Mechanism for the Unfolding and Refolding of Ribonuclease A. Kinetic Studies Utilizing Spectroscopic Methods†

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ABSTRACT: The kinetics for the base-line unfolding and refolding reactions of RNase A in urea solutions have been studied by absorbance, fluorescence, and double-jump techniques. All of the data have been analyzed in terms of three separate molecular processes: a fast ND phase attributed directly to the large conformational changes which take place, a slow CT phase which is due to the isomerization of proline-93, and a slow XY phase of unknown origin. The refolding amplitudes are independent of the initial urea concentration from 3 to 10 M, suggesting that both of the slow phases involve urea-insensitive structural changes in the unfolded state. In agreement with their subtle nature, there is no intrinsic change in absorbance associated with either the CT or XY process, and there is no intrinsic change in fluorescence for the XY process. All three processes give rise to counterpart relaxations in the unfolding and refolding directions, suggesting that all three equilibria are simply driven in opposite directions. Comparative relaxation times for unfolding and refolding agree nicely with this simple analysis. The relaxation time for the CT phase is weakly dependent on urea concentration while that of the XY phase is strongly dependent on urea concentration. At low final urea concentrations the CT phase accounts for 30% of the total absorbance amplitude for refolding while the XY phase accounts for 50%. At intermediate urea concentrations, much of the CT phase becomes rate limited by the XY phase, so that the entire slow phase plots as a single-exponential function. However, at urea concentrations above 4 M a much smaller (7%) CT phase reappears as a faster process superimposed on the dominant (73%) XY phase. In the unfolding direction, both the CT and XY phase can be seen in double-jump experiments and the CT phase can also be seen directly with fluorescence monitoring. All of these experimental data can be nicely accounted for without assuming the appearance of true structural intermediates during base-line refolding.

The preceding paper (Lin & Brandts, 1983b) established that, when refolding RNase A into low-urea solutions, 30% of the enzymic activity is recovered in a slow phase which is rate limited by the isomerization of that 30% fraction of unfolded molecules which have proline-93 in the incorrect trans configuration. The 70% fraction which have proline-93 in the cis configuration refold more quickly under low-urea conditions. However, it is known from previous studies (Brandts et al., 1975; Garel et al., 1976) that only 20% of the unfolded molecules are capable of "very fast" refolding when the reaction is monitored by changes in tyrosine absorbance. Thus, the entire 80% slow phase in refolding cannot be completely accounted for by the isomerization of proline-93.

The purpose of this paper is to experimentally characterize all of the kinetic phases which can be detected in RNase refolding and unfolding reactions, utilizing various spectroscopic monitoring including tyrosine absorbance, fluorescence, and double-jump methods. It will be seen that there is another slow phase in refolding which occurs concurrently with the isomerization of proline-93 and that the relaxation time for this phase depends very strongly on the urea concentration in the refolding buffer. From the double-jump experiments and fluorescence experiments, it is concluded that these same two slow phases are present during the unfolding reaction. Both of the slow phases are inherently "invisible" in terms of changes in absorbance, and only the phase involving proline-93 can be seen directly by fluorescence measurements. Both slow phases are associated with equilibrations which occur in the unfolded protein and are not due to structural intermediates. Although these two phases seem to occur independently of each other in the unfolding direction, a very curious interdependent pathway can be shown to dominate the refolding reaction at high urea concentrations.

Materials and Methods

Materials. RNase was purchased from Sigma Chemical Co. (type XII-A, lot 49C-8047). Urea (Ultra Pure) was obtained from Schwarz/Mann Co. All other chemical are reagent grade and have been described in previous papers (Lin & Brandts, 1983a,b; Lin & Brandts, 1978).

Absorbance Measurements of RNase Refolding and Unfolding (Manual Mixing). 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 A refolding. The sensitive 0.1-A slide wire was used. The experiments were carried out at two temperatures, 10.5 and 16 °C. For refolding, the unfolded RNase (in high urea concentration, at low pH) was diluted into sodium acetate buffer to different final urea concentrations in the presence or absence of $(NH_4)_2SO_4$, and then the solution was mixed with a spatula for a few seconds before observation. The mixing dead time was about 5–10 s. Extrapolation to zero time was employed to obtain the total absorbance change of the slow refolding phases, which was always found to be close to 80% of total absorbance change. In cases where more than one kinetic phases were observed, the peel-off method was used to resolve the separate phases.

A double-jump method similar to that of Brandts et al. (1975) was used to obtain the rates of the slow phases of RNase unfolding in high-urea solution. The procedures were as follows: A small amount of native RNase in H_2O (30 μL, 150 mg/mL) at 10.5 °C was unfolded by pipetting into a urea solution (170 μL, 5.6 M, pH 2.0) also at 10.5 °C for predetermined time intervals. Then, 167 μL of solution was quickly pipetted into a quartz cuvette containing 2.5 mL of 0.1 M

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0006-2960/83/0422-0564$01.50/0

sodium acetate buffer (pH 5.6, thermostated at 10.5 °C) and mixed for a few seconds with a spatula. The change in absorbance (at 287 nm) as a function of time was recorded. Two kinetic phases were resolved and the amplitude of each phase at various times of unfolding was quantitated. A sample of RNase unfolded at room temperature for 10 min before cooling to 10.5 °C (1 min) was also examined in the identical way, and these amplitudes were used to obtain the 100% reference amplitudes for subtraction. Another double-jump experiment in which RNase was unfolded in 8.0 M urea, pH 2, solution and refolded in a 0.5 M urea–0.39 M $(NH_4)_2SO_4$ solution was also carried out at 10.5 °C. Only one slow phase was detected and quantitated, due to the small relaxation time of the faster refolding phase.

Fluorescence Measurements of RNase Refolding and Unfolding (Manual Mixing). The measurements were made on a Perkin-Elmer MPF-44 fluorescence spectrophotometer by using a 1-cm quartz cell thermostated at 10.5 or 16 °C. The instrument was run in the energy mode with both slits set at 5 nm. The excitation wavelength was either 277 or 268 nm, while the emission wavelength was 305 nm. The refolding reaction was carried out in the identical way as that for absorbance measurements. For the unfolding reaction, RNase in H_2O was added into a 8.5 M urea, pH 2.0, or 5.0 M urea, pH 2.0, solution. The mixing dead time is about 10 s.

Absorbance and Fluorescence Measurements of RNase Refolding and Unfolding Using a Stopped-Flow Spectrophotometer. The detailed instrumentation has been described in a previous paper (Lin & Brandts, 1978). However, a H_2/D_2 lamp instead of a xenon lamp was used here. The cuvette was also insulated to allow the experiments to be carried out at 10.5 and 16 °C. Both absorbance and fluorescence modes were used. For absorbance measurements, the wavelength was set at 287 nm with a 0.1-mm slit. For fluorescence measurements, the excitation wavelength was set at 277 or 268 nm with a 5-mm slit, and an optical filter (Corning, 26-4622) was placed before the photomultiplier tube. A pair of syringes with a volume ratio of 1 to 15 was used for both refolding and unfolding experiments. The mixing dead time was found to be in the range of 5–10 ms.

Results and Discussion

In order to correlate spectroscopic studies with earlier results (Lin & Brandts, 1983b) from the direct measurements of isomerization of proline-93, we used urea exclusively as the denaturing agent rather than the more popular denaturant Gdn·HCl. Both fluorescence and absorbance measurements were carried out by using either the Durrum stopped-flow instrument or manual mixing. Both methods were necessary because of the large spread in relaxation times. The results from the different methods were in very good agreement when they could be compared directly, as will be seen. Almost all experiments were carried out at 10.5 °C, except as noted. For refolding experiments, the initial buffer was 5 M in urea, pH 2, and for unfolding experiments, the native RNase was initially in water. Where two or more kinetic phases were indicated in the data, the individual relaxation times were estimated by peeling off exponentials.

(1) Refolding. The last 5% or so of the refolding reaction, with either absorbance or fluorescence detection, is complicated by a small and very slow kinetic phase which is also apparent in earlier data from other laboratories (Cook et al., 1979; Schmid, 1981). It is only seen for refolding into low urea concentration and is not apparent in the data at high urea concentration either in the presence or in the absence of $(NH_4)_2SO_4$. The amplitude and apparent relaxation time are not very reproducible. It is seen most clearly in the time range from about 7 min, after all other phases are essentially completed, to about 30 min when the base line finally flattens out completely. The change in absorbance during this time span amounts to ca. 1.5–2% of the total change in absorbance for all other phases. In treating data where this very slow phase was present, relaxation times were determined by using the linear portions of semilogarithmic plots over the time span when this very slow phase made no apparent contribution to our data. No serious effort was made to characterize this small phase.

(a) Absorbance Measurements. The kinetic refolding pattern for RNase is strongly dependent on the final urea concentration in the refolding buffer. When refolding into buffers containing a low urea concentration of 0.31 M, three kinetic phases can be seen. There is one very fast phase with a relaxation time, which we will refer to as τ_{ND}, in the 10-ms range at 10.5 °C. It has been shown that this phase is due to the direct refolding of the denatured form to the native form (Garel et al., 1976; Nall et al., 1978). This conversion occurs with intrinsic changes in both absorbance and fluorescence. In addition to this fast phase, there are two slow phases with comparable relaxation time in the 100-s time region at 10.5 °C. The slowest of these at low urea concentration of 0.31 M, whose relaxation time will be referred to as τ_{CT}, will be shown to be due to the trans-to-cis isomerization of proline-93 in the unfolded protein. This phase involves no intrinsic change in absorbance but does have a large change in fluorescence. Thus, it is visible in absorbance decay curves in refolding experiments only because it is coupled by mass action effects to the τ_{ND} relaxation. The other slow phase, designated as τ_{XY}, is slightly faster than the τ_{CT} phase at low urea but much slower at high urea. The molecular events which give rise to this phase are unknown. However, it will be shown that in all likelihood this phase gives rise to no intrinsic change in either absorbance or fluorescence.

At 0.31 M urea, the fast phase accounts for 20% of the total amplitude and has a relaxation time τ_{ND} of about 30 ms at 10.5 °C and 20 ms at 16 °C (see Table I). The relaxation times of the two slow phases (τ_{XY} = 23 s and τ_{CT} = 85 s at 10.5 °C), calculated from the curve in Figure 1, differ by only a factor of 4 but are ca. 1000 times slower than the fast phase. The amplitude of the τ_{XY} phase is 50% while that of the τ_{CT} phase is 30%. The relaxation times and amplitudes of the two slow phases obtained from stopped-flow monitoring (Table I) are in good agreement with those obtained by manual mixing.

When refolding into a higher urea concentration of 1 M urea, the rate of refolding becomes slower. The fast phase still accounts for 20% of the total amplitude but the relaxation time τ_{ND} increases to 70 ms. Two slow phases can still be seen in the data of Figure 1, but the separation of these phases is more difficult since urea has a higher proportional effect in slowing down the τ_{XY} phase than the τ_{CT} phase.

At urea concentrations of 2 and 3.1 M, the entire slow phase shown in Figure 1 plots as a single-exponential function which accounts for ca. 80% of the total amplitude, since the τ_{ND} amplitude remains constant at 20%. The relaxation time of this single slow phase is 300 s at 2 M and 510 s at 3.1 M urea. As implied in the sequence of curves in Figure 1 at progressively higher urea concentrations and as will become evident from later data, the molecular events that are involved in the τ_{XY} relaxation in low urea become much slower than those involved in the τ_{CT} relaxation when the urea concentration is 2 M or higher. That these data at higher urea concentration plot as a single-exponential function is due to the fact that,

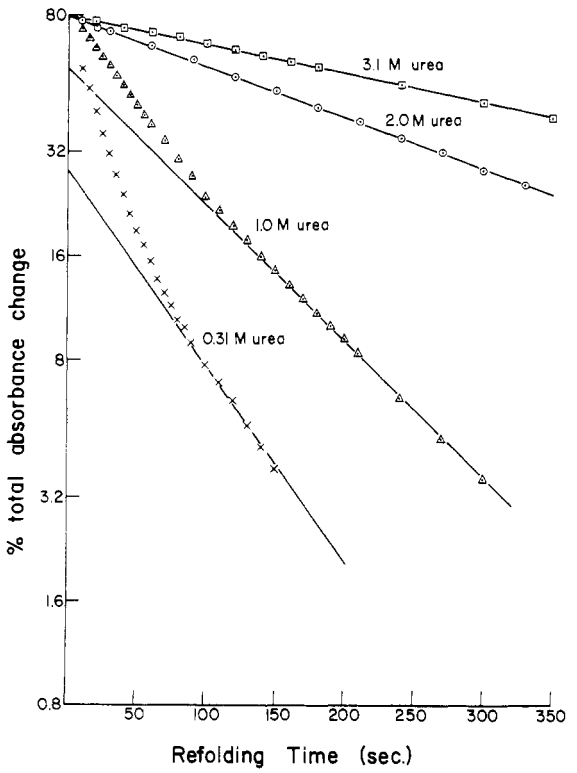

FIGURE 1: Refolding kinetics of RNase at different final urea concentrations at 10.5 °C. RNase (1.1×10^{-3} M in 5 M urea, pH 2) solution was diluted by a factor of 16 into 0.05 M sodium acetate buffer (pH 5.6) containing various amounts of urea. The final urea concentrations are as indicated. The final pH was 5.1 ± 0.1. The experiments were carried out on a Cary 14 by using hand mixing. A fast phase (~20%) is not seen. Two phases are seen when jumping into low urea concentrations (0.31 and 1.0 M). The faster one was assigned as the τ_{XY} phase and the slower one as the τ_{CT} phase. One phase is seen at higher urea concentrations (2.0 and 3.1 M), which was assigned as the τ_{XY} phase. See the text for detailed explanation.

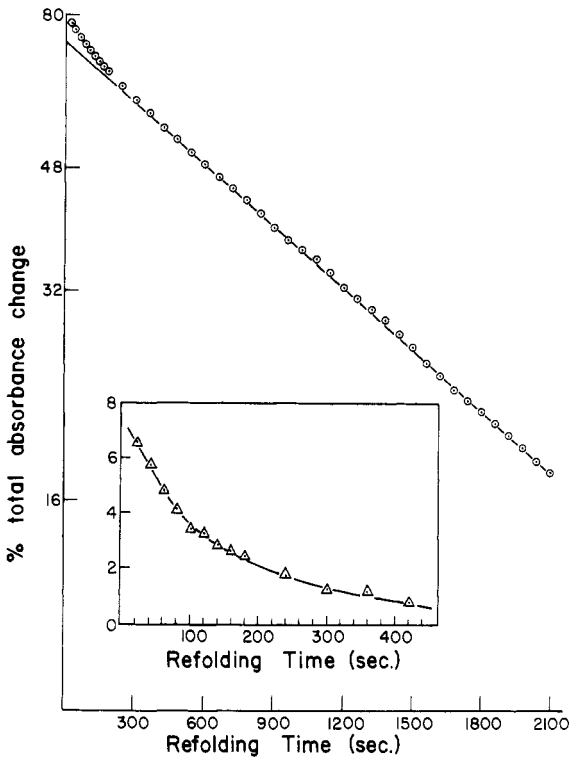

FIGURE 2: Refolding kinetics of RNase at high urea concentration at 10.5 °C. RNase (9.5×10^{-4} M in 5.0 M urea, pH 2.0) was diluted by a factor of 16 into a 0.05 M sodium acetate buffer, pH 5.4, solution containing 5.2 M urea. The slow phase was assigned as the τ_{XY} phase. The inset (triangles) show more clearly the small faster phase (assigned as the τ_{CT} phase, obtained after peeling off the τ_{XY} phase) which occurs during the first 400 s.

for a substantial fraction of unfolded molecules, the molecular processes associated with *both* relaxations must occur before refolding can take place. For these molecules, a separate relaxation for the τ_{CT} process is not seen at 2 and 3.1 M since their refolding is rate limited by the slower τ_{XY} process so the single-exponential decay is for this coupled process. In recognition of its rate-limiting role, we will designate the single slow-phase relaxation time observed at 2 and 3.1 M as corresponding to τ_{XY} in Table I and provide further justification later (Lin & Brandts, 1983c).

A very curious phenomenon occurs at yet higher urea concentrations. At 4.1 M urea (data not plotted but summarized in Table I), the slow phase becomes even slower (τ_{XY} = 780 s) but there is a very small deviation from linearity during the first 250 s of refolding. At 5.2 M urea, this nonlinearity is even more pronounced as seen in Figure 2 and, particularly, in the inset to Figure 2. The data can be quantitated nicely in terms of two exponentials. The major slow phase, accounting for ca. 73% of the total refolding amplitude, has a τ_{XY} of 1500 s. The smaller slow phase, accounting for but 7% of the total amplitude, has a 7-fold shorter relaxation time of 220 s. We will designate the relaxation time of this minor phase as τ_{CT}, since it will be shown that this is in all likelihood due to a minor population of the unfolded protein which must undergo *only* the CT structural changes before refolding can take place (Lin & Brandts, 1983c). The amplitude and relaxation time for this minor CT phase seen in high-urea refolding are completely predictable from the larger CT phase seen in low-urea refolding.[1]

The presence of ammonium sulfate greatly accelerates the rate of refolding of RNase (Schmid, 1981). We have utilized this additive to provide a better experimental characterization of the τ_{XY} and τ_{CT} phases and the way in which they sequentially couple and uncouple during refolding. Some of the data utilizing ammonium sulfate are shown in Figure 3 and others are included in Table I. Figure 3A shows data which compare the slow refolding kinetics at 10.5 °C in the presence and absence of 0.56 M $(NH_4)_2SO_4$ when the final urea concentration is 0.31 M. It is evident that the relative amplitudes of the two slow phases are unchanged by ammonium sulfate. While the relaxation time for the slower τ_{CT} phase is also unaffected, ammonium sulfate causes the relaxation time of the τ_{XY} phase to decrease 10-fold from 22 s in the absence to 2.2 s in the presence of 0.56 M $(NH_4)_2SO_4$.[2]

The data in Figure 3B show the same comparative experiment, except that the concentration of urea in the refolding buffer was 2 M. Under these conditions, as discussed earlier, the slow phase occurs as a single-exponential process in the absence of ammonium sulfate. The addition of ammonium sulfate reestablishes the two-phase behavior by speeding up the XY process (τ_{XY} = 17 s) so it is faster than the CT process, which then permits determination of the τ_{CT} value of 130 s. Since it is known from the experiment (Figure 3A) in 0.31

[1] A much faster phase, in the time range of seconds, was also partially seen in manual mixing experiments when RNase was refolded in 4.1 or 5.2 M urea, but these data are not plotted in Figure 2. This was presumed to be the τ_{ND} phase.

[2] It was observed that the total absorbance change for all three refolding phases seen in stopped-flow experiments is greatly affected by the presence of $(NH_4)_2SO_4$, even though the relative amplitudes are unchanged. For example, the total amplitude for refolding into 0.31 M urea is decreased by ca. 30% by the addition of 0.56 M $(NH_4)_2SO_4$.

Table I: Kinetic Data of Refolding of RNase in Urea Solution

final conditions[a,b]									
urea (M), pH	$(NH_4)_2SO_4$ (M)	method[c]	temp (°C)	τ_{ND} phase amplitude	τ_{ND} phase τ (s)	τ_{XY} phase[d] amplitude	τ_{XY} phase[d] τ (s)	τ_{CT} phase amplitude	τ_{CT} phase τ (s)
0.31, 5	0	A(M)	10.5			0.50	22	0.30	85
0.31, 5	0	A(SF)	10.5	0.20	0.028	0.52	21	0.28	85
0.31, 5	0	A(SF)	16.0	0.20	0.20	0.50	17	0.30	48
0.31, 5	0.56	A(SF)	10.5	0.20	0.020	0.53	2.2	0.27	80
0.31, 5	0	F(M)	10.5			0.37	23	0.48	85
0.31, 5	0	F(M)	16.0			0.34	18	0.51	47
0.31, 5	0	F(SF)	10.5	0.15	0.025	0.37	24	0.48	85
0.31, 5	0	F(SF)	16.0	0.15	0.018	0.32	17	0.53	48
0.31, 5	0.56	F(SF)	10.5	0.15	0.020	0.38	2.5	0.47	80
1.0, 5.1	0	A(M)	10.5			0.16	38	0.64	120
1.0, 5.1	0	A(SF)	10.5	0.20	0.070	0.20	35	0.60	115
1.0, 5.1	0.	F(M)	10.5			0.10	40	0.75	110
1.0, 5.1	0	F(SF)	10.5	0.15	0.080			0.72	120
2.0, 5.2	0	A(M)	10.5			0.80	300		
2.0, 5.2	0	A(SF)	10.5	0.20	0.40	0.80	270		
2.0, 5.2	0	F(M)	10.5			0.80	290		
2.0, 5.2	0	F(SF)	10.5	0.15	0.38	0.85	280		
2.0, 5.2	0.56	A(M)	10.5			0.54	17	0.26	130
3.1, 5.2	0	A(M)	10.5			0.80	510		
4.1, 5.3	0	A(M)	10.5			0.74	780	0.060	190
5.2, 5.4	0	A(M)	10.5			0.73	1500	0.070	220
2.85, 5.2	0.83	A(M)	10.5			0.55	14	0.25	160
3.0, 5.2	0.56	A(M)	10.5			0.53	35	0.27	180
3.63, 5.2	1.17	A(M)	10.5			0.55	14	0.25	180
4.0, 5.2	0.94	A(M)	10.5			0.54	35	0.26	190

	activation energy (kcal/mol)		
	τ_{ND} phase	τ_{XY} phase	τ_{CT} phase
fluorescence	10	9	17
absorbance	10	7	17

[a] Initial conditions were always 1×10^{-3} M RNase–5 M urea, pH 2.0. [b] Final solutions were buffered in 0.05–0.1 M acetate. [c] A(M) = absorbance with manual mixing; A(SF) = absorbance with stopped-flow mixing; F(M) = fluorescence with manual mixing; F(SF) = fluorescence with stopped-flow mixing; A(DJ) = absorbance following double-jump mixing.

M urea that ammonium sulfate does not affect its value, we assume that τ_{CT} would also have a value of 130 s were it to appear as a separate phase in the upper curve of Figure 3B which contains no ammonium sulfate. Since there is no indication of its presence and since the relaxation time observed in the upper curve is much larger (i.e., 300 s) than the value of τ_{CT}, we must conclude that refolding is rate limited by the XY process in 2 M urea and the absence of ammonium sulfate. This supports the idea of a refolding mechanism in 2 M urea in which the slower XY process ($\tau_{XY} \cong 300$ s) rate limits the occurrence of the intrinsically faster CT process ($\tau_{CT} = 130$ s), as alluded to earlier.

Using this procedure, it is possible to obtain values for both τ_{XY} and τ_{CT} as a function of urea concentration. The values of τ_{XY} are obtained directly from data at various urea concentrations in the absence of ammonium sulfate, while the values of τ_{CT} are obtained either directly (where two phases can be seen experimentally) or indirectly (where only one phase exists with no ammonium sulfate) by adding ammonium sulfate to accelerate the XY process so it is no longer rate limiting. The resultant values are shown in Figure 4. The τ_{CT} relaxation (filled circles) shows a small, but significant deacceleration in response to the addition of urea, with the τ_{CT} value increasing by more than a factor of 2 from 0 to 5 M urea. Note that the point at 5.2 M urea, obtained directly from the small τ_{CT} phase of 7% amplitude shown in Figure 2, agrees very nicely with the rest of the refolding data obtained from the larger τ_{CT} phase of ~30% amplitude seen either directly at low urea or indirectly at high urea in the presence of ammonium sulfate. In contrast to the τ_{CT} phase, the τ_{XY} phase (open circles) shows a very strong dependence of relaxation time on urea concentration, increasing by nearly 100-fold over the concentration range 0.3–5 M.

By referring back to data from the preceding paper (Lin & Brandts, 1983b), it is clear that the CT process, characterized here by absorbance changes, is in fact the isomerization of proline-93. The relaxation time for isomerization of proline-93 during refolding was measured directly by isomer-specific proteolytic methods at 0.31, 1.0, and 2.0 M urea, and the values are shown (X's) in Figure 4. At the low urea concentrations, the relaxation times for isomerization agree exactly with those for the τ_{CT} phase in absorbance, as does the amplitude of 30%. At 2 M urea, the relaxation time for isomerization coincides with the relaxation time for the τ_{XY} phase in absorbance, as expected, since most of the CT process becomes rate limited by the slower XY process during refolding in 2 M urea and the τ_{CT} phase is not seen as a separate relaxation. In reality, a small amount (ca. 7% of the total amplitude) of the CT process is not rate limited by the XY process in 2 M urea (Lin & Brandts, 1983c), but this cannot be seen as a separate relaxation in 2 M urea since τ_{CT} and τ_{XY} are not sufficiently different to allow experimental resolution of this minor phase. It is only at higher urea concentrations, where τ_{CT} and τ_{XY} are vastly different, that it can be separately resolved.

One final series of absorbance experiments were conducted, which examined the refolding reaction at different initial urea concentrations but at the same final urea concentrations of 0.33 M. The results from the manual mixing experiments are shown in Figure 5, for initial urea concentrations of 3, 5, and 10 M. As expected, the relaxation times τ_{XY} and τ_{CT} show no dependence on initial concentrations. More importantly,

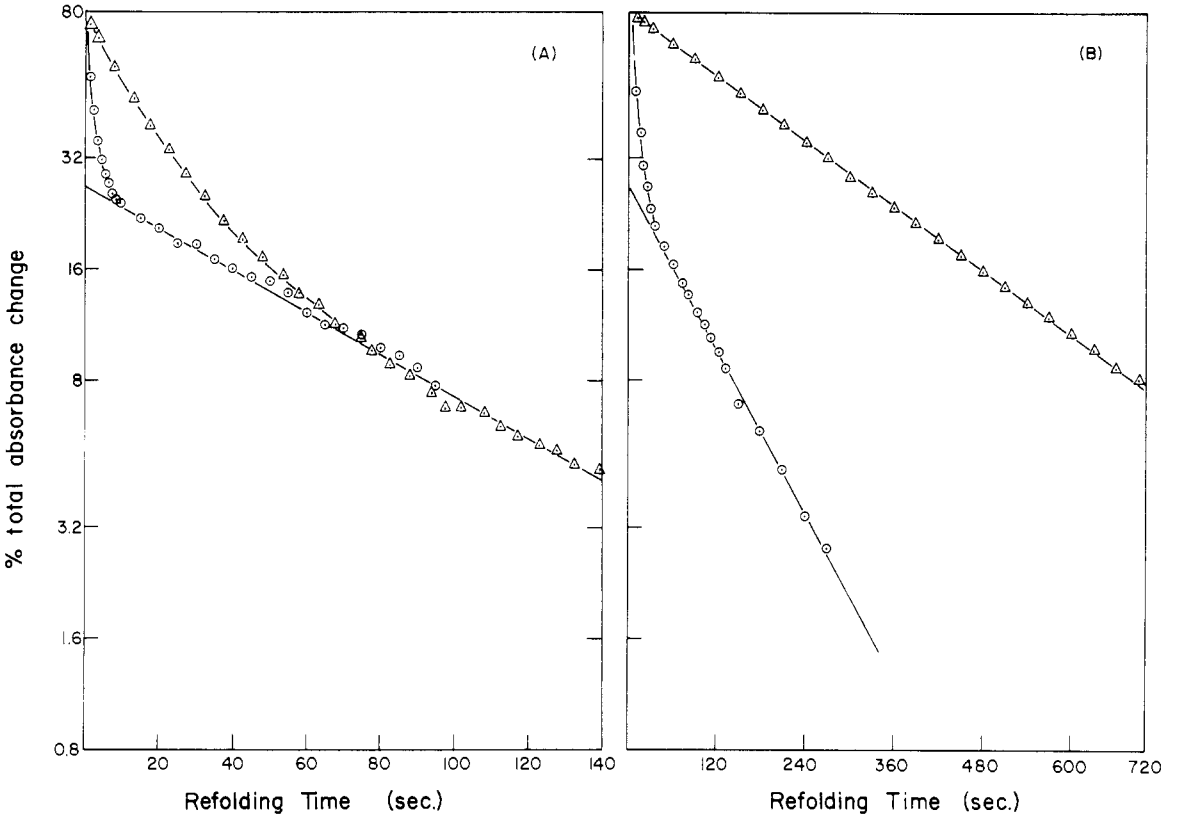

FIGURE 3: Comparisons of refolding kinetics of RNase in the presence and absence of $(NH_4)_2SO_4$ at two different final urea concentrations at 10.5 °C. (A) Final urea concentration of 0.3 M, pH 5.0. (Triangles) No $(NH_4)_2SO_4$; (circles) with 0.56 M $(NH_4)_2SO_4$. The experiment was carried out by using stopped-flow mixing. (B) Final urea concentration of 2 M, pH 5.2. (Triangles) No $(NH_4)_2SO_4$; (circles) with 0.56 M $(NH_4)_2SO_4$. The experiment was carried out on a Cary 14 by using hand mixing. RNase (1.1×10^{-3} M in 5 M urea, pH 2) was diluted by a factor of 16 with a 0.05 M acetate buffer solution.

it is seen that the relative amplitudes associated with each of the two slow phases are invariant with changes in initial urea concentration up to 10 M. Also, stopped-flow experiments could not detect any significant changes in the relative amplitude of the fast τ_{ND} phase over this same concentration range. Thus, the molecular species which are present in unfolded RNase and which contribute to the τ_{ND}, τ_{XY}, and τ_{CT} phases are apparently not altered significantly in concentration by the addition of urea even up to concentrations of 10 M.

(*b*) *Fluorescence Measurements.* Both the manual mixing and stopped-flow experiments which utilized fluorescence were carried out under much the same conditions as those which utilized absorbance monitoring. Many of these experiments will not be discussed directly, but the data are summarized in Table I. There is, however, some additional information obtainable from fluorescence, and sufficient data will be shown to illustrate differences between the two methods of detection. The data in Figure 6 show the kinetic pattern of the slow refolding phase (in 0.31 M urea) for absorbance and fluorescence. Both sets of data can be resolved into two slow kinetic phases with the individual relaxation times derived from fluorescence being experimentally indistinguishable (see Table I) from those derived from absorbance. The same is true for the fast τ_{ND} phase (data not plotted). The only difference between data derived by the two techniques is in terms of amplitudes. At 0.31 M urea, the relative fluorescent amplitudes for the τ_{ND}, τ_{XY}, and τ_{CT} phases are ca. 15%, 35%, and 50%, respectively, while the corresponding absorbance amplitudes are 20%, 50%, and 30%. The sole reason for this difference, as will become more apparent when discussing the unfolding experiments, is because the CT process is associated with a change in intrinsic fluorescence of RNase whereas it is not in absorbance measurements.

The effects of urea and ammonium sulfate on the refolding kinetics detected by fluorescence are exactly the same as those observed from absorbance measurements, except for the amplitude effect noted above (cf. Table I). Under the same final conditions, relaxation times for all of the refolding phases are experimentally indistinguishable. As seen with absorbance, increasing urea causes the two slow phases in fluorescence to combine into a single phase. It was also observed with fluorescence that ammonium sulfate affects only the τ_{XY} relaxation time, with no detectable change in τ_{CT}.

(*II*) *Unfolding.* (*a*) *Absorbance Measurements.* In base-line refolding experiments, all fast and slow phases can be seen in either absorbance or fluorescence measurements. This is true even for those phases which have no intrinsic change in absorbance or fluorescence since they become coupled by mass action effects to the fast ND conformation process which is the last step in refolding. However, in base-line unfolding experiments, the fast ND process occurs first and thereby becomes uncoupled, so the slow phases will only be seen if there are intrinsic changes in the variable used for detection. Absorbance measurements during base-line unfolding show only a single phase, whose relaxation time will be designated as τ_{ND}' since this phase is clearly the analogue of the τ_{ND} phase seen in refolding. No slow phases are seen in absorbance. Data in Table I, taken from stopped-flow experiments, show that τ_{ND}' is 50 ms for unfolding into 4.7 M urea (pH 2.0, 10.5 °C), while it is 45 ms in 8.0 M urea.

(*b*) *Double-Jump Absorbance Experiments.* Direct measurement of the isomerization of proline-93 (Lin & Brandts, 1983b) shows that this process does occur as a slow phase in unfolding experiments. Our failure then to see the unfolding

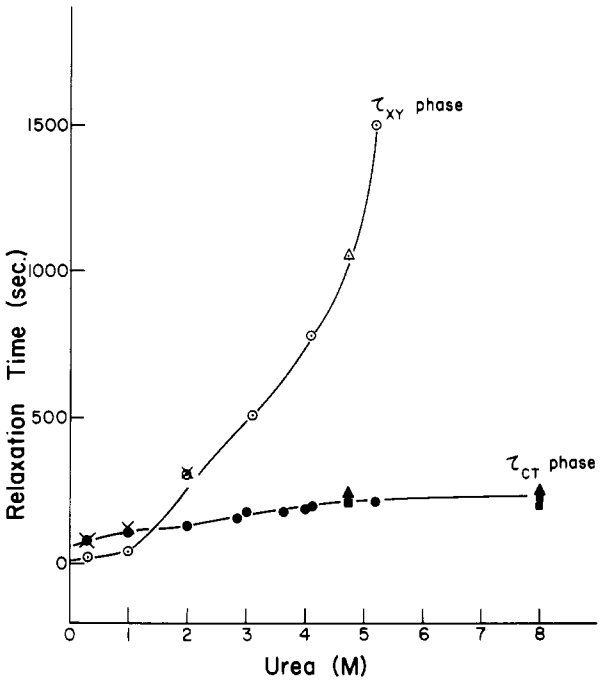

FIGURE 4: Plots of relaxation time for τ_{XY} and τ_{CT} phases as a function of urea concentration. Open and filled circles represent τ_{XY} and τ_{CT}, respectively, obtained directly from refolding experiments. Triangles represent the one-way refolding relaxation time predicted from two-way relaxation time (τ_{XY}') obtained for unfolding from double-jump experiments. Squares represent τ_{CT} predicted from τ_{CT}' obtained from fluorescence unfolding method. Crosses represent the relaxation time obtained directly from the isomeric-specific proteolytic method (Lin & Brandts, 1983b) for isomerization of proline-93.

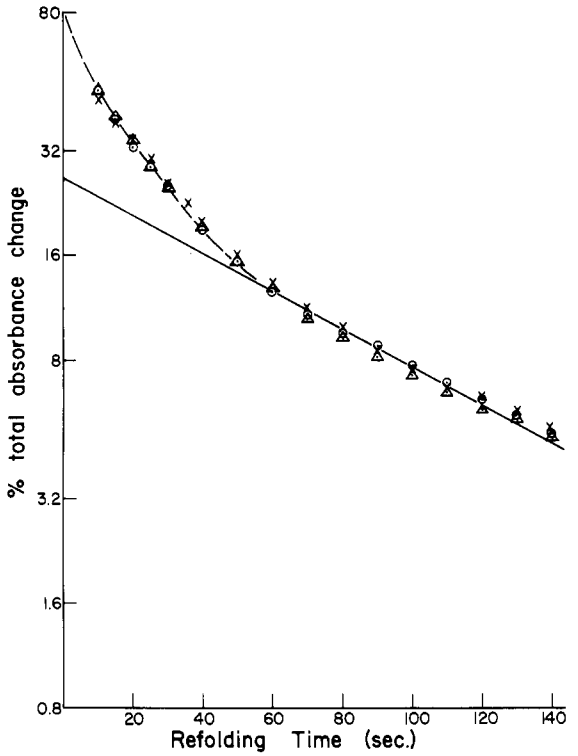

FIGURE 5: Refolding kinetics of RNase with different initial urea concentrations at 10.5 °C. (Triangles) RNase initially in 10 M urea, pH 2.3; (circles) RNase initially in 5 M urea, pH 2.0; (crosses) RNase initially in 3 M urea, pH 1.96. Final conditions are the same: 0.33 M urea, 6.0×10^{-5} M RNase A, pH 5.2. The experiments were carried out on a Cary 14 by using hand mixing.

analogue of the CT refolding process in absorbance measurements simply means it is silent. Double-jump experiments

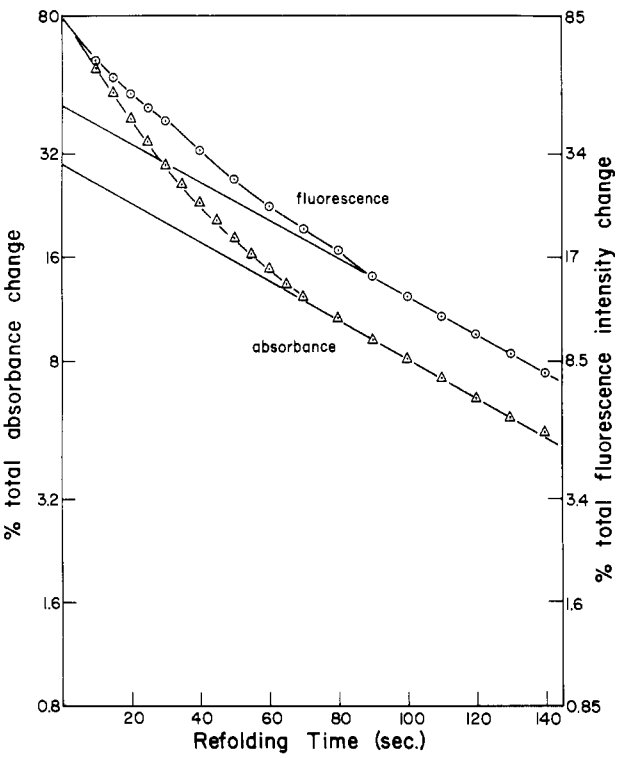

FIGURE 6: Comparisons of kinetic patterns of the slow refolding phases (τ_{CT} and τ_{XY}) obtained from absorbance and fluorescence measurements at 10.5 °C. (Circles) Fluorescence measurement; (triangles) absorbance measurement. Initial conditions: RNase (1.0×10^{-3} M) in 5 M urea, pH 2.0. Final conditions: RNase (6.25×10^{-5} M) in 0.31 M urea, pH 5.1. The experiments were carried out by using hand mixing.

(Brandts et al., 1975) using absorbance monitoring can be used to confirm its existence in unfolding. It is also of interest as to whether the XY process seen in refolding experiments has a silent counterpart in unfolding. Since the initial urea concentrations up to 10 M have no effect on the amplitude of the τ_{XY} phase seen in refolding, we would certainly anticipate that it might still occur in the high urea concentrations used for unfolding experiments.

When the double-jump experiments were carried out, native RNase was first placed in a denaturing buffer containing 5.6 M urea (pH 2.0, 10.5 °C), allowed to unfold for a variable amount of time t, and then transferred to a native-state buffer containing low urea (0.3 M urea, pH 5.0, 10.5 °C), and the slow refolding was then monitored on the Cary 14. Under these low-urea refolding conditions, the individual amplitudes of both the τ_{XY} and τ_{CT} refolding phases can be measured as a function of the unfolding time t. Plots of these data are shown in Figure 7. The growth of both the τ_{XY} and τ_{CT} refolding phases, as a function of unfolding time t, are first-order processes within errors. The relative amplitudes of the two phases, after long unfolding times, are identical with those obtained during simple refolding experiments. However, the growth of the τ_{XY} refolding phase during unfolding occurs much slower, with a relaxation time ($\tau_{XY}' = 300$ s) that is almost twice as long as that for the growth of the CT refolding process ($\tau_{CT}' = 170$ s). In a separate experiment for unfolding in 8 M urea, and refolding into 0.5 M urea, and 0.39 M $(NH_4)_2SO_4$, τ_{CT}' was found to be 180 s (Table I). In this latter experiment, τ_{XY}' could not be measured by manual mixing as the XY refolding process was too fast.

Direct measurements of the cis-to-trans isomerization of proline-93 (Lin & Brandts, 1983b) during unfolding in 8.5 M urea showed a relaxation time of 140 s and an involvement

Table II: Kinetic Data of Unfolding of RNase in Urea Solution

final conditions,[a] urea (M), pH	methods[b]	temp (°C)	τ_{ND}' phase amplitude	τ_{ND}' phase τ (s)	τ_{XY}' phase amplitude	τ_{XY}' phase τ (s)	τ_{CT}' phase amplitude	τ_{CT}' phase τ (s)
4.7, 2.0	F(M)	10.5						155
8.0, 2.0	F(M)	10.5						165
8.0, 2.0	F(M)	16.0						80
4.7, 2.0	F(SF)	10.5	0.70	0.050			0.30	150
8.0, 2.0	F(SF)	10.5	0.70	0.046			0.30	150
4.7, 2.0	F(SF)	16.0	0.70	0.036			0.30	76
8.0, 2.0	F(SF)	16.0	0.70	0.030			0.30	75
4.7, 2.0	A(SF)	10.5	1.0	0.050				
8.0, 2.0	A(SF)	10.5	1.0	0.045				
4.8, 2.0	A(DJ)[c]	10.5				300		170
8.0, 2.0	A(DJ)[d]	10.5						180

	activation energy (kcal/mol)	
	τ_{ND}' phase	τ_{CT}' phase
fluorescence	11	21

[a] Initial conditions: 1.0×10^{-3} M RNase in H_2O; dilution factor, 1:15. [b] Methods are as designated in Table I. [c] Assayed by diluting in 0.1 M acetate buffer solution; final urea concentration, 0.30 M. [d] Assayed by diluting in 0.1 M acetate–0.42 M $(NH_4)_2SO_4$ solution; final conditions, 0.5 M urea and 0.39 M $(NH_4)_2SO_4$.

of 30% of the RNase molecules. The faster slow phase seen in unfolding, monitored by the double-jump method, shows a 30% amplitude and a relaxation time τ_{CT} of 180 s in 8.0 M urea. Since there are rather large inherent errors in both the isomer-specific proteolysis method and in the double-jump method, the agreement is very satisfactory. This allows an unequivocal identification of the τ_{CT}' unfolding phase with the isomerization of proline-93. Since the τ_{CT} refolding phase in absorbance was previously shown to coincide with direct measurements of the isomerization of proline-93 during refolding, the τ_{CT} and τ_{CT}' phases must arise from driving the proline-93 isomerization process in opposite directions during unfolding and refolding, respectively. Knowing the value of τ_{CT}' from the double-jump experiments (which correspond to two-way cis-to-trans isomerization) and knowing the equilibrium cis/trans ratio for proline-93 in unfolded RNase from direct measurements, one can then calculate what the expected value would be for the one-way τ_{CT} process during refolding. These calculated values are plotted (filled triangles) at 4.8 and 8 M urea in Figure 4, along with the actual measured values of τ_{CT} from refolding experiments. These predicted values for refolding, calculated from unfolding experiments, agree nicely with the values measured directly during refolding.[3] This shows that the isomerization of proline-93 is not significantly catalyzed during the refolding of RNase but is exactly what is predicted from the measured relaxation time during unfolding. Our results therefore disagree with the previous suggestion that the presence of a structural intermediate along the refolding pathway speeds up proline isomerization during refolding (Nall et al., 1978; Cook et al., 1979; Schmid, 1981; Schmid & Blaschek, 1981).

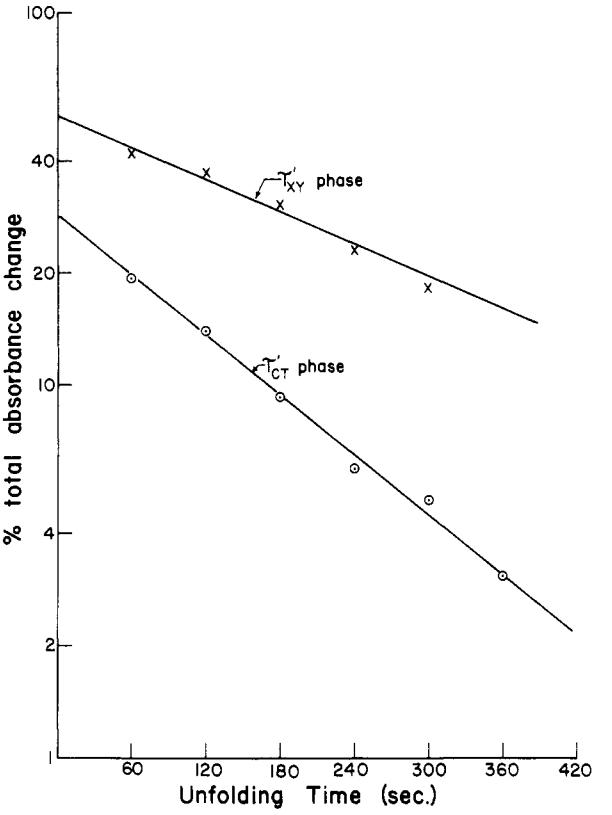

FIGURE 7: Rates of slow unfolding phases (τ_{XY}' and τ_{CT}') of RNase at 10.5 °C detected by the double-jump method. (Circles) τ_{CT}' phase; (crosses) τ_{XY}' phase. The time scale on the abscissa corresponds to the time allowed for unfolding, prior to refolding. The ordinate corresponds to the percent of absorbance change of each phase for refolding subtracted from that of the equilibrated unfolded sample.

The other slow phase seen in unfolding by the double-jump method, i.e., the τ_{XY}' phase, is correlated with the growth of the τ_{XY} refolding phase and we therefore feel that these two are also counterparts arising from driving the same silent structural process in opposite directions. By use of the measured value of the two-way relaxation time from double-jump experiments (τ_{XY}' = 300 s) and the equilibrium concentration of species derived from the simple model in the following paper

[3] Refolding and unfolding experiments were carried out at different pH values, as noted in Tables I and II, so that the direct comparison of these relaxation times in Figure 4 might reasonably be questioned. There are additional data, however, which show that the relaxation times for the slow phases in refolding are reasonably independent of pH so long as the final conditions are in the native-state base-line region and not too close to the transition point. For example, refolding studies (0.31 M urea, 10.5 °C) at eight different pH values in the range 2.7–6.0 all gave a τ_{CT} value of 100 ± 10 s, with an amplitude of 28 ± 2%. Over a pH range from 3.3 to 6.0 under similar conditions, values of τ_{XY} were 20 ± 5 s and the amplitude of the XY phase was 50 ± 3%. At lower pH values as refolding conditions become closer to the transition point, both τ_{CT} and τ_{XY} become larger (e.g., τ_{CT} = 200 s and τ_{XY} = 55 s at pH 2.65) and their relative amplitudes change markedly. Because of the pH independence of refolding relaxation times outside the transition region, the direct comparison of base-line unfolding data to base-line refolding data in Figure 4 is probably justified, even though pH values under final conditions are different.

(Brandts & Lin, 1983c) the expected one-way relaxation time for refolding, for the same process seen in unfolding, is predicted to be 1050 s in 4.8 M urea. This predicted value is plotted (open triangle) in Figure 4 and falls precisely in sequence with the actual measured values of τ_{XY} for the refolding process.

It will be recalled that, in the refolding direction, most of the τ_{CT} process becomes rate limited by the τ_{XY} process when the urea concentration was increased, implying a coupled mechanism of refolding whereby the molecular events for both processes must occur before refolding takes place. It is interesting then that this does not happen in the unfolding direction since the observed relaxation time τ_{CT}' is shorter than the observed relaxation time τ_{XY}' in 4.8 M urea.

(*c*) *Fluorescence Measurements.* Two stopped-flow kinetic phases are seen by fluorescence monitoring during unfolding, while only the slower one can be studied by manual mixing (Table II). Both phases are strictly first order in their time dependence. The relative amplitudes are 70% and 30%, with the fast phase being the larger. The faster phase corresponds exactly in relaxation times to the single phase seen in absorbance unfolding, with a τ_{ND}' value of 50 ms at 10.5 °C and 30 ms at 16 °C with 8 M urea in the final buffer. The relaxation times for the slower fluorescent phase at two different urea concentrations are in excellent agreement with the τ_{CT}' relaxation times at the same urea concentrations (Table II) obtained by the double-jump method. Thus, the extra phase seen in fluorescence and not seen in absorbance is the τ_{CT}' phase. This phase then involves a substantial inherent change in fluorescence even though it is silent in absorbance. This agrees with the previous suggestion that the isomerization of proline-93 occurs with large changes in the fluorescence of tyrosine-92 (Schmid, 1981; Schmid & Blaschek, 1981).

The XY process, seen in unfolding reactions monitored by the double-jump method, is not seen in fluorescence unfolding experiments which must mean that it is silent in fluorescence.

(*III*) *Activation Energies.* In all cases where it was possible, activation energies were measured for each of the kinetic phases seen in the unfolding and refolding reactions. These are shown in Tables I and II. In some cases, both fluorescence and absorbance monitoring were used with satisfactory agreement between the two estimates. The large values of E_a obtained for the τ_{CT} refolding phase at 0.31 M urea (ca. 17 kcal/mol) and the τ_{CT}' unfolding phase at 8 M urea (ca. 21 kcal/mol) confirm the counterpart nature of these two relaxations and agree with the assignment of these phases to the isomerization of proline-93. Likewise, the activation energy for the refolding τ_{ND} (ca. 10 kcal/mol) and the unfolding τ_{ND}' phase (ca. 11 kcal/mol) are very similar. Although the activation energy for the τ_{XY}' unfolding phase could not be accurately measured since it is not visible in either absorbance or fluorescence, the τ_{XY} refolding phase has an activation energy of ca. 8 kcal/mol.

Our assignment of activation energies to the slow phase of RNase refolding differs substantially from other treatments in the literature. Nall et al. (1978) reported an activation energy of only 5 kcal/mol for slow refolding in low Gdn·HCl and suggested that this small value argued against the idea (Brandts et al., 1975) that the slow phase is rate limited by the free isomerization of proline residues. Their value was obtained from the temperature dependence of the half-time for the *entire* slow phase of refolding, whereas our data were obtained under conditions where the τ_{CT} and τ_{XY} relaxations could be separately analyzed. Although it is true that the activation energy for the XY process is somewhat smaller than expected for the free isomerization of peptide bonds, the activation energy for the CT process is exactly what would be expected.

Further Discussion

Using absorbance, fluorescence and double-jump methods, three separate relaxation times, τ_{ND}, τ_{XY}, and τ_{CT}, can be resolved in the refolding direction and three separate relaxation times, τ_{ND}', τ_{XY}', and τ_{CT}', can be resolved in the unfolding direction. Many previous studies have shown that the very fast τ_{ND} and τ_{ND}' relaxations are associated with large alterations in RNase conformation, since both are accompanied by substantial intrinsic changes in many different structural parameters. These must result directly from driving the major conformational changes in different directions, as has been frequently assumed. Therefore, our attention here will focus primarily on the other four relaxations of less certain origin.

The principle of microscopic reversibility necessitates that a reaction must progress through the same succession of states as mass flows in either the forward or reverse direction under *constant* conditions. However, when data from base-line refolding experiments are compared with data from base-line unfolding experiments, the final conditions are substantially different so it cannot be assumed that states important in one direction are necessarily the same states which are important in the other direction. Nevertheless, the evidence presented in the accompanying papers (Lin & Brandts, 1983b,c) supports the contention that the τ_{CT} and τ_{CT}' are counterpart relaxations which result from driving the isomerization of proline-93 in opposite directions during base-line unfolding and refolding reactions. Likewise, the evidence suggests that τ_{XY} and τ_{XY}' are counterpart relaxations arising from a subtle structural change of unknown origin.

The important characteristics of the slow-phase relaxations can be summarized as follows.

(I) (A) Double-jump experiments show that the τ_{CT}' unfolding phase is directly associated with the formation of the molecular species which is responsible for the τ_{CT} refolding phase. Both phases are first order, indicating a simple relationship between them. (B) Double-jump experiments suggest that the same molecular species are involved in the unfolding τ_{XY}' phase and in the refolding τ_{XY} phase, for the same reasons given above.

(II) (A) From the relaxation time τ_{CT}' during unfolding, one can calculate (Lin & Brandts, 1983c) what would be the expected relaxation time for the same process if it is merely driven in the opposite direction during refolding. This calculated relaxation time agrees almost exactly with the relaxation times which were actually measured for the τ_{CT} refolding phase (Figure 4). (B) From the measured relaxation time τ_{XY}' for unfolding, the calculated refolding relaxation time for the counterpart process also agrees nicely with the experimentally measured values for the τ_{XY} refolding phase (Figure 4).

(III) (A) The τ_{CT}' unfolding phase is silent in absorbance but has a large change in fluorescence. Presumably, there will also then be no intrinsic change in absorbance for the τ_{CT} refolding phase, so its visibility is due to mass-action coupling to the τ_{ND} phase. It will be shown later that this assumption is consistent with all amplitude data (Lin & Brandts, 1983c). (B) The τ_{XY}' unfolding phase is silent in absorbance and in fluorescence. We assume then that there is also no intrinsic change in absorbance or fluorescence for the τ_{XY} refolding phase, if it is indeed the counterpart process.

(IV) (A) The relative amplitude of the τ_{CT} refolding phase is independent of initial urea concentrations up to 10 M (Figure 5), when refolding into low urea. (B) The relative

amplitude of the τ_{XY} refolding phase is also independent of initial urea concentrations up to 10 M.

(V) The amplitude and relaxation time for the τ_{CT} refolding phase, measured by absorbance, are identical within errors (Figure 4) to the amplitude and relaxation time measured independently for the isomerization of proline-93 during refolding by using isomer-specific proteolysis (Lin & Brandts, 1983b). The relaxation time for the τ_{CT}' unfolding phase, measured either by double-jump or fluorescence techniques, is also in excellent agreement with that measured directly for the isomerization of proline-93 during unfolding of RNase (Figure 4). In addition, the large activation energies of ca. 20 kcal/mol for the τ_{CT} and τ_{CT}' phases are consistent with the involvement of isomerization.

(VI) (A) The relaxation time τ_{CT}, and presumably τ_{CT}' as well, shows a small dependence on urea concentration (Figure 4). (B) The relaxation time τ_{XY}, and presumably τ_{XY}' as well, shows a large dependence on urea concentration (Figure 4).

(VII) The relative absorbance amplitude associated with the fast τ_{ND} refolding phase is constant at ca. 20% for all conditions.

(VIII) There is an interesting relationship between the absorbance amplitudes of the two slow phases in refolding experiments. When the CT process is much slower than the XY process, there is 30% amplitude associated with τ_{CT} and 50% amplitude associated with τ_{XY}. When the CT process is much faster than the XY process, there is only 7% amplitude associated with τ_{CT} and 73% amplitude associated with τ_{XY}. Only one slow relaxation is detected under intermediate conditions, and this has a relaxation time close to that expected for τ_{XY}. This amplitude behavior is quantitatively predictable if it is assumed that the CT and XY processes occur independently of one another (Lin & Brandts, 1983c).

(IX) For refolding into low urea the fluorescence amplitudes for the τ_{ND}, τ_{XY}, and τ_{CT} processes are 15%, 35%, and 50%, respectively, while they are 20%, 50%, and 30% for absorbance. For unfolding the corresponding amplitudes are 70%, 0%, and 30% for fluorescence and 100%, 0%, and 0% for absorbance.

Three independent sources of evidence show that the four slow-phase relaxations are actually due to two counterpart pairs of molecular processes. First, the double-jump experiments (item I) demonstrate directly the relationship between the τ_{CT}' unfolding relaxation and the τ_{CT} refolding relaxation and between the τ_{XY}' unfolding relaxation and the τ_{XY} refolding relaxation. Second, for both the CT and XY processes, the refolding relaxation time can be accurately predicted from the unfolding relaxation time if it is assumed that they are counterpart processes (item II). Finally, it will be shown in the following paper (Lin & Brandts, 1983c) that, assuming the counterpart nature of the relaxations, both the absorbance and fluorescence amplitudes (item IX) for the two slow refolding relaxations can be accurately predicted knowing only the fast-phase amplitude and the equilibrium cis/trans ratio for proline-93 which was determined directly for unfolded RNase.

Two sources of evidence show that both the CT and XY processes are associated with very local changes in the structure of the polypeptide chain in the unfolded state and are not involved directly in the large conformational changes which occur during unfolding and refolding. First of all, the lack of any intrinsic change in absorbance or fluorescence for the XY process (item III) and the lack of any intrinsic change in absorbance for the CT process rule out any extensive conformational changes. The substantial change in intrinsic fluorescence for the CT process has already been attributed (Schmid, 1981; Schmid & Blaschek, 1981) to a local effect of the isomerization of proline-93 on the fluorescence characteristics of tyrosine-92. The fact that complete *unfolding* (as measured by absorbance change) can occur before the CT and XY processes begin to equilibrate, while the XY and CT processes act to delay the *refolding*, must mean that these processes occur in the unfolded form of RNase. Second, the refolding amplitudes for both the XY and CT relaxations are independent of the initial urea concentrations in the unfolding buffer up to concentrations of 10 M (item IV). This means that the equilibrium distribution of XY and CT states in the unfolded RNase are not affected by urea. Urea solutions of 10 M are thought to be very close to "complete random coil" solvents (Tanford, 1968) so that if significant changes in secondary and tertiary structure were involved in either of these processes, then these high urea concentrations surely would shift the equilibrium populations and thereby affect the amplitudes during refolding. On the other hand, it is known that strong protein denaturants do not cause any changes in the cis/trans equilibria for model peptides. For example, only a small change in cis/trans ratio can be detected for (trifluoroacetyl)proline from 0 to 6 M Gdn·HCl by NMR (unpublished observations from this laboratory).

As implied above and as further documented in item V, there is every reason to believe that the CT process involves no significant structural changes other than the isomerization of proline-93. There is, however, a very small dependence of the relaxation time τ_{CT} on urea concentration [item (VI) (A)], changing by a factor of ca. 2.5 from 0 to 8 M urea. It has been suggested (Nall et al., 1978) that processes which are rate limited by the free isomerization of proline residues should exhibit no significant dependence of relaxation times on the concentration of denaturants such as Gdn·HCl. The experimental basis for this suggestion was the lack of any dependence for the isomerization of the dipeptide Ala-Pro on Gdn·HCl up to 2 M. However, it was shown in previous papers (Lin & Brandts, 1983a,b) that the isomerization of proline-93 can be significantly influenced by overall chain dynamics which would not be present in dipeptides. For example, the relaxation time for isomerization varies by a factor of 7 between various fragments of RNase containing proline-93, depending on the length of the chain and on the presence or absence of disulfide bonds. Denaturants such as urea or Gdn·HCl are known to interact strongly with polypeptides, and we do not think it is unreasonable that they will thereby exert at least a small effect on rates of isomerization for long polypeptides through their effects on chain dynamics.

One final point should be emphasized. Our data show [item (II) (A)] that the measured rate of isomerization during refolding is exactly that which is expected on the basis of the rate of isomerization during unfolding. It was previously suggested that proline isomerization for RNase during refolding may be up to 40 times faster than expected from the rate during unfolding (Cook et al., 1979), and it was later suggested (Schmid, 1981; Schmid & Blaschek, 1981; Rehage & Schmid, 1982) that the relative rates for proline-93 may show such a discrepancy. The reason for the disagreement between our conclusion and these earlier suggestions has to do not with differences in experimental data but with the method of data analysis. Earlier studies assumed that the entire slow phase could be treated as being due to proline isomerization, and the half-times for this entire phase were compared for the unfolding and refolding processes. The present results show that there are two slow phases and that the phase due to isomerization of proline-93 is the slower of

the two phases in refolding to low urea but is the faster of the two phases for unfolding into high urea. Only after separation of the two phases can the isomerization of proline-93 be accurately determined. In order to explain the apparent discrepancy of isomerization rates during unfolding and refolding, it was proposed that refolding occurred through an enzymatically active structural intermediate I_N (Nall et al., 1978; Cook et al., 1979), which contains one or more prolines in the incorrect isomeric form. Our data rule out the specific suggestion (Schmid, 1981; Schmid & Blaschek, 1981) that it is proline-93 which is in the incorrect form in I_N and in general provide no positive support for the existence of I_N.

In contrast to the CT process, very little is known about the structural changes involved in the XY relaxation in unfolded RNase, except that it does not involve changes in equilibrium conformational features that are urea sensitive. In spite of the lack of a urea effect on the equilibrium distribution of XY species, urea does exert an enormous effect on the relaxation time τ_{XY} [item (VI) (B)]. Its value is increased by nearly 100 times in going from 0.3 to 5 M urea, implying that the activated state lying on the pathway between the equilibrium XY species is seriously destabilized by urea even though the initial and final states are not. The XY process bears many similarities to the CT process, noted above, and conceivably could be due to the isomerization of a peptide bond(s) other than the proline-93 bond. However, because of this large sensitivity of relaxation time to urea concentration, which is not seen for the CT process, and because of the small E_a of 8 kcal/mol, we are inclined not to suggest this interpretation at present.

In the following paper (Lin & Brandts, 1983c), a very simple model is proposed which will account for all of the above experimental characteristics of the RNase unfolding and refolding reactions, including the interesting dependence of refolding amplitudes on the relative values of relaxation times τ_{CT} and τ_{XY} (item VIII).

Registry No. RNase A, 9001-99-4; urea, 57-13-6.

References

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953.

Cook, K. H., Schmid, F. X., & Baldwin, R. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6157.

Garel, J. R., Nall, B. T., & Baldwin, R. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1853.

Lin, L.-N., & Brandts, J. F. (1978) *Biochemistry 17*, 4102.

Lin, L.-N., & Brandts, J. F. (1983a) *Biochemistry* (companion paper in this issue).

Lin, L.-N., & Brandts, J. F. (1983b) *Biochemistry* (second paper of four in this issue).

Lin, L.-N., & Brandts, J. F. (1983c) *Biochemistry* (following paper in this issue).

Nall, B. T., Garel, J. R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317.

Rehage, A., & Schmid, F. X. (1982) *Biochemistry 21*, 1499.

Schmid, F. X. (1981) *Eur. J. Biochem. 114*, 105.

Schmid, F. X. & Blaschek, H. (1981) *Eur. J. Biochem. 114*, 111.

Tanford, C. (1968) *Adv. Protein Chem. 23*, 122.

Mechanism for the Unfolding and Refolding of Ribonuclease A. Simulations Using a Simple Model with No Structural Intermediates†

Lung-Nan Lin and John F. Brandts*

ABSTRACT: A simple mechanism is proposed to account for the folding and unfolding of RNase A in aqueous urea solution. This is based on the existence of three independent structural processes, each of which gives rise to counterpart relaxations in base-line unfolding and base-line refolding experiments. The fast ND process involves the actual conformational changes which take place, while the slower CT and XY processes are due to urea-insensitive chain equilibrations in the denatured state. The CT process is identified as the cis–trans isomerization of proline-93, while the origin of the XY process is not known. If numerical values for the minimal set of parameters are assumed, the model can be used to simulate base-line unfolding and baseline refolding experiments. The relaxation times and amplitudes of the different phases seen in the simulated kinetics agree under all conditions with the corresponding experimental parameters, within the experimental error. This includes values obtained from absorbance, fluorescence, and double-jump experiments in both the unfolding and refolding directions and over a concentration range of urea from 0.3 to 8.0 M. It is concluded that none of the kinetic phases which can be seen directly in stopped-flow or manual-mixing experiments are due to populating structural intermediates along the folding pathway. There is, however, evidence from amplitude measurements that there is a urea-sensitive process which occurs too fast to be seen directly by stopped-flow methods. It is suggested that this is due to a fast equilibrium between the urea-denatured form, closely akin to a random chain, and the water-denatured form, which has less-extended dimensions and possibly some local ordered structure.

There is an extremely large amount of data in the literature on the unfolding and refolding of RNase A, much more so than for any other protein. It is in many ways the archetypical globular protein for the testing of mechanisms for folding. Most of the thermodynamic evidence (Lumry et al., 1966; Brandts & Hunt, 1967; Tanford, 1968; Jackson, 1970; Privalov & Khechinashvili, 1974; Brandts et al., 1975; Y. Saito and A. Wada, unpublished experiments) is quantitatively consistent with the idea that the equilibrium unfolding and refolding closely approximates a two-state process with no detectable concentration of true structural intermediates. On the other

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0006-2960/83/0422-0573$01.50/0