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Folding of Ribonuclease T_1. 2. Kinetic Models for the Folding and Unfolding Reactions†

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Received August 11, 1989; Revised Manuscript Received October 25, 1989

ABSTRACT: The slow refolding of ribonuclease T_1 was investigated by different probes. Structural intermediates with secondary structure are formed early during refolding, as indicated by the rapid regain of a native-like circular dichroism spectrum in the amide region. This extensive structure formation is much faster than the slow steps of refolding, which are limited in rate by the reisomerization of incorrect proline isomers. The transient folding intermediates were also detected by unfolding assays, which make use of the reduced stability of folding intermediates relative to that of the native protein. The results of this and the preceding paper [Kiefhaber et al. (1990) *Biochemistry* (preceding paper in this issue)] were used to propose kinetic models for the unfolding and refolding of ribonuclease T_1. The unfolding mechanism is based on the assumption that, after the structural unfolding step, the slow isomerizations of two X-Pro peptide bonds occur independently of each other in the denatured protein. At equilibrium a small amount of fast-folding species coexists with three slow-folding species: two with one incorrect proline isomer each and another, dominant species with both these prolines in the incorrect isomeric state. In the mechanism for refolding we assume that all slow-folding molecules can rapidly regain most of the secondary and part of the tertiary structure early in folding. Reisomerizations of incorrect proline peptide bonds constitute the slow, rate-limiting steps of refolding. A peculiar feature of the kinetic model for refolding is that the major unfolded species with two incorrect proline isomers can enter two alternative folding pathways, depending on which of the two reisomerizes first. The relative rates of reisomerization of the respective proline peptide bonds at the stage of the rapidly formed intermediate determine the choice of pathway. It is changed in the presence of prolyl isomerase, because this enzyme catalyzes these two isomerizations with different efficiency and consequently leads to a shift from the very slow to the intermediate refolding pathway.

Unfolding and refolding of ribonuclease T_1 (RNase T_1)[1] from *Aspergillus oryzae* are complex processes. The loss of ordered structure during unfolding ($N \rightarrow U_F$) is followed by slow equilibration processes of the denatured polypeptide chains, which create at least two different slow-folding (U_S) species. These slow reactions show a number of properties that are characteristic of proline isomerization. In our working model (Kiefhaber et al., 1990) we therefore assume that unfolded RNase T_1 consists of several species that differ in the cis/trans conformation of one or more prolyl peptide bonds. There is a small amount (3.5%) of fast-folding (U_F) molecules which refold within the dead time of manual mixing; we suppose that they contain the correct proline isomers. The 96.5% slow-folding U_S species should have one or more important prolines in the nonnative, incorrect isomeric state. They give rise to two predominant phases in refolding, an intermediate[2] process with a time constant of 300 s and a very slow process with $\tau = 6500$ s (at pH 5.0, 10 °C).

A number of properties of the slow refolding reactions are not readily explained by a simple proline model. Refolding is strongly dependent on the employed conditions, and the amplitudes observed for the two slow phases depend on the probes that are used to monitor folding. In particular, the relative amplitude of the very slow phase is much larger in reactivation experiments than in absorbance- or fluorescence-detected refolding. The amplitudes are also strongly

† This work was supported by grants from the Deutsche Forschungsgemeinschaft (to U.H. and F.X.S.) and the Fonds der Chemischen Industrie (to T.K. and F.X.S.).

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[1] Abbreviations: RNase T_1, Lys25 isoenzyme of ribonuclease T_1 from *Aspergillus oryzae*; GpC, guanylyl(3′→5′)cytidine; GdmCl, guanidinium chloride; N and U, native and unfolded protein, respectively; I, folding intermediates.

[2] The two slow rate-limiting phases that are observed in the slow refolding of RNase T_1 are labeled "intermediate" (faster process with time constant τ_2) and "very slow" (slower process with time constant τ_1), respectively.

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dependent on pH. The very slow phase dominates the folding kinetics at pH 5; however, with increasing pH this phase decreases in amplitude at the expense of a concomitant increase in the size of the intermediate folding reaction. A similar shift toward the intermediate phase was observed when the temperature was raised from 10 to 25 °C. These initial results suggest that the slow refolding reactions are complex processes and probably involve the formation of structural intermediates.

It is our aim here to elucidate the kinetic mechanism of slow refolding of RNase T_1. To detect and characterize putative partially folded intermediates, we use four different approaches. (i) The folding conditions are varied from "strongly native" (in the presence of NaCl) to "marginally native" (in the presence of increasing concentrations of GdmCl), in order to stabilize or destabilize putative intermediates. (ii) Circular dichroism (CD) in the amide region is used to monitor the formation of secondary structure during slow refolding. (iii) An unfolding assay (Schmid, 1983, 1986) is employed to screen for partially folded intermediates which are less stable than the native protein and consequently unfold under conditions where completely folded RNase T_1 remains stable. This assay is used in further experiments to monitor the transient accumulation of such intermediates in the course of slow refolding. (iv) Prolyl isomerase (Fischer et al., 1984) is employed as a diagnostic tool to locate proline-controlled folding steps. The information from these experiments is then combined with the results of the preceding work (Kiefhaber et al., 1990) to propose a kinetic model for the slow refolding of RNase T_1. We assume in the model that two proline peptide bonds are of major importance for slow folding and that partially folded intermediates are formed early in refolding. Their stability is dependent on the conditions, in particular on pH.

Materials and Methods

Materials. For the experiments the same materials were used as described in the preceding paper (Kiefhaber et al., 1990).

Methods. A Perkin-Elmer Lambda 5 spectrophotometer, a Perkin-Elmer LS-5B fluorimeter, a Hitachi F-4010 fluorimeter with integrated magnetic stirrer, and a Jasco J-500A spectropolarimeter were used for optical measurements.

Refolding Kinetics. Unfolded RNase T_1 (in 6.0 M GdmCl and 0.1 M glycine hydrochloride, pH 1.7, or in 8.0 M urea and 0.1 M Tris-HCl, pH 8.0) was diluted 40-fold into the appropriate refolding buffer in a thermostated cuvette, to initiate refolding. The following buffers (each 0.1 M) were employed: glycine hydrochloride (pH 2 and 3); NaOAc/acetic acid (pH 4 and 5); sodium cacodylate (pH 6 and 7); Tris-HCl (pH 8). Refolding in the presence of NaCl was carried out in 10 mM NaOAc/acetic acid, pH 5, containing various amounts of NaCl. Refolding was detected by the increase in fluorescence at 320 nm (after excitation at 268 nm), by the increase in absorption at 287 nm, or by the change of the CD signal at 225 nm. The concentrations of RNase T_1 depended on the probes employed and are stated in the figure legends. Catalyzed refolding reactions were carried out by adding various amounts of the enzyme peptidyl-prolyl cis–trans isomerase to the refolding buffer.

Unfolding Assays To Monitor the Transient Formation of Folding Intermediates. Unfolded RNase T_1 (in 5.0 M GdmCl, 67 mM glycine hydrochloride, and 33 mM NaOAc/acetic acid, pH 2.1) was refolded by 35-fold dilution into 0.1 M NaOAc/acetic acid, pH 5.0 at 10 °C. After various times of refolding (t_i) samples were withdrawn and diluted 3-fold to give final conditions of 3.4 M GdmCl and 0.1 M NaOAc/acetic acid, pH 5.0 at 10 °C. The unfolding of the intermediate was detected by the decrease of the fluorescence at 320 nm after excitation at 268 nm. The amplitude of the unfolding reaction is a measure for the relative amount of intermediate present at the time t_i. Aliquots of the refolding solutions were allowed to refold completely and subjected to the same unfolding assays. Here no decrease in fluorescence could be detected. The concentration of RNase T_1 was 0.37 μM in the unfolding assay.

Unfolding Assays To Monitor the Formation of Native Molecules. Unfolded RNase T_1 (in 5.8 M GdmCl and 0.1 M glycine hydrochloride, pH 1.7) was diluted 40-fold into 0.1 M Tris-HCl, pH 8.0 at 10 °C, to initiate refolding. After various times of refolding (t_i) samples were withdrawn and diluted 10-fold to final conditions of 5.6 M GdmCl and 0.1 M glycine hydrochloride, pH 2.0, at 10 °C in a fluorescence cuvette. The resulting kinetics of unfolding were detected by the decrease in fluorescence at 320 nm after excitation at 268 nm. The amplitude of the unfolding reaction (A_t) is a measure for the amount of native protein present in the refolding solution at t_i. Part of the refolding solution was unfolded under the same conditions after refolding was completed (t_∞) in order to determine the total unfolding amplitude (A_∞). The ratio of A_t to A_∞ gives the percentage of native molecules present after refolding time t_i. Protein concentrations ranged from 6.3 to 7.5 μM in the refolding step. Additionally, refolding in the presence of 0.7 and 1.0 μM prolyl isomerase was probed by unfolding assays. In these experiments the protein was initially unfolded by 8 M urea and 50 mM Tris-HCl, pH 8.0, in order to prevent inactivation of prolyl isomerase by residual GdmCl in the refolding solution.

CD Spectrum of the Early Folding Intermediate. Unfolded RNase T_1 (in 6 M GdmCl and 0.1 M glycine hydrochloride, pH 1.7) was diluted 40-fold into 0.1 M NaOAc/acetic acid, pH 5.0, and 10 °C in a thermostated 1-mm CD cell. Refolding kinetics were monitored at various wavelengths from 210 to 235 nm, and the slow kinetics were extrapolated back to zero time in order to evaluate the CD signal of the early folding intermediate before the onset of the slow refolding reactions. The concentration of RNase T_1 in the refolding solution was 95 μM.

Results

Slow Refolding of RNase T_1 Depends on the Folding Conditions. In the preceding paper (Kiefhaber et al., 1990) we showed that the rates and amplitudes of the two slow refolding reactions of RNase T_1 are strongly pH-dependent. In addition, different relative amplitudes were observed for these two reactions depending on the probe used to monitor refolding. These properties appear to be incompatible with the simple proline model for folding. In this model the relative amplitudes of the individual refolding reactions are determined by the equilibrium distribution of the different proline isomers in the unfolded polypeptide chains (Brandts et al., 1975). Therefore, they should depend neither on the refolding conditions nor on the probe used to monitor folding.

We have investigated the dependence on the concentrations of GdmCl and of NaCl, respectively, of the slow refolding reactions of RNase T_1 to examine whether the peculiar kinetic properties of these processes could be due to the presence of partially folded intermediates during refolding. Structural intermediates should be less stable than native RNase T_1 and hence be susceptible to changes in the "stabilizing" (NaCl) or "destabilizing" (GdmCl) character of the refolding solution. The dependence on GdmCl of the slow folding kinetics as monitored by absorbance and by fluorescence is summarized

Table I: Dependence on GdmCl of the Slow Refolding Reactions of RNase T_1[a]

		intermediate phase		very slow phase	
GdmCl (M)	method[b]	A_2[c]	τ_2 (s)	A_1[c]	τ_1 (s)
0.15	Fl	0.39	75	0.42	1100
0.15	Abs	0.45	80	0.18	900
0.50	Fl	0.48	85	0.40	1300
1.00	Fl	0.50	100	0.40	1200
1.00	Abs	0.60	100	0.24	1200
1.50	Fl	0.49	105	0.44	730
2.00	Fl	0.37	250	0.57	680
2.00	Abs	0.19	290	0.73	670

[a] Initial unfolding conditions were 6.0 M GdmCl and 0.1 M glycine hydrochloride, pH 1.7. Refolding was started by a 40-fold dilution to 0.1 M NaOAc/acetic acid, pH 5.0, and the indicated final concentrations of GdmCl in a spectrometer cuvette. Refolding temperature was 25 °C. The final RNase T_1 concentrations were 0.37 μM (fluorescence) and 7 μM (absorbance). The error limits are about ±5% for the amplitudes and about ±10% for the time constants. [b] Fl = fluorescence at 320 nm after excitation at 268 nm; Abs = absorbance at 287 nm. [c] A_1 and A_2 are the relative amplitudes of the very slow and intermediate refolding phases, respectively. The amplitudes are given as fractions of the total change in fluorescence or absorbance as observed in the equilibrium unfolding transitions.

Table II: Dependence on NaCl of the Slow Refolding Reactions of RNase T_1[a]

		intermediate phase		very slow phase	
NaCl (M)	method[b]	A_2[c]	τ_2 (s)	A_1[c]	τ_1 (s)
0	Fl	0.34	66	0.47	1100
0	Abs	0.45	80	0.19	900
0.02	Fl	0.32	67	0.47	1200
0.20	Fl	0.27	60	0.49	1100
2.00	Fl	0.16	63	0.52	1800
2.00	Abs	0.31	51	0.26	1500

[a] All refolding experiments were carried out at 25 °C. Initial unfolding conditions were 6.0 M GdmCl and 0.1 M glycine hydrochloride, pH 1.7. Refolding was initiated by a 40-fold dilution into 10 mM NaOAc/acetic acid and the indicated concentrations of NaCl in a spectrometer cuvette. Error limits are as indicated in Table I. [b] Fl = fluorescence at 320 nm after excitation at 268 nm; Abs = absorbance at 287 nm. [c] A_1 and A_2 are the amplitudes of the very slow and intermediate refolding phases, respectively. The amplitudes are given as fractions of the total change in fluorescence or absorbance as observed in the equilibrium unfolding transitions.

in Table I. The rates of the two slow refolding reactions are affected by GdmCl in very different ways. (i) The intermediate (τ_2) phase becomes slower with increasing concentrations of GdmCl. Such a behavior was observed for other protein folding reactions as well, and it reflects an increasing difference in free energy between the transition state for folding and the unfolded or intermediate state that precedes it (Tanford, 1970; Matthews & Hurle, 1987; Kuwajima et al., 1989). (ii) The behavior of the very slow (τ_1) phase is different. This refolding reaction actually becomes faster with increasing concentrations of GdmCl; i.e., the less favorable the folding conditions are, the faster is refolding on this particular pathway. A similar behavior was found in the urea dependence of this reaction (Thomson et al., 1989). The sum of the amplitudes of the two slow refolding reactions increases with GdmCl. At 0.15 M GdmCl only about 65% of the total change in absorbance occurs in the two slow phases. This number increases to more than 90% in the presence of 2.0 M GdmCl. As there are 96% U_S molecules in unfolded RNase T_1 (Kiefhaber et al., 1990), the amplitude data suggest that there is a rapid initial change in absorbance and fluorescence at the beginning of refolding in the presence of low concentrations of GdmCl, but not at high concentrations of GdmCl. The ratio of the amplitudes (A_1/A_2) increases with the concentration of GdmCl in both absorbance and fluorescence; i.e., the very slow phase gains in amplitude at the expense of the intermediate phase.

Native RNase T_1 is strongly stabilized by NaCl (Oobatake et al., 1979; Pace et al., 1988; Kiefhaber et al., unpublished work). Therefore, a variation of NaCl concentration was used to investigate the influence of increasingly "stabilizing" conditions on the refolding of RNase T_1. The results are shown in Table II. The time constant of the intermediate (τ_2) phase is almost independent of the NaCl concentration, whereas the very slow (τ_1) phase is decelerated with increasingly "native" conditions. The sum of the amplitudes for the two slow phases decreases slightly with increasing NaCl concentration. This is in large part due to a marked decrease in the amplitude of the faster (τ_2) reaction.

In summary, the data in Tables I and II show that under favorable folding conditions a rapid change in absorbance and fluorescence occurs prior to the two slow reactions. This leads to a decrease in the relative amplitudes of these two phases from over 90% (in 2 M GdmCl) to less than 65% (below 0.5 M GdmCl). The simplest explanation for this observation is that the two slow refolding phases are preceded by rapid formation of folding intermediates with partially ordered structure. A second remarkable feature is the unusual dependence on the folding conditions of the very slow phase, which decreases progressively in rate with increasingly native conditions. This indicates that rapid formation and stabilization of (possibly incorrect) intermediate structure decelerates a late, rate-limiting step of folding.

Transient Intermediates Are Formed Rapidly during Slow Refolding of RNase T_1. The presence of transient, partially folded intermediates in the course of the slow refolding reactions of RNase T_1 was investigated by an assay that was originally developed for the folding of pancreatic RNases (Schmid, 1983). The assay is based on the assumption that any partially folded intermediates on the renaturation pathway are less stable toward GdmCl-induced unfolding than the native molecules. The assay consists of two steps. First, the protein is allowed to refold for a limited period of time under favorable folding conditions in order to populate putative intermediates. Then the refolding is interrupted and the conditions are changed to a GdmCl concentration close to the onset of the unfolding transition of the native protein. Under this condition completely refolded molecules will remain native; any partially folded intermediates, however, should no longer be stable and consequently unfold transiently to U_S. These U_S molecules will then refold again in a very slow reaction since the folded state is still stable under the assay conditions. The result of such an unfolding assay for RNase T_1 is shown in Figure 1. In the first step of the experiment the protein was allowed to refold in a test tube for 7 min at pH 5.0, 10 °C, in the presence of 0.14 M GdmCl. Then the sample was transferred in the second step to 3.4 M GdmCl, pH 5.0, 10 °C (in the fluorimeter cell). Under these conditions native RNase T_1 is at the edge of its stability (Kiefhaber et al., 1990). The observed changes in fluorescence resulting from the shift in conditions are shown in Figure 1. The test for partially folded, less stable intermediate(s) is positive. More than half of the fluorescence change between the unfolded and the native state has already occurred after 7 min of refolding. However, only a small part of the fluorescence increase originates from the formation of stable, native RNase T_1. A major fraction of the fluorescence is due to the presence of partially folded intermediates, which are unstable at 3.4 M GdmCl. They unfold in the assay with a time constant of 65 s and give rise

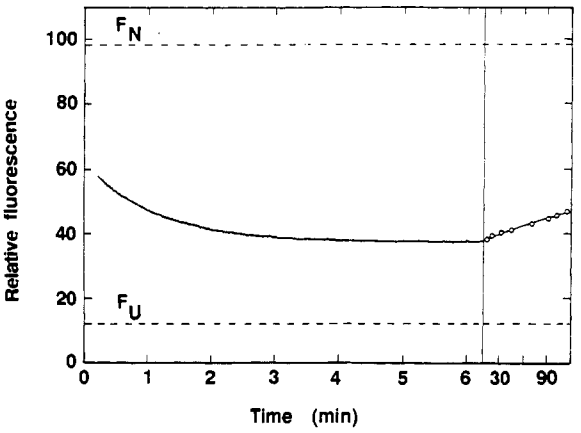


FIGURE 1: Kinetics of denaturation of partially folded intermediates in the presence of 3.4 M GdmCl and 0.1 M NaOAc/acetic acid, pH 5.0, at 10 °C. The intermediates were populated by a 7-min refolding pulse (at 0.14 M GdmCl and 0.1 M NaOAc/acetic acid, pH 5.0, 10 °C). Unfolding of the intermediates was monitored by the decrease in fluorescence at 320 μM. Under these conditions that intermediates unfold with a time constant of 65 s. In the late time region the beginning of the extremely slow refolding of the transiently formed unfolded protein is shown. Fluorescence was recorded at discrete time intervals rather than continuously to minimize radiation damage of the sample. The fluorescence emission of the native protein was regained after about 24 h. The fluorescence intensities of native and of unfolded RNase T_1 as obtained from the equilibrium unfolding transition (Kiefhaber et al., 1990) are shown as broken lines for comparison.

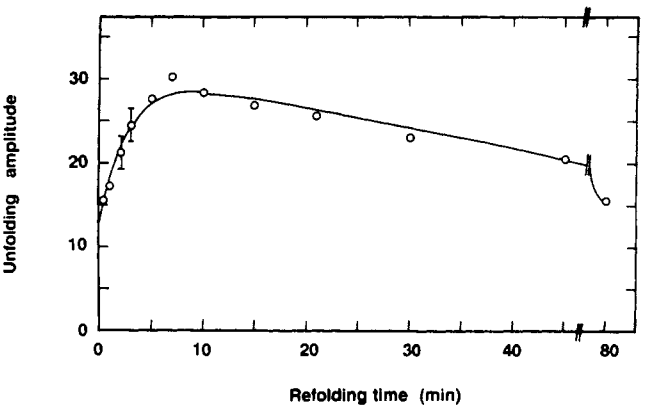


FIGURE 2: Transient formation of folding intermediates (O) during refolding of RNase T_1 at pH 5.0 (0.1 M NaOAc/acetic acid and 0.14 M GdmCl) and 10 °C. The relative amount of intermediates was measured by unfolding assays as in Figure 1. The amplitude of the unfolding reaction of the intermediates is shown as a function of the duration of refolding. The solid line represents the theoretical time course for the intermediates with time constants of 170 s for the formation and 6500 s for the decay of the intermediate. About 30% of the maximal population of the intermediate is already present within the dead time of the experiment. The concentration of RNase T_1 was 1.2 μM in the refolding step.

to the pronounced fluorescence decrease in the first 6 min of the assay (Figure 1). This unfolding reaction is followed by an extremely slow increase in fluorescence, caused by refolding to the N state. After about 24 h the fluorescence of native RNase T_1 is regained. When refolding is allowed to go to completion in the first step, such a decrease in fluorescence, following the jump to 3.4 M GdmCl, is no longer observed because native RNase T_1 is stable under these conditions. The results show that after 7 min of refolding intermediate structure is present with fluorescence properties intermediate between the native and the unfolded protein. Assays carried out under the same conditions as in Figure 1, but by using absorbance at 287 nm to monitor unfolding, gave similar results, indicating that tyrosine residues are partially buried already in the intermediate structure. Unlike native RNase T_1, this transient structure is unstable in the presence of 3.4 M GdmCl and is unfolded with a time constant of 65 s.

Unfolding assays as shown in Figure 1 can also be used to monitor the kinetics of formation of such partially folded intermediates in the course of refolding. In these experiments the duration of the refolding pulse in the first step is varied and the amplitude of the unfolding reaction of the intermediates in the subsequent assay at 3.4 M GdmCl is then used as a measure of the relative amount of intermediate species present at the time of sample transfer. The dependence on refolding time of these unfolding amplitudes is shown in Figure 2. As expected for transient intermediates on sequential kinetic pathways, the concentration of partially folded species first increases in the early time region of refolding, reaches a maximum after about 10 min, and then slowly decreases again when native protein is formed. The kinetics of formation of intermediates are complex: part of the intermediate species is populated rapidly within the dead time of the experiment (30 s), whereas the other part is formed slowly within about 10 min. The reaction from the intermediate state(s) to the native state is very slow.

The results in Figure 2 suggest that partially folded intermediates are produced by at least two reactions. In the first reaction I is formed very rapidly; in the second one I is formed with a time constant of about 170 s. This reaction is somewhat faster than, but still similar in rate to, the intermediate phase of slow refolding under these conditions (τ = 300 s). This 170-s reaction presumably contributes to the intermediate folding phase. The rate of disappearance of at least the major fraction of the intermediates in Figure 2 coincides with the slowest phase of refolding (τ = 6500 s). The absolute concentrations of the intermediate species cannot be derived from these experiments, because their fluorescence properties are not known precisely.

Secondary Structure Is Formed Rapidly in the Course of Slow Refolding. The results in Figures 1 and 2 strongly suggest that partially folded intermediates with altered fluorescence properties are indeed formed transiently during the slow refolding reactions of RNase T_1. This is confirmed by the comparison of the refolding kinetics monitored by CD in the peptide region (Figure 3a) and by tyrosine absorbance at 287 nm (Figure 3b). During refolding in the presence of 0.15 M GdmCl (pH 5.0, 25 °C) the (negative) CD signal at 225 nm increases within the dead time of mixing (30 s) to a value that is already more negative than the CD of the completely folded protein. The final CD signal of native RNase T_1 is then reached in two very slow phases, which occur in the time region of the rate-limiting slow refolding reactions (as observed by absorbance, Figure 3b). In the presence of 1.0 M GdmCl this "overshoot" phenomenon is still apparent. However, the formation of secondary structure is decelerated and occurs with a time constant of about 70 s. Again, the two rate-limiting reactions are correlated with an apparent decrease of the CD signal. When folding is carried out in the presence of 2.0 M GdmCl, both slow refolding reactions are accompanied by an increase in secondary structure, and the overshoot is no longer observable. However, there is still a rapid regain of part of the CD signal (about 40%) at the beginning of folding, which suggests either that part of the secondary structure can still be formed rapidly under these conditions or else that a fraction of the unfolded molecules can still form their secondary structure in a fast reaction even at 2.0 M GdmCl. Under these conditions no initial rapid change in absorbance is observed (Figure 3b). In summary, the data in Figure 3 demonstrate that the early intermediates are

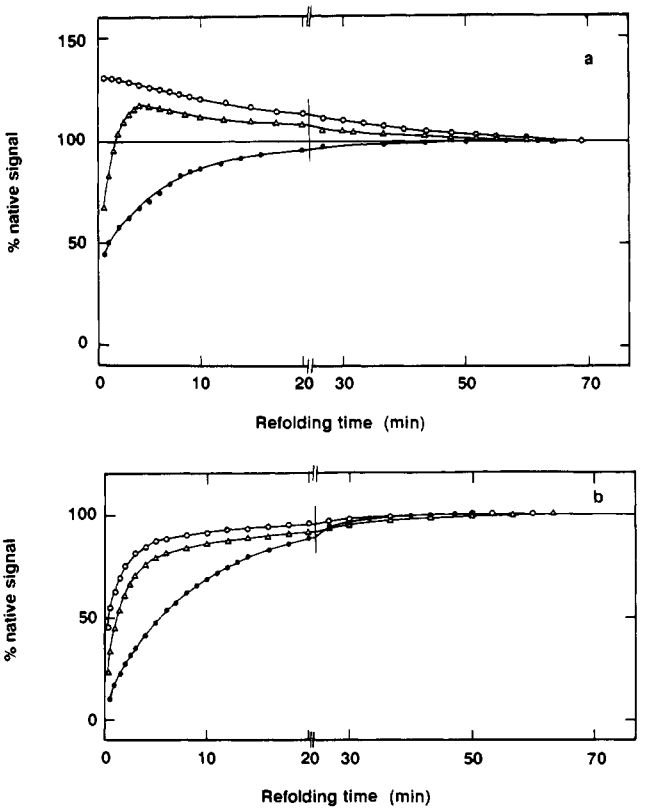


FIGURE 3: Refolding of RNase T_1 in the presence of 0.15 M (○), 1.0 M (△), and 2.0 M (●) GdmCl at pH 5 and 25 °C, detected by (a) CD at 225 nm and (b) absorbance at 287 nm. 0% and 100% represent the CD and absorbance signals of the unfolded and the native protein, respectively, as observed in the equilibrium unfolding transitions. Unfolded RNase T_1 (in 6.0 M GdmCl and 0.1 M glycine hydrochloride, pH 1.7) was refolded by 40-fold dilution to 0.1 M NaOAc/acetic acid, pH 5.0, containing the appropriate amount of GdmCl, in the spectrometer cell at 25 °C. Protein concentrations were 90 μM in the CD measurements and 5.5–13 μM for absorbance-detected refolding.

sensitive to GdmCl. Rapid formation of a high amount of secondary structure and part of the tertiary structure in most if not all refolding species is observed only at low final concentrations of GdmCl, i.e., under strongly native conditions.

One explanation for the "overshoot" observed in the kinetic experiment in Figure 3a is that the intermediates have a more extensive secondary structure than the native protein. However, aromatic residues are known to contribute to the CD of native proteins around 225 nm as well (Adler et al., 1973). Since RNase T_1 contains a high fraction of aromatic residues (Takahashi & Moore, 1982), it appears more likely that the slowly formed positive contribution to the CD around 225 nm oritinates from structure formation around the aromatic residues of the protein and thus occurs in the same time region as the slow changes in absorbance and in fluorescence. This interpretation is supported by results obtained by CD at 238 nm. Refolding kinetics observed at this wavelength are identical with absorbance- and fluorescence-detected kinetics. At this wavelength (where the CD signals of the secondary structural elements are very weak) CD is clearly dominated by the aromatic contributions.

The rapid formation of extensive secondary structure is sensitive to pH. A fast generation of the native-like CD signal with an "overshoot" similar to the results at pH 5.0 shown in Figure 3a is observed when refolding is carried out in the pH range from pH 3 to pH 7. Below pH 3 and above pH 7 the amplitude of the rapid change in CD is progressively decreased. These results indicate that the stability of this intermediate

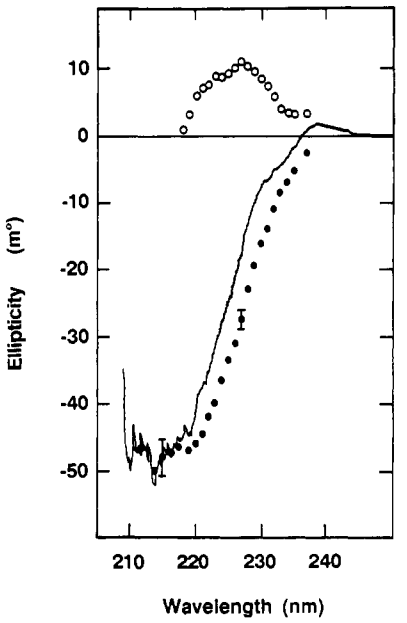


FIGURE 4: CD spectrum of an early folding intermediate (●), which is formed rapidly after the initiation of refolding. The solid line represents the spectrum of RNase T_1 after complete refolding. Additionally, the difference between the spectra of the intermediate and renatured RNase T_1 is shown (○). Unfolded RNase T_1 (in 6.0 M GdmCl and 0.1 M glycine hydrochloride, pH 1.7) was refolded by 40-fold dilution to final conditions of 0.1 M NaOAc/acetic acid and 0.15 M GdmCl, pH 5.0, at 10 °C in the CD cuvette. The resulting slow refolding kinetics were measured at various wavelengths and extrapolated back to zero time of refolding, in order to obtain the CD values of the intermediate structure which is formed rapidly before the onset of the slow refolding reactions. The protein concentration was 95 μM. For the experiments 1-mm thermostated cuvettes were used.

secondary structure depends on ionizable groups that titrate below pH 3 and above pH 7, respectively. The decrease in intermediate formation at high pH is paralleled by a reciprocal increase in the CD changes that occur during the two slow, rate-limiting phases of folding.

The Intermediates Show a Native-like CD Spectrum. To get information about the secondary structure of the rapidly formed intermediates, their CD spectrum was reconstructed by monitoring refolding at pH 5.0, 10 °C, at different wavelengths in the far-UV region. The slow phases of the refolding kinetics were then extrapolated back to time zero to obtain the CD signal of the rapidly formed folding intermediates at the individual wavelengths [cf. Kuwajima et al. (1985)]. The "CD spectrum" determined in such a way is shown in Figure 4 in comparison to the CD spectrum of completely refolded RNase T_1 under the same conditions. In their overall shape both spectra are very similar, suggesting that the intermediates already contain a major fraction or all of the secondary structural elements of native RNase T_1. This holds in particular for the extended amino-terminal α-helix, which dominates the CD spectrum of this protein. As discussed above, the deviations of the intermediates' spectrum from the native CD in the 220–235-nm region (indicated by the open dotted curve in Figure 4) probably originate from positive contributions of the numerous aromatic residues to the spectrum of native RNase T_1. These residues are presumably still in a less ordered environment in the intermediates.

The Intermediate Phase of Refolding Is a Complex Reaction. The slow refolding of RNase T_1 shows two properties that are difficult to explain. (i) The relative amplitudes for the two slow refolding reactions depend strongly on the probe used to monitor folding. The assays for the formation of native

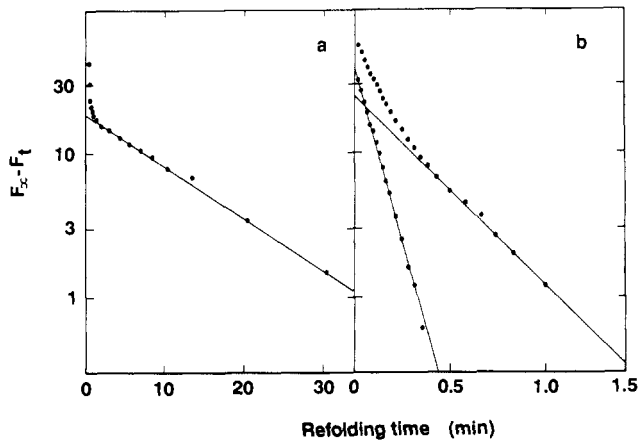


FIGURE 5: Refolding kinetics of RNase T_1 (6.8 μM) in the presence of 1.0 μM prolyl isomerase at pH 8.0 and 10 °C. (a) Semilog plot of the refolding kinetics; τ_1 = 720 s was obtained by extrapolation of the terminal phase to zero time as indicated. (b) Replot of the fast components of panel a. The drawn lines yield the time constants τ_2 = 21 s and τ_3 = 5.5 s. RNase T_1 was unfolded in 8.0 M urea and 0.1 M Tris-HCl, pH 8.0. Refolding was initiated by a 40-fold dilution to final conditions of 0.1 M Tris-HCl and 0.2 M urea, pH 8.0, at 10 °C in a 1-cm fluorimeter cuvette. The refolding solution additionally contained 1.0 μM prolyl isomerase. Refolding was monitored by the change in fluorescence at 320 nm after excitation at 268 nm. The curves were analyzed as the sum of three exponentials by "peeling off" exponentials.

RNase T_1 during folding show that about 70% of all molecules refold on the slowest pathway, and only 25% use the intermediate pathway. In contrast, most of the fluorescence and absorbance changes occur in the course of this intermediate phase of folding. (ii) Formation of native RNase T_1 (Kiefhaber et al., 1990) as well as formation of an intermediate (Figure 2) occurs in the time region of the intermediate phase, indicating that this refolding phase might actually be composed of several reactions with similar time constants. To test such a possibility, we added prolyl isomerase to the refolding solution to increase selectively the rate of reactions which are limited in rate by the isomerization of prolyl peptide bonds. We had noted before that in the presence of prolyl isomerase the intermediate phase is accelerated significantly, but it was difficult to resolve as a single exponential (Fischer et al., 1989). Figure 5 shows the time course of slow refolding of RNase T_1 in the presence of 1.0 μM prolyl isomerase. Slow folding under the same conditions, but in the absence of isomerase, is governed by two phases with time constants τ_1 = 3400 s (amplitude = 0.20) and τ_2 = 500 s (amplitude = 0.46). The accelerated refolding reaction (Figure 5) cannot be described as the sum of only two exponentials. As shown by the semilog plots in Figure 5 the reaction appears to be triphasic with time constants of τ_1 = 720 s (amplitude = 0.08), τ_2 = 21 s (amplitude = 0.11), and τ_3 = 6 s (amplitude = 0.16). This analysis of the folding kinetics indicates that all slow processes in RNase T_1 folding are accelerated in the presence of prolyl isomerase and hence should involve proline isomerization. The intermediate phase appears indeed to be a mixture of at least two processes which are catalyzed by the isomerase with different efficiencies. The relative amplitude of the slowest phase of folding is decreased in the presence of prolyl isomerase. The finding of three kinetic phases that are catalyzed by prolyl isomerase does not necessarily mean that three different proline residues are involved in these reactions. A single incorrect proline residue can give rise to two different kinetic phases when it reisomerizes in two different structural environments (created, for instance, by a second proline that exists in two isomeric states as well).

Prolyl Isomerase Affects the Choice of Folding Pathway for the Slow-Folding Molecules. The results in Figure 5 suggest that the presence of prolyl isomerase changes the amplitudes of the slow refolding reactions. However, spectroscopic techniques, such as fluorescence, cannot be used for an unambiguous determination of the number of molecules that refold on a particular pathway, because part of the spectral properties can change in early folding steps. The formation of native protein on the individual pathways can be measured quantitatively by unfolding assays which detect only the formation of completely folded protein (Schmid, 1983; Kiefhaber et al., 1990). We have used this technique here to investigate the effect of increasing concentrations of prolyl isomerase on the distribution of refolding RNase T_1 on the different refolding pathways. The experiments in presence of prolyl isomerase were carried out at pH 8.0 at a residual concentration of 0.2 M urea. These are conditions where prolyl isomerase shows a high activity. The results (Figure 6) show that native protein is formed on three major pathways. In addition to the small amount of 3.5% U_F molecules there are two slow pathways similar to the results obtained for refolding at pH 5.0 (Kiefhaber et al., 1990). In the absence of prolyl isomerase, 66% of all molecules refold on the very slow pathway (τ = 3400 s) and 31% on the intermediate pathway. This pathway is best approximated by a sequential reaction involving two steps with time constants of 190 s and 500 s, respectively. The addition of increasing concentrations of prolyl isomerase (Figure 6b,c) leads to an acceleration of both pathways. The intermediate pathway is catalyzed very efficiently: in the presence of 1.0 μM prolyl isomerase the native state is regained completely within 30 s. This suggests that both sequential slow steps that are apparent in the absence of prolyl isomerase (Figure 6a) involve proline isomerization. The very slow phase is accelerated as well, albeit to a lesser extent (cf. also Figure 5).

In addition to the acceleration of both folding pathways, the presence of prolyl isomerase leads to a pronounced redistribution of molecules from the very slow to the intermediate pathway. In the absence of the isomerase 66% of all RNase T_1 molecules refold on the very slow pathway. This number is decreased to 35% in the presence of 1 μM prolyl isomerase. Concomitantly, the amount of molecules that refold on the intermediate pathway increases from 31% to 62%. The simplest explanation for this shift involves the molecules with two incorrect proline isomers. These molecules can enter either the very slow or the intermediate pathway, depending on which proline reisomerizes first; i.e., the distribution on the two pathways is determined in a competition reaction by the relative rates of the two isomerization reactions at the stage of the rapidly formed early intermediate. The catalysis by prolyl isomerase of the reaction that feeds into the intermediate pathway is apparently more efficient, thus leading to the observed increase in the amplitude of this refolding reaction in the presence of 1 μM prolyl isomerase.

Discussion

Kinetic Models for Unfolding and Refolding of RNase T_1. The experimental results on the folding of RNase T_1 from this and the preceding paper are used here to propose kinetic models for the unfolding mechanism of this protein and for the refolding on individual kinetic pathways. These refolding pathways are then tentatively integrated into an overall mechanism for refolding, and the molecular basis of crucial steps of folding is discussed.

Model for Unfolding. Unfolded RNase T_1 consists of a mixture of fast and slow refolding species. There is a small

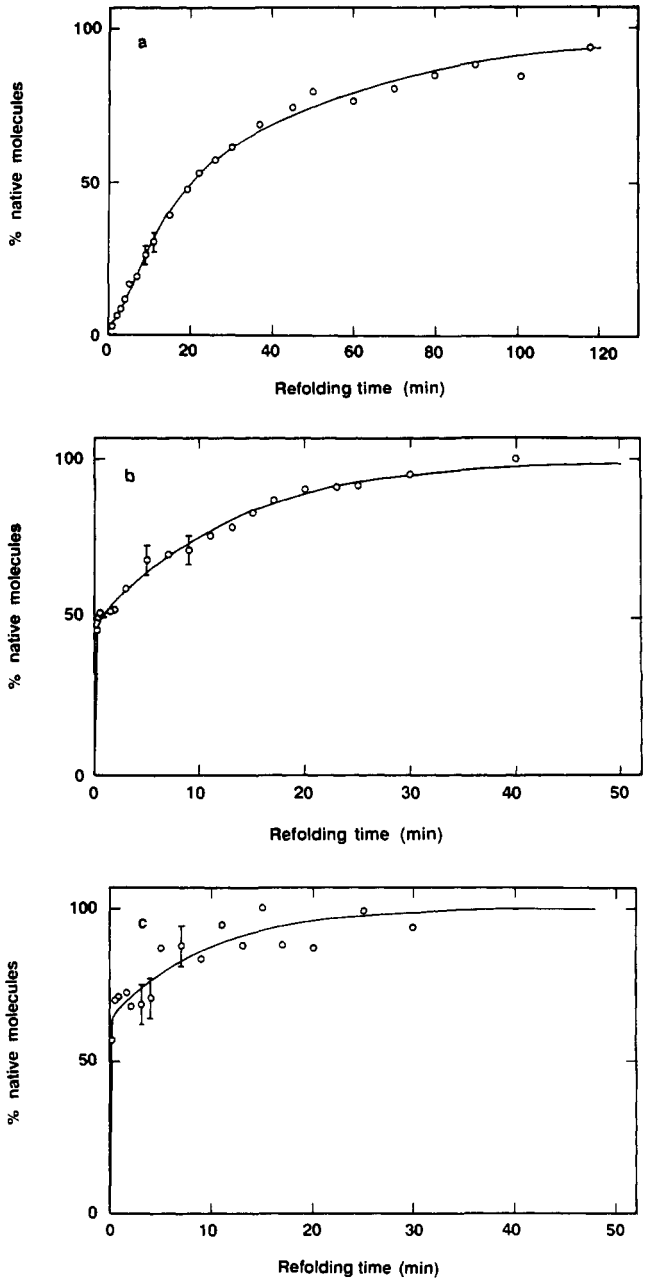
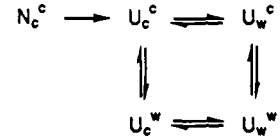


FIGURE 6: Time course of formation of native RNase T_1 at pH 8.0 and 10 °C in the presence of 0 μM (a), 0.7 μM (b), and 1.0 μM (c) prolyl isomerase. The concentrations of RNase T_1 were 7.5, 6.8, and 6.3 μM, respectively. The percentage of native molecules after various refolding times was measured by unfolding assays as described under Materials and Methods. Refolding conditions were 0.1 M Tris-HCl and 0.15 M GdmCl (a) or 0.2 M urea (b and c), pH 8.0, and 10 °C. Theoretical curves (solid lines) were calculated on the basis of Scheme II with the following time constants and amplitudes: (a) τ_1 = 3000 s, τ_2 = 500 s, τ_3 = 100 s, τ_4 = 190 s, A_1 = 66%, A_2 = 31%; (b) τ_1 = 780 s, τ_2 = 2 s, τ_3 = 27 s, τ_4 = 5 s, A_1 = 51%, A_2 = 46%; (c) τ_1 = 550 s, τ_2 = 2 s, τ_3 = 20 s, τ_4 = 4 s, A_1 = 35%, A_2 = 62%. A_1 and A_2 refer to the relative amounts of molecules refolding via the "upper" and the "lower" pathways, respectively, of Scheme II. For all calculations 3% fast refolding molecules were assumed.

amount of 3.5% U_F molecules, which, after transfer to folding conditions, regain the native state within the dead time of manual mixing. The existence of this species was demonstrated by unfolding assays for native RNase T_1, carried out after short times of refolding (Kiefhaber et al., 1990). The slow part of the refolding kinetics occurs in the time range of minutes to hours on two major parallel pathways, which originate from different forms of the unfolded protein. An alternative sequential model, where the two slow phases represent consecutive steps on a single folding pathway, could be ruled out. The species that give rise to these two slow folding reactions are produced by slow isomerizations of the unfolded polypeptide chains, which are very similar, if not identical, in rate. The formation of one U_S species is characterized by an initial lag phase. The equilibrium distribution of the various unfolded molecules appears to be independent of the pH and of the denaturant used to induce unfolding. The kinetics of formation of U_S are governed by an experimental activation energy of 88 kJ/mol.

Scheme I: Kinetic Model To Explain the Isomerization Reactions of Unfolded RNase T_1[a]



[a] At equilibrium 3.5% of all molecules are in the U_c^c (=U_F) state. The apparent time constants for the vertical and the horizontal isomerization are in the range of about 250 s at 10 °C and 1000 s at 0 °C.

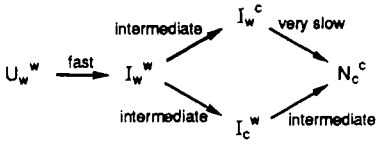
These properties of the unfolded state can be explained by a model in which the kinetic heterogeneity of the folding of RNase T_1 originates from the presence of incorrect proline isomers in the slow-folding (U_S) species. The existence of more than one pathway for slow refolding suggests that the isomerizations of at least two proline residues are involved. Such a model requires that at least three different U_S species exist, two with either one proline in the nonnative state and one with both prolines incorrect. A model to explain the unfolding of RNase T_1 and the independent isomerizations of the unfolded chain on the basis of the proline hypothesis is presented in Scheme I. Analogous models have been proposed to explain the unfolding of pancreatic RNases from different species (Lin & Brandts, 1983; Schmid et al., 1984; Grafl et al., 1986). According to the model in Scheme I we assume that after the rapid $N \rightarrow U_F$ unfolding reaction two proline isomerizations occur independently of each other in the unfolded protein chain. As we do not know which proline residues are responsible for these reactions, we simply denote the two isomeric states as c (=correct, as in the native protein) and w (=wrong, the nonnative proline isomer). The subscript and superscript refer to the states of the two involved prolines, respectively. As an example, U_c^w stands for a species with the first proline in the correct state and the second one in the incorrect state. U_c^c with two correct isomers is equivalent to the fast-folding U_F species. At equilibrium we find only 3.5% U_c^c molecules. This suggests that both isomerizations of the unfolded protein favor the incorrect isomer and hence lead to a predominance of U_S molecules. The relative amounts of the individual U_S species cannot be measured by refolding assays, because the molecules with two incorrect isomers, U_w^w, can fold on alternative pathways, depending on the relative rates of reisomerization during refolding (see below). If the two isomerizations were governed by the same equilibrium constant, then a fraction of 0.035 for U_c^c would be observed for a common c:w ratio of 0.19:0.81 at the two proline peptide bonds. Such distributions are frequently observed for cis/trans equilibria in proline-containing oligopeptides. At present, we do not know whether the two cis proline residues in RNase T_1 (which are expected to be largely trans in the unfolded chains) are indeed responsible for the formation of the different U_S species. One of the two isomerizations shows a time constant of 250 s (at 10 °C), as measured by the double jump refolding assays (Kiefhaber et al., 1990). The other isomerization is probably slightly faster. Its rate is reflected in the

lag phase which was observed in the formation of a fraction of the U_w^w molecules. The actual unfolding mechanism may be more complex since isomerizations of the two trans prolines may cause minor slow reactions as well.

Kinetic Models for the Individual Refolding Reactions. The kinetic model for unfolding and isomerization in Scheme I suggests that one fast- and three slow-folding species are present in unfolded RNase T_1. The first model to consider for the refolding of the slow-folding U_S species would be a simple reversal of the unfolding model (Scheme I), where the slow reisomerizations, w → c, of the incorrect proline isomers in the unfolded protein chains constitute slow, rate-limiting steps, which are followed by rapid chain folding in the subsequent $U_c^c \rightarrow N$ step. Such a simple mechanism of initial reisomerization, w → c, followed by rapid chain folding is clearly ruled out by the experimental data on the slow refolding of RNase T_1. In the course of refolding of the U_S species rapid changes in the spectral properties are observed, which can occur within the dead time of the experiments, much faster than the rate-limiting slow steps of refolding. This is most clearly demonstrated by the CD-detected kinetics. Within a few seconds after transfer to native conditions, the slow refolding molecules regain a CD spectrum in the amide region which is already very similar to the CD spectrum of the native protein (Figure 4). The simplest explanation for this finding is that all slow-folding species are able to form extensive native-like secondary structure early in refolding, well in advance of the slow reisomerizations of the incorrect proline isomers. These intermediates with secondary structure but with incorrect proline isomers are sensitive to the folding conditions: they are formed only under conditions where folded proteins show a high stability ("strongly native" conditions). In the presence of increased residual concentrations of denaturant rapid structure formation is decreased. These results are easiest explained by two-step kinetic models for the folding of the individual U_S species. In the first steps partially ordered intermediates (I_w^w, I_c^w, I_w^c) are formed rapidly without a change in the isomeric state of the proline residues. In the second, slow steps the incorrect isomers are reversed to the native conformation and the fully folded state of RNase T_1 is achieved. These models for the individual U_S species are shown in eqs 1–3.

$$U_c^w \xrightarrow{\text{fast}} I_c^w \xrightarrow{\text{intermediate}} N_c^c \qquad (1)$$

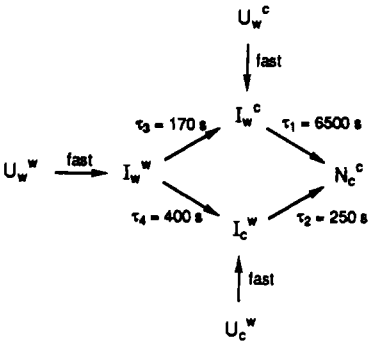
$$U_w^c \xrightarrow{\text{fast}} I_w^c \xrightarrow{\text{very slow}} N_c^c \qquad (2)$$



(3)

Refolding of the two U_S species with only one incorrect isomer (U_c^w and U_w^c) is thus a sequential two-step process (eqs 1 and 2). The refolding mechanism for the U_S species with two incorrect isomers (U_w^w) is more complex (eq 3). We assume that, as for the other U_S species, initial structure formation is possible, leading to the rapid formation of a partially ordered intermediate with two incorrect isomers, I_w^w. This species can refold further on two alternative pathways, depending on which of the two incorrect prolines isomerizes first. The resulting products are either the intermediate I_c^w as in the pathway of eq 1 or alternatively the intermediate I_w^c as in eq 2. Reisomerization of the other incorrect proline then leads to the final native state. The choice between the two alternative pathways of the model in eq 3 should depend on

Scheme II: Kinetic Model for the Slow Refolding Reactions of RNase T_1 under Strongly Native Conditions[a]



[a] U stands for unfolded species, I for intermediates of refolding, and N for the native protein. The subscript and the superscript indicate the two involved proline peptide bonds in the correct (c) and the wrong (w) isomeric state, respectively. The time constants given for the individual steps refer to folding conditions of 0.15 M GdmCl, pH 5.0, 10 °C.

the relative rates of reisomerization of the incorrect prolines in the intermediate I_w^w. These rates need not necessarily be identical with the corresponding rates in the unfolded protein because prefolding (in the $U_w^w \rightarrow I_w^w$ step) can lead to changes in the kinetic properties of proline isomerization. This kinetic competition model for the folding of the U_w^w molecules (eq 3) explains the experimental observation that the relative amplitudes of the two slow refolding reactions are changed in the presence of prolyl isomerase. The progressive shift from the upper, very slow pathway (eq 3) to the lower, intermediate pathway in the presence of increasing amounts of prolyl isomerase indicates that at the stage of I_w^w the "lower" w → c isomerization ($I_w^w \rightarrow I_c^w$) is catalyzed more efficiently than the "upper" isomerization ($I_w^w \rightarrow I_w^c$). This difference could originate either from the different sequence context of the two prolines or else from differences in steric accessibility of the two prolines. Accessibility is presumably a major factor for the efficiency of catalysis. This is revealed by a comparison of the two processes $I_w^w \rightarrow I_c^w$ (lower branch, eq 3) and $I_w^c \rightarrow N_c^c$ (upper branch), which are both limited by the same proline isomerization. In the presence of 1 μM prolyl isomerase, $I_w^w \rightarrow I_c^w$ is accelerated from τ = 200 s to τ = 4 s (i.e., about 50-fold), whereas $I_w^c \rightarrow N_c^c$ is accelerated from τ = 3000 s to τ = 550 s (i.e., about 5-fold). The simplest explanation for this difference is that the intermediate with only one incorrect proline isomer (I_w^c) is more extensively folded than the intermediate with two incorrect isomers (I_w^w) and therefore the accessibility for prolyl isomerase is decreased.

Integrated Kinetic Model for the Slow Refolding Reactions. An integration of the folding pathways for the three slow refolding species (eqs 1–3) into an overall kinetic mechanism is presented in Scheme II. It resembles basically the refolding mechanism given for the species with two incorrect isomers, U_w^w (eq 3). The two reactions from the unfolded forms with a single incorrect isomer, U_w^c and U_c^w, to the two partially folded intermediates, I_w^c and I_c^w, respectively (cf. eqs 1 and 2), are included as well. U_w^c and U_c^w enter the branched pathway (eq 3) at these intermediates. An important feature of this model is the formation on all pathways of partially folded molecules, I_i^i, with incorrect isomers. These rapid processes lead to the fast regain of an essentially native-like CD spectrum of RNase T_1 within the dead time of refolding (cf. Figure 4). At the same time up to 50% of the changes in absorbance and fluorescence (depending on the folding conditions) occur in this rapid phase. This indicates that, in addition to a high amount of secondary structure, these intermediates already contain significant tertiary structure which

leads to a partial shielding of aromatic residues from the aqueous solvent. The steps in Scheme II that are coupled with proline isomerization are slow. The proposed folding mechanism (Scheme II) explains why only two slow refolding phases are observed, although three unfolded species are involved: it is caused by the competition reaction at the intermediate I_w^w and the merging of the pathways at the intermediates I_w^c and I_c^w.

It was found experimentally that native RNase T_1 is formed on two slow pathways. In the absence of prolyl isomerase 72% of the N molecules are formed by a very slow process with a time constant of 6500 s (at pH 5, 10 °C). We assign this reaction to the $I_w^c \rightarrow N$ step in the upper branch of the model (Scheme II). It is extremely slow, and therefore no kinetic coupling is observed with the reactions that produce I_w^c from U_w^c and from I_w^w, respectively. Since most of the unfolded molecules use the upper refolding pathway and since the final $I_w^c \rightarrow N$ step is very slow, the intermediate I_w^c is strongly populated during refolding. This is shown by the results in Figure 2. I_w^c is formed by two reactions, a fast one which largely represents the $U_w^c \rightarrow I_w^c$ step and a slow one (τ = 170 s), which is limited by the $I_w^w \rightarrow I_w^c$ reaction. This process is slow because it involves proline isomerization. The decay of the intermediates is determined by the time constants of the $I_w^c \rightarrow N$ reaction (τ = 6500 s), which is limited in rate by the strongly decreased isomerization rate of the second proline.

The remaining 25% N molecules are formed more rapidly. The time course of their production is best approximated by a two-step sequential process with time constants of 400 s and of 250 s, respectively (cf. Figure 6). We assign these two steps to the lower branch of the model, i.e., to the $I_w^w \rightarrow I_c^w$ and the $I_c^w \rightarrow N$ reactions. The rapid formation of a fraction of the I_c^w molecules by the $U_c^w \rightarrow I_c^w$ path is not evident in these results. However, its contribution to the lower folding pathway may be fairly small. Unlike the upper pathway, the lower pathway consists of two steps that are similar in rate. Therefore, the formation of native RNase T_1 on this branch is characterized by an initial lag (cf. Figure 6). Both sequential steps are limited by proline isomerization. This is clearly demonstrated by their increased rates in the presence of prolyl isomerase.

The refolding model in Scheme II explains why the intermediate folding phase shows a large and variable amplitude in absorbance and fluorescence. The time constants of three steps in Scheme II ($I_w^w \rightarrow I_w^c$, τ = 170 s; $I_w^w \rightarrow I_c^w$, τ = 400 s; $I_c^w \rightarrow N_c^c$, τ = 250 s) are very similar, and therefore, all three reactions contribute to the intermediate phase. The relative amplitudes are variable, because the structure and the stability of the different intermediates strongly depend on the folding conditions. Under "unfavorable" conditions, for instance, I_w^c is more "unfolded-like" by fluorescence and absorbance. Consequently, the amplitude of the very slow $I_w^c \rightarrow N_c^c$ step is increased at the expense of the preceding $I_w^w \rightarrow I_w^c$ step. The composite nature of the intermediate phase is confirmed by the results in the presence of prolyl isomerase, which improves the resolution between the two competing reactions at the stage of the intermediate I_w^w (cf. Figure 5).

Some of the intermediates in the kinetic model (Scheme II) are catalytically active. This is indicated by the experimental finding that, at pH 5, 10 °C, 25% of the enzymatic activity returns within the dead time of the refolding experiment (about 20 s). Since only a small amount of this activity originates from the fast folding reaction (3.5%), the remaining activity (more than 20%) has to be assigned to one or more of the intermediates in Scheme II. At present, we do not know whether all or only part of these intermediates contribute to this rapid formation of enzymatic activity. When refolding of RNase T_1 is carried out at pH 8 (where secondary structure formation is slow) instead of pH 5, only about 11% of the activity is regained early in slow refolding.

A remarkable feature emerges from the proposed mechanism for the slow refolding of RNase T_1 in Scheme II. The overall rate of refolding of U_S molecules with two wrong proline isomers (the U_w^w species) depends strongly on the order of reisomerization of the incorrect prolyl peptide bonds. If the "upper" proline isomerizes first, then an intermediate is formed (I_w^c) which strongly decelerates the second isomerization into the 6500-s range (at 10 °C). Apparently, this incorrect isomer is "locked" into the wrong conformation by the ordered structure of I_w^c. However, when this proline isomerizes first (in the $I_w^w \rightarrow I_c^w$ step of the lower pathway), then refolding is much faster and both proline isomerizations occur in the time range of 200–400 s. Previously it was found that the formation of ordered structure in a folded intermediate can lead to an increase in the reisomerization rate of an incorrect proline peptide bond (Schmid & Blaschek, 1981). The results on RNase T_1 suggest that the opposite can occur as well: rapid structure formation can lead to a strong decrease in the reisomerization rate; i.e., the nonnative proline isomer is "trapped" by structure formation.

Concluding Remarks. Two proline residues appear to be important for the unfolding and refolding kinetics of RNase T_1. The respective cis/trans isomerizations (i) lead to the formation of slow-folding species in the unfolded protein and (ii) constitute rate-limiting steps of the slow refolding reactions. In refolding, proline reisomerization is not the first step, but it is preceded by formation of secondary and also some tertiary structure. The early formation of structure modifies the kinetics of proline isomerization. This is reflected in a dependence on the folding conditions of both slow phases. The unusual increase in rate of the very slow process with GdmCl suggests that this particular reisomerization is actually hindered by the formation of ordered structure. This structure is labile: it is only observed under "strongly native" folding conditions.

Slow refolding occurs basically on two parallel pathways. They originate in part from the two unfolded species with one incorrect proline isomer each. The major unfolded species has two incorrect isomers and can enter either one of the two pathways depending on which of the incorrect prolines isomerizes first.

The rapidly formed intermediate states all resemble native RNase T_1 in the amide CD spectrum. This indicates that they already show extensive secondary structure. In particular, the long amino-terminal α-helix is likely to be formed early in refolding, because the CD in the far-UV region is dominated by the strong contribution of this helix. The helix can apparently be formed irrespective of the isomeric state of the proline residues. The cis proline residues of native RNase T_1 are not located near the helix. Whether early helix formation is a key step for the folding pathways of RNase T_1 remains to be determined. Less is known about the extent and time course of β-sheet formation, because it is difficult to sort out the weak CD signals of the β-sheet. This holds in particular for proteins, such as RNase T_1, with a high content of aromatic residues, which contribute to the CD in the far-UV region. The rapid generation of partial enzymatic activity during refolding suggests that at least a fraction of the early intermediates should have correct β-sheet structure because the

active site of RNase T_1 resides within the β-sheet region of this protein. The results on RNase T_1 support folding models, such as the framework model (Kim & Baldwin, 1982), which assume that the secondary and presumably part of the tertiary structure are formed early in folding (Schmid & Baldwin, 1979; Kuwajima et al., 1985; Udgaonkar & Baldwin, 1988; Roder et al., 1988). Such intermediates are sometimes referred to as "molten globular" states (Kuwajima, 1989).

A novel folding mechanism emerges from the studies on RNase T_1. Unfolded molecules with two incorrect isomers can enter two alternative folding pathways depending on which proline reisomerizes first. When the "upper" proline reverts back to the native state first, then coupled structure formation or stabilization leads to a "locking-in" of the remaining incorrect "lower" proline isomers, which then can regain the native state only in an extremely slow reaction (cf. Scheme II). This indicates that rapid structure formation can actually lead to a deceleration of folding. Consequently, "unfavorable" conditions, such as an increase in GdmCl concentration, lead to an acceleration of such folding reactions, as was observed for the very slow folding phase.

Several lines of indirect evidence (such as the large fraction of U_S molecules) suggest that isomerizations of the two cis proline residues (at positions 39 and 55) are involved into the slow folding of RNase T_1. Pro55 is part of a solvent-exposed loop structure in the native protein; Pro39 is near the active site. Its neighboring residues Tyr38 and His40 are in contact with the inhibitor 2′GMP, bound at the catalytic center of the protein. Both the main-chain and the side-chain mobilities (as indicated by the crystallographic temperature factors) of Pro39 are much smaller than those of Pro55 (Arni et al., 1988). It appears likely that, because of these different properties, incorrect trans isomers of Pro39 and of Pro55 influence protein folding in a different way. It is conceivable that Pro39, when locked into a prefolded structure in the incorrect trans state, can lead to the observed strong deceleration of folding. Decisive answers about the role of the individual proline residues for the folding of RNase T_1 should be provided by the study of mutant proteins with alterations at proline positions.

Acknowledgments

We thank U. Heinemann, H.-J. Hinz, S. Huber, R. Jaenicke, H. H. Kohler, W. Saenger, and R. Schönbrunner for fruitful discussions.

Registry No. RNase T_1, 9026-12-4; Pro, 147-85-3.

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