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Folding of Ribonuclease T_1. 1. Existence of Multiple Unfolded States Created by Proline Isomerization†

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ABSTRACT: It is our aim to elucidate molecular aspects of the mechanism of protein folding. We use ribonuclease T_1 as a model protein, because it is a small single-domain protein with a well-defined secondary and tertiary structure, which is stable in the presence and absence of disulfide bonds. Also, an efficient mutagenesis system is available to produce protein molecules with defined sequence variations. Here we present a preliminary characterization of the folding kinetics of ribonuclease T_1. Its unfolding and refolding reactions are reversible, which is shown by the quantitative recovery of the catalytic activity after an unfolding/refolding cycle. Refolding is a complex process, where native protein is formed on three distinguishable pathways. There are 3.5% fast-folding molecules, which refold within the millisecond time range, and 96.5% slow-folding species, which regain the native state in the time range of minutes to hours. These slow-folding molecules give rise to two major, parallel refolding reactions. The mixture of fast- and slow-folding molecules is produced slowly after unfolding by chain equilibration reactions that show properties of proline isomerization. We conclude that part of the kinetic complexity of RNase T_1 folding can be explained on the basis of the proline model for protein folding. This is supported by the finding that the slow refolding reactions of this protein are accelerated in the presence of the enzyme prolyl isomerase. However, several properties of ribonuclease T_1 refolding, such as the dependence of the relative amplitudes on the probes, used to follow folding, are not readily explained by a simple proline model.

The elucidation of the molecular mechanism of protein folding from a disordered polypeptide chain to the specific native state remains to be one of the major challenges in biochemistry. The information for the native three-dimensional structure and for the folding pathway to this state is apparently encoded in the amino acid sequence of the protein chain. In order to improve our understanding of protein folding and stability, it will be necessary to combine results from theoretical and statistical studies, from protein structure determinations as well as from experiments on the stability and the folding kinetics. Studies of the kinetics of protein folding aim at the characterization of rate-limiting events on the folding pathway, the detection of partially folded intermediate states, and the elucidation of their relevance for the folding mechanism. Other aspects of the protein folding problem are the role of individual segments of the polypeptide chain for the folding pathway and the formation of secondary structural elements early in folding. Experimental evidence obtained for several small proteins points to an ordered folding mechanism, where secondary structure can indeed be formed rapidly at an early stage. This is followed by slow steps in which ultimately the compact native state is reached (Kim & Baldwin, 1982; Jaenicke, 1987). Similar to the stability of the native protein, the folding mechanism may depend strongly on the solvent conditions chosen for folding. For some proteins, isomerizations of incorrect X–Pro peptide bonds were suggested as rate-limiting

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slow steps for refolding (Brandts et al., 1975; Schmid & Baldwin, 1978; Nall, 1985; Lang et al., 1987).

A good model protein for folding studies should have several features that are important for the design and the meaningful interpretation of folding experiments. (i) Unfolding should be reversible under a wide variety of conditions, in order to avoid complication of kinetics by side reactions such as aggregation. (ii) The amino acid sequence and the three-dimensional structure of the protein itself and of related or homologous proteins should be known. (iii) In order to test specific predictions about the role of individual amino acid residues or of defined chain segments for folding, the gene of the protein should be available for the production of variants by site-directed mutagenesis.

In our studies we use the Lys25 isoenzyme of ribonuclease T_1 (RNase T_1)[1] from *Aspergillus oryzae* (Takahashi & Moore, 1982; Heinemann & Hahn, 1989) as a model to investigate the molecular mechanism of protein folding. It is a small single-domain protein with 104 amino acid residues, and its unfolding transition is reversible. The amino acid sequence of RNase T_1 and those of a number of other, homologous RNases of microbial origin are known (Hill et al., 1983), and the three-dimensional structures of several complexes of RNase T_1 with nucleotides have been solved at high resolution (Heinemann & Saenger, 1982, 1983; Arni et al., 1987, 1988; Koepke et al., 1989; Sugio et al., 1988). The secondary structure of RNase T_1 consists of one extended α-helix of 4.5 turns and two antiparallel β-sheets, which are composed of two (β_1–β_2) and of five (β_3–β_7) strands, respectively. The elements of secondary structure are connected by extended "loop" regions. The protein chain is held together by two disulfide bonds which form a small (2–10) and a large (6–103) covalently linked loop. RNase T_1 contains four proline peptide bonds, two of which (Trp59–Pro60 and Ser72–Pro73) are trans and two of which (Tyr38–Pro39 and Ser54–Pro55) are cis in the native protein. X-ray structures of several related RNases are known as well (Hill et al., 1983). The gene for RNase T_1 was chemically synthesized and introduced into an *Escherichia coli* expression/secretion system. The protein can be isolated from the *E. coli* periplasm in good yield (Quaas et al., 1988a,b).

Equilibrium unfolding transitions of RNase T_1 are reversible under a wide variety of conditions. Remarkable features of this protein are its very low isoelectric point and its strong stabilization in the presence of increasing concentrations of NaCl (Oobatake et al., 1979; Pace & Grimsley, 1988). Unfolded protein with reduced disulfides spontaneously reoxidizes to the native state under suitable conditions (Pace & Creighton, 1986). Reduced or reduced and carboxymethylated RNase T_1 still shows ordered structure and enzymatic activity under favorable conditions such as low temperature and high concentrations of NaCl (Pace et al., 1988).

The kinetic mechanism of folding of RNase T_1 has not yet been investigated. Preliminary data indicate that refolding is dominated by slow kinetic phases, which depend on the final urea concentration (Thomson et al., 1989). It is our aim here to elucidate the molecular nature of the rate-limiting steps of unfolding and refolding of wild-type RNase T_1. In our experiments we first search for suitable conditions to unfold and refold the protein. Then we ask whether folding occurs on a sequential pathway or, alternatively, whether the apparent complexity of folding originates from the presence of multiple unfolded species, giving rise to fast and slow phases in refolding. Also, tests are presented to examine whether proline isomerization plays an important role in the folding mechanism of this protein. The results should form a basis for asking specific questions about the molecular processes that underlie the kinetic mechanism. These questions should then be approached by employing mutants of the protein with amino acid substitutions at predetermined sites.

Materials and Methods

Materials. RNase T_1 was purified from *E. coli* cells, transformed with a plasmid carrying a chemically synthesized gene, which was cloned and expressed in *E. coli*, as described elsewhere (Quaas et al., 1988a,b). GpC was purchased from Sigma, St. Louis, MO. GdmCl and urea, both ultrapure, were from Schwarz/Mann, Orangeburg, NY. Sodium cacodylate was from Serva, Heidelberg, West Germany. All other substances were purchased from Merck, Darmstadt, West Germany.

Methods. The concentration of RNase T_1 was calculated by using an absorption of 1.9 at 278 nm for a 1 mg/mL solution (Takahashi et al. 1970). For optical measurements a Jasco J-500A spectropolarimeter, a Perkin-Elmer Lambda 5 spectrophotometer, a Perkin-Elmer LS-5B fluorimeter, and a Hitachi F-4010 fluorimeter with integrated magnetic stirrer were used. All spectrometers had thermostatable cell holders.

GdmCl-Induced Unfolding Curves. Native RNase T_1 was incubated in the presence of 0.1 M NaOAc/acetic acid, pH 5.0, and various concentrations of GdmCl (0–6.5 M at 25 °C and 0–7.0 M at 10 °C) until the equilibrium was reached (24–120 h, depending on temperature). Unfolding was detected by fluorescence at 320 nm (after excitation at 268 nm), absorbance at 287 nm, and CD at 222 nm. Protein concentrations were 1.4 μM in the fluorescence measurements and 12.6 μM in the absorbance and CD measurements. Absorbance and fluorescence were measured in 10-mm cells; CD was measured in 1-mm cells.

In the transition region the equilibrium constant (K_D) for the unfolding reaction was calculated by using

$$K_D = f(D)/f(N) \quad (1)$$

where $f(D)$ represents the fraction of unfolded protein and $f(N)$ is the fraction of native protein, where $f(D) + f(N) = 1$. The equilibrium constant in pure solvent was obtained by linear extrapolation of ln K_D to 0 M GdmCl (Schellman, 1978; Pace, 1986). This constant was used to calculate the free energy of unfolding, ΔG_D.

Activity Assay for RNase T_1. The activity of RNase T_1 was measured by using the cleavage of the dinucleotide GpC. RNase T_1 was diluted 40-fold into a GpC solution (in 10 mM Tris-HCl and 2 mM EDTA, pH 7.8) at 10 °C. The concentration of GpC was adjusted such that the absorbance at 257 nm was 0.8 in a 10-mm cell. The resulting increase in absorbance at 257 nm caused by the cleavage of GpC by RNase T_1 was recorded for 5 min. The value of ΔA_{257}/min was used as a measure for the enzymatic activity of RNase T_1. The activity increases linearly with increasing protein concentration up to a value of about 5 μM RNase T_1.

Unfolding Kinetics. Unfolding was initiated by a 40-fold dilution of native RNase T_1 to a final concentration of 5.9 M GdmCl at 10 °C and various pH values. The following buffers (each 0.1 M) were employed: glycine hydrochloride (pH 1.5 to 3); NaOAc/acetic acid (pH 4 and 5); sodium cacodylate (pH 6 and 7); Tris-HCl (pH 8). The protein concentration was 0.37 μM. Unfolding was monitored by the decrease in

[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.

fluorescence at 320 nm after excitation at 268 nm.

Reactivation Kinetics. Unfolded RNase T_1 (in 8.0 M urea and 0.1 M Tris-HCl, pH 8.0) was refolded by a 40-fold dilution with 0.1 M Tris-HCl, pH 8.0, at 10 °C. After various times of refolding 20-μl samples were withdrawn and diluted 40-fold into a cuvette containing 0.8 mL of the GpC solution, and the activity was measured at 10 °C. In addition, 4.8 μM trypsin was present, to degrade unfolded RNase T_1 molecules and thus inhibit further folding during the activity assay. The concentration of RNase T_1 in the refolding step was 0.37 μM. Catalyzed reactivation experiments were carried out by adding 0.97 μM prolyl isomerase to the refolding buffer.

Refolding Kinetics. Unfolded RNase T_1 (in 8.0 M urea, 0.1 M Tris-HCl, pH 8.0, or in 6.0 M GdmCl and 0.1 M glycine hydrochloride, pH 1.7) was diluted 40-fold with the appropriate folding solutions in the spectrophotometer cell. In the various pH ranges the same buffers were used as described for the unfolding kinetic experiments. The time course of renaturation was detected by the increase in fluorescence at 320 nm (5-nm bandwidth) after excitation at 268 nm (2.5-nm bandwidth) or by the increase in absorbance at 287 nm. Protein concentrations were 0.37 μM for the fluorescence measurements and 5.6 μM for the absorbance measurements.

Unfolding Assays To Monitor the Formation of Native Molecules. Denatured RNase T_1 (in 5.0 M GdmCl, 67 mM glycine hydrochloride, and 33 mM NaOAc/acetic acid, pH 2.1) was diluted 30-fold into 0.1 M NaOAc/acetic acid, pH 5.0, at 10 or 25 °C to initiate refolding. After various times of refolding (t_i) samples were withdrawn and diluted 4-fold in a fluorimeter cell to final unfolding conditions of 5.25 M GdmCl, 75 mM glycine hydrochloride, and 25 mM NaOAc/acetic acid, pH 2.1, in the experiments at 10 °C and to 4.0 M GdmCl, 75 mM glycine hydrochloride, and 25 mM NaOAc/acetic acid, pH 2.4, in the experiments at 25 °C. The resulting unfolding kinetics were detected by the decrease in fluorescence at 320 nm after excitation at 268 nm. The amplitudes of unfolding (A_t) are a measure for the amount of native molecules present during refolding after t_i. Part of the protein was allowed to refold completely. The respective unfolding assay (at $t = t_\infty$) yields the maximal amplitude (A_∞), which is proportional to the total amount of native molecules. The ratio of A_t to A_∞ gives the percentage of native molecules formed after t_i. The concentration of RNase T_1 was 1.5 μM in the refolding step and 0.37 μM in the unfolding assay.

Double Jump Experiments To Monitor the Formation of the Slow Refolding Species. Native RNase T_1 was diluted 9-fold to final conditions of 6.0 M GdmCl and 0.1 M glycine hydrochloride, pH 1.5. Under these conditions RNase T_1 unfolds rapidly. After various times (t_i) samples of the unfolded protein were withdrawn and diluted 40-fold into 0.1 M NaOAc/acetic acid, pH 5.0, at 25 °C to initiate refolding. The refolding reaction was monitored by the change in fluorescence at 320 nm after excitation at 268 nm. The protein concentration was 0.37 μM in the refolding assays. The unfolding reactions were carried out at various temperatures between 0 and 25 °C.

Results

Stability of RNase T_1. The unfolding of RNase T_1 can be monitored conveniently by the decrease in absorbance of the tyrosine side chains at 287 nm (Thomson et al., 1989) and by the decrease in tryptophan fluorescence emission at 320 nm (Pace, 1986). The thermal unfolding transition as measured by absorbance at pH 5.0 shows a midpoint at 62 °C and a molar decrease in tyrosine absorption at 287 nm of 3600 M^{-1} cm^{-1}. The change in absorbance is reversible upon cooling, unless the protein is kept at temperatures above 80 °C for an extended time (≥1 h). Thermal unfolding can adequately be described by a simple two-state model, as shown by the calorimetric test (Kiefhaber et al., unpublished work). A two-state thermodynamic analysis (based on the calorimetrically determined values for ΔH and ΔC_p) gives a value of 46 kJ/mol for the Gibbs free energy of unfolding, ΔG_D, at pH 5 and 25 °C.

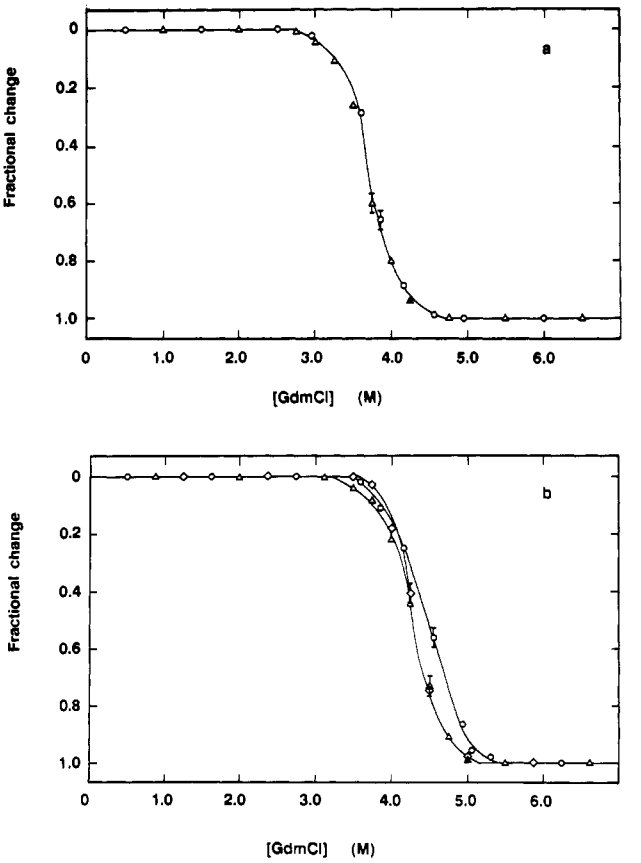

FIGURE 1: GdmCl-induced unfolding transitions of RNase T_1 in 0.1 M NaOAc/acetic acid, pH 5.0, at 25 °C (a) and 10 °C (b). Unfolding was monitored by the decrease in absorbance at 287 nm (Δ), by the decrease in fluorescence at 320 nm after excitation at 268 nm (○), and by the increase of the molar ellipticity at 222 nm (◇). The concentration of RNase T_1 was 13 μM in the absorbance and CD experiments and 1.35 μM in the fluorescence experiments. The fractional changes in the spectroscopic signals were calculated by a linear extrapolation of the base lines from the native and unfolded regions into the transition region.

The unfolding transitions induced by GdmCl at 10 °C and at 25 °C (pH 5.0) are shown in Figure 1. The transitions were measured by absorbance at 287 nm and by fluorescence at 320 nm and at 10 °C also by CD at 222 nm. At 25 °C the absorbance- and fluorescence-detected transitions coincide, as expected for a two-state process. The midpoint is at 3.67 M GdmCl (Figure 1a). The molar decrease in absorption at 287 nm upon unfolding by GdmCl is 4800 M^{-1} cm^{-1}. This value is significantly higher than the corresponding value of 3600 M^{-1} cm^{-1} found for thermal unfolding (Kiefhaber et al., unpublished work). This difference may originate from the aromatic residues being less accessible to solvent in the thermally unfolded state as compared to the GdmCl-unfolded state. Similar differences have been observed for other proteins (Tanford, 1968). The cooperativity parameter $m = d\Delta G_D/d[GdmCl]$ is −12.0 kJ/(mol·M). At 10 °C the coincidence of the individual unfolding transitions is less perfect (Figure 1b). In particular, the transition observed by far-UV CD appears to be slightly more cooperative than the fluorescence-

Table I: Time Constants for the Slow Unfolding of RNase T_1 by 5.9 M GdmCl[a]

pH	τ (s)	pH	τ (s)
1.6	5	5.0	14100
2.0	100	6.0	950
4.0	2170	8.0	250

[a] Measurements were carried out at 10 °C. Initial conditions were 13.5 μM native RNase T_1 and 0.1 M Tris-HCl, pH 8.0. Unfolding experiments were initiated by a 40-fold dilution to final conditions of 5.9 M GdmCl at the indicated pH values in the fluorimeter cell; buffers used were 0.1 M glycine hydrochloride (pH 1.6 and 2.0), 0.1 M NaOAc/acetic acid (pH 4.0 and 5.0), 0.1 M sodium cacodylate (pH 6.0), and 0.1 M Tris-HCl (pH 8.0). Unfolding was monitored by the change in fluorescence at 320 nm after excitation at 268 nm. The error limit of the time constants is about ±10%.

and absorbance-detected transitions. Consequently, different midpoints of 4.30 M GdmCl (CD), 4.35 M (absorbance), and 4.42 M (fluorescence) are found for the transitions. The same samples were used at 10 and 25 °C; therefore, the deviations observed at 10 °C should not originate from differences in GdmCl concentration. Because of the small variations in shape the cooperativity parameter m cannot be given. Linear extrapolation (Schellman, 1978; Pace, 1986) of the data at 25 °C yields ΔG_D = 44 kJ/mol in the absence of GdmCl. This value is in good agreement with ΔG_D obtained from the extrapolation of the thermal unfolding data to 25 °C.

Kinetics of Unfolding. The kinetics of unfolding in the presence of 5.9 M GdmCl were measured at 10 °C as a function of pH. Unfolding of RNase T_1 as monitored by the decrease in tryptophan fluorescence is a monophasic reaction at all employed pH values. Its amplitude accounts for 80–100% of the change in fluorescence that was observed in the equilibrium unfolding transition at pH 5.0 (cf. Figure 1). The rate of the unfolding reaction is strongly pH-dependent (Table I). It is slowest at the pH of optimum stability (around pH 5) with a time constant of τ = 14100 s. An increase in pH to pH 8 leads to a 56-fold increase in the unfolding rate. Decreasing of the pH accelerates unfolding as well. This is most pronounced around pH 2. At pH values smaller than 1.6 unfolding by 5.9 M GdmCl (10 °C) is complete within the dead time of manual mixing (10 s).

Reactivation of RNase T_1. Unfolding of RNase T_1 is a reversible process. After complete unfolding in the presence of 8.0 M urea, pH 8.0, enzymatic activity as assayed by the hydrolysis of the dinucleotide GpC is completely restored following a 40-fold dilution to 0.2 M urea at pH 8.0 and 10 °C. To measure the time course of reactivation, samples of the refolding protein were withdrawn after various time intervals and added to a GpC assay solution, which contained 4.8 μM trypsin. Trypsin cleaves unfolded RNase T_1 species rapidly and thus inhibits further refolding during the time required for the assay. Native RNase T_1 is not inactivated by trypsin under these conditions. The time course of reactivation (Figure 2) is complex; it consists of fast and slow processes. About 10% of the activity returns within the dead time of the experiment (20 s). Further reactivation is slow: an additional 20% of the RNase T_1 activity is regained with a time constant of 300 s, whereas the remaining 70% reactivates very slowly, with a time constant of 2100 ± 500 s.

Slow Refolding Reactions of RNase T_1. In addition to the reactivation experiments of Figure 2, refolding was monitored by structural probes, i.e., by the increase in absorbance at 287 nm and in fluorescence at 320 nm. The fluorescence-detected slow refolding reactions, observed after a 40-fold dilution of unfolded RNase T_1 (in 8.0 M urea, pH 8.0) to folding conditions of 0.2 M urea at pH 5.0 and at pH 8.0 (10 °C), are

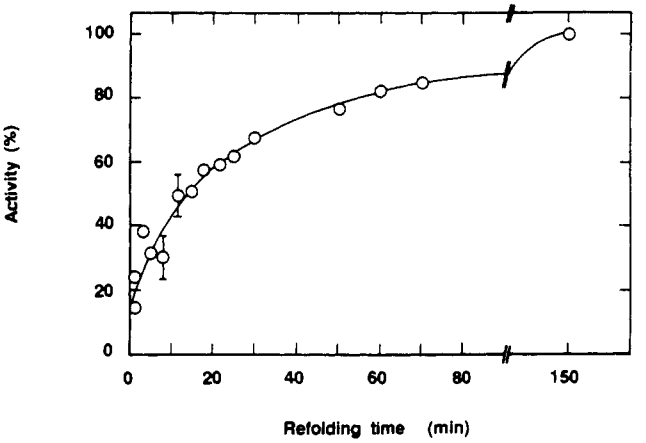

FIGURE 2: Time course of reactivation of unfolded RNase T_1 at pH 8.0 and 10 °C. The protein was unfolded by 8.0 M urea in 0.1 M Tris-HCl, pH 8.0, at 10 °C. Refolding was started by a 40-fold dilution to 0.2 M urea and 0.1 M Tris-HCl, pH 8.0, at 10 °C. After various times of refolding, aliquots were withdrawn from the refolding solution and the activity was measured, using the cleavage of the dinucleotide GpC at 10 mM Tris-HCl, pH 7.8, at 10 °C. The assay solution contained a 500-fold excess of trypsin, to prevent unfolded or partially folded molecules from refolding during the assay. The concentration of RNase T_1 in the refolding step was 0.37 μM. (—) Theoretical curve, assuming biphasic kinetics with τ_1 = 2500 s (A_1 = 68%) and τ_2 = 300 s (A_2 = 21%).

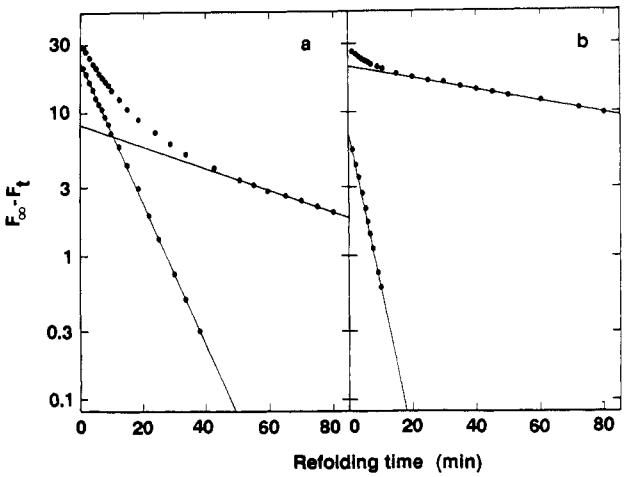

FIGURE 3: Semilog plots of the refolding of RNase T_1 in the presence of 0.2 M urea at pH 8.0 (a) and pH 5.0 (b) at 10 °C. Refolding was measured by the increase in fluorescence at 320 nm after excitation at 268 nm. Unfolded RNase T_1 (in 8.0 M urea and 0.1 M Tris-HCl, pH 8.0) was refolded by a 40-fold dilution with 0.1 M Tris-HCl, pH 8.0, or with 0.1 M NaOAc/acetic acid, pH 5.0, at 10 °C in the fluorimeter cell. The final concentration of RNase T_1 was 0.37 μM. The curves were analyzed as the sums of two exponentials with τ_1 = 3400 s, τ_2 = 500 s at pH 8.0 and τ_1 = 6500 s, τ_2 = 250 s at pH 5.0.

shown in Figure 3. At both pH values slow refolding can be modeled by a biphasic process; however, both the rates and the relative amplitudes of the two phases depend strongly on pH. In the following we label these two phases as "intermediate" and "very slow" refolding reactions. Table II gives a summary of the observed time constants and relative amplitudes for the slow refolding processes in the range from pH 3 to pH 8. The time constant of the intermediate phase is almost insensitive to pH; it remains in the 250–400-s range from pH 3 to pH 8. The very slow phase, however, is strongly decelerated at pH 3 (τ = 14300 s), and it becomes faster with increasing pH. At pH 8.0, its time constant is 3000 s. The amplitudes of the two refolding phases show a complex and compensatory dependence on pH, irrespective of the probe used

Table II: Kinetic Data for the Slow Refolding of RNase T_1[a]

refolding conditions	method[b]	intermediate phase		very slow phase		
		A_2[c]	τ_2 (s)	A_1[c]	τ_1 (s)	A_1/A_2
0.2 M urea, pH 3.0	Fl	0.18	300	0.31	14300	1.70
0.2 M urea, pH 4.0	Fl	0.14	290	0.30	11200	2.21
0.2 M urea, pH 5.0	Fl	0.16	250	0.43	6500	2.59
0.15 M GdmCl, pH 5.0	Fl	0.13	230	0.47	7400	3.70
0.15 M GdmCl, pH 5.0	Abs	0.16	360	0.34	7500	2.10
0.2 M urea, pH 5.0	Act	0.30	400	0.45	6500	1.50
0.2 M urea, pH 6.0	Fl	0.28	300	0.38	4800	1.37
0.2 M urea, pH 7.0	Fl	0.35	350	0.26	4000	0.76
0.2 M urea, pH 8.0	Fl	0.46	500	0.20	3400	0.43
0.15 M GdmCl, pH 8.0	Fl	0.42	350	0.20	3400	0.49
0.15 M GdmCl, pH 8.0	Abs	0.36	400	0.22	2500	0.61
0.2 M urea, pH 8.0	Act	0.21	300	0.68	2500	3.23

[a] Initial conditions were 6.0 M GdmCl and 0.1 M glycine hydrochloride, pH 1.7, or 8.0 M urea and 0.1 M Tris-HCl, pH 8.0. Refolding was initiated by a 40-fold dilution to the indicated folding conditions (at 10 °C); buffers used were 0.1 M glycine hydrochloride (pH 3.0), 0.1 M NaOAc/acetic acid (pH 4.0 and 5.0), 0.1 M sodium cacodylate (pH 6.0 and 7.0), and 0.1 M Tris-HCl (pH 8.0). Error limits are about ±5% for the amplitudes and about ±10% for the time constants. [b] Fl = fluorescence measurements at 320 nm (after excitation at 268 nm); Abs = absorbance measurements at 287 nm; Act = activity measurements as described under Materials and Methods and in the legend to Figure 2. [c] A_1 and A_2 are the amplitudes of the intermediate and the very slow refolding phases, respectively. They are given as fractions of the total change in fluorescence or absorbance as observed in the equilibrium unfolding transition. In the case of activity the amplitudes are the fraction of activity regained in the respective phase.

Table III: Influence of the Unfolding Conditions on the Slow Refolding Kinetics of RNase T_1

unfolding conditions[a]	refolding conditions[b]	intermediate phase		very slow phase	
		A_2[c]	τ_2 (s)	A_1	τ_1 (s)
6.0 M GdmCl, pH 8.0, 25 °C	0.15 M GdmCl, pH 5.0	0.34	74	0.42	1000
6.0 M GdmCl, pH 1.5, 25 °C	0.15 M GdmCl, pH 5.0	0.38	77	0.42	1050
6.0 M GdmCl, pH 8.0, 10 °C	0.15 M GdmCl, pH 5.0	0.38	78	0.42	1000
6.0 M GdmCl, pH 1.5, 10 °C	0.15 M GdmCl, pH 5.0	0.39	68	0.42	1000
8.0 M urea, pH 8.0, 25 °C	0.20 M urea, pH 5.0	0.33	72	0.45	1050

[a] Buffers used for unfolding were 0.1 M glycine hydrochloride (at pH 1.5) and 0.1 M Tris-HCl (at pH 8.0). [b] Refolding was carried out at 25 °C by 40-fold dilution of unfolded RNase T_1 into 0.1 M NaOAc/acetic acid, pH 5.0 in a fluorimeter cuvette. The resulting kinetics were measured by the increase in fluorescence at 320 nm after excitation at 268 nm. [c] A_1, A_2; relative amplitudes of the very slow and the intermediate refolding phase, respectively. The amplitudes are given as fractions of the total change in fluorescence as observed in the equilibrium unfolding transition. Error limits are as indicated in Table II.

to monitor the reaction. Below pH 5 the very slow phase dominates refolding. Between pH 5 and pH 8 the amplitude of this phase decreases in parallel with a concomitant increase of the amplitude of the intermediate phase, which dominates slow refolding at pH 8. A comparison of the kinetics monitored by absorbance and by fluorescence at pH 5 and 8 indicates that to a first approximation the time constants for the two phases are independent of the probe used.

The sum of the amplitudes for the two slow refolding reactions accounts for approximately 50% (at pH 5.0) and 60% (at pH 8.0) of the entire change in absorbance as derived from the equilibrium unfolding transition. Similar values are found for fluorescence. There is a pronounced difference in the relative amplitudes observed in the spectroscopic and in the reactivation experiments. At pH 8, only 20% of the activity is regained in the intermediate phase and 70% in the very slow phase. This is independent of whether reactivation was carried out in the presence of residual 0.2 M urea or 0.15 M GdmCl. In the spectroscopic refolding experiments under the same conditions, however, most of the observed signal change occurs in the intermediate phase (Table II). The rates of both refolding phases are markedly dependent on temperature as well, and the relative amplitude of the intermediate phase increases with temperature.

The amplitudes of the two slow refolding reactions do not depend on the unfolding conditions, provided that they warrant complete denaturation of RNase T_1: the slow refolding kinetics (as measured at pH 5.0 and 25 °C) are independent of the pH and the temperature of the 6.0 M GdmCl unfolding solution (Table III). Furthermore, the same refolding kinetics are observed in the presence of residual 0.2 M urea (after unfolding in 8.0 M urea, pH 8.0) or of residual 0.15 M GdmCl (after unfolding in 6.0 M GdmCl, pH 1.5).

Time Course of Formation of Native Molecules during Refolding. The results in Tables II and III indicate that slow refolding of RNase T_1 is a complex process, where the relative amplitudes depend on the conditions and on the probes used to monitor folding. This could originate from a sequential reaction, where the two slow phases of refolding are successive steps on a single kinetic pathway, or alternatively from the refolding of different unfolded species on two parallel pathways. In the first case only the slow phase should yield native protein; in a parallel mechanism, however, native RNase T_1 should be formed in both slow phases. To measure the formation of native protein during refolding, we use an unfolding assay that was originally developed for bovine pancreatic RNase A (Schmid, 1986). The assay is based on the finding that the native protein unfolds under a fixed unfolding condition much more slowly than any partially folded, less stable, intermediate species (Schmid, 1983, 1986). The assay consists of three steps. (i) The protein is incubated under th desired unfolding condition for a sufficiently long period of time to warrant complete equilibration of the different unfolded species. (ii) Refolding is started at time $t = 0$ by dilution into suitable conditions. (iii) Aliquots of the refolding protein are withdrawn after variable time intervals, t_i, and the amount of native protein, N, present at t_i is determined by a kinetic unfolding assay under standard conditions. The amplitude of this reaction is proportional to the amount of N formed after $t = t_i$. The increase of this amplitude with the folding time, t_i, gives the kinetics of formation of native protein under the conditions of step ii. This assay was used to measure the

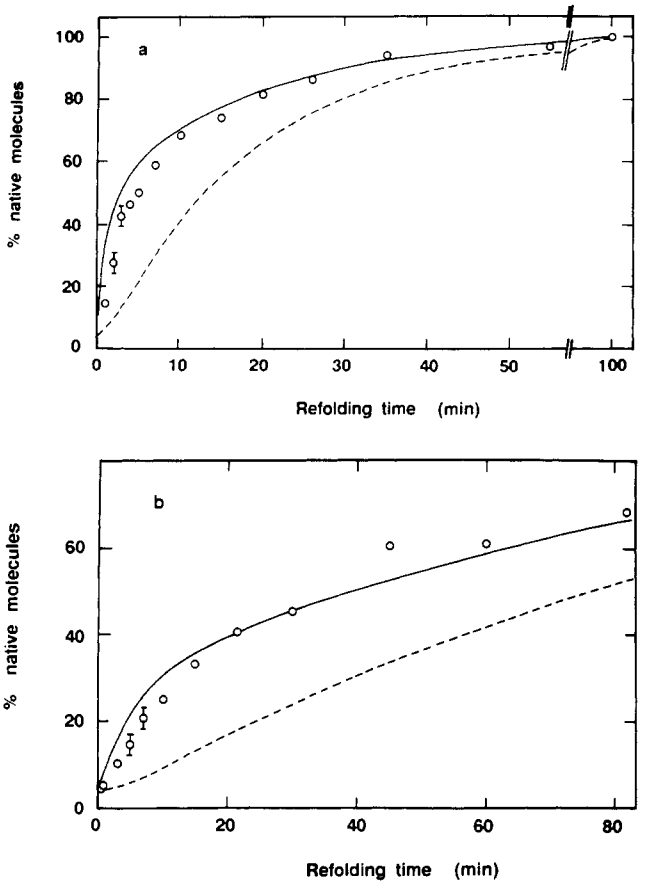

FIGURE 4: Time course of formation of native RNase T_1 at pH 5.0, measured by unfolding assays as described under Materials and Methods. (a) 25 °C; (b) 10 °C. The refolding buffer contained 0.1 M NaOAc/acetic acid and 0.2 M GdmCl. The concentration of RNase T_1 in the refolding step was 1.4 μM. The experimental data (O) are compared with theoretical curves for the formation of native molecules either on two parallel pathways (according to eqs 3a and 3b; solid lines) or alternatively on a sequential pathway (according to eq 2; dashed lines). The following time constants were used for the calculation. (a) 25 °C: τ_1 = 1100 s (A_1 = 53%); τ_2 = 70 s (A_2 = 43%). (b) 10 °C: τ_1 = 6500 s (A_1 = 72%); τ_2 = 300 s (A_2 = 24%).

formation of native RNase T_1 molecules in the course of slow refolding. RNase T_1 was refolded in the presence of 0.17 M GdmCl, pH 5.0, at 25 °C (Figure 4a) and at 10 °C (Figure 4b). After the indicated time intervals of refolding, samples were transferred to unfolding conditions of 5.25 M GdmCl, pH 2.1, 10 °C (or 4.0 M GdmCl, pH 2.4, for the experiments at 25 °C), and the relative amplitudes of the resulting unfolding reactions are shown as a function of the refolding time in Figure 4. The observed time courses of formation of native RNase T_1 in panels a and b of Figure 4 are each compared with two theoretical kinetic curves. In the first, sequential model it is assumed that the two slow phases of the reactivation of RNase T_1 (Figure 3 and Table II) originate from a single slow-folding species, which refolds on a two-step sequential pathway with a partially active intermediate I, as outlined in eq 2. The two steps in the model are equivalent to the two phases observed in slow refolding. The second, parallel kinetic model represents a mechanism in which native protein is formed on two slow pathways, originating from different unfolded states (eqs 3a and 3b).

$$U \xrightarrow{\tau_2} I \xrightarrow{\tau_1} N \qquad (2)$$

$$U_x \xrightarrow{\tau_2} N \qquad (3a)$$

$$U_y \xrightarrow{\tau_1} N \qquad (3b)$$

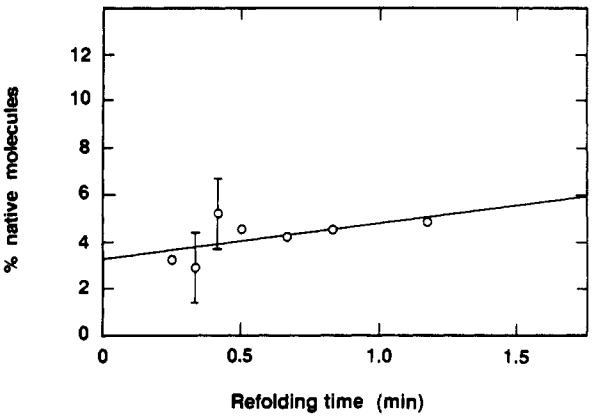

FIGURE 5: Time course of formation of native RNase T_1 at pH 5.0 and 10 °C. Measurements in the early time region under the conditions of Figure 4b. The extrapolation of the data to the start of refolding gives the amount of fast-folding U_F species, which are present at equilibrium in the unfolded protein and refold within the dead time of the experiment.

The comparison of the experimental data at 10 °C and at 25 °C with these two models rules out the strictly sequential mechanism of eq 2. Slow refolding is much better described by the assumption of two parallel reactions which both yield native RNase T_1 (eqs 3a and 3b). At both temperatures, however, formation of native protein shows a small lag phase at the beginning, which is not predicted by the simple parallel model (eqs 3a and 3b). This could originate from the formation of intermediate structure on the faster or on both slow folding pathways.

Detection of Fast-Folding Molecules. The early time region of the kinetics in Figure 4 indicate that a small amount of native RNase T_1 is formed very rapidly, i.e., that a small amount of fast-folding species U_F is present. In order to get a quantitative estimate for the fraction of U_F molecules, we use the same conditions and assays as in Figure 4, except we use very short refolding pulses in step ii. In each experiment an aliquot of the protein is allowed to refold completely to determine the total concentration of the refolding protein in the sample. The ratio of the amplitudes of these two unfolding assays is equal to the fraction of U_F molecules present in the fully unfolded protein. The results of these assays (Figure 5) indicate that there is indeed a small amount of about 3.5% fast-folding species in unfolded RNase T_1. After the rapid $U_F \rightarrow N$ refolding reaction a very slow increase in the amount of native molecules is observed (Figure 5) at the onset of the slow refolding reactions. This increase coincides with the early time region of Figure 4b.

The unfolding assays for native protein as used in Figures 4 and 5 represent a method which "counts molecules" that regain the native state on the individual refolding pathways. The quantitative analysis of the kinetic data in Figures 4 and 5 suggests that under the given refolding conditions (0.17 M GdmCl, pH 5.0, 10 or 25 °C) 3.5% of all molecules refold on the $U_F \rightarrow N$ pathway. In the course of the slow reaction, 24% refold on the intermediate (eq 3a) and 72% on the very slow (eq 3b) pathway. This distribution is to a first approximation independent of the temperature of refolding between 10 and 25 °C.

Slow Isomerization Reactions of the Unfolded Protein. The simplest explanation for the occurrence of fast and slow refolding reactions is provided by the proline hypothesis (Brandts et al., 1975; Schmid & Baldwin, 1978). We therefore asked the question whether proline isomerization could be the molecular origin of the kinetic heterogeneity of the folding of

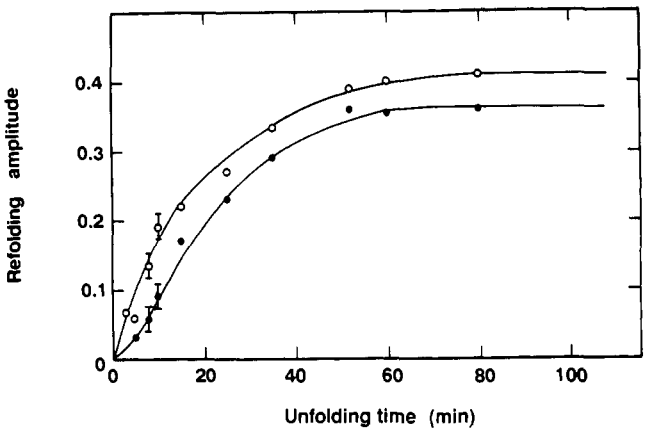

FIGURE 6: Time course of formation of the very slow (○) and the intermediate (●) refolding phase after rapid and complete denaturation in 6.0 M GdmCl and 0.1 M glycine hydrochloride, pH 1.5, at 0 °C. After various times of denaturation refolding assays were carried out by a 40-fold dilution with 0.1 M NaOAc/acetic acid, pH 5.0, at 25 °C. Renaturation was measured by the increase in fluorescence at 320 nm after excitation at 268 nm. The amplitudes of the intermediate (●) and the very slow (○) refolding phases are shown as a function of the incubation time under unfolding conditions. The concentration of RNase T_1 in the refolding assays was 0.37 μM.

RNase T_1. To test this hypothesis, we investigated whether the slow phases observed in refolding originate from slow isomerization reactions of the unfolded protein chains and determined the corresponding activation energy. Proline isomerization is expected to occur only after the rapid N → U_F unfolding reaction has taken place. In the test we first unfolded RNase T_1 in the presence of 6.0 M GdmCl, pH 1.5 at 0 °C. Under these conditions the N → U_F reaction is very rapid (cf. Table I) and proline isomerization should be slow (Cheng & Bovey, 1977; Brandts et al., 1975; Schmid, 1986). Then, at variable time intervals after unfolding, samples were withdrawn and transferred to standard refolding conditions of 0.15 M GdmCl, pH 5 at 25 °C, and the amplitudes of the resulting slow refolding reactions were measured. The results are shown in Figure 6. After very short incubation times under unfolding conditions most of the molecules are still in the U_F state. In the refolding assay they revert rapidly to N within the dead time of mixing, and the amplitudes of the slow refolding reactions are very small. With extended incubation under unfolding conditions the amount of U_F decreases and the amplitudes of the two slow refolding phases slowly increase. After approximately 80 min they have reached their final equilibrium value. The formations of the two slow-folding phases show very similar time courses; however, the formation of the intermediate refolding phase shows a lag at the beginning. Analysis of the data in terms of first-order kinetics yields a time constant of 1000 s for the formation of the very slow phase and for the late time region of the formation of the intermediate refolding phase. The dependence on temperature of this time constant was determined between 0 and 25 °C. It yields an apparent activation energy of 88 kJ/mol, as expected for processes that are limited by proline isomerization (Brandts et al., 1975; Schmid & Baldwin, 1978).

Slow Reactivation Is Catalyzed by Prolyl Isomerase. Peptidyl-prolyl cis–trans isomerase is able to catalyze some slow protein folding reactions that are limited in rate by proline isomerization (Lang et al., 1987). The fluorescence-detected slow phases of RNase T_1 refolding are accelerated by this enzyme, albeit with different efficiency (Lin et al., 1988; Fischer et al., 1989). The relative amplitudes of the two slow refolding reactions of RNase T_1 are strongly different, depending on whether folding is followed by fluorescence or by

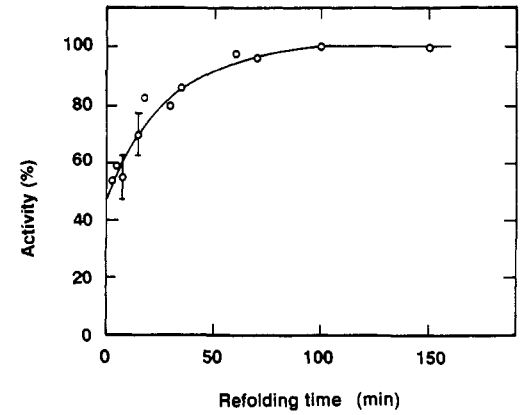

FIGURE 7: Time course of reactivation of RNase T_1 in the presence of prolyl isomerase. The experiments were carried out at pH 8.0 and 10 °C, as described in Figure 2. The refolding buffer additionally contained 0.97 μM prolyl isomerase.

the regain of enzymatic activity (cf. Table II). To determine whether the steps catalyzed by prolyl isomerase are on the pathways to the native protein, we have carried out refolding in the presence of prolyl isomerase and measured the time course of reactivation. Catalysis is observed. The comparison of the results in Figure 2 (without prolyl isomerase) and in Figure 7 (in the presence of 1.0 μM prolyl isomerase) shows that the catalyzed steps (i.e., proline isomerization steps) are indeed rate-limiting for the regain of catalytically active, native protein. The intermediate reaction is complete within the dead time of the experiment (30 s) in presence of prolyl isomerase. The very slow reaction, which accounts for most of the activity regain, is accelerate about 1.5-fold. Its relative amplitude appears to be smaller than in the absence of prolyl isomerase. Together with the data in Figures 2 and 4, this indicates that most of the unfolded RNase T_1 refolds on the slow pathway, although this reaction has only a small amplitude in absorbance or fluorescence.

Discussion

It was the aim of this investigation to present an initial characterization of the unfolding and refolding kinetics of RNase T_1. Our results confirm that folding is reversible under a wide variety of conditions, a conclusion that has also been drawn from equilibrium unfolding experiments on this protein (Pace et al., 1988; Pace & Laurents, 1989). The observed folding kinetics are easiest explained by assuming a coexistence of different unfolded species, which give rise to fast and slow refolding reactions. We suggest that this kinetic heterogeneity of the unfolded state is caused by the slow isomerization of proline peptide bonds. This hypothesis is supported by the finding that the slow refolding species (U_S) are created after the rapid unfolding process (N → $_F$) by subsequent slow reactions which occur in the unfolded protein chain. They show properties of proline isomerization, such as an activation energy of 88 kJ/mol. There are two major slow refolding phases, and we propose that they originate from at least two U_S species which differ in the number and/or location of the incorrect proline isomers. The generation of the U_S species which refolds most slowly after unfolding occurs with a time constant of 1000 s at 0 °C. The formation of the intermediate refolding reaction is complex: it shows a lag at the beginning, but in the late time region it becomes very similar to the formation of the species, which refolds very slowly. A tentative explanation for this phenomenon could be that the formation of both U_S species is limited in rate by the isomerization of the same proline peptide bond. In one case, however, this process occurs

in the presence of another incorrect proline, the isomerization of which would be responsible for the observed lag phase. At first glance, the unexpected consequence of such a model would be that U_S species with only one incorrect proline isomer refold more slowly than a U_S species that has an additional wrong proline. The proline model for the folding of RNase T_1 is supported by the finding that the slow refolding reactions are catalyzed with different efficiency by the enzyme prolyl isomerase (Fischer et al., 1989).

There is no evidence yet on the molecular identity of the proline residues that are involved in the slow folding pathways. Possible candidates for an important role in folding are prolines 39 and 55, which are in the cis conformation in native RNase T_1 (Arni et al., 1987, 1988). Cis prolines are expected to isomerize largely to the more favorable trans conformation after unfolding. Therefore, they should give rise to high concentrations of unfolded molecules with the respective incorrect isomers, as was observed in Figures 4 and 5.

Three major phases are detected in the refolding of RNase T_1. There is a small amount (3.5%) of fast-folding U_F species which under all employed conditions refold in the dead time of manual mixing. The presence of U_F was demonstrated by an unfolding assay for native molecules. This assay is exlusively sensitive for native protein (it counts native molecules), because any partially folded intermediates unfold more rapidly than the completely refolded molecules. This highly discriminative property of the unfolding assay is related to the observation that generally the activated state for folding is close to the native state (Segawa & Sugihara, 1984) and hence that the unfolding rate of a protein is very sensitive to small perturbations of its stability. The unfolding assays are a better technique to determine the concentrations of the individual fast- and slow-folding species of a protein than, e.g., reactivation experiments, because partially active folding intermediates have been observed for several proteins (Rudolph et al., 1976; Schmid & Blaschek, 1981).

The small amount of less than 4% U_F molecules in unfolded RNase T_1 suggests that the proline peptide bonds that are responsible for formation of the slow-folding species are predominantly in the nonnative state in the unfolded protein. Pancreatic RNase A, which also has two cis and two trans prolines as RNase T_1, shows 20% fast-folding species (Garel & Baldwin, 1973).

In the simplest model for the role of proline isomerization in protein folding the amplitudes and rates of slow refolding reactions should reflect the distribution of the different unfolded species prior to refolding and the intrinsic rates of isomerization as observed in the denatured protein chain. The kinetic properties of the slow refolding reactions of RNase T_1 are in apparent contradiction to this model. The rates of the two slow phases, as well as their amplitudes, strongly depend on the folding conditions (in particular on pH). In addition, the observed amplitudes depend on the probes that were used to monitor folding. Clearly, slow refolding is more complex than would be expected by a simple reversal of the unfolding mechanism. Possibly these peculiar kinetic properties originate from the rapid formation of partially folded intermediates during the slow refolding reactions, i.e., from mutual interdependence between protein folding and proline isomerization events. Further experiments will be required to assay for the presence of such putative intermediates during slow refolding and to elucidate their possible role for the folding pathway (Kiefhaber et al., 1990).

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

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

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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

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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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