (in 5 M urea, pH 2.1) was diluted 16 times into sodium acetate buffer in the presence or absence of $(NH_4)_2SO_4$. The absorbance change at 287 nm was continuously monitored until total equilibrium was achieved (35–40 min). The mixing dead time was about 10–25 s. The final RNase concentrations were ca. 7.6×10^{-5} M. Procedures for stopped-flow experiments were as described earlier (Lin & Brandts, 1983b).

Fluorescence Measurement of RNase Refolding. These hand-mixing measurements were made on a Perkin-Elmer MPF-44 fluorescence spectrophotometer by using a 1-cm quartz cell thermostated at 10 °C. The instrument was run in the ratio mode with both slits set at 5 nm. The excitation and emission wavelengths were 268 and 305 nm, respectively. The final RNase concentration was either 2×10^{-5} M or 5×10^{-6} M. The stopped-flow experiments were carried out as described earlier (Lin & Brandts, 1983b).

RNase Refolding Detected by Fluorescence Unfolding Assay. The same instrument settings described above were used. The experimental procedures were similar to those of Schmid et al. (1986) except that urea and sodium acetate buffer, instead of guanidine hydrochloride and sodium cacodylate buffer, were used in our experiments. To initiate the refolding reaction, 25 μL of equilibrated unfolded RNase (~22 mg/mL, in 5 M urea, pH 2.1, at 10 °C) was diluted 16 times into 375 μL of 0.1 M sodium acetate buffer (pH 5.8, at 10 °C), in the presence or absence of 0.5 M $(NH_4)_2SO_4$. At various time intervals, 250-μL aliquots of the refolding solution were quickly pipetted into a cuvette (thermostated at 11 °C) containing 2.5 mL of 5.5 M urea solution (pH 2.1). After the solution was mixed with a spatula for 10 s, the increase of fluorescence intensity was continuously monitored until equilibrium. The total amplitude changes at various time intervals of refolding were obtained by semilogarithmic extrapolation to zero time. Absorbance of each of the final solutions was obtained to correct for concentration variations in each assay.

Assay for Isomerization of the Tyr-92–Pro-93 Bond of RNase in the Presence and Absence of PPI. This experiment is similar to one previously described (Lin & Brandts, 1983b). The detailed procedures are as follows: For initiation of the reaction, 20 μL of the unfolded RNase (~6×10^{-3} M, in 4.5 M urea, pH 2.1, at 10 °C) was pipetted into 90 μL of pepsin solution (120 mg/mL, in 4.5 M urea, pH 2.1, at 10 °C). After reaction for 20 s, 50 μL of the solution was quickly pipetted into 2.25 mL of trypsin solution (7.5 mg/mL in 0.05 M veronal buffer, pH 8.2, at 10 °C) in the presence or absence of PPI (final pH 7.9). The solution was mixed thoroughly with a stirring bar. At suitable time intervals, 150 μL of reaction mixture was quickly pipetted into a centrifuge tube containing 50 μL of soybean trypsin inhibitor solution (40 mg/mL, in 0.05 veronal buffer, pH 8.6, at room temperature) and mixed with a vibrator to stop trypsin activity. Then, 100 μL of aminopeptidase P solution (~10 units/mL, in 0.05 M veronal buffer with citrate – Mn^{2+} reagent, pH 8.6) was added to each tube, and the solution was incubated at 35 °C for 1.5 h to release tyrosine-92 from the cleaved peptide fragment. The solutions were heated in a water bath at 85 °C for 3 min and then dried at room temperature by nitrogen. The resulting residue was taken up in 0.1 mL of H_2O, and 1.5 mL of absolute ethanol was added to precipitate proteins and peptides. After centrifigation at 7500 rpm for 20 min, the supernatants were quantitatively transferred to test tubes and dried again with nitrogen. The residues were dissolved by adding 250 μL of 0.4 M borate buffer, pH 9.5, and were filtered with the aid of a centrifugal filter to remove any particles. The solutions were analyzed for free tyrosine-92 by using high-performance liquid chromatography as described previously (Lin & Brandts, 1983a). The sample that reacted with trypsin for 30 min was used as the "infinite time" hydrolysis sample, and the amount of tyrosine released from this sample (after correcting for the small peak in the control) was used as the 100% reference for quantitating the time dependence of hydrolysis. The control run was carried out with RNase subjected only to pepsin and trypsin activity, with and without PPI but without APP activity, in the identical way.

Analysis of Refolding Kinetic Data. Statistical fittings of decay curves were performed on an IBM-PC with multiple

exponential decay analysis software (EMF Software, Knoxville, TN). The iterative fitting routine uses a modified version of the Simplex algorithm. For most of the fittings, only the relaxation times and relative amplitudes of each phase were treated as adjustable parameters, and various initial values were assigned to them as a check on convergence. In some fittings, the infinite-time base line was also treated as an adjustable parameter, which led to very similar results. The reported parameter values are those that minimized the sum of squared residuals. Fitting errors and analysis of the quality-of-fit were carried out by using the F test (Draper & Smith, 1966), as described under Results.

Results

Data and Data Analyses. Because of large differences in the time constants for the various kinetic processes involved, a complete statistical analysis of the slow refolding reaction of RNase A necessitates having high-quality data over a time range from about 1 s to nearly 1000 s. However, stopped-flow data are accurate only below about 100 s, because of poor long-term base line stability associated with slow thermal mixing and inherent spectrophotometric limitations, while the dead time for hand-mixing experiments limits their usefulness to times longer than about 20–30 s. To overcome this problem, the following analyses have been carried out on combined stopped-flow data (0–100 s) and hand-mixing data (30 s and longer) taken on solutions under identical conditions and after precise normalization on concentration and amplitude. In the overlap region from 30 to 100 s, both types of data were included in the data set.

The experimental refolding data obtained from either fluorescence or absorbance monitoring, were fit to the equation

$$A_t = A_\infty + \sum_1^n A_i \exp(t/\tau_i)$$

where n is the number of kinetic phases used to express the data, t is the time, A_i and τ_i are the amplitude and relaxation time for the ith phase, and A_∞ is the final equilibrium amplitude assigned directly from the instrumental reading after a time of ca. 30–40 min. The "best values" of parameters were determined by an iterative nonlinear least-squares program by floating all $2n$ fitting parameters simultaneously until minimization had occurred. To ascertain that the absolute minimum was achieved, different sets of initialization parameters were chosen and the minimization was repeated several times.

To obtain error estimates, a single parameter was fixed at a value which deviated a small amount from its best value, and the other $2n - 1$ fitting parameters were floated until the new minimum was reached. This fit using $2n - 1$ adjustable parameters always showed a larger sum of squared residuals (SSR) than the best fit using $2n$ adjustable parameters, as expected. However, the two fits were compared by using the F test, which takes into account the difference in the number of adjustable parameters in order to ascertain whether the difference in the quality of fit was statistically significant. The "probable error" for a parameter was assigned according to the fixed deviation from the best value that was necessary before the F test indicated a statistically inferior fit at the 90% confidence level. This error analysis was repeated for each parameter.

As would be anticipated, it was always found for any given data set that the SSR became smaller as n, the number of phases included in the fit, was increased. To determine how many phases were necessary to arrive at the best fit in a statistical sense, the SSR of the various fits were compared with one another by using the F test, which takes into account the different number of fitting parameters that were used. In this way, one can decide the minimum number of phases that must be used to statistically fit the data.

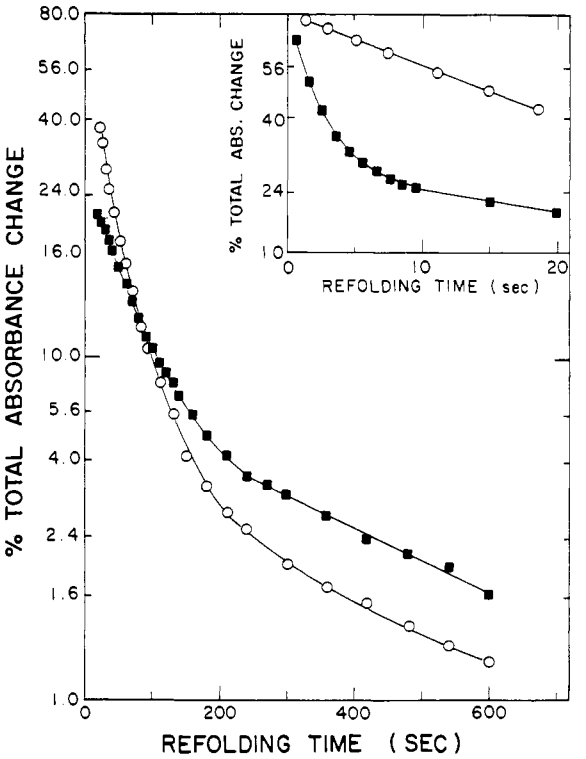

FIGURE 1: Refolding kinetics (10 °C, 0.31 M urea, pH 5.6) of RNase A in the absence of ammonium sulfate (circles) and in the presence of 0.56 M ammonium sulfate (squares), plotted semilogarithmically. The inset at the top of the figure shows an expanded-scale presentation of the first 20 s of the refolding reactions.

Determination of Number of Phases in Slow Refolding of RNase A. Some of the refolding data on RNase A, obtained by absorbance monitoring, are shown in Figure 1 as semilogarithmic plots. Note that the decay curve in the presence of 0.56 M ammonium sulfate is much steeper in the time interval of a few seconds than is the decay curve in the absence of ammonium sulfate, showing that at least one of the kinetic phases is being accelerated by the presence of the salt. A similar acceleration was seen in comparable decay curves (not shown) monitored by fluorescence rather than absorbance.

As discussed in the introduction, nonstatistical analysis of RNase A decay curves have in the past led to an assumption of either two or three slow phases. Schmid's laboratory (Schmid, 1981; Schmid et al., 1986) has decomposed the slow refolding reaction into a minor phase (I) and a major phase (II) corresponding to the refolding of the putative species U_s^I and U_s^{II}. Lin and Brandts (1984), on the other hand, have proposed the existence of three slow phases (i.e., the XY, CT, and ct phases) corresponding to the refolding of species with an incorrect trans isomer for proline-93 (CT), an incorrect trans isomer for proline-114 (ct), and an incorrect Y configuration in an as yet unknown XY chain rearrangement. In an attempt to distinguish between these two representations, both absorbance and fluorescence refolding data in the presence and absence of ammonium sulfate have been statistically analyzed in terms of two slow phases and in terms of three slow phases. The results from the two-phase fits are summarized in Table I, which includes both relaxation times and amplitudes of the minor phase and the major phase, the SSR for each data set as well as statistical fitting errors for each

Table I: Analysis of Slow Refolding of RNase A by Absorbance and Fluorescence, Assuming Two Kinetic Phases[a]

data set	detection	τ_{II}	α_{II}	τ_{I}	α_{I}	SSR
A: 0 M $(NH_4)_2SO_4$	Abs	24.6 ± 1.5	67.4 ± 3.5	155 ± 35	12.6 ± 2.5	2.98×10^{-5}
B: 0 M $(NH_4)_2SO_4$	Fl	39.6 ± 2	72.6 ± 3	276 ± 60	12.5 ± 3	1.38
C: 0.56 M $(NH_4)_2SO_4$	Abs	2.3 ± 0.3	53.7 ± 1.5	97.6 ± 11	26.3 ± 1	2.46×10^{-4}
D: 0.56 M $(NH_4)_2SO_4$	Fl	2.5 ± 0.3	41.6 ± 1.5	74.0 ± 4	43.4 ± 1	2.66

[a] Relaxation times are given in seconds. Phase amplitudes are given as percent of the total refolding amplitude. For absorbance (Abs) data, the sum of the slow-phase amplitudes is taken as 80% of the total amplitude to take into account the absence of the 20% fast phase. For fluorescence (Fl) data, the fast-phase amplitude is only 15%, so the sum of the slow-phase amplitudes is taken as 85%.

Table II: Analysis of Slow Refolding of RNase A by Absorbance and Fluorescence, Assuming Three Kinetic Phases[a]

data set	detection	τ_{XY}	α_{XY}	τ_{CT}	α_{CT}	τ_{ct}	α_{ct}	SSR
A: 0 M $(NH_4)_2SO_4$	Abs	20.0 ± 2	51.0 ± 6	59.4 ± 10	26.0 ± 5	618 ± 200	$3.0^{+1.5}_{-0.5}$	2.9×10^{-6}
B: 0 M $(NH_4)_2SO_4$	Fl	24.4 ± 4	30.1 ± 10	61.2 ± 10	49.2 ± 9	590 ± 200	5.7 ± 1.3	0.116
C: 0.56 M $(NH_4)_2SO_4$	Abs	2.1 ± 0.1	52.2 ± 0.3	61.9 ± 4	23.0^{-2}_{+1}	600 ± 200	4.8^{+2}_{-1}	4.7×10^{-6}
D: 0.56 M $(NH_4)_2SO_4$	Fl	2.3 ± 0.2	40.9 ± 1	62.7 ± 3	40.9 ± 1	485^{+300}_{-100}	$3.2^{1.5}_{-0.8}$	0.177

[a] Relaxation times are given in seconds. Phase amplitudes are given as percent of the total refolding amplitude. For absorbance (Abs) data, the sum of the slow-phase amplitudes is taken as 80% of the total amplitude to take into account the 20% fast phase in in refolding. For fluorescence (Fl) data, the fast-phase amplitude is only 15%, so the sum of the slow-phase amplitudes is taken as 85%.

parameter obtained from the F test at the 90% confidence level. Several characteristics of these results are worth noting:

(1) Within fitting errors, the relaxation times for the major and minor phase are different for fluorescence refolding than for absorbance refolding (cf. data sets A vs. B and C vs. D). The only exception to this is the relaxation time for the major phase in the presence of ammonium sulfate, which is statistically identical in absorbance and fluorescence data.

(2) Within errors, the addition of ammonium sulfate changes both the amplitude and the relaxation time of both the major phase and of the minor phase in absorbance decay (cf. A vs. C) as well as in fluorescence decay (cf. B vs. D), without exception.

(3) For all four data sets, the SSR values are quite large and the deviations of the experimental data from the calculated curve show a very systematic and persistent pattern. The residuals at each folding time are shown in Figure 2. It is apparent from this that deviations tend to be systematically positive at intermediate times and systematically negative at long times. Not only is this pattern seen in absorbance data from sets A and C, but the same pattern appears in the fluorescence data of sets B and D (not shown).

The identical data sets were also analyzed, assuming three-phase kinetics, and the corresponding results are shown in Table II for the XY, CT, and ct phases. The important points may be summarized as follows:

(1) Within fitting errors, the relaxation times for all three phases are identical for absorbance monitoring and fluorescence monitoring, in both the absence (cf. data sets A vs. B) and presence (cf. C vs. D) of ammonium sulfate. The amplitudes are significantly different, however, since the amplitude of the XY phase is smaller and that of the CT phase is larger in fluorescence decay than in absorbance decay. This is particularly apparent in the presence of ammonium sulfate, where the fitting errors are smaller because of the greater separation in relaxation times of the XY and CT phases.

(2) With one important exception, the relative amplitudes and relaxation times are all unaffected by the addition of ammonium sulfate for both adsorbance (cf. A vs. C) and fluorescence decay (cf. B vs. D). The single exception is the relaxation time of the XY phase, which changes quite drastically from a value of ca. 20 s in the absence of ammonium sulfate to 2 s in the presence of ammonium sulfate.

(3) The SSR values for the three-phase fits are markedly smaller than those for the two-phase fits, amounting to relative factors of 10, 12, 52, and 15 for data sets A, B, C, and D,

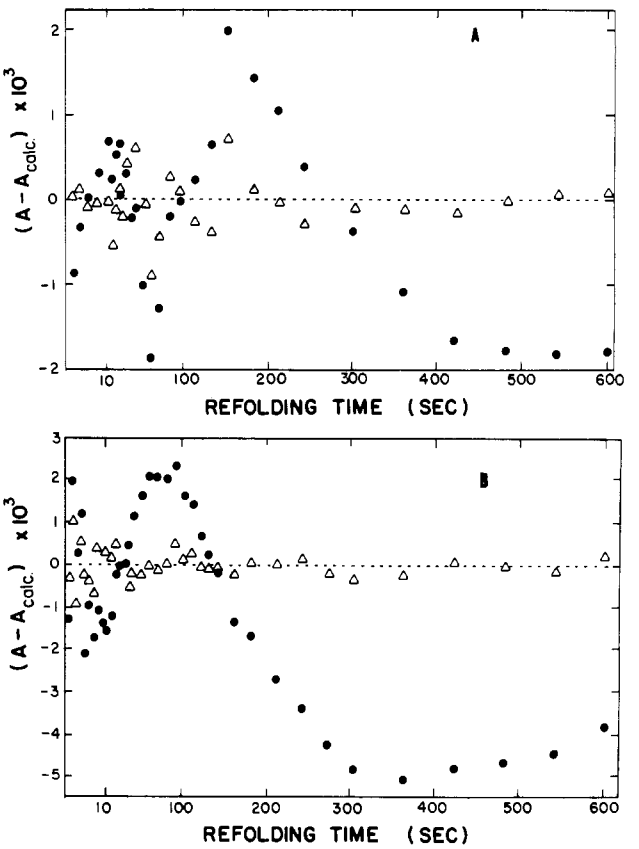

FIGURE 2: Deviation of experimental points from calculated refolding curve vs. refolding time. The circles correspond to deviations with a two-phase refolding model and the triangles represent deviations with a three-phase refolding model. Frame A is for data set A in the absence of ammonium sulfate, while frame B is for data set C in the presence of 0.56 M ammonium sulfate. Note that the time scale during the first 10 s of refolding has been enlarged so that the appropriate deviations can be plotted properly.

respectively. Equally important, the deviations between experimental points and calculated curve occur nearly randomly along the time axis, as seen in parts A and B of Figure 2 for data sets A and C, respectively, and this is markedly contrasted to the strong systematic deviations for the two-phase fits.

To be certain that no unforeseen errors were introduced by using data sets that were a combination of stopped-flow and hand-mixing results, the same analyses depicted in Tables I and II were carried out on the corresponding hand-mixing data only. In the absence of ammonium sulfate, all of the three-

phase parameters were statistically identical with those obtained from the combined data. However, the fitting errors for the relaxation times and relative amplitudes of the XY and CT phases were about twice as large as those shown in Table II, reflecting the less extensive data base, while the errors in the ct phase were comparable. In the presence of ammonium sulfate, the dead time for hand mixing precluded seeing the fast XY phase but the relaxation times and relative amplitudes for the CT and ct phase were statistically equivalent to those shown in Table II. For the two-phase fits of hand-mixing data only, the situation was very similar.

The differences in the quality of fit between the two-phase analyses and the three-phase analyses in Tables I and II, respectively, were compared directly by using the *F* test, which takes into account that there are only four adjustable parameters in the two-phase fit while there are six adjustable parameters in the three-phase fit. For each of the data sets A–D, the *F* test showed that the three-phase fit was statistically superior to the two-phase fit at the 99% confidence level or higher. A similar situation was found to exist for the two- and three-phase fits of the hand-mixing data only in the absence of ammonium sulfate since, for data sets A and B, the three-phase fit was statistically superior to the two-phase fit at the 95% confidence level.

We were interested in whether the addition of a fourth kinetic phase would further improve the statistical fit even beyond that obtained with three phases. A number of attempts at four-phase fitting were tried with each of the data sets A–D, and these invariably led to only small decreases of ca. 10% in the SSR values over those obtained in the corresponding three-phase fits. This is in sharp contrast to the 10–50-fold differences in SSR values between two- and three-phase analyses. Furthermore, the four-phase fits invariably contained three phases with statistically identical relaxation times and relative amplitudes to the phases found in the three-phase fits, with the addition of a fourth phase of very small positive or negative amplitude. Application of the *F* test showed that the four-phase fits were statistically no better than the three-phase fits at the 99% confidence level in all cases.

The conclusion to be drawn from these analyses is that the two-phase model does not provide an adequate statistical representation of the slow refolding reaction of RNase A in either the presence or absence of ammonium sulfate at 10 °C. At least three slow phases are required to properly express the data, and nothing is gained statistically by adding a fourth phase. This does not prove that there are only three phases, but it does say that information about additional phases cannot be obtained from kinetic decay curves similar to those discussed above.

Refolding Detected by Fluorescence Change upon Unfolding. It has been shown (Rehage & Schmid, 1982; Lin & Brandts, 1983b) that the isomerization of proline-93 from cis to trans during unfolding leads to a slow fluorescence change, even though unfolding itself is very fast. Schmid et al. (1986) proposed that double-jump experiments (i.e., refolding followed by unfolding) can then be used to monitor the isomerization of proline-93 during refolding with no interference from other refolding processes. Figure 3 shows the semilogarithmic plot of the difference in the unfolding intensity (slow phase only) between the fully refolded sample (refolded for 2 h; arbitrary fluorescence intensity of 515) and other samples with various refolding times (shown on the ordinate). The two experiments shown were identical except that the refolding buffer contained no ammonium sulfate in one case and 0.5 M in the other case. In both cases, the data show two kinetic phases with relative

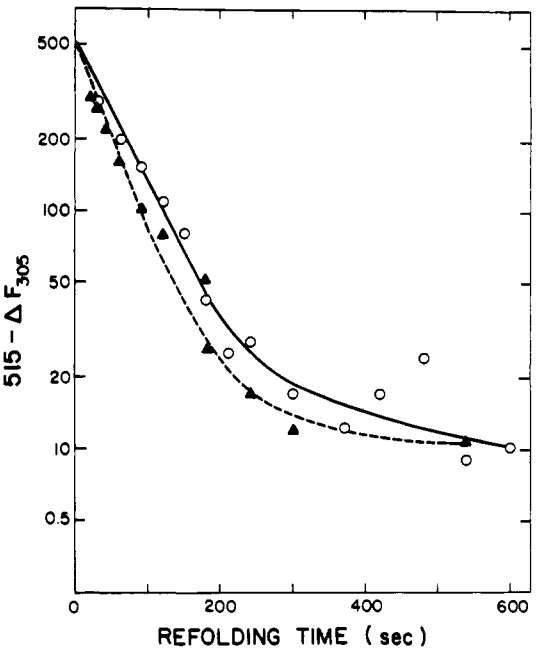

FIGURE 3: Refolding kinetics (10 °C, 0.31 M urea, pH 5.6) of RNase in the presence and absence of $(NH_4)_2SO_4$, detected by fluorescence unfolding assay: 0 (○) and 0.5 M (▲) $(NH_4)_2SO_4$. RNase concentration in refolding solution is ca. 1.0×10^{-4} M. The total fluorescence change for the fully refolded sample is 515 in arbitrary units.

amplitude of ca. 10 to 1. The indicated relaxation time for the faster phase is somewhat smaller (50 vs. 65 s) in 0.5 M $(NH_4)_2SO_4$, but this may not be experimentally significant. Because of its small amplitude, the slower phase is difficult to quantitate, but its relaxation time is in the range of a few hundred seconds.

Since it is thought that the slow unfolding phase seen in fluorescence detects exclusively the isomerization of the Tyr-92–Pro-93 bond, then the major phase seen in Figure 3 should correspond to the CT phase. Indeed, the relaxation times noted above are experimentally indistinguishable from those determined above for the CT phase in both absorbance-detected and fluorescence-detected decay curves (cf. Table II) in both the absence and presence of ammonium sulfate.

The minor phase seen in the data of Figure 3 apparently corresponds to the ct phase, since the relaxation time is in the appropriate range. Since the ct phase is thought to be rate-limited by the isomerization of proline-114, it is at first surprising that it would be seen by a method which apparently monitors only the isomerization of proline-93. Statistically, however, the frequency of occurrence of the incorrect trans isomer of proline-93 is ca. 30% among the slow-refolding molecules (i.e., corresponding to an amplitude fraction of 25/80 for the CT phase), so it is to be expected that about one-third of the unfolded molecules with proline-114 in the trans configuration will also have proline-93 in the trans configuration. For these molecules, the isomerization of proline-93 will be rate-limited by the slower isomerization of proline-114 so that their decay will be seen in the ct phase rather than in the CT phase.

Since it was previously shown in Table II that the relaxation time for the XY phase is extremely sensitive to ammonium sulfate, the lack of any significant effect of 0.5 M $(NH_4)_2SO_4$ on the relaxation times or total amplitude change for the data of Figure 3 means that the XY phase is silent in this fluorescence assay. This is consistent with the idea that the XY phase does not involve the isomerization of proline-93,

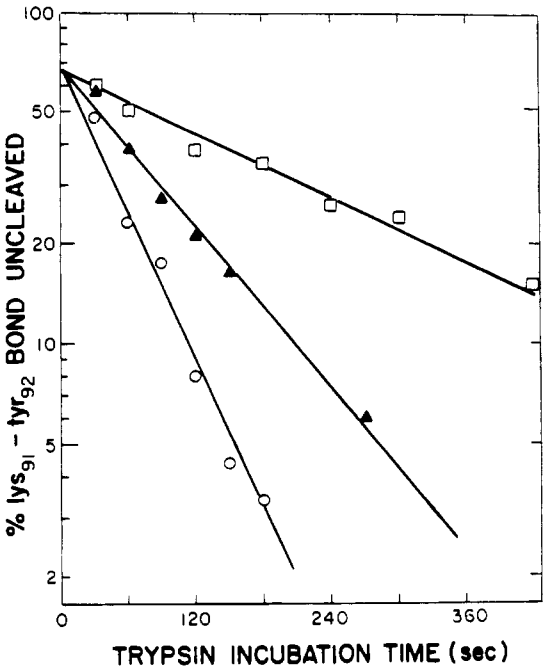

FIGURE 4: Semilog plots of rates of cis-to-trans isomerization of proline-93 in the pepsin-generated fragment of RNase in the presence and absence of PPI at 10 °C: 0 (□), 25 (▲), and 73 units (○) of PPI.

which must therefore occur as the rate-limiting step exclusively in the CT phase. This conclusion conflicts directly with the suggestion of Schmid (1986) that the isomerization of proline-93 may occur only in the XY phase and not in the CT phase.

Isomer-Specific Proteolytic (ISP) Measurements of Isomerization of Tyr-92–Pro-93 in the Presence of PPI. These experiments were carried out on equilibrated, unfolded RNase (4.5 M urea, pH 2.1) immediately after it was irreversibly unfolded by a short, intense pepsin pulse. Figure 4 shows the semilogarithmic plots for the percent of the Lys-91–Tyr-92 bond cleaved as a function of the incubation time with trypsin. Since trypsin can only cleave the Lys-91–Tyr-92 bond when the following Tyr-92–Pro-93 bond is in the trans form, the fast-phase amplitude in Figure 4 should correspond to the amount of trans form for the proline-93 bond while the slow-phase amplitude should correspond to the amount of cis form, and the relaxation time for the slow phase corresponds to the cis-to-trans isomerization of the Tyr-92–Pro-93 bond in the pepsin-generated fragment of RNase.

The assay for trypsin cleavage of the Lys-92–Tyr-92 bond is based on the release of free tyrosine that results upon the addition of aminopeptidase P subsequent to inhibition of the trypsin activity. Aminopeptidase P is specific for cleavage of an amino-terminal residue of a polypeptide chain but only if the second residue is prolyl. Since there is only one Tyr-Pro sequence in RNase, the method should be absolutely specific for the release of tyrosine-92.

The original study utilizing this method (Lin & Brandts, 1983a) reported that unfolded RNase contained ca. 30% of the incorrect trans isomer at equilibrium. Because of the suggestion (Schmid et al., 1986) that the entire slow phase in refolding might involve molecules with proline-93 in the trans configuration, we decided to reinvestigate the state of isomerization of proline-93. In two different experiments (one shown in Figure 4), the previously reported amplitudes and relaxation times were duplicated. As a further check on the method, it was decided to repeat the same experiments but in the presence of PPI, an enzyme that specifically catalyzes proline isomerization. These results are also shown in Figure 4. As would be expected if the assay were working correctly, the amplitude of the slow phase did not change, but the relaxation time did change from 4.5 min in the absence of PPI to 1.8 min in the presence of 25 units of PPI and to 1.0 min in the presence of 73 units of PPI. Control experiments where PPI was replaced by an equivalent concentration of serum albumin showed no rate enhancement. Since the only known activity of PPI is to catalyze the isomerization of prolyl peptide bonds, these results further support the idea that this ISP method does in fact monitor the release of a tyrosine residue whose cleavage depends on isomerization of an adjacent proline residue. Proline-93 seems to be the only logical possibility.

Discussion

The slow refolding reaction of RNase, as observed by either absorbance or fluorescence monitoring, is best resolved into a minimum of three kinetic phases: the XY, CT, and ct phases. This is true in the presence or absence of ammonium sulfate, which has little or no effect on the relative amplitudes of the three phases or on the rates for the CT or ct phases. Only the rate of the XY phase is highly sensitive to ammonium sulfate.

Since experimentally identical relaxation times are found for each of these three phases by analysis of fluorescence and absorbance decay curves, this strongly suggests that both detection methods are monitoring the same three rate-limiting steps during refolding. The fact that "fluorescence-detected kinetics are slower than absorbance-detected kinetics" is, in our opinion, not due to the fact that the two methods monitor different processes (Schmid, 1981; Schmid et al., 1986) during refolding but is a trivial effect that arises because the relative amplitude of the slower of the two large phases (i.e., the CT phase) is enhanced in fluorescence by intrinsic local fluorescent changes due to the isomerization of proline-93, which is silent in absorbance. In fact, it has been shown (Lin & Brandts, 1983c) that the enhancement in the fluorescent amplitude of the CT phase, relative to its absorbance amplitude, in the refolding direction can be quantitatively predicted from the measured changes in fluorescence that occur as the result of the isomerization of proline-93 in the unfolding direction, with no additional assumptions required.

Furthermore, the fact that the same three phases are seen in absorbance and fluorescence decay curves argues against the idea that the transition from the nativelike intermediate to the native state is seen by fluorescence but not by absorbance (Schmid et al., 1986). We feel that the transition $I_N \rightarrow N$, which is the $N_{cCY} \rightarrow N_{cCX}$ transition in our terminology (Lin & Brandts, 1984), is nearly silent in both fluorescence and absorbance.

Inspection of the deconvolution data in Tables I and II shows that neither the major nor the minor phase derived from the two-phase analysis can be consistently equated to any of phases derived from the three-phase analysis. The relaxation time of the major phase is intermediate between the XY and CT phases, while that of the minor phase is intermediate between the CT and ct phases; i.e., the two-phase analysis leads to hybridization of the three kinetic processes that are taking place during refolding. This mixing of phases is particularly prominent in the absence of ammonium sulfate, where the relaxation times of the XY and CT are more similar. Since both the major and minor phases are contaminated by events that occur in the CT phase, this may have contributed to the conclusion of Schmid et al. (1986) that both U_s^{II} (major phase) and U_s^{I} (minor phase) involve the isomerization of proline-93 as they refold.

Earlier ISP results (Lin & Brandts, 1983a, 1984) also support the idea that a minimum of three rate-limiting processes are taking place during refolding. These results showed that ca. 25–30% of the unfolding molecules contain proline-93 in the incorrect trans state and 5% contain proline-114 in the incorrect trans state. Since 80% of the unfolded molecules participate in the slow refolding reaction, this leaves nearly 50% of the slow amplitude unaccounted for and it must be assigned to a third phase (or phases). The new ISP results reported here show that the proline isomerase enzyme PPI acts to catalyze the slow phase in trypsin hydrolysis of RNase, which provide further support for the analytical method used to study proline-93.

The double-jump fluorescence assay developed by Schmid et al. (1986) apparently is absolutely specific for the isomerization of proline-93, and all other processes that occur during refolding are silent by this technique. By use of this assumption, our results indicate that isomerization of proline-93 is involved as the *rate-limiting step* only in the CT phase. The lack of any significant effect of ammonium sulfate on the refolding kinetics monitored by this technique shows clearly that proline-93 does not isomerize in the XY phase, as Schmid (1986) recently suggested, since this phase was shown to be extremely sensitive to ammonium sulfate. The very small amplitude seen in the ct phase is consistent with the statistical expectation that about one-third of the unfolded molecules that contain a trans form for proline-114 will also contain a trans form for proline-93, which will then isomerize during the ct phase as a non-rate-limiting step.

The method of analysis of kinetic data used here is clearly preferable to the method of peeling-off exponentials that we and others used in the past, since errors are properly accounted for and inadvertent bias is eliminated. For RNase, where relaxation times are rather closely spaced, the final results do depend quite strongly on having a complete data set, an accurate value for the equilibrium base line, and very precise data. Even with all these precautions, the estimates for relaxation times and amplitudes have moderately large fitting errors when relaxation times are closely spaced, due to strong interdependence of parameters.

In summary, these new data support our previous three-phase analysis of the refolding reaction of RNase. The trans-to-cis isomerization of proline-93 occurs as the rate-limiting step only in the CT phase, which involves ca. 25% of the unfolded molecules. Isomerization of proline-93 definitely does not occur in the XY phase, which involves the formation of the nativelike intermediate I_N, nor is there any direct evidence to suggest that it occurs subsequent to this in the transition from I_N to the native state.

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