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Isomerization of Proline-93 during the Unfolding and Refolding of Ribonuclease A†

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ABSTRACT: Using the method of isomer-specific proteolysis, the isomerization of proline-93 has been monitored directly during the time course of the unfolding and refolding reactions of RNase A. It has been found that proline-93 is 100% cis in the native protein and 70% cis in the reversibly unfolded protein. During the unfolding reaction, the change from 100% to 70% cis occurs as a first-order process with a relaxation time of 140 s in 8.5 M urea, 10 °C. For refolding, the change from 70% to 100% cis also occurs as a first-order process, with a relaxation time (10 °C) of 90 s in 0.3 M urea, 130 s in 1.0 M urea, and 310 s in 2.0 M urea. Parallel experiments which measured the recovery of enzyme activity during refolding were also conducted. These show that 30% of the activity recovers in a slow phase with a first-order relaxation time (10 °C) of 100 s in 0.3 M urea. Because of the excellent agreement of both the amplitude and relaxation time for trans-to-cis isomerization and for activity recovery, it is concluded that the slowest phase in the recovery of enzyme activity is rate limited by the isomerization of proline-93. These results demonstrate that proline-93 must be cis before refolding to the active form can take place, in contrast to previous suggestions, and argue against the existence of a nativelike intermediate form on the refolding pathway which contains proline-93 in the incorrect trans configuration.

It was first proposed in 1975 (Brandts et al., 1975) that the unfolding and refolding of globular proteins are rate limited by cis–trans isomerization of those peptide bonds that contain the imide nitrogen of prolyl residues. According to this suggestion in its simplest form, the slow phase in folding reactions arises because a sizable fraction of denatured molecules have one or more proline residues in the incorrect configuration. The actual conformational changes (i.e., changes in ψ and ϕ angles) were assumed to occur rapidly once all of the prolines are in the correct native configuration. Although a simple process in itself, the possible occurrence of isomerization can prove to be an enormous experimental complication when trying to elucidate the "pathway" of folding for proteins containing proline. Being both very slow and highly visible in terms of phase amplitudes, proline isomerization in the unfolded chain can either obscure or be mistaken for other kinetic phases which might be attributable to true structural intermediates along the folding pathway.

Although considerable indirect evidence now suggests that proline isomerization acts to slow down protein folding and unfolding (Brandts et al., 1977; Lin & Brandts, 1978; Schmid & Baldwin, 1978; Cook et al., 1979), there is still some question as to the precise way in which this might occur for particular proteins. Most of the critical data has been obtained on RNase A. Nall et al. (1978) and Cook et al. (1979) concluded that, in contrast to unfolding, the slow phase in refolding does not possess the appropriate characteristics for a reaction which is rate limited by proline isomerization. In particular, they suggest that the relaxation time is 40-fold faster than expected, the activation energy in low Gdn·HCl is much too small, and the strong dependence of relaxation time on Gdn·HCl is inconsistent with data on the isomerization of small model compounds. Their data were interpreted in terms of a model whereby unfolded RNase refolded quickly to a nativelike intermediate I_N which contains one or more prolines in an incorrect nonnative configuration. This proposed intermediate is able to bind 2′-CMP with high affinity, suggesting that the active site is intact. Furthermore, it was assumed that the pathway through the nativelike intermediate was responsible for a large acceleration in the rate of isomerization during refolding, as well as for the small activation energy for the slow phase. Later, it was suggested that proline-93 is in the incorrect trans configuration in I_N (Schmid, 1981; Schmid & Blaschek, 1981; Rehage & Schmid, 1982).

Results from the preceding paper (Lin & Brandts, 1983a) show that, by using trypsin and aminopeptidase P (APP) in tandem, it is possible to quantitatively measure the cis/trans ratio for proline-93 in oxidized RNase because of the stereospecificity of trypsin proteolysis. This technique does not work directly for native RNase or for reversibly unfolded RNase, since they are not good substrates for trypsin in the native-state buffers which must be used to maintain high trypsin activity. However, the technique will work if the native or reversibly unfolded RNase is treated first with pepsin in a buffer (4.5 M urea, pH 2) which is a native-state buffer for pepsin but a denaturing buffer for RNase. Thus, the technique which is employed here will consist of treatment of RNase with a short pepsin pulse, followed by a trypsin pulse of variable duration followed by a long APP pulse. The pepsin pulse causes irreversible unfolding and limited degradation, thereby facilitating the cleavage of RNase by trypsin in a native-state buffer. Trypsin will then cleave the Lys_{91}-Tyr_{92} bond only for those RNase molecules having the Tyr_{92}–Pro_{93} bond in the

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trans form. The tyrosine-92 in those molecules that have been cleaved by trypsin can then be released quantitatively as the free amino acid by long-term treatment with APP. Measurement of released tyrosine is carried out by HPLC. By using high concentration of proteases, and working at moderately low temperatures, we can carry out the pepsin and trypsin cleavages quickly relative to the time required for proline-93 to isomerize, so the method determines the fraction of trans isomer present at the time immediately preceding the introduction of RNase into the pepsin solution. To correct for small amounts of isomerization which do occur during treatment, we measured the release of tyrosine-92 as a function of trypsin cleavage time and extrapolated it back to zero time.

This then constitutes a fairly direct method for following isomerization of proline-93. As will be seen, the results show that proline-93 is important in controlling the velocity of the slow phase of RNase folding, as has been previously suggested. However, we find that the recovery of enzyme activity *requires* that proline-93 be in the native cis form, and our results argue against the existence of an active, nativelike intermediate form of RNase in the folding pathway which contains proline-93 in the incorrect trans configuration.

Materials and Methods

Materials. Bovine RNase A (catalog R-5500, lot no. 49C-8047), purchased from Sigma Chemical Co., was directly used without further purification. Pepsin (from porcine stomach mucosa), also obtained from Sigma Chemical Co., was passed through a Sephadex G-25 column to remove autolysis products (Rose & Richards, 1979) and then lyophilized. Urea (ultrapure grade) was purchased from Schwarz/Mann Co. Cytidine 2′,3′-monophosphate (sodium salt) and tris(hydroxymethyl)aminomethane were obtained from Sigma Chemical Co. All other enzymes, proteins, and chemicals have been described in the preceding paper (Lin & Brandts, 1983a) or in earlier papers (Lin & Brandts, 1979a,b, 1980).

Assay for Isomeric State of the Tyr_{92}–Pro_{93} Bond. (*a*) *Native RNase.* For determination of native RNase, it was first irreversibly denatured by pepsin at low pH and in 4.5 M urea solution, and then trypsin was added to detect the isomeric state of the Tyr_{92}–Pro_{93} bond. The detailed procedures are as follows: For initiation of the reaction, 10 μL of native RNase (3.5×10^{-3} M, in H_2O) at 10 °C was pipetted into 80 μL of pepsin solution (120 mg/mL in 4.5 M urea, pH 1.9), incubated at 10 °C, and mixed thoroughly with a stirring bar. After reaction for 20 s, 4.9 mL of trypsin solution (3.5 mg/mL, in 0.05 M veronal buffer, pH 8.6, at 10 °C) was added to the incubation solution and mixed well with the stirring bar. At suitable time intervals (30 s, 1 min, 3 min, 5 min, 7 min, and 30 min), 0.8 mL of reaction mixture was quickly pipetted into a centrifuge tube containing 50 μL of soybean trypsin inhibitor (86 mg/mL, in 0.05 M veronal buffer, pH 8.6) and mixed with a vibrator to stop trypsin activity. Then 0.12 mL of aminopeptidase P (APP) solution (8 units/mL, in 0.05 M veronal buffer with citrate–Mn^+ 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 centrifugation at 7500 rpm for 20 min, the supernatants were quantitatively transferred to test tubes and dried again with nitrogen. The residues were finally dissolved in 0.25 mL of boric acid buffer. A sample incubated with trypsin for 30 min was used as the "infinite time" hydrolysis sample, and the amount of tyrosine released from this sample was used as the 100% reference for quantitating the time dependence of hydrolysis. A control run with RNase subjected only to pepsin and trypsin activities but without APP activity was also carried out in the identical way. The final solutions were analyzed for free tyrosine-92 by using HPLC as described previously (Lin & Brandts, 1983a). Another control run was carried out in which a standardized tyrosine solution, instead of RNase A, was subjected to the identical experimental procedures including pepsin and trypsin treatment, precipitation by ethanol, etc. It was found that the recovery of tyrosine subsequent to these manipulations was greater than 95%, as determined by HPLC.

Before the above procedures were established, an experiment was conducted in which native RNase was subjected to the same pepsin activity for various times from 10 s to 30 min, before being subjected to 30-min pulses with trypsin and APP. It was found that the amount of tyrosine released from the sample treated with pepsin for 20 s was identical with that released from samples treated up to 30 min. However, if the pepsin pulse was omitted entirely, almost no tyrosine was released by the combined action of trypsin and APP. This shows that native RNase can be completely and irreversibly unfolded with only a 20-s pepsin pulse, thereby rendering it susceptible to trypsin cleavage.

(*b*) *Reversibly Denatured RNase.* The experimental procedures for assaying the isomeric state of the Tyr_{92}–Pro_{93} bond in the unfolded protein are identical with those for the native except that denatured RNase (in 8.5 M urea, pH 2.0) instead of native was mixed with pepsin to initiate the reaction.

(*c*) *Refolding and Unfolding.* The experimental procedures are very similar to those described above. The only difference is that RNase was unfolded or refolded for certain periods of time before addition to the pepsin solution. For refolding experiments, the unfolded, equilibrated RNase (in 8.5 M urea, pH 4.0) was diluted to different final urea concentrations (0.3 M, 1 M, and 2 M) to initiate refolding. At various times, pepsin solution was added for 20 s to irreversibly denature all RNase, and then trypsin was added to assay for isomers at the proline-93 bond. For unfolding experiments, native RNase in H_2O (3 μL, 150 mg/μL) was unfolded with addition of 30 μL of 9.3 M urea solution (pH 2.0). At various times of unfolding, pepsin solution was added in the identical way, followed by trypsin, soybean trypsin inhibitor, and APP as described above.

Assay for Regain of Activity of Unfolded RNase A. Our experimental procedures are similar to those of Crook et al. (1960) and Schmid & Blaschek (1981). The details are as follows: First, 20 μL of unfolded RNase (1.8×10^{-2} M, equilibrated in 8.5 M urea, pH 4.0) was diluted 26 times with 0.05 M sodium acetate solution (pH 5.6) at 10 °C to initiate the refolding. At various times of refolding, 200 μL of the RNase solution was quickly pipetted into a thermostated quartz cuvette (at 10 °C) containing 2.2 mL of cytidine 2′,3′-monophosphate solution (0.5 mg/mL, in 0.1 M Tris–4 M urea, pH 7.2). After mixing for a few seconds with a spatula, we followed the change in absorbance at 290 nm with time for 2 min with a Cary 14 spectrophotometer. The dead time for mixing was about 5–10 s. A sample with a long refolding time (30 min) served as the fully reactivated RNase. The relative activity of solutions at various refolding times was quantitated from the initial slope of absorbance change of each sample vs. that of fully reactivated sample.

Results

It is well-known that native RNase A is resistant to trypsin

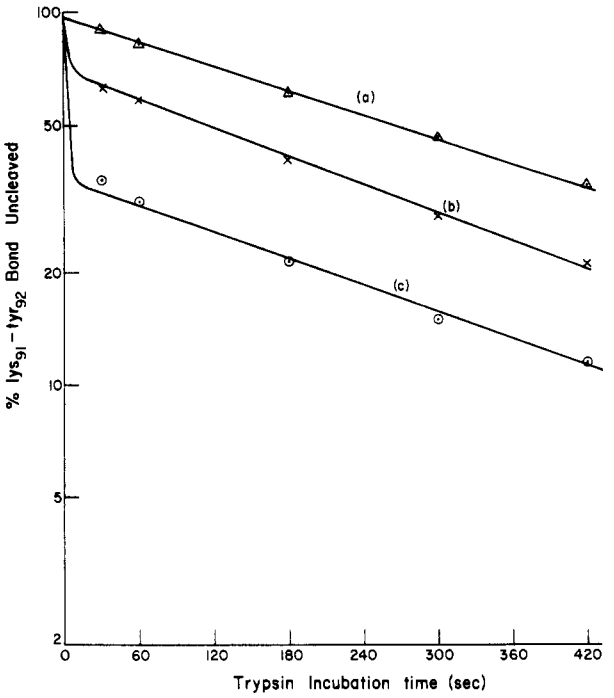

FIGURE 1: Rates of hydrolysis of the Lys_{91}–Tyr_{92} bond of RNase catalyzed by trypsin at 10 °C. Initial states of RNase: (a) native form; (b) reversibly denatured form; (c) oxidized form. All samples were first treated with pepsin for 20 s immediately before the addition of trypsin at zero time. See the text for experimental details.

attack and only the unfolded protein can be cleaved by trypsin. So before the state of the Tyr_{92}–Pro_{93} bond can be determined by the isomeric specificity of trypsin, RNase must first be unfolded and must remain unfolded so that trypsin will exhibit its maximum activity. Although a high concentration of denaturants and low pH will unfold RNase, the activity of trypsin in such a solution is greatly decreased, so it is unable to cleave the peptide bond fast enough to detect the state of isomerization. Our data (unpublished) show that even at low Gdn·HCl concentration (~0.05 M) trypsin activity toward oxidized RNase is very low, since the guanidinium ion is a competitive inhibitor of trypsin. However, we found that trypsin does exhibit high activity in the presence of low urea concentrations (0.1 M or below). We therefore chose urea as a denaturant throughout this work. We also found that pepsin, an acid endopeptidase which has high activity at low pH and in the presence of moderately high urea concentrations (up to 4.5 M), can render all RNase (>97%) irreversibly unfolded after just a short incubation of 20 s at 10 °C. A large volume of trypsin solution (to dilute out the urea), buffered near neutral pH, can then be added to the pepsin–RNase reaction mixture to assay for the isomeric state of the Tyr_{92}–Pro_{93} peptide bond.

Characterization of States. Figure 1 shows the semilog plots of the percent of free tyrosine-92 released as a function of the incubation time with trypsin, when the initial state of RNase is either the native, reversibly denatured, or the oxidized form. The 100% reference, always very close to 1.0 mol of tyrosine/mol of RNase, has been determined from a sample having 30-min incubation with trypsin. Since trypsin can only cleave the Lys_{91}-Tyr_{92} bond when the following Tyr_{92}–Pro_{93} bond is trans (Lin & Brandts, 1983a), the slow kinetic phases seen in Figure 1 can be attributed to the cis-to-trans isomerization of the Tyr_{92}–Pro_{93} bond and subsequent hydrolysis of the Lys_{91}–Tyr_{92} bond. The fast hydrolysis, implicit in the data of Figure 1, corresponds to the rapid cleavage of the trans form present at the time the incubations began. The total amplitude of the slow phase, obtained by extrapolation to zero time, is then the percent of cis form present in the original, intact RNase molecule. The estimates of cis form are 97% for the native, 70% for the reversibly denatured with disulfide bonds intact, and 36% for oxidized RNase.[1] The relaxation time for the slow phase is nearly identical for all three species, as expected, with a value of 6.0 ± 0.5 min. However, it should be noted that these relaxation times are not for the whole RNase polypeptide chain, but for the fragment containing the Tyr_{92}–Pro_{93} bond resulting from pepsin cleavage. According to the work of Hirs et al. (1960), this is probably the fragment which contains residues 56 to 108, and which will still be cross-linked to other fragments by disulfide bridges except for oxidized RNase.

Our data then show that native RNase in solution has proline-93 entirely in the cis form, in agreement with the X-ray data (Wyckoff et al., 1970) on the crystalline form of RNase S. Also, the estimate of 36% cis for the oxidized form agrees well with the earlier estimate of 33% (Lin & Brandts, 1983a) which was obtained by a similar procedure using trypsin and APP but which omitted the pretreatment with pepsin.

The cis content of 70% for the reversibly denatured form is somewhat of a surprise, suggesting that the presence of intact disulfide bonds nearly doubles the equilibrium concentration of cis form in the unfolded state. This is probably due to steric effects and/or local interactions involved with disulfide bond 40–95.

Isomerization during Refolding. The isomerization of the Tyr_{92}–Pro_{93} bond during RNase refolding (or unfolding) was also investigated by using the same experimental approach. RNase was refolded (or unfolded) for a period of time before the pepsin–trypsin treatment was initiated, in order to determine the cis–trans content at that particular moment along the reaction pathway. For each refolding (or unfolding) time, six different incubation times with trypsin were examined and the data were extrapolated to zero trypsin incubation time, in the same manner as shown in Figure 1. Only the final extrapolated values for each refolding (or unfolding) time will be shown in subsequent figures.

Refolding experiments are shown in Figure 2. In each case, RNase was equilibrated in 8.5 M urea at 10 °C, pH 4, to achieve denaturation. At zero time, the denatured RNase was transferred to a native-state buffer at 10 °C, pH 5, which contained low urea concentrations of either 0.3, 1.0, or 2.0 M. The abscissa represents the time allowed for refolding before the pepsin was added. These semilog plots are linear and show that the isomerization of the Tyr_{92}–Pro_{93} bond during refolding is a first-order process. It is seen that the relaxation time for

[1] There is a small amount of uncertainty involved in the extrapolation to "zero time". The native RNase, containing nearly 100% cis form, is rapidly converted to unfolded RNase upon incubation with pepsin. The pepsin-cleaved unfolded form will have a smaller equilibrium cis content than the native, probably lying somewhere between that measured for the reversibly unfolded form (70%) and that measured for the oxidized form (36%). Thus, some conversion of cis to trans will occur during the 20-s incubation with pepsin even before the trypsin is added. This will be less of a problem with unfolded forms than with the native, since pepsin cleavage will cause less change in the equilibrium cis fraction, so isomerization will not occur to any great extent until the equilibrium is disturbed by trypsin hydrolysis of the trans form. The uncertainty for native RNase can be imagined to be due to an uncertainty in the location of "zero time" on the abscissa of Figure 1, with the "correct" value lying somewhere between −20 s (when pepsin is added) and 0 s (when trypsin is added). Using the first value leads to an estimate of 99.5% cis content in the native form, while the latter leads to 97% cis. Since these two estimates are closer to each other than the expected experimental error, we shall not worry in detail about choosing the correct "zero time" and will arbitrarily select the point at which trypsin is added.

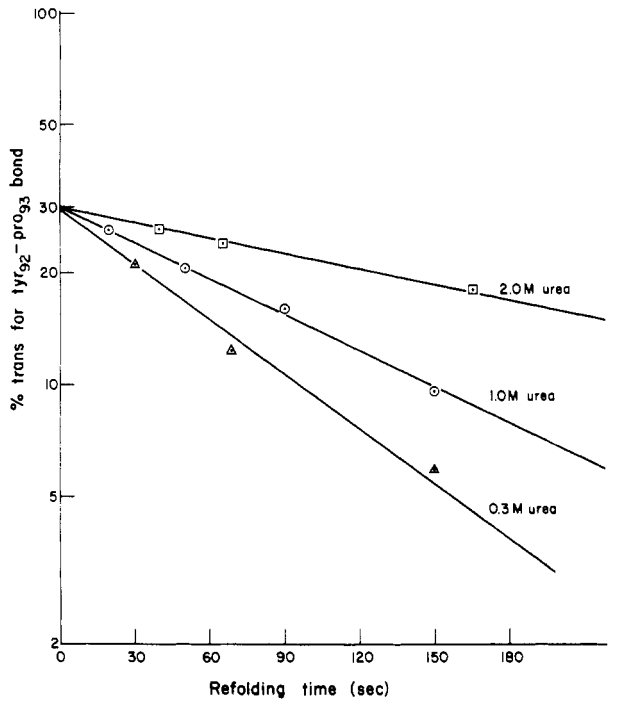

FIGURE 2: The semilog plot of trans-to-cis isomerization of Tyr_{92}–Pro_{93} bond during RNase refolding at 10 °C. RNase was initially unfolded in 8.5 M urea solution, pH 4.0, and then refolded in 0.3 M (triangles), 1 M (circles), and 2 M (rectangles) urea solution by dilution. The content of the trans form of the Tyr_{92}–Pro_{93} bond at various refolding times was assayed by the method described for Figure 1.

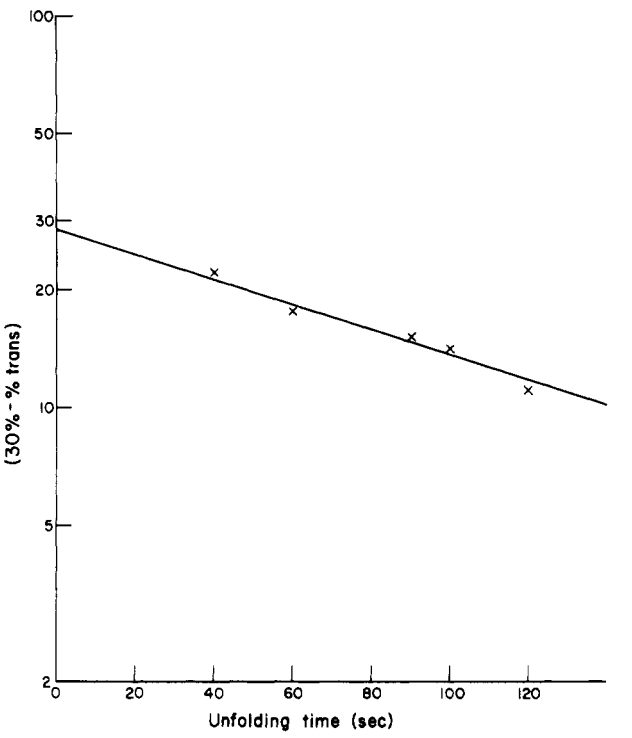

FIGURE 3: The semilog plot of cis-to-trans isomerization of Tyr_{92}–Pro_{93} bond during RNase unfolding at 10 °C. Native RNase in H_2O was unfolded in 8.5 M, pH 2, urea solution. At various times, the amounts of cis and trans forms of Tyr_{92}–Pro_{93} bond were determined by the method described for Figure 1.

disappearance of the trans isomer during refolding varies significantly with urea concentration, ranging from 90 s at 0.3 M to 130 s at 1 M to 310 s at 2 M urea. The extrapolated amplitudes are identical, about 30%, which is consistent with the previous finding from data in Figure 1 that reversibly unfolded RNase has a 30% trans content.

It was previously suggested (Nall et al., 1978; Cook et al., 1979) that the relaxation time for proline isomerization in RNase refolding was dependent on the concentration of strong denaturants such as Gdn·HCl. Our results confirm that this is also true for proline-93 isomerization in urea. As will be seen from data presented in the following papers (Lin & Brandts, 1983b,c), however, the bulk of this effect of urea on the rate of isomerization of proline-93 arises indirectly, as the result of an effect of urea on another process which becomes rate limiting at high urea concentrations.

Isomerization during Unfolding. Experiments similar to those described above for refolding of RNase have been carried out in the unfolding direction and are shown in Figure 3. The time of unfolding does, in this case, include the 20-s pepsin incubation since unfolding and isomerization will clearly continue to occur in this buffer before trypsin addition. For final denaturing conditions of 8.5 M urea, 10 °C, and pH 2.0, a relaxation time of 140 s is obtained from the first-order plot of Figure 3.

Recovery of Enzyme Activity during Refolding. It is important to determine the kinetics of recovery of enzymatic activity during refolding which can then be compared to the recovery of the native cis form of the proline-93 peptide bond. If the protein can refold into an active nativelike intermediate which still has the proline-93 bond in the trans configuration (Schmid & Blaschek, 1981), then it would be expected that recovery of activity will occur more quickly than recovery of the native cis isomer. On the other hand, if the protein is unable to refold into an active form while the proline-93 bond is trans, then the recovery curves for activity and for the cis

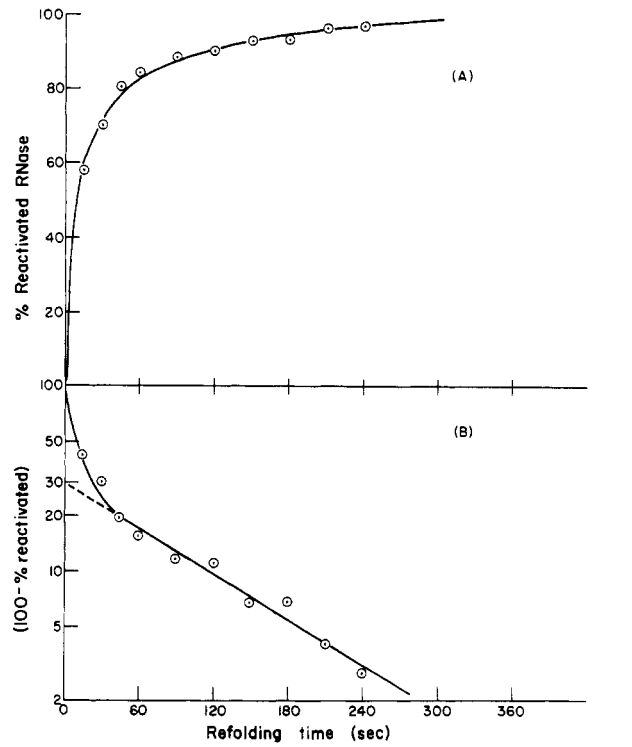

FIGURE 4: Recovery of enzymatic activity during RNase refolding at 10 °C: (A) percent of reactivated RNase vs. refolding time; (B) semilog plot of percent of nonreactivated RNase vs. refolding time. See the text for experimental details.

isomer should coincide if indeed the isomerization of proline-93 is rate limiting.

The data in Figure 4 show the first-order plot for activity regain. The initial conditions were again 8.5 M urea, pH 4, and 10 °C. The RNase was then placed in a refolding buffer (0.3 M urea, pH 5.0, and 10 °C) and allowed to refold for

a certain time. It was then quickly transferred to the assay buffer containing 2′,3′-CMP at 10 °C. The measured activities shown in Figure 4 are plotted as a function of refolding time allowed prior to assay. Extrapolation of the slow phase in activity regain back to zero refolding time shows that 30% of the RNase activity reappears in the slowest phase which, under these conditions, exhibits a relaxation time of 100 s. Under nearly identical conditions, the data of Figure 2 show that unfolded RNase contains 30% of proline-93 residues in the incorrect trans form which then isomerizes to the cis form during refolding with a relaxation time of 90 sec. Since the recovery of activity and the recovery of cis form of proline-93 occur coincidently within errors, we conclude that proline-93 must be in the cis form before RNase can fold into an active structure. It seems highly likely that, under these conditions, the isomerization of proline-93 is the rate-limiting step in the recovery of activity in the slow refolding phase.

During the first 30 s or so of the activity recovery shown in Figure 4B, there is evidence of another slow phase which is somewhat faster than isomerization of proline-93. This second slow phase will be investigated in more detail in the following paper (Lin & Brandts, 1983b).

Discussion

The data show that the Tyr_{92}–Pro_{93} bond in RNase solutions is in the 100% cis form, consistent with the X-ray structure for RNase S. The method can be used to study other proteins, especially where X-ray structures are not available or where it is suspected that a dynamic cis–trans equilibrium may be important in controlling solution properties of a protein (Brown et al., 1977; Marsh et al., 1979).

Reversibly unfolded RNase in 8.5 M urea contains 70% of the Tyr_{92}–Pro_{93} bond in the cis form, compared to 35% for oxidized RNase. Local interactions or steric hindrance associated with the disulfide bond 40–95 could be responsible. These results emphasize the fact that isomerization can be strongly influenced by residues other than those directly involved in the peptide bond, even in a "random coil" solvent. The conventional assumption that denatured proteins have prolines which are only ca. 10–20% cis seems to be unjustified, at least for those prolines known to be cis in the native form.

Another indication of long-range effects on isomerization is evident from comparing two-way relaxation times for isomerization of proline-93 in various polypeptide chains. These values range from ca. 7 min in fragment 92–98 to 3.2 min in oxidized RNase to 1.8 min for the pepsin fragment[2] with disulfides intact to 1.1 min for reversibly unfolded RNase A.

The regain of enzymatic activity during refolding of RNase completely parallels the regain of the cis form for proline-93. About 30% of the unfolded RNase molecules regain activity slowly (τ = 100 s). Also, 30% of the unfolded molecules were shown by independent measurements to be in the trans form and to isomerize slowly (τ = 90 s) to the cis form under identical refolding conditions. The most reasonable interpretation of these results is that the 30% slow phase in recovery of activity is rate limited by isomerization of proline-93. There is no indication in our results that any RNase molecules can fold into an active nativelike intermediate state which has proline-93 in the incorrect trans form, as suggested (Schmid & Blaschek, 1981).

From the measured relaxation time for two-way isomerization of proline-93 during *unfolding* (140 s) in 8.5 M urea and from the measured cis/trans equilibrium ratio in unfolded RNase, it would be predicted that the one-way relaxation time for trans-to-cis isomerization during *refolding* would be ca. 185 s at the same urea concentration of 8.5 M. The actual measured rates for isomerization at lower urea concentrations of 0.3, 1, and 2 M are 90, 130, and 310 s, respectively. It will be shown in the following paper (Lin & Brandts, 1983b) that the comparison of unfolding and refolding relaxation times is complicated by two factors. First, there is a small inherent effect of urea on both the unfolding and refolding rates of isomerization of proline-93. Second, the entire slow refolding phase for RNase, which accounts for 80% of the amplitude in absorbance measurements, involves another slow process in addition to the isomerization of proline-93. In refolding experiments, the isomerization of proline-93 becomes rate limited by the other slow process when the urea concentration exceeds 1 M. Once these effects are accounted for, it will be seen that there is excellent agreement between the isomerization rates in the unfolding and refolding directions. The previous suggestion (Cook et al., 1979; Schmid & Blaschek, 1981) that isomerization in the refolding direction is greatly accelerated, relative to that in the unfolding direction, by the existence of structural intermediates does not appear to be valid for proline-93.

Registry No. RNase A, 9001-99-4; L-proline, 147-85-3; urea, 57-13-6.

[2] The amount of cis isomer present at equilibrium was not actually measured for the pepsin fragment, as it was for the other RNase derivatives. To estimate the two-way relaxation time for the pepsin fragment, we assumed that the equilibrium cis fraction was the same as for reversibly unfolded RNase (i.e., 70%).

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