Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 872–876, February 1986
Biochemistry

Role of proline peptide bond isomerization in unfolding and refolding of ribonuclease

(protein folding/folding intermediates)

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Communicated by Robert L. Baldwin, September 30, 1985

ABSTRACT **The isomerization of the proline peptide bond between tyrosine-92 and proline-93 in bovine pancreatic ribonuclease A has been investigated in the unfolded protein as well as during the slow refolding process. This bond is in the *cis* state in the native protein. By comparison of various homologous ribonucleases we show that isomerization of proline-93 is associated with a change in fluorescence of tyrosine-92. This provides a spectroscopic probe to monitor this process in the disordered chain after unfolding as well as its reversal in the course of slow refolding. In unfolded ribonuclease incorrect *trans* isomers of proline-93 are found in both slow-folding species. *trans* → *cis* reversal of isomerization of this proline peptide bond during refolding shows kinetics that are identical with the time course of formation of native protein. Isomerization of proline-93 is slower than the formation of a native-like folded intermediate that accumulates on the major slow refolding pathway. Models to explain these results are discussed.**

The rapid unfolding reactions ($N \rightarrow U_F$) of many native proteins (N) are followed by slow, presumably local, isomerization reactions of the denatured polypeptide chains, which lead to an equilibrium distribution of fast-refolding (U_F) and slow-refolding (U_S) species (Eq. 1).

$$N \rightarrow U_F \rightleftharpoons U_S \quad [1]$$

The proline hypothesis proposed by Brandts and co-workers provided a plausible explanation for the molecular nature of these isomerizations (1). After rapid unfolding, *cis* ⇌ *trans* isomerizations of Xaa-proline peptide bonds occur and create molecules with incorrect proline isomers. These are the U_S species.

U_F and U_S were detected for bovine RNase A by Garel and Baldwin several years ago (2). The overall $U_F \rightleftharpoons U_S$ equilibration reaction of unfolded RNase A displays many properties expected for a proline-limited process (3–5). There are two main slow-refolding species of RNase A: a major U_S^{II} species (65–70% of all unfolded molecules) and a minor U_S^{I} species (10–15%). Under conditions strongly favoring the native state, refolding of U_S^{II} involves structural intermediates (Eq. 2). I_1 is an open, hydrogen-bonded intermediate, which is formed rapidly. I_N is a native-like, enzymatically active species that is still separated from the fully native state, N, by a slow isomerization process (5–7). Little is known about the folding mechanism of the very slow refolding U_S^{I} species (Eq. 3) primarily because of its small amplitude.

$$65\text{–}70\%\quad U_S^{II} \xrightarrow{\text{fast}} I_1 \xrightarrow{\text{variable}} I_N \xrightarrow{\text{slow}} N \quad [2]$$

$$10\text{–}15\%\quad U_S^{I} \xrightarrow{\text{very slow}} N \quad [3]$$

U_S^{I} actually may consist of several minor species (8). This multiplicity of states suggests that more than one isomerization occurs in the unfolded protein (RNase A contains four proline residues in its sequence). A model involving two independent isomerization reactions of unfolded RNase has been proposed (9). Isomer-specific proteolysis experiments designed by Lin and Brandts (8, 10) led to the unexpected conclusion that the major isomerization, creating the dominant U_S^{II} species of unfolded RNase, might not be correlated with a proline isomerization event but might instead originate from an as yet unspecified "XY" process. From their data they concluded that only the minor U_S^{I} species are created by proline isomerization (i.e., of Pro-93 and Pro-114) in the unfolded chain. They suggested that the unspecified XY process probably does not involve proline isomerization and is responsible for the formation of the major slow refolding U_S^{II} species (8–11).

Recently we observed that one of the isomerizations that occurs in unfolded RNase is correlated with a specific increase in tyrosine fluorescence (12). To probe the molecular origin of this fluorescence-detected isomerization we now employ homologous RNases from related species, which differ in sequence at or around individual tyrosine residues. The results strongly indicate that tyrosine fluorescence is sensitive to the isomeric state of the proline peptide bond connecting Tyr-92 and Pro-93. We then use this distinctive property as a tool to characterize the role of this particular isomerization for unfolding and refolding of RNase.

MATERIALS AND METHODS

Materials. Bovine RNase A (type XII A) and cytidine 2′,3′-(cyclic)phosphate (2′,3′-CMP) were from Sigma; guanidinium chloride (GdmCl) (ultrapure) was from Schwarz/Mann, and sodium cacodylate was from Fluka. All other chemicals used were from Merck (Darmstadt). Porcine RNase was isolated from porcine pancreas by following published procedures (13, 14). Concentrations of RNase solutions were determined spectrophotometrically, using a molar absorbtivity at 277 nm of 9700 M^{-1} cm^{-1} (15) for the bovine enzyme. RNase A from guinea pig was isolated by following the procedure developed for red deer RNase (14). RNases from black rat (*Rattus rattus*), cuis (*Galea musteloides*), capybara (*Hydrochoerus hydrochoeris*), and red kangaroo (*Macropus rufus*) were isolated and sequenced as described (16–18).

Methods. Kinetics of folding were monitored by absorbance at 287 nm, using a Cary 118C spectrophotometer, or by fluorescence at 305 nm (10-nm slit) with excitation at 268 nm (4-nm slit), using a Hitachi Perkin–Elmer MPF 44 fluorescence spectrophotometer. Final concentrations in the

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Abbreviations: U_F, U_S^{I}, U_S^{II}, fast- and two different slow-refolding species, respectively; I_1, open intermediate; I_N, native-like folding intermediate; N, native protein; GdmCl, guanidinium chloride.

absorbance experiments were in the range of 30 μM RNase, in fluorescence experiments, about 10 μM.

Acid catalysis of the fluorescence-detected isomerization was measured by a two-step procedure. Unfolding and isomerization were initiated by adding 45 μl of concentrated $HClO_4$ to 5 μl of native RNase (60 mg/ml in H_2O) as described in ref. 3. After various times 20-μl aliquots were withdrawn and added to 0.98 ml of 0.3 M glycine/5.0 M GdmCl at 10°C in the fluorimeter cell. The final pH was 2.3. The amplitudes of the remaining fluorescence-detected isomerizations were determined and plotted as a function of the time of incubation in $HClO_4$. This gave the time course of isomerization in the concentrated $HClO_4$ solution.

The modified assay for the decrease of RNase molecules with incorrect isomers during the individual two slow refolding reactions was carried out in the following manner. Refolding at 0°C was initiated by mixing 5 μl of unfolded RNase A (70 mg/ml in 0.1 M glycine, pH 1.8/4.0 M GdmCl at 0°C) with 95 μl of 0.42 M ammonium sulfate in 0.05 M sodium cacodylate, pH 6.5, to give refolding conditions of 0.4 M ammonium sulfate and 0.2 M GdmCl, pH 6.2, and 0°C. After different times of refolding, aliquots were taken, and the refolding molecules were rapidly converted into a mixture of U_F, U_S^I, and U_S^{II} by complete unfolding, using a 3-fold dilution into a final solution of 5.1 M GdmCl, pH 1.9, at 0°C for 55 s. The amounts of U_S^I and U_S^{II} in this mixture were then determined by a refolding assay (10-fold dilution) under final conditions of 7 μM RNase in 0.5 M GdmCl/0.05 M sodium cacodylate, pH 6.2, at 10°C. Refolding was monitored by the decrease in tyrosine fluorescence at 305 nm (excitation at 268 nm). The kinetic traces were analyzed as the sum of two exponentials. The observed time constants (τ) for the two phases (55 ± 5 and 240 ± 20 s; ± is SD) were independent of the duration of refolding in the first step. The $U_F \rightarrow N$ reaction is complete within the time of manual mixing. The observed amplitudes of the slow refolding reactions were proportional to the amounts of U_S^{II} and U_S^I produced by the rapid unfolding step at pH 2. The dependence of these amplitudes on the duration of the initial refolding step reflects the disappearance of molecules with incorrect isomers on the $U_S^{II} \rightarrow N$ and the $U_S^I \rightarrow N$ pathway, respectively. The zero time point was obtained from an experiment in which the initial refolding step was omitted.

RESULTS AND DISCUSSION

Slow Fluorescence-Detected Isomerization of Unfolded RNases Probably Originates from Tyr-92. One major slow isomerization reaction of unfolded bovine RNase A is correlated with an increase in tyrosine fluorescence (9, 12). Specific long-range interactions are no longer operative in the completely unfolded protein; therefore this fluorescence change probably originates from a local change in chain geometry in the vicinity of one or more tyrosine residues. Here we have used seven homologous RNases that differ in sequence at or around Tyr-92 and Tyr-115 to probe the origin of this slow fluorescence change. In bovine RNase these two tyrosine residues are adjacent to Pro-93 and Pro-114, respectively. The fluorescence-detected isomerization can be measured conveniently after unfolding at pH 2 in high concentrations of GdmCl, because the actual protein unfolding reaction ($N \rightarrow U_F$) is extremely rapid at low pH for all these RNases (12). Fig. 1 shows this isomerization after unfolding of bovine RNase A at 10°C, pH 2, in the presence of 5.0 M GdmCl. RNases from pig, cuis, and kangaroo, which have a Tyr-Pro sequence at positions 92–93 but lack either Tyr-115 or Pro-114, display slow fluorescence-detected isomerizations as well. They are very similar to the corresponding reaction of the bovine enzyme in their rates and in their amplitudes (Table 1). In the RNases from capybara and from guinea pig, Tyr-92 is replaced by Phe-92 (17, 21), and in these two proteins the slow isomerizations of the unfolded chain are not coupled to a change in tyrosine fluorescence (Table 1). Tyr-92 is the only tyrosine residue that is absent from capybara and guinea pig RNases as compared to the bovine enzyme. Substitution of this particular tyrosine is sufficient to abolish the slow increase in fluorescence after complete unfolding, indicating that the emission properties of the remaining five tyrosines (including the nearby Tyr-97) are not affected by the above process. In other words, this slow isomerization is a strictly local event, which occurs in the immediate vicinity of position 92 in the unfolded chain. If position 92 is occupied by tyrosine, then changes in the fluorescence of this residue can be used as a specific probe for this isomerization.

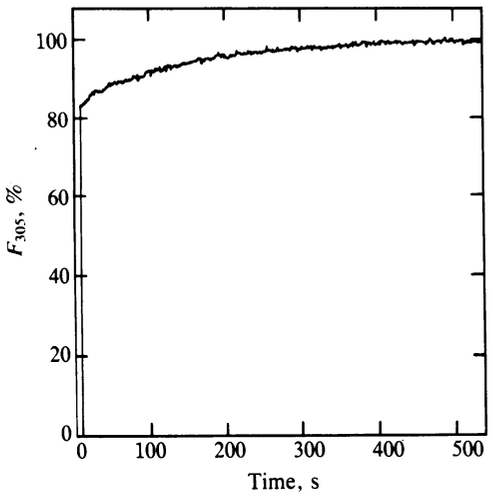

FIG. 1. Time course of the fluorescence-detected isomerization of bovine RNase A after unfolding in 0.1 M glycine, pH 2, in the presence of 5.0 M GdmCl at 10°C. Unfolding and isomerization were initiated by a 20-fold dilution of native RNase A (160 μM) to a final concentration of 8 μM enzyme. The increase in fluorescence was monitored at 305 nm (10-nm slit) with excitation at 268 nm (4-nm slit). The final value was set arbitrarily as 100%.

Fluorescence-Detected Isomerization Shows Properties of

Table 1. Kinetics of the slow fluorescence-detected isomerization of various RNases

Enzyme source	Sequences at positions* -92-93-	-114-115-	No. of tyrosines	τ, s	ΔF,† %
Ox	-Tyr-Pro-	-Pro-Tyr-	6	160	17
Rat	-Tyr-Pro-	-Pro-Tyr-	4	140	20
Pig	-Tyr-Pro-	-Pro-Pro-	4	200	16
Cuis	-Tyr-Pro-	-Pro-Ser-	4	180	20
Kangaroo	-Tyr-Pro-	- ‡ -Tyr-	5	230	10
Capybara	-Phe-Pro-	-Leu-Tyr-	5	—	—
Guinea pig A	-Phe-Pro-	-Pro-Tyr-	5	—	—

The kinetics of isomerization were measured after rapid unfolding ($N \rightarrow U_F$) in 0.1 M glycine, pH 2, in the presence of 5.0 M GdmCl at 10°C. Unfolding and isomerization were initiated by a 20-fold dilution of the native proteins (in H_2O) to final 3–5 μM enzyme. The increase in fluorescence after unfolding was monitored at 305 nm (excitation at 268 nm).

*References to the sequence data are as follows: ox (19), rat (16), pig (20), cuis and capybara (17), kangaroo (18), and guinea pig (21).

†Relative amplitude of the fluorescence-detected isomerization; the final fluorescence of the protein unfolded at equilibrium is set as 100%.

‡Residue 114 is deleted in kangaroo RNase (18).

Proline Isomerization. Tyr-92 is located next to Pro-93 in the RNase sequence. The simplest explanation for the molecular origin of the fluorescence-detected isomerization is that it reflects isomerization of the peptide bond between Tyr-92 and Pro-93 after $N \rightarrow U_F$ unfolding from the all-*cis* state to an equilibrium mixture of *cis* and *trans* configurations. If this interpretation is correct, then the fluorescence-detected isomerization should show properties characteristic of proline isomerization. Previously we had shown already that both the rate and the amplitude of this reaction are independent of pH in the range of pH 2 to pH 7 as well as independent of the concentration of GdmCl (provided that sufficient denaturant is present to keep the protein unfolded) (12). This independence of the particular unfolding conditions implies that the fluorescence-detected reaction is not limited in its rate by a structural unfolding process, but rather by an isomerization that is insensitive to changes in conditions that strongly affect protein unfolding reactions. (The $N \rightarrow U_F$ unfolding reaction of RNase A is strongly accelerated by increasing the concentration of GdmCl.)

The activation energy for the fluorescence-detected isomerization is 22 kcal/mol (1 kcal = 4.18 kJ), independent of the concentration of GdmCl used for unfolding (12). This value is well within the range expected for a proline-controlled reaction.

The *cis* $\rightleftharpoons$ *trans* isomerization of proline peptide bonds is catalyzed by strong acids (22, 23). The fluorescence-detected isomerization is acid catalyzed as well (Fig. 2). Catalysis starts at about 5 M $HClO_4$. The rate of the catalyzed reaction and its dependence on $HClO_4$ concentration are identical with the major faster reaction observed in the acid catalysis of the overall isomerization (3). Acid catalysis, together with the evidence presented above, strongly suggests that the reaction that changes the fluorescence of Tyr-92 is proline isomerization. The simplest explanation is that the fluorescence-detected process in unfolded RNase is isomerization of the peptide bond between Tyr-92 and Pro-93 after unfolding from the native all-*cis* state to a mixture of *cis* and *trans* configurations. This conclusion has already been proposed by us and others (7, 9, 12). The present results provide major support for it. Isomerization of the Tyr-Pro bond between residues 92 and 93 probably leads to a marked change in orientation of the tyrosyl chromophore relative to the neighboring disulfide bond between residues 40 and 95 and thus to a change in the efficiency of fluorescence quenching.

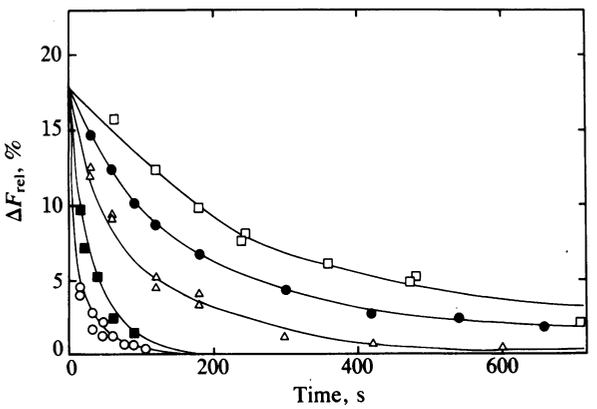

FIG. 2. Acid catalysis of the fluorescence-detected isomerization of unfolded bovine RNase. At zero time, native RNase (60 mg/ml in H_2O) was diluted 10-fold with concentrated $HClO_4$ solution at 0°C to give 3.3 M $HClO_4$ (□), 6.4 M $HClO_4$ (●), 7.9 M $HClO_4$ (△), 9.0 M $HClO_4$ (■), or 10.5 M $HClO_4$ (○). Fluorescence is quenched by high concentrations of $HClO_4$. Therefore the isomerization was not measured directly in $HClO_4$ but by a sampling procedure. At the indicated times 20-μl aliquots were withdrawn and added to 0.98 ml of 5.0 M GdmCl/0.3 M glycine at 10°C in the fluorimeter cell, to give a final pH of 2.3. The decrease of the amplitude of the remaining fluorescence-detected isomerization, ΔF_{rel}, is shown as a function of the time of incubation in $HClO_4$.

Reversal of the Fluorescence-Detected Isomerization of Tyr-Pro at Residues 92–93 During Refolding Can Be Monitored by Unfolding Assays. The reversal of the fluorescence-detected isomerization in the course of slow refolding can be measured by unfolding assays in the following way. After various times of folding, t_i, samples are withdrawn from the refolding solution, transferred to unfolding conditions of pH 2, 4 M GdmCl, 10°C, and the amplitudes of the resulting slow fluorescence-detected isomerizations are determined. These amplitudes are proportional to the fraction of RNase molecules that had already reversed the fluorescence-detected isomerization during the refolding step; consequently, in the unfolding assay, they isomerize back to the equilibrium distribution of the unfolded state. The kinetics of the reversal of the fluorescence-detected isomerization during refolding in 0.2 M GdmCl, pH 6, at 10°C are shown in Fig. 3. According to the proline model, the fluorescence amplitudes reflect the fraction of RNase molecules that have already reversed the isomerization of the peptide bond between Tyr-92 and Pro-93 during the refolding time t_i. Our assay thus measures the kinetics of *trans* → *cis* conversion at Tyr-92 and Pro-93 during the slow refolding reactions.

Previously Cook *et al.* (6) developed a two-step assay that measures the overall amount of RNase molecules with any incorrect isomers at different times during the slow refolding process. The reversal of the fluorescence-detected isomerization of the Tyr-Pro peptide bond at residues 92–93 correlates well with the overall decrease of the number of molecules with incorrect isomers (Fig. 3).

Reversal of Pro-93 Isomerization Occurs During Both Slow Refolding Reactions. The fluorescence assays for the isomeric state of Tyr-Pro at residues 92–93 can be used to locate the stage of slow refolding at which *trans* → *cis* reversal of the

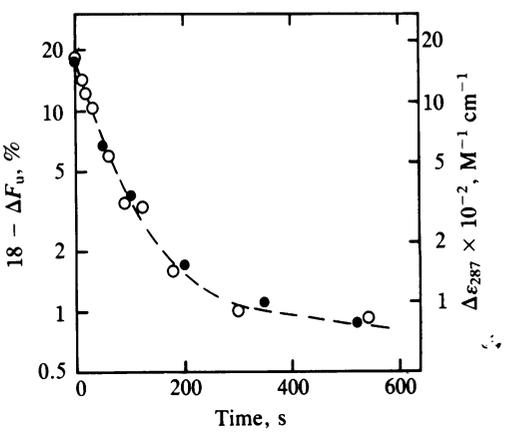

FIG. 3. Comparison of the kinetics of the reversal of the fluorescence-detected isomerization (○) with the kinetics of the overall decrease of species with incorrect isomers (●) during refolding of bovine RNase A. Reversal of the fluorescence-detected isomerization during refolding at 0.2 M GdmCl and 10°C was initiated by a 20-fold dilution of unfolded RNase A (1.6 mM in 0.1 M glycine/4.0 M GdmCl, pH 6.2). After various times of refolding, unfolding assays were performed by a 10-fold dilution to final 4.0 M GdmCl/0.1 M glycine, pH 2.0. The dependence of the amplitude of the slow fluorescence isomerization after unfolding on the duration of the refolding step is shown. The amplitudes are given as percentage of the final fluorescence emission, $\Delta F_u = \Delta F \cdot 100/F_\infty$ (%). The assay performed with completely refolded material yielded $\Delta F_u = 18\%$. The data for overall isomerization, expressed as decrease of the amplitudes of the slow refolding assays (left ordinate), are taken from figure 2 of ref. 6.

isomerization occurs. Under conditions strongly favoring the native conformation most of the U_S molecules refold according to the mechanism in Eq. 2, which involves the population of the native-like intermediate I_N (6, 7, 24). I_N closely resembles the native protein in its catalytic activity and in its tyrosine absorbance. However, I_N molecules still have incorrect isomers and consequently they revert back to U_S species upon rapid unfolding at low pH (5, 6, 24). Fig. 4*A* shows that at 0°C, in the presence of 0.4 M ammonium sulfate, pH 6 (these conditions strongly favor native folding), I_N is populated rapidly during slow refolding. Formation of I_N is monitored by the major faster phase (τ = 11 s) of the absorbance-detected kinetics (cf. also ref. 7). Reversal of Tyr-Pro isomerization at position 92–93 is much slower; as in Fig. 3, its correspondence with the overall decrease of molecules with incorrect isomers as measured by the two-step assay of Cook *et al.* (6) is good. *trans* → *cis* isomerization of Pro-93 evidently occurs in the last step of refolding in parallel to the formation of the native protein.

The two-step assay of Cook *et al.* (6) measures the overall decrease of molecules with incorrect isomers during refolding. The individual isomerization steps in the course of the major $U_S^{II} \rightarrow N$ and the minor $U_S^{I} \rightarrow N$ pathways can be separated and measured individually by a modification of the two-step assay. This is achieved by performing the final refolding step of the assay under conditions that allow a kinetic separation of the two slow refolding reactions. We used this modified assay to probe refolding under the strongly native conformation-favoring conditions employed in Fig. 4*A*. The results (shown in Fig. 4*B*) demonstrate that reversal of incorrect isomers on the $U_S^{II} \rightarrow N$ pathway dominates the overall process. It accounts for about 80% of all U_S molecules and proceeds with a time constant of about 130 s. The wrong isomers of the very slow refolding U_S^{I} species are converted to their native state more slowly with a time constant of about 450 s. The sum of both individual isomerizations is also plotted in Fig. 4*B*. It matches the overall isomerization process as determined by the original two-step assay (the broken line in Fig. 4*A*).

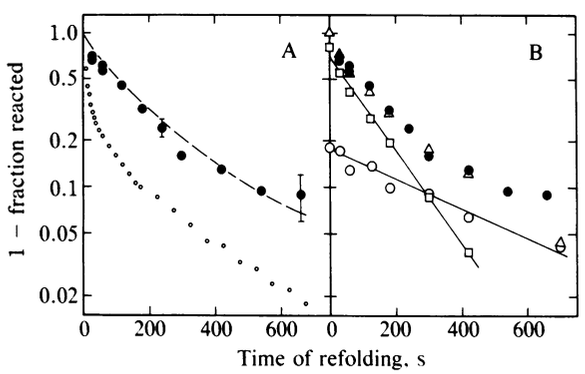

FIG. 4. Isomerization reactions during the slow refolding of RNase A. (*A*) Comparison of the kinetics of the reversal of the fluorescence-detected isomerization of the peptide bond between Tyr-92 and Pro-93 (●) with the absorbance-detected kinetics of refolding (○○○) at 0°C in 0.2 M GdmCl/0.4 M ammonium sulfate, pH 6.2. *trans* → *cis* isomerization of Tyr-Pro at residues 92–93 was measured by unfolding assays after the indicated times of refolding. Assays were performed in 4.0 M GdmCl/0.1 M glycine, pH 2.0. The amplitude of the slow fluorescence isomerization is given as a fraction of the total change that is observed with completely refolded RNase; the total amplitude amounts to 18% of the fluorescence of the unfolded protein. Absorbance-detected refolding was monitored at 287 nm at a protein concentration of 30 μM. The absorbance kinetics can be approximated by two exponentials (τ = 130 s, 29% amplitude; τ = 11 s, 71% amplitude). The broken line indicates the overall decrease of RNase species with incorrect isomers, taken from ref. 7. (*B*) Decrease of species with incorrect isomers in the time course of the $U_S^{I} \rightarrow N$ (○) and the $U_S^{II} \rightarrow N$ (□) refolding reactions under the conditions of *A*. △, Sum of both processes. The data are compared to the kinetics of Pro-93 *trans* → *cis* isomerization (●, same data as in *A*).

In Fig. 4*B* the kinetic curve of *trans* → *cis* isomerization at Pro-93 is compared to the modified two-step assay. The best correlation is clearly observed with the sum of these two isomerizations. This demonstrates that *trans* → *cis* isomerization of Pro-93 occurs with the same kinetics as the formation of N in the $U_S^{II} \rightarrow N$ and the $U_S^{I} \rightarrow N$ refolding pathways.

Taken together, the results in Fig. 4 show that U_S^{II} species with an incorrect *trans* Pro-93 are able to refold up to a native-like, catalytically active state. *trans* → *cis* isomerization then occurs in the largely folded state during the $I_N \rightarrow N$ step. This reaction is more rapid than the corresponding process in the unfolded protein, but it still shows properties characteristic of proline isomerization and it is coupled to a decrease in tyrosine fluorescence (6, 7, 25). Hence folding is possible with an incorrect Pro-93 isomer, but the folding rate is decreased from the millisecond to the second time range. *trans* → *cis* isomerization of Pro-93 takes place during the $U_S^{I} \rightarrow N$ reaction as well; however, it is much slower than on the $U_S^{II} \rightarrow N$ pathway (Fig. 4).

Proline Isomerization and Protein Folding. The role of particular prolines for refolding may be determined primarily by two factors: (*i*) by the location in the three-dimensional structure and (*ii*) by the refolding conditions. Under favorable conditions incorrect prolines such as a *trans*-Pro-93 in RNase may be tolerated in structural folding intermediates (26–30), and the rate of isomerization can be increased by the rapid formation of ordered structure prior to isomerization. This model for *in vitro* refolding may be relevant for *in vivo* folding as well. The newly synthesized all-*trans* polypeptide chain is able to refold rapidly to a compact ordered state such as I_N, followed by the *trans* → *cis* isomerization of those β-bend prolines that are *cis* in the native protein (7).

CONCLUSIONS

The fluorescence-detected isomerization of various unfolded RNases is related to the presence of Tyr-92, which is adjacent in sequence to Pro-93. It shows many properties that are characteristic for proline peptide bond isomerization, such as catalysis by strong acid and an activation enthalpy of 22 kcal/mol (12). We suggest that this isomerization originates from *cis* ⇌ *trans* equilibration of the Tyr-Pro peptide bond at residues 92–93, which is all-*cis* in the native protein. Incorrect *trans* isomers of Pro-93 are found in both U_S^{II} and U_S^{I}. About 85 ± 10% of the species with a *trans* Pro-93 reisomerize in the course of the $U_S^{II} \rightarrow N$ refolding reaction and about 15 ± 10% during the minor $U_S^{I} \rightarrow N$ reaction. These numbers correlate reasonably well with the observed ratio of U_S^{II} to U_S^{I} in unfolded RNase A (4:1; cf. Eqs. 2 and 3). The formation of native protein on both slow refolding pathways and the corresponding *trans* → *cis* isomerization of Pro-93 show identical kinetics. During the major $U_S^{II} \rightarrow N$ reaction, reisomerization proceeds with the same rate as the final $I_N \rightarrow N$ step.

These results can be explained by a model that assumes that most, if not all, slow-folding molecules actually contain an incorrect *trans* Pro-93. The U_S^{II} molecules have a nonnative isomer only at Pro-93 and under favorable conditions they can refold with a *trans* Pro-93 to the native-like I_N state. The final slow $I_N \rightarrow N$ step is *trans* → *cis* isomerization of Pro-93, which is accelerated by prior structure formation. Isomerization of Pro-93 on the minor $U_S^{I} \rightarrow N$ pathway is significantly slower. The presence of additional incorrect isomers could impair or decelerate the formation of I_N-like structure on this pathway and thereby decrease the rate of refolding as well as of *trans* → *cis* at Pro-93.

On the basis of isomer-specific proteolysis experiments, Lin and Brandts (9–11) concluded that no more than 30% of

all unfolded molecules—i.e., only the very slow refolding U_S^I species (U_S^I corresponds to the $U_{TX} + U_{TY}$ species of ref. 11) contained a *trans* Pro-93. Hence in their model for unfolding and refolding of RNase (11) only the $U_S^I \rightarrow N$ reaction involves *trans* → *cis* isomerization of this proline peptide bond. Folding steps prior to isomerization are definitely excluded in this model and consequently the reaction *trans* → *cis* of Pro-93 as it occurs in the unfolded chain has to be the first and rate-limiting step before folding of U_S^I can begin. This model is not supported by our findings (*i*) that *trans* → *cis* isomerization of Pro-93 occurs on both slow refolding pathways, $U_S^I \rightarrow N$ and $U_S^{II} \rightarrow N$, and (*ii*) that most incorrect isomers of Pro-93 revert back to *cis* with the same rate as the final $I_N \rightarrow N$ step of the $U_S^{II} \rightarrow N$ pathway. Further experiments with homologous or mutated RNases that differ at proline positions should help to resolve the discrepancy between these two different models for the folding of RNase.

We thank Dr. R. Jaenicke, K. Lang, Dr. R. Rudolph, and N. Schülke for many discussions. In particular our thanks are due to Dr. J.-R. Garel for helpful comments and suggestions. Pancreatic tissue from guinea pig was a generous gift from VonHeyden GmbH, Regensburg. This research was supported by the Deutsche Forschungsgemeinschaft (Schm 444/2-1 and 2-2).

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