Refolding of Ribonuclease in the Presence and Absence of Ammonium Sulfate Pulses. Comparison between Experiments and Simulations†

Lung-Nan Lin and John F. Brandts*
Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003
Received July 28, 1986; Revised Manuscript Received October 23, 1986

ABSTRACT: Experiments have been carried out on ribonuclease A in which refolding in high concentrations of guanidine hydrochloride is either preceded or not preceded by a short ammonium sulfate pulse. Application of the pulse causes the rapid formation of the nativelike intermediate, and the effect of this pulse was determined by using three different methods for monitoring the subsequent refolding reaction: direct absorbance, direct fluorescence, and a double-jump fluorescence unfolding assay which is specific for the isomerization of proline-93. The effect of the pulse is quite different depending on the method of detection. With absorbance detection, the pulse causes a large reduction in the refolding amplitude with no change in the kinetics of the decay curve, while with the fluorescence unfolding assay, the pulse causes no change in the refolding amplitude but produces a large acceleration in the decay kinetics. The results with direct fluorescence are intermediate with some reduction seen in the refolding amplitude and some acceleration in the decay kinetics. The results of these experiments are simulated by using the simple model of Lin and Brandts (1984) [Lin, L.-N., & Brandts, J. F. (1984) *Biochemistry 23*, 5713] in which proline-93 must be in the correct cis configuration before folding to the native or nativelike state can occur. In all cases, the simulations accurately predict the experimental results for all three methods of detection, without any adjustment of parameter values from those published earlier. It is concluded that our simple model is in excellent agreement with results from these pulse experiments and that the acceleration seen in the decay curves with fluorescence and fluorescence unfolding monitoring is probably not due to an inherent increase in the rate of isomerization of proline-93 in the nativelike intermediate as suggested by others but rather to expected mass action effects that can be accurately predicted from our model.

Schmid (1986) recently developed a new double-jump assay for following the kinetics of ribonuclease (RNase) refolding, based on fluorescence changes during subsequent unfolding. This assay method appears to see only the isomerization of proline-93 and is silent toward all other structural changes. Using this assay, he found that the refolding kinetics in 2 M guanidine hydrochloride (Gdn·HCl) are greatly accelerated when the RNase is subjected to a short pulse of $(NH_4)_2SO_4$ just prior to refolding, relative to refolding under the same final conditions in the absence of the pulse. He concluded from this that refolding to the nativelike intermediate I_N occurs with proline-93 in the incorrect trans isomer and that trans to cis isomerization is accelerated 5 times in the nativelike intermediate relative to isomerization in the unfolded form. He further concluded that "these findings rule out the simple model of Lin and Brandts ... in which Pro-93 trans → cis isomerization must take place before any folding occurs".

In this paper, we report results of refolding experiments under the same conditions as those of Schmid (1986), not only using the fluorescence unfolding assay for monitoring refolding but also using direct fluorescence and absorbance monitoring. We have found good agreement with Schmid's experimental results using the fluorescence unfolding assay and have in addition shown that pretreatment with the $(NH_4)_2SO_4$ pulse causes a substantial acceleration in refolding kinetics monitored by direct fluorescence changes but causes no change in the kinetics monitored by direct absorbance measurements.

Simulations of these experiments are carried out by using the simple model of Lin and Brandts (1983c, 1984) and using values for all parameters from the original publications where possible. These simulations are able to reproduce the experimental data not only for monitoring with Schmid's fluorescence unfolding assay but also for monitoring with direct fluorescence and with direct absorbance. Thus, all experimental results are in excellent agreement with the predictions of our simple model and, rather than ruling out the model, go a long way toward providing justification for it.

Materials and Methods

Materials. Bovine RNase A (catalog no. R-5500, lot no. 25F-80501), purchased from Sigma Chemical Co., was directly used without further purification. Cacodylic acid (sodium salt, catalog no. C-0250, lot no. 37C-0121) was also obtained from Sigma Chemical Co. Ultrapure guanidine hydrochloride (Gdn·HCl) is the product of Schwarz/Mann Co. All other chemicals are of reagent grade.

RNase Refolding Experiments. The refolding experiments are very similar to those of Schmid (1986) for comparison purposes. The refolding reaction was monitored by three probes. In addition to the fluorescence unfolding assay of Schmid (1986), refolding was also monitored directly by absorbance and fluorescence change. The instrumentation for RNase refolding experiments has been previously described (Lin & Brandts, 1983b, 1987). The detailed experimental procedures are as follows:

(*1*) *RNase Refolding in 2 M Gdn·HCl/0.16 M $(NH_4)_2SO_4$ Solution at 10 °C.* For refolding detected by absorbance and fluorescence change, 500 μL of equilibrated, unfolded RNase (in 4 M Gdn·HCl/0.1 M glycine, pH 2.0, at 10 °C; concentration ca. 3.7×10^{-4} M for absorbance measurement, 5.6×10^{-5} M for fluorescence measurement) was diluted 5 times into a cuvette (thermostated at 10 °C) containing 2 mL of 1.5 M Gdn·HCl, 0.2 M $(NH_4)_2SO_4$, and 0.05 M cacodylate buffer

† This work was supported by NIH Grant GM 11071.

0006-2960/87/0426-1826$01.50/0

solution (pH 6.5). The absorbance or fluorescence change was continuously followed until equilibrium. For refolding detected by the fluorescence unfolding assay, 50 μL of the unfolded RNase (in 4 M Gdn·HCl/0.1 M glycine, pH 2.0, at 10 °C; concentration 3×10^{-4} M) was also diluted 5 times into a test tube (thermostated at 10 °C) containing 200 μL of 1.5 M Gdn·HCl, 0.2 M $(NH_4)_2SO_4$, and 0.05 M cacodylate buffer (pH 6.5) for a predetermined time interval of refolding. Then, a 200-μL aliquot of the solution was quickly pipetted into a cuvette containing 1.8 mL of 5.0 M Gdn·HCl/0.1 M glycine, pH 1.95 (at 11 °C), and mixed for a few seconds with a spatula. The change in fluorescence intensity was continuously followed until equilibrium. The fluorescence unfolding amplitude at each refolding time interval was obtained by extrapolation to zero unfolding time in a semilogarithmic plot (Schmid, 1986; Lin & Brandts, 1986).

(2) RNase Subjected to a Short Pulse in 0.8 M $(NH_4)_2SO_4$ at 0 °C Immediately before Refolding in 2 M Gdn·HCl/0.16 M $(NH_4)_2SO_4$ Solution at 10 °C. For refolding detected by absorbance and fluorescence measurements, 70 μL of unfolded RNase (in 4 M Gdn·HCl/0.1 M glycine, pH 2.0, at 0 °C; concentration ca. 3.7×10^{-3} M for absorbance measurement, 5.6×10^{-4} M for fluorescence measurement) was diluted 10 times into a test tube (thermostated at 0 °C) containing 630 μL of 0.888 M $(NH_4)_2SO_4$/0.0555 M cacodylate buffer (pH 6.1) for 12–15 s. Then, 500 μL of solution was quickly pipetted into a cuvette (thermostated at 10 °C) containing 2.0 mL of 2.4 M Gdn·HCl/0.0375 M cacodylate buffer (pH 6.0) and mixed for a few seconds with a spatula. The absorbance or fluorescence change was continuously followed until equilibrium. For refolding detected by the fluorescence unfolding assay, 30 μL of unfolded RNase (in 4 M Gdn·HCl/0.1 M glycine, pH 2.0, at 0 °C; concentration 3.0×10^{-3} M) was diluted 10 times into a test tube (thermostated at 0 °C) containing 270 μL of 0.888 M $(NH_4)_2SO_4$/0.0555 M cacodylate buffer (pH 6.1) for 12–15 s. Then, 50 μL of the solution was diluted 5 times into a test tube (thermostated at 10 °C) containing 200 μL of 2.4 M Gdn·HCl/0.0375 M cacodylate buffer (pH 6.0) for a predetermined time interval of refolding. Then a 200-μL aliquot of solution was quickly pipetted into a cuvette (thermostated at 11 °C) containing 1.8 mL of 5.0 M Gdn·HCl/0.1 M glycine, pH 1.95, and mixed for a few seconds with a spatula. The change in fluorescence intensity was continuously followed until equilibrium.

In addition to the above refolding experiments in high Gdn·HCl solution, refoldings with and without a $(NH_4)_2SO_4$ pulse detected by fluorescence unfolding assay were also carried out in the same fashion in lower Gdn·HCl solution [i.e., 0.08 M Gdn·HCl, 0.16 M $(NH_4)_2SO_4$, and cacodylate buffer solution, pH 5.9] at 11 °C.

Results

Refolding in High Gdn·HCl. All of these refolding experiments were carried out with the same final conditions [i.e., 2 M Gdn·HCl, 0.16 M $(NH_4)_2SO_4$, and 0.04 M cacodylate, pH 5.9, at 10 °C] using three different detection methods (i.e., direct absorbance, direct fluorescence, and fluorescence unfolding assays) to monitor the refolding process. For each detection method, two different experiments were performed. The first was to refold the unfolded RNase (in 4 M Gdn·HCl/0.1 M glycine, pH 2.0) directly in the final solution without pretreatment. The second was to pre-refold the unfolded RNase in 0.8 M $(NH_4)_2SO_4$ at 0 °C for 12–15 s before refolding in the 2 M Gdn·HCl/0.16 M $(NH_4)_2SO_4$ solution. The exposure to the short ammonium sulfate pulse causes a fraction of the denatured molecules to convert quickly to the nativelike intermediate I_N (Lin & Brandts, 1984; Schmid, 1983).

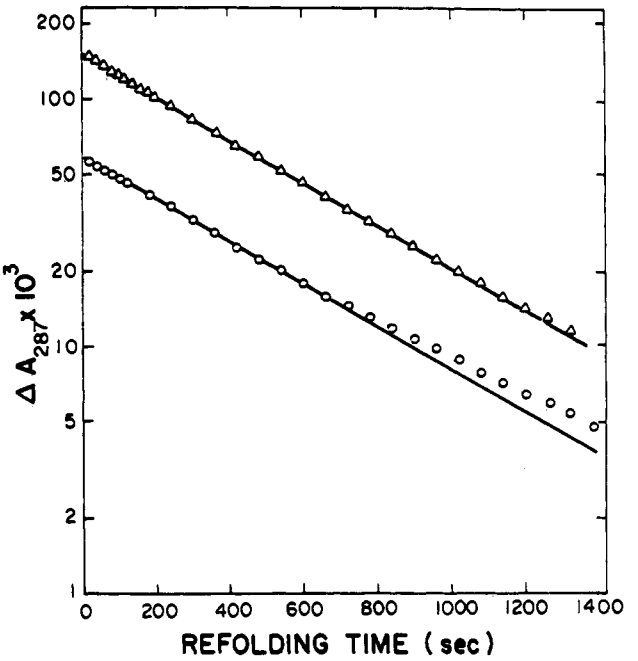


FIGURE 1: Comparison of the simulated refolding kinetics of RNase with that obtained from experimental data detected by direct absorbance measurements in 2 M Gdn·HCl. The model of eq 1 was used for simulation. The solid lines represent the simulated data. The circles and triangles represent the experimental data obtained for refolding with and without a $(NH_4)_2SO_4$ pulse, respectively. See text for details.

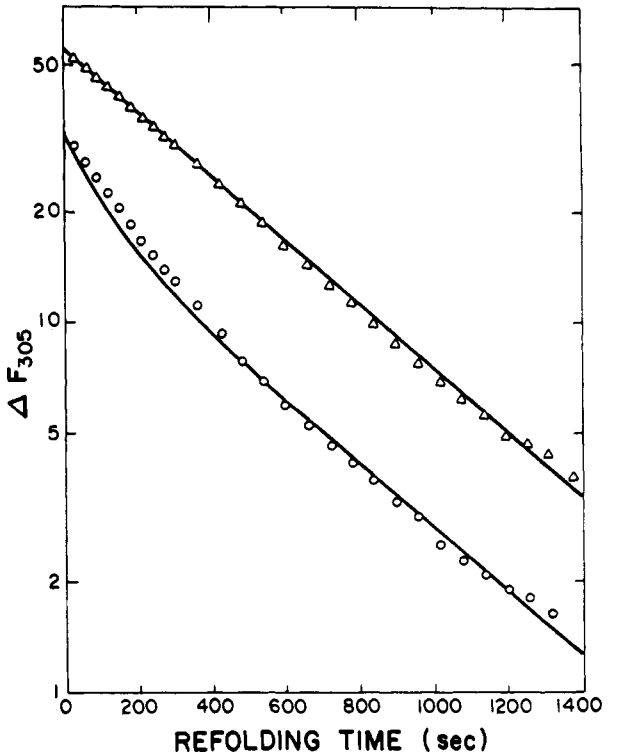


FIGURE 2: Comparison of the simulated refolding kinetics of RNase with that obtained from experimental data detected by direct fluorescence measurements in 2 M Gdn·HCl. The model of eq 1 was used for simulation. The solid lines represent the simulated data. The circles and triangles represent the experimental data obtained for refolding with and without the $(NH_4)_2SO_4$ pulse, respectively.

The results of these experiments are shown in Figures 1–3 as semilogarithmic plots, where the triangles correspond to direct refolding and the circles are for refolding with a prior

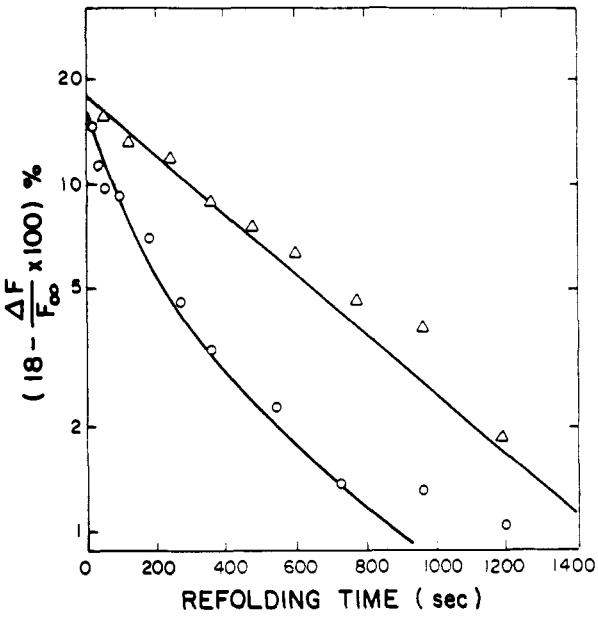


FIGURE 3: Comparison of the simulated refolding kinetics of RNase with that obtained from experimental data detected by fluorescence unfolding assay for refolding in 2 M Gdn·HCl. The model of eq 1 was used for simulation. The solid lines represent the simulated data. The circles and triangles represent the experimental data obtained for refolding with and without a $(NH_4)_2SO_4$ pulse, respectively.

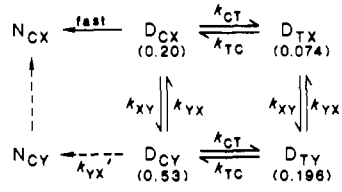
$(NH_4)_2SO_4$ pulse. The data for absorbance monitoring are in Figure 1. These results show that the kinetic patterns for refolding either with or without the $(NH_4)_2SO_4$ pulse are very similar. The only major difference is in the total refolding amplitude. The direct refolding has an amplitude of 0.154 absorbance unit, while that with a prior $(NH_4)_2SO_4$ pulse has an amplitude of 0.059 absorbance unit. The final RNase concentrations for both experiments are 7.4×10^{-5} M. Careful examination of both sets of data indicates that they are not strictly single-phase reactions. The reason for the slight deviations will be explained later. However, a relaxation time of ca. 500 s would be obtained for both experiments if they were treated as a single first-order reaction. The solid lines are calculated curves from simulations and will be discussed later.

Figure 2 shows the results of the identical refolding experiments detected by direct fluorescence measurements. Besides having a large difference in the total amplitude between the two refolding experiments (55 vs. 33, in arbitrary units), their kinetic patterns, as shown in Figure 2, are also quite different. For refolding without a prior $(NH_4)_2SO_4$ pulse, the refolding kinetics are very similar to those detected by absorbance measurements and can be treated approximately as a single-phase, first-order reaction with a relaxation time of 500 s. For refolding with a prior $(NH_4)_2SO_4$ pulse, the kinetics are faster, and two phases are apparent. The relaxation times were estimated to be 500 and 170 s, and the amplitudes for the slower and faster phase are 20 and 13, respectively. As will be shown later in discussing the simulations, the differences in the kinetic pattern for the two experiments are due to a change in the rate-limiting step caused by the $(NH_4)_2SO_4$ pulse and to the unequal intrinsic fluorescence values between unfolded RNase species.

Figure 3 shows refolding data detected by the fluorescence unfolding assay. The differences in the unfolding fluorescence change (slow phase only) between the fully refolded sample (which is always ca. 18% of the total fluorescence intensity) and other samples with various refolding times (shown on the ordinate) are plotted on the abscissa. Since each data point in Figure 3 is obtained by extrapolation to zero time of the fluorescence unfolding reaction, the data have more scatter and are subject to larger errors than direct methods. Unlike those data shown in Figures 1 and 2 measured by direct absorbance and fluorescence change, the total amplitude for refolding is not affected by the $(NH_4)_2SO_4$ pulse. This suggests that an unfolded species, which accounts for more than 50% of the total refolding amplitude observed by absorbance change, and which refolds quickly during a $(NH_4)_2SO_4$ pulse, is not seen at all in the fluorescence unfolding assay.

To a greater extent than observed in the direct fluorescence measurement, the kinetic patterns for the two refolding experiments are quite different. While refolding without a prior $(NH_4)_2SO_4$ pulse has the same kinetic pattern seen by absorbance measurements, the kinetics of refolding with a prior $(NH_4)_2SO_4$ pulse are very much faster than without the pulse, and there is strong indication of two kinetic phases. Due to the scattering of data, the two kinetic phases could not be unambiguously resolved.

Simulations. A somewhat abbreviated form of our latest RNase folding model (Lin & Brandts, 1984) will be used for digital simulations of the above experiments, i.e.



(1)

The small ct phase, with only ca. 5% of the refolding amplitude, has been omitted for simplicity. In this model, the CT transition occurs horizontally among denatured forms and corresponds to the isomerization of proline-93, while the XY transition occurs vertically and is due to some as yet unknown process which probably is not proline isomerization (Lin & Brandts, 1984). The nativelike intermediate N_{CY} (analogous to the I_N state in Baldwin–Schmid terminology) differs from the native form N_{CX} in that it has an incorrect Y configuration rather than the native X configuration, but it does possess the correct cis form for proline-93. Under strongly native conditions and particularly in the presence of high concentrations of $(NH_4)_2SO_4$, the D_{CY} state refolds very quickly via the N_{CY} path, while it refolds slowly via the D_{CX} path in the presence of relatively high concentrations of Gdn·HCl or urea. Thus, the short $(NH_4)_2SO_4$ pulse employed in the earlier experiments acts to completely depopulate the D_{CY} state without significantly changing the concentrations of the other two slow-refolding species, D_{TX} and D_{TY}.[1]

When this model was first formulated in 1983, equilibrium concentrations of the four unfolded species were estimated, and these are shown in parentheses in eq 1. At the same time, relative intrinsic values of absorbance and fluorescence were estimated for all species. All D states were determined to have the same absorbance and were assigned a relative value of 1.0 relative to 0 for the two N states. On the other hand, the two unfolded species having the incorrect trans form of proline-93, D_{TX} and D_{TY}, were found to have a higher intrinsic fluorescence of 2.5 relative to 1.0 for D_{CX} and D_{CY} and 0 for N_{CX} and N_{CY}. For the fluorescence unfolding assay, these same values will apply for the unfolded states, but a relative value

[1] The T → C reaction, by which D_{TX} and D_{TY} are unpopulated during refolding, was found to have a relaxation time of 180 s under the conditions of the $(NH_4)_2SO_4$ pulse, which prompted us to correct the simulations for an estimated 8% decrease in the initial concentrations of the D_{TX} and D_{TY} species which will occur during the 12–15-s pulse.

of 1.0, rather than 0, must be assigned to N_{CX} and N_{CY} since the unfolding fluorescence assay does not see the D → N conformational step (Schmid, 1986) in contrast to the direct fluorescence assay. In accordance with the above, the N_{CY} → N_{CX} transition is silent for all methods of detection.

Rather than attempting to achieve the best fit between experiments and simulations by adjusting parameter values, we will simply use these earlier estimates to demonstrate semiquantitative agreement. In addition, values of 150 s for k_{TC}^{-1} and 420 s for k_{YX}^{-1} will be used for all simulations in 2 M Gdn·HCl, and these are in close agreement with those measured earlier (Lin & Brandts, 1983b) in equivalent concentrations of urea. With this assignment of parameters, the only difference among simulations is that D_{CY} is assigned a fractional population of 0.53 at zero refolding time in the absence of the $(NH_4)_2SO_4$ pulse and a fractional population of 0 in the presence of the pulse.

The simulated absorbance kinetics are shown as the solid lines in Figure 1, and it is seen that these pass through the data points quite closely both in the absence and in the presence of the $(NH_4)_2SO_4$ pulse. The simulations predict nicely both the large loss in refolding amplitude caused by the pulse and also the absence of any effect on the shape of the decay curve. The tailing off of experimental data at long refolding times is probably due to the small ct phase, which is neglected in the simulations.

The solid lines in Figure 2 represent the corresponding simulations for detection by direct fluorescence measurements, and they are also in satisfactory agreement with the experiment considering that all parameters are identical with those used in Figure 1 except for the values of intrinsic observables. The moderately large loss in experimental amplitude caused by the pulse is present in the simulations, which also show the pulse-induced acceleration in fluorescence decay and the strong indication of two-phase behavior not seen in the absorbance simulations.

The simulated curves in Figure 3 are for refolding detected by the fluorescence unfolding assay of Schmid. The extremely large experimental effect of the pulse on the velocity of decay, the strong two-phase behavior, and the lack of any effect on the amplitude are fully accounted for in the simulations.

There is a simple mechanistic explanation for the fact that hardly any change in decay pattern (neglecting amplitude changes) is produced by the $(NH_4)_2SO_4$ pulse when monitoring by absorbance while a significant change is seen in direct fluorescence which becomes even more exaggerated when monitoring by the fluorescence unfolding assay. In the absence of the pulse, the D_{TY} and D_{CY} species are at equilibrium (see eq 1) when refolding is initiated, so that the D_{TY} → D_{CY} conversion (k_{TC}^{-1} = 150 s) is rate limited by the slower D_{CY} → D_{CX} step (k_{YX}^{-1} = 420 s) due to mass action effects. After pretreatment by the $(NH_4)_2SO_4$ pulse, all of the D_{CY} species is removed from the equilibrium, and when refolding is resumed in 2 M Gdn·HCl, the D_{TY} → D_{CY} conversion is not rate limited by mass action effects associated with the slower XY phase but proceeds at its own pace. Since the intrinsic fluorescence of D_{TY} and D_{CY} are different while their intrinsic absorbance is identical, this acceleration in the D_{TY} → D_{CY} conversion is seen with fluorescence monitoring but not with absorbance monitoring. The effect is more pronounced in the fluorescence unfolding assay than in direct fluorescence since the conformational step D → N is superimposed in the latter and is silent in the former.

Refolding in Low Gdn·HCl. In accordance with the above interpretation, our model makes the rather specific prediction

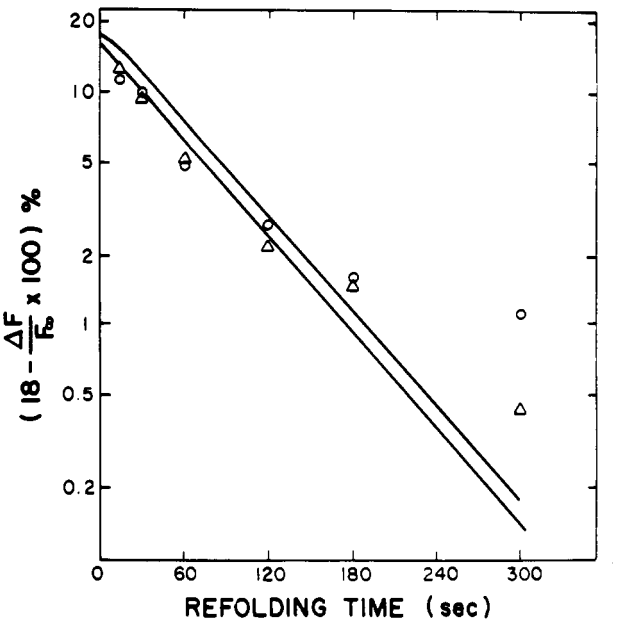


FIGURE 4: Comparison of the simulated refolding kinetics of RNase with that obtained from experimental data detected by fluorescence unfolding assay for refolding in 0.08 M Gdn·HCl. The model of eq 1 was used for simulation. The solid lines represent the simulated data. The circles and triangles represent the experimental data obtained for refolding with and without a $(NH_4)_2SO_4$ pulse, respectively.

that identical pretreatment with the $(NH_4)_2SO_4$ pulse will produce virtually no effect on the shape of the decay curve for any detection method if the refolding is then resumed in a buffer where the CT process is not rate limited by the XY process. Since the XY process becomes considerably faster than the CT process in low concentrations of denaturants (Lin & Brandts, 1983b), this prediction can be easily tested.

Using the fluorescence unfolding assay, we repeated the experiments shown in Figure 3 exactly except the final refolding was carried out in 0.08 M rather than 2 M Gdn·HCl. The results are shown in Figure 4. In accordance with the model prediction, and in strong contrast to the results in Figure 3, experimentally identical kinetics were obtained in the presence and absence of the $(NH_4)_2SO_4$ pulse. The solid lines show that the model simulates the results of both experiments quite well. These simulations were carried out exactly as before except a k_{TC}^{-1} value of 60 s and a $k_{YX}'^{-1}$ value of 10 s were used[2] which are in good agreement with those reported earlier (Lin & Brandts, 1983b, 1987) under similar conditions.

Discussion

The simulated refolding curves, based on our existing model and previously published parameters, show excellent agreement with these new experimental data both in the presence and in the absence of the $(NH_4)_2SO_4$ pulse, using three different methods of detection and two quite different sets of final refolding conditions. We feel that the behavior seen in these pulse experiments, rather than ruling out our model as suggested by Schmid (1986), actually goes a long way toward establishing its validity.

[2] In these simulations, we assumed that D_{CY} is refolded by the D_{CY} → N_{CY} process which is in agreement with other information suggesting the nativelike intermediate is the primary XY path to the native state at low denaturant concentration, in contrast to the situation at high denaturant concentration. However, identical simulated results would be obtained by making the opposite assumption that the primary path is D_{CY} → D_{CX} and the value of k_{YX}^{-1} is 10 s.

Although the experimental data obtained by using the fluorescence unfolding assay (Figure 3) are virtually the same as those obtained by Schmid (1986) using the same conditions, the interpretations are very different. According to these simulations, the faster kinetics observed after the pulse are due to acceleration of the isomerization of proline-93 in the *unfolded state* (i.e., in the $D_{TY} \rightarrow D_{CY}$ step) because of predictable mass action effects associated with the pulse removal of the existing D_{CY} species. If this is true, then the $(NH_4)_2SO_4$ pulse should not alter the kinetics when the XY phase is faster than the CT phase, and this was shown to be true by carrying out the same experiments in a different buffer containing less Gdn·HCl.

Schmid (1986) suggested that the faster kinetics induced by the $(NH_4)_2SO_4$ pulse are due to catalysis of the trans to cis isomerization of proline-93 in the *nativelike intermediate*, which in our model already contains proline-93 in the correct cis form. Thus, Schmid (1986) feels that it is the larger refolding phase (i.e., the XY phase in our terminology) which definitely involves isomerization of proline-93, while the smaller refolding phase may (Schmid et al., 1986) or may not (Schmid, 1986).

The fundamental differences then between the models are that ours assumes that only 25–30% of the unfolded molecules have proline-93 in the incorrect trans configuration and that these molecules must first isomerize before they can fold via the CT phase into a native or nativelike state, while Schmid's interpretation requires ca. 65–80%, depending on the assumption regarding the small phase, of the unfolded molecules to have the incorrect trans form for proline-93 and most of these molecules refold to a nativelike structure in the major phase before they isomerize under strongly native conditions. The transition from the nativelike intermediate to the native state is seen in direct fluorescence studies according to Schmid, whereas we believe it is silent or nearly silent.

In addition to the simulations, we feel there are other factors favoring our interpretations. Most importantly, direct measurements of the isomerization of proline-93 by isomer-specific proteolysis (ISP) have shown that only ca. 30% of the unfolded molecules have proline-93 in the trans form and that these molecules isomerize during refolding with a relaxation time identical with that of the CT phase, which is known to involve 25–30% of the refolding amplitude in terms of UV changes or 2′-CMP binding (Lin & Brandts, 1983a, 1984).

Implicit in the results of the simulations is the fact that there are two quite different ways in which ammonium sulfate pulses can "catalyze" the rate of isomerization of proline-93: (1) a *direct* catalysis of the isomerization of proline-93 in the nativelike intermediate, and/or (2) an *apparent* catalysis resulting from trivial mass action effects among those forms that are still unfolded after the pulse is applied. The trivial, nonstructural catalysis will exist for any mechanism of refolding which has coupled equilibria among the unfolded forms. For our particular model, the trivial effect by itself is adequate to completely account for the experimentally observed degree of catalysis, so there is no need to invoke direct catalysis of proline-93 in the nativelike intermediate. Schmid (1986) assumed that all experimentally observed catalysis arises from direct catalysis in the nativelike intermediate. However, in failing to even consider mass action effects on his own model of refolding, this interpretation must be open to question.

Registry No. RNase A, 9001-99-4; guanidine hydrochloride, 50-01-1; ammonium sulfate, 7783-20-2.

REFERENCES

Lin, L.-N., & Brandts, J. F. (1983a) *Biochemistry 22*, 559.
Lin, L.-N., & Brandts, J. F. (1983b) *Biochemistry 22*, 564.
Lin, L.-N., & Brandts, J. F. (1983c) *Biochemistry 22*, 573.
Lin, L.-N., & Brandts, J. F. (1984) *Biochemistry 23*, 5713.
Lin, L.-N., & Brandts, J. F. (1987) *Biochemistry* (in press).
Schmid, F. X. (1983) *Biochemistry 22*, 4690.
Schmid, F. X. (1986) *FEBS Lett. 198*, 217.
Schmid, F. X., Grafl, R., Wrba, A., & Beintema, J. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 872.