Protein Science (1992), *1*, 1162–1172. Cambridge University Press. Printed in the USA.

Structure of a rapidly formed intermediate in ribonuclease T1 folding

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(RECEIVED March 3, 1992; REVISED MANUSCRIPT RECEIVED April 13, 1992)

Abstract

Kinetic intermediates in protein folding are short-lived and therefore difficult to detect and to characterize. In the folding of polypeptide chains with incorrect isomers of Xaa-Pro peptide bonds the final rate-limiting transition to the native state is slow, since it is coupled to prolyl isomerization. Incorrect prolyl isomers thus act as effective traps for folding intermediates and allow their properties to be studied more easily. We employed this strategy to investigate the mechanism of slow folding of ribonuclease T1. In our experiments we use a mutant form of this protein with a single cis peptide bond at proline 39. During refolding, protein chains with an incorrect trans proline 39 can rapidly form extensive secondary structure. The CD signal in the amide region is regained within the dead-time of stopped-flow mixing (15 ms), indicating a fast formation of the single α-helix of ribonuclease T1. This step is correlated with partial formation of a hydrophobic core, because the fluorescence emission maximum of tryptophan 59 is shifted from 349 nm to 325 nm within less than a second. After about 20 s of refolding an intermediate is present that shows about 40% enzymatic activity compared to the completely refolded protein. In addition, the solvent accessibility of tryptophan 59 is drastically reduced in this intermediate and comparable to that of the native state as determined by acrylamide quenching of the tryptophan fluorescence. Activity and quenching measurements have long dead-times and therefore we do not know whether enzymatic activity and solvent accessibility also change in the time range of milliseconds. At this stage of folding at least part of the β-sheet structure is already present, since it hosts the active site of the enzyme. The trans to cis isomerization of the tyrosine 38–proline 39 peptide bond in the intermediate and consequently the formation of native protein is very slow (τ = 6,500 s at pH 5.0 and 10 °C). It is accompanied by an additional increase in tryptophan fluorescence, by the development of the fine structure of the tryptophan emission spectrum, and by the regain of the full enzymatic activity. This indicates that the packing of the hydrophobic core, which involves both tryptophan 59 and proline 39, is optimized in this step. Apparently, refolding polypeptide chains with an incorrect prolyl isomer can very rapidly form partially folded intermediates with native-like properties.

Keywords: circular dichroism; folding kinetics; folding pathway; fluorescence lifetime; fluorescence quenching; protein folding; stopped-flow

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Abbreviations: RNase T1, Lys 25 isoenzyme of ribonuclease T1 from *Aspergillus oryzae*; (S54GP55N)-RNase T1, ribonuclease T1 with Ser 54 and Pro 55 replaced by Gly and Asn, respectively; PPIase, peptidyl-prolyl cis–trans isomerase; ΔG, free energy of folding; $\Delta\Delta G$, difference in the free energies of folding of the wild-type protein and the variant; GpC, guanylyl(3′–5′)cytidine; GdmCl, guanidinium chloride; N and U, native and unfolded protein, respectively; I, folding intermediate; U_S and U_F, slow- and fast-folding molecules, respectively.

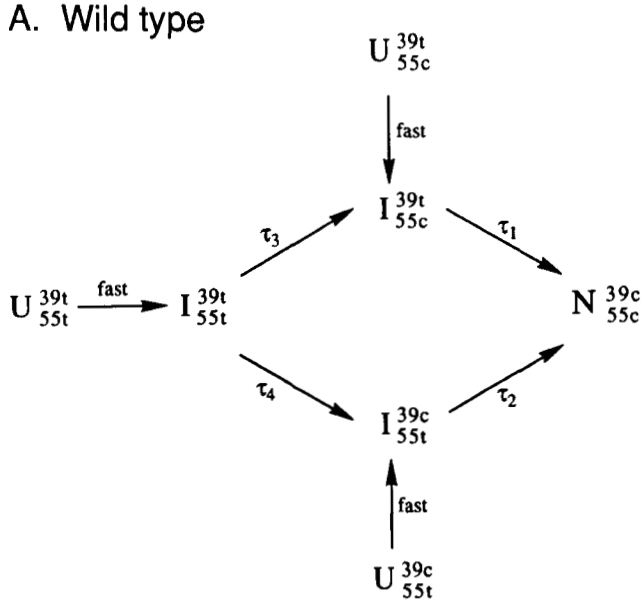
The mechanisms that govern the folding of a polypeptide chain into its native, three-dimensional structure are still largely unsolved. The acquisition of the native state occurs probably on defined folding pathways with distinct intermediate states (Baldwin, 1990; Kim & Baldwin, 1990). The structural characterization of such folding intermediates is difficult. Due to the high cooperativity of folding transitions, intermediates are rarely populated at equilibrium, and kinetic intermediates accumulate only transiently in

the time course of folding. Rapid pulse-labeling of amide protons in combination with high-resolution NMR analysis allowed the detection and characterization of early folding intermediates of RNase A (Udgoankar & Baldwin, 1988, 1990), cytochrome c (Roder et al., 1988), and barnase (Bycroft et al., 1990). These studies revealed that secondary structure can form very rapidly during refolding and, in the case of cytochrome c, two α-helices located at the opposite ends of the polypeptide chain can interact at a very early stage in folding (Roder et al., 1988). These rapid steps, which occur in the millisecond time range, are often followed by slow, rate-limiting steps that may include the formation of the correct disulfide bonds (Creighton, 1990; Weissman & Kim, 1991) and/or the cis–trans isomerization of Xaa-Pro peptide bonds (Brandts et al., 1975; Kim & Baldwin, 1990; Kiefhaber et al., 1990a). These reactions typically occur within minutes to hours.

We are using RNase T1 (see Kinemage 1) as a model system to investigate the properties of folding intermediates that are formed prior to the rate-limiting steps of folding, which in the case of RNase T1 involve prolyl isomerization. The folding of RNase T1 is a complex kinetic process and comprises several fast and slow reactions (Kiefhaber et al., 1990b,c). Isomerizations of the two cis prolyl peptide bonds of RNase T1 (at Pro 39 and at Pro 55) are of major importance as slow steps both in the unfolded protein and during refolding (Kiefhaber et al., 1990a, 1992). Under unfolding conditions 3.5% fast-folding molecules ($U_F = U^{39c}_{55c}$) are present and coexist with three different classes of slow-folding molecules (U^x_S; cf. Scheme 1A). The U_F molecules have all prolyl peptide bonds in the same conformation (cis or trans) as the native state and therefore they refold rapidly under favorable conditions. Refolding of the U_S molecules involves the slow trans to cis isomerization of one (in U^{39t}_{55c} and U^{39c}_{55t}) or two (in U^{39t}_{55t}) prolyl peptide bonds at a late stage of folding. All slow-folding species can rapidly form partially folded intermediates as judged by the fast recovery of the far-UV CD signal (Kiefhaber et al., 1990c). The kinetic and structural analysis of these folding intermediates was complicated, however, by the presence of at least three intermediate species that differ in the isomeric state of the prolyl peptide bonds (cf. Scheme 1A). Also, it was not possible yet to assess the importance of the trans prolines 60 and 73 during refolding of RNase T1, since their contributions are probably small in amplitude and masked by the isomerizations of the cis prolines, which are correlated with large amplitudes.

To simplify the structural analysis of the folding intermediates we investigated a variant of RNase T1, which lacks one of the two cis prolines (Pro 55; Kinemage 3). In this mutant, (S54G,P55N)-RNase T1, the prolyl peptide bond Ser 54–cis Pro 55 is replaced by Gly 54–Asn 55. This mutation has only minor effects on the stability of the protein ($\Delta\Delta G^0 = -0.9$ kcal/mol); it leads, however,

A. Wild type



B. Variant

$$U^{39t} \xrightarrow{\text{fast}} I^{39t} \xrightarrow{\tau_1'} N^{39c}$$

Scheme 1. Kinetic models for the slow-refolding reactions of wild-type (**A**) and (S54GP55N)-RNase T1 (**B**) under strongly native conditions. U is the unfolded protein, I represents intermediates, and N is the native protein. The superscripts and subscripts denote the isomeric states of proline 39 and proline 55, respectively, in the native cis (c) or in the nonnative trans (t) conformation. The relaxation times for wild-type RNase T1 are $\tau_1 = 3{,}000$ s, $\tau_2 = 500$ s, $\tau_3 = 100$ s, and $\tau_4 = 190$ s at pH 8 and 10 °C and $\tau_1 = 7{,}000$ s, $\tau_2 = 250$ s, $\tau_3 = 170$ s, and $\tau_4 = 400$ s at pH 5.0 and 10 °C. For (S54GP55N)-RNase T1 τ_1' is 2,700 s at pH 8 and 10 °C and 6,500 s at pH 5.0 and 10 °C (adopted from Kiefhaber et al., 1990a,c). Additionally, 3.5 ± 0.5% of unfolded wild-type protein and 17 ± 2% of unfolded (S54G,P55N)-RNase T1 have all X-Pro peptide bonds in the same isomeric state as the native protein and thus refold very rapidly (U_F species)

to a simplified folding mechanism since only a single major slow-folding species with an incorrect trans Pro 39 remains (Kiefhaber et al., 1990a; cf. Scheme 1B). Consequently, in refolding only one dominant folding intermediate is formed in which the Tyr 38–Pro 39 peptide bond is still in its nonnative trans conformation and has to undergo a trans to cis isomerization to finally reach the native state. This reaction was found to be unusually slow when compared to other proline-limited folding reactions (Kiefhaber et al., 1990a,b), probably because a "premature" interaction of Pro 39 with Trp 59 occurs in a folding intermediate when Pro 39 is still in the incorrect trans conformation (Kiefhaber et al., 1992).

In this article we show that the intermediate with an incorrect trans Pro 39 shows extensive secondary and tertiary structure, a hydrophobic core with low solvent accessibility, and partial enzymatic activity. The regain of

the secondary structure and at least part of the tertiary structure of this intermediate is complete within a few milliseconds of refolding.

Results

Slow-folding kinetics

The folding kinetics of (S54G,P55N)-RNase T1 were monitored under various conditions and monitored by using different spectroscopic and functional probes. The observed time constants and the corresponding amplitudes are summarized in Table 1. Refolding is clearly dominated by a very slow reaction ($\tau \approx 6{,}500$ s at pH 5.0 and 10 °C). Both the amplitude and the rate constant of this reaction depend on pH in the range from pH 5 to pH 8. Above pH 8, unfolding of RNase T1 becomes progressively irreversible with increasing pH. An additional slow-refolding phase with a small amplitude and a time constant in the 300–500-s range (at 10 °C) is detected by most probes. It is, however, not monitored by unfolding assays that are sensitive for the formation of native protein (Kiefhaber et al., 1990a). This suggests that a folding intermediate rather than native protein is formed in this step. This reaction is catalyzed by the enzyme peptidyl-prolyl cis-trans isomerase and is probably caused by the cis to trans isomerization at one or both trans proline residues (at Pro 60 and Pro 73) in molecules that also have an incorrect trans isomer at Pro 39. Significant fractions of the native fluorescence as well as part of the enzymatic activity are regained rapidly after the start of refolding. This is revealed by a comparison of the amplitude of the slow-folding reactions of (S54G,P55N)-RNase T1 with the signal changes measured in equilibrium unfolding experiments (Table 1). These rapid changes have too large amplitudes to be accounted for by the refolding of about 17% U_F molecules that are present in the unfolded protein (Kiefhaber et al., 1990a; Table 1). Rather, they are correlated with the fast formation of partially folded intermediates (Kiefhaber & Schmid, 1992).

Table 1. *Slow-folding kinetics of (S54G,P55N)-RNase T1 detected by various methods*[a]

Refolding conditions	Probe[b]	τ_1 (s)	τ_2 (s)	A_1[c]	A_2
pH 5.0, 25 °C	Fl.	700	35	0.54	0.09
pH 5.0, 10 °C	Fl.	7,300	340	0.51	0.11
pH 8.0, 25 °C	Fl.	270	84	0.42	0.12
pH 8.0, 10 °C	Fl.	3,000	450	0.28	0.15
pH 8.0, 10 °C, 0.5 μM PPI	Fl.	780	30	0.31	0.17
pH 5.0, 10 °C	Unf.	6,500	–	0.83	–
pH 8.0, 10 °C	Unf.	2,700	–	0.86	–
pH 5.0, 10 °C	Act.	5,000	–	0.50	–
pH 8.0, 10 °C	Act.	4,500	300	0.63	0.06

[a] Refolding was initiated by 40-fold dilution of unfolded RNase T1 (in 6 M GdmCl, 0.1 M NaAc, pH 5.0) into the given refolding conditions. In all measurements the residual GdmCl concentration was 0.15 M. Error limits are ±5% for the amplitudes and ±10% for the relaxation times. In the activity experiments error limits are 10% for the amplitudes and 15% for the relaxation times.

[b] Fl., fluorescence measurements at 320 nm after excitation at 268 nm; Unf., unfolding assays to monitor formation of native molecules as described under Materials and methods; Act., activity measurements as described under Materials and methods.

[c] Amplitudes are given as fractions of the total change in fluorescence as observed in the equilibrium unfolding transitions. In the activity measurements the amplitude is the fraction of activity that is regained in the respective reaction. In unfolding assays the amplitude represents the fraction of native molecules that are formed in the respective kinetic phase. The fraction of the total refolding amplitude that is not recovered in the displayed slow phases is regained too fast to be detected in the deadtime of the respective experiments (1 s for fluorescence measurements and 10 s for unfolding assays and reactivation measurements).

Time course of the folding intermediate

Unfolding assays provide a sensitive technique to detect transient folding intermediates and to measure the time course of their formation and decay during refolding. Partially folded intermediates are less stable than the native protein, and therefore they unfold under conditions where the native protein is still marginally stable (Cook et al., 1979; Schmid, 1983; Kiefhaber et al., 1990c). In the experimental protocol, folding intermediates are populated by a short refolding pulse and then transferred to conditions where the native protein is only marginally stable. Although intermediates unfold under these conditions, molecules that have already reached the native state remain folded. The amplitude of the observed unfolding reaction therefore is a measure of the amount of intermediates that was initially present at the time, when refolding was interrupted. Figure 1 shows the GdmCl-induced unfolding transition of (S54G,P55N)-RNase T1 at pH 5.0 and 10 °C. The transition from the native to the unfolded form of the protein occurs in the range from 3 to 5 M GdmCl with a midpoint at 4.00 ± 0.05 M. To detect folding intermediates, unfolding assays were performed at 2.8 M GdmCl, where the native protein is still stable. Refolding of (S54G,P55N)-RNase T1 was initiated by dilution of the unfolded protein to 0.1 M Na acetate, 0.15 M GdmCl at pH 5.0 and 10 °C. After various time intervals of refolding (t_i), aliquots were withdrawn and transferred to 2.8 M GdmCl at pH 5 and 10 °C. Under these conditions the transiently formed intermediates unfold with a relaxation time of 15 s, whereas the native molecules remain stable. The amplitude of the unfolding reaction depends on the duration of the refolding pulse (t_i) and is a measure of the amount of intermediates that are present after t_i. As an example, in Figure 2A we show the time course of fluorescence for a sample that had refolded for 5 min and was then transferred to 2.8 M GdmCl. The decrease in fluorescence within the first minute originates from the unfolding of intermediates. The

subsequent slow increase in fluorescence ($\tau = 3{,}500$ s) reflects the very slow refolding of the transiently unfolded molecules to the native state at 2.8 M GdmCl. Experiments as in Figure 2A, carried out after varying time intervals, were then used to follow the time course of formation and decay of intermediates during refolding. The respective time course is shown in Figure 2B. Most of the unfolded molecules (71%) form the intermediate in a rapid reaction that occurs in the dead-time of the experiment (10 s). An additional 12% of the denatured molecules form the intermediate in a slow process ($\tau = 170$ s at pH 5.0 and 10 °C). This reaction is similar in rate to the minor slow phase that was also detected by fluorescence under the same conditions ($\tau = 340$ s; cf. Table 1). This could indicate that a small fraction of the U_S molecules of (S54G,P55N)-RNaseT1 contains a second incorrect prolyl isomer (possibly a cis Pro 60 and/or a cis Pro 73) in addition to the incorrect trans Pro 39. Apparently, these molecules can form or stabilize the intermediate only after these prolyl residue(s) have isomerized to the native (trans) conformation.

The kinetics of formation of native molecules under the same conditions is also shown in Figure 2B (cf. also

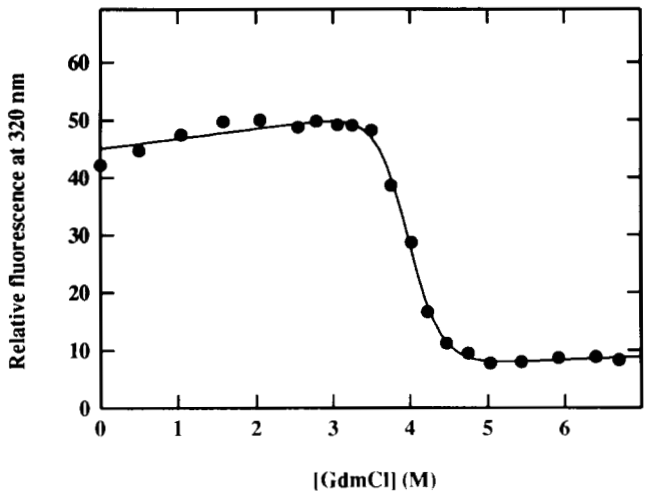


Fig. 1. GdmCl-induced unfolding transition of (S54G,P55N)-RNase T1 at pH 5.0 and 10 °C. RNase T1 was incubated in 0.1 M Na acetate, pH 5.0, at the indicated GdmCl concentrations. After 24 h the fluorescence of the samples was measured at 320 nm after excitation at 268 nm. A nonlinear least-squares fit of the transition gave a free energy of stabilization, ΔG^0, of -46.9 ± 2.5 kJ/mol at zero denaturant and an m-value of 11.73 ± 0.65 kJ/(mol * M). The protein concentration was 0.46 μM.

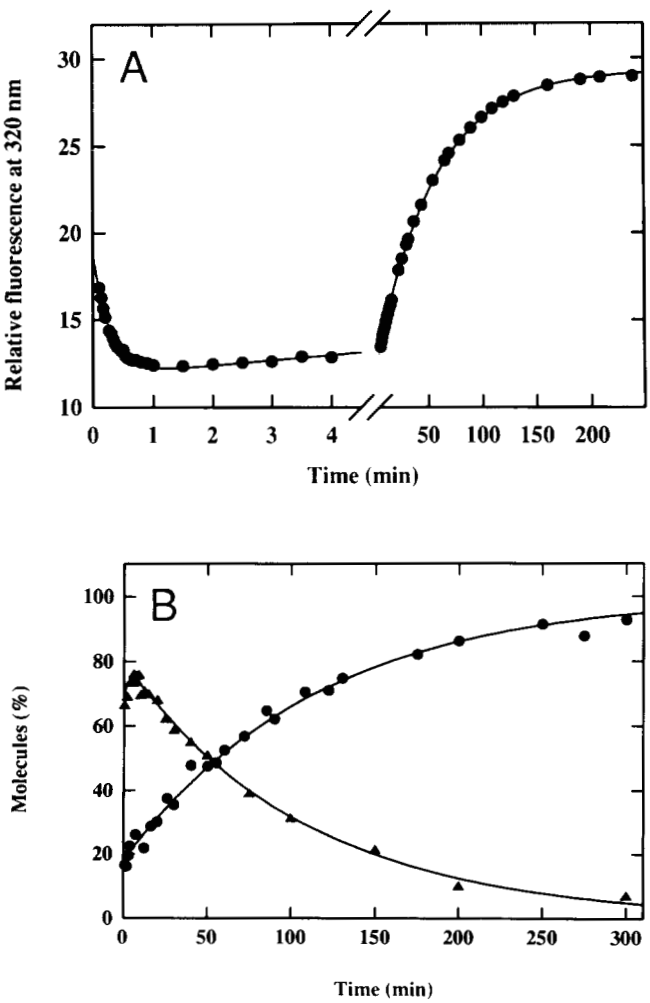


Fig. 2. A: Unfolding of the transient folding intermediate of (S54G, P55N)-RNase T1 in 2.8 M GdmCl, 0.1 M Na acetate, pH 5.0 at 10 °C. Unfolded RNase T1 (in 6.0 M GdmCl, 0.1 M glycine, pH 2) was diluted 40-fold into 0.1 M Na acetate, pH 5.0 at 10 °C to initiate refolding. After 5 min refolding was interrupted, the sample was transferred to 2.8 M GdmCl, and the unfolding reaction of the folding intermediate was monitored by the decrease in tryptophan fluorescence at 320 nm after excitation at 268 nm. The slow increase in fluorescence following the unfolding reaction of the intermediate originates from the slow refolding of denatured molecules to the native protein at 2.8 M GdmCl. Nonlinear least-squares fit of the data gave relaxation times of 15 s for the unfolding reaction of the intermediate and of 3,500 s for the subsequent slow-refolding reaction. The protein concentration was 3.2 μM in the refolding step. **B**: Time course of formation and decay of the folding intermediate (▲) and kinetics of formation of native molecules (●) during refolding of (S54G,P55N)-RNase T1 in 0.1 M Na acetate, 0.15 M GdmCl, pH 5.0 at 10 °C. The amount of intermediate was measured after various times of refolding according to the method shown in Figure 2A. The amount of intermediate was normalized assuming that the intermediate has 40% of the fluorescence of the native protein at 320 nm (cf. Fig. 4). The amount of native protein was determined by diluting unfolded RNase T1 (in 6.0 M GdmCl, 0.1 M glycine, pH 2.0) 40-fold with 0.1 M Na acetate, pH 5.0 at 10 °C. After various times of refolding (t_i) aliquots of the refolding solution are transferred into 5.2 M GdmCl, 0.1 M glycine, pH 2.0. Under these conditions native RNase T1 unfolds with a relaxation time of 11 s, whereas the unfolding reaction of the folding intermediates is too fast to be measured. The observed unfolding amplitude is therefore a direct measure of the amount of native protein that was present in the refolding solution at the time t_i. Unfolding of the native protein was measured by the decrease in tryptophan fluorescence at 320 nm after excitation at 268 nm. The protein concentration was 3.2 μM in the refolding step. The solid lines represent nonlinear least-squares fits of the data, which give relaxation times of 170 s for the slow formation of the intermediate (amplitude = 12%), of 6,500 s for the decay of the intermediate, and of 6,500 s for the formation of native protein. Additionally, 17% of the unfolded molecules form the native protein and 71% form the intermediate within the dead-time of the refolding experiments.

Table 1). It can be described by a single first-order reaction that is undergone by 83 ± 2% of all molecules (τ = 6,500 s at pH 5.0 and 10 °C). This reaction correlates to the kinetics of disappearance of the folding intermediate. This strongly suggests that the intermediate lies on the folding pathway and that it is converted to native protein in the rate-limiting step of folding. A lag phase in the formation of native molecules is not observed, since the formation of the preceding folding intermediate is much faster than its conversion to the native state. The remaining 17 ± 2% of the native protein are regained rapidly in the dead-time of the experiment in the course of the fast $U_F \rightarrow N$ reaction.

Structural properties of the folding intermediate

The rapid formation of the intermediate and its slow conversion to the native protein facilitate the characterization of its structural properties. The far-UV CD spectra of the intermediate and of the completely refolded protein are compared in Figure 3A. (S54G,P55N)-RNase T1 was refolded at pH 5 and 10 °C. Under these conditions the intermediate forms very rapidly and is then converted to the native protein with a relaxation time of 6,500 s (cf. Fig. 2B). After 20 s of refolding a CD spectrum in the peptide region was recorded. As in wild-type RNase T1 the ellipticity of the intermediate is slightly more negative than the CD of the native protein (Fig. 3A). This difference in CD is most pronounced near 227 nm (Fig. 3A) and it probably originates from an altered environment of the tryptophan and/or of tyrosine residues at the stage of the folding intermediate (Kiefhaber et al., 1990c). Ordering of aromatic residues can contribute significantly to the CD of native proteins near 230 nm (Adler et al., 1973; Manning & Woody, 1989; Khan et al., 1989; Kuwajima et al., 1991). Spectra recorded after 20 s and after 10 min of refolding, when the additional formation of the intermediate via the slow reaction (τ = 170 s) is complete (cf. Fig. 2B) are identical. After the rapid formation of the native like peptide CD spectrum, the ellipticity around 227 nm slowly decreases in magnitude and finally matches the value of the native protein. This decrease follows the same kinetics as the slow phase observed in fluorescence measurements and in unfolding assays (τ = 6,500 s at pH 5 and 10 °C). A similar rapid regain of a native-like CD spectrum was also observed when refolding was performed at pH 8 both at 10 and 25 °C. Above pH 8, however, only a small amount of the peptide CD signal is restored after 20 s, and the spectrum of the native protein is not completely recovered at the end of refolding.

A similar procedure was used to obtain CD spectra of the folding intermediate in the aromatic region. Again, the spectra after 20 s and after 10 min of refolding are very similar and resemble the spectrum of the native protein in the region between 275 and 320 nm. Pronounced

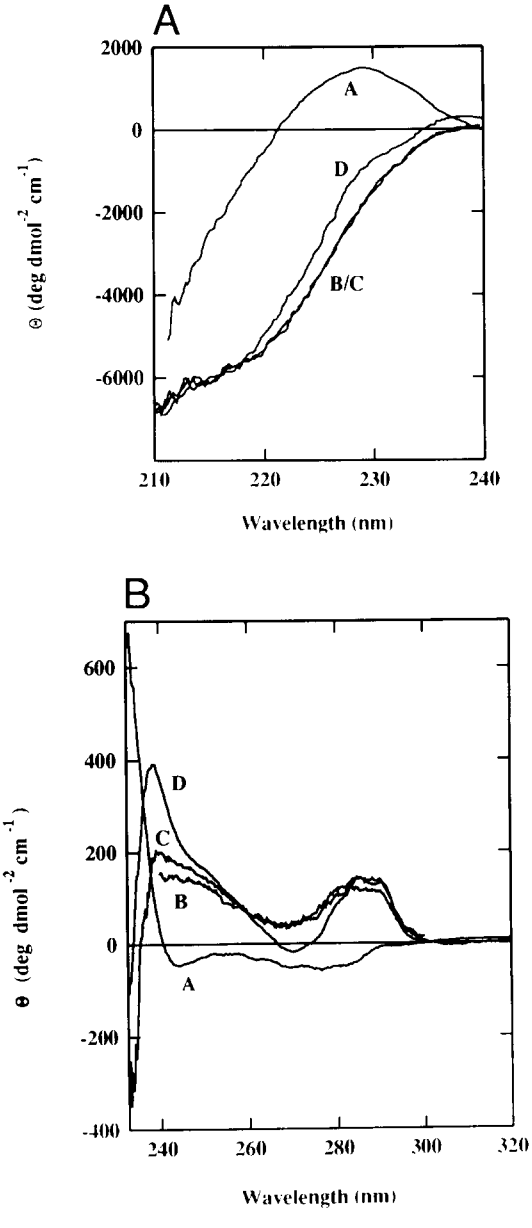


Fig. 3. **A**: CD spectra in the peptide region of (S54G,P55N)-RNase T1 of the unfolded protein (A), of refolding molecules after 20 s (B) and after 10 min (C) of refolding, and of the refolded protein (D). The spectrum of the refolded protein is identical with the CD spectrum of the native protein before denaturation. The spectrum of the unfolded protein was recorded in 6.0 M GdmCl, 0.1 M Na acetate, pH 5.0 at 10 °C, all other samples were in 0.15 M GdmCl, 0.1 M Na acetate, pH 5.0 at 10 °C. All spectra are corrected for the respective buffers. Refolding of denatured RNase T1 (in 6.0 M GdmCl, 0.1 M Na acetate, pH 5.0) was initiated by 40-fold dilution with 0.1 M Na acetate, pH 5.0 at 10 °C in the CD cuvette. Spectra were recorded from 240 to 210 nm with a scan speed of 20 nm/min and a time constant of 1 s in 0.1-cm thermostatted cuvettes. The spectra of the native and of the unfolded protein were accumulated eight times. The protein concentration was 27 μM. **B**: CD spectra of (S54G,P55N)-RNase T1 in the aromatic region of the unfolded protein (A), of refolding molecules after 20 s (B) and after 10 min (C) of refolding, and of the refolded protein (D). The spectrum of the refolded protein is identical with the spectrum of the native protein. For the experiments 1-cm thermostatted cuvettes were used; all other experimental conditions were as in Figure 3A.

differences between the spectra of the native protein and the folding intermediate were observed near the minimum at 270 nm and near the maximum at 230 nm. The native CD in these regions is restored only during the final very slow reaction from the intermediate to the native structure. The fast recovery of the native-like aromatic CD above 275 nm is observed at 10 and at 25 °C, both at pH 5 and at pH 8.

The enzymatic activity of the folding intermediate of (S54G,P55N)-RNase T1 was determined after 20 s and after 10 min of refolding. These measurements showed that 50 ± 5% of the RNase activity is regained at the stage of the folding intermediate. At this stage, however, only about 20% native molecules are present in the refolding solution (Fig. 2B). This suggests that the folding intermediate is about 40% as active as the native protein.

Fluorescence of the folding intermediate

The tryptophan fluorescence of native RNase T1 is blue-shifted with a maximum at 320 nm and shoulders near 328 nm and 307 nm. The fine structure of the fluorescence spectrum is indicative of the strongly hydrophobic environment of the single tryptophan residue (Trp 59) of RNase T1 (Lakowicz, 1983). To compare the fluorescence spectra of the folding intermediate and of the native protein the same experimental procedure as in the CD measurements was applied. Fluorescence emission spectra were recorded after 20 s and after 10 min of refolding. Figure 4 shows the spectra of the folding intermediate at pH 5 and 10 °C, of the native protein and of the protein in the denatured state (in 6 M GdmCl). The emission maximum is shifted from 349 nm (in the unfolded state) to 325 nm within the first few seconds of folding. The prominent shoulders near 328 nm and 307 nm are, however, absent and the maximum is broadened. The fine structure of the spectrum is regained in the slow interconversion of the folding intermediate into native protein. This indicates that Trp 59 is already in a hydrophobic environment at the stage of the folding intermediate, although the packing of the residues around Trp 59 might not be as close and rigid as in the native protein.

The local environment of tryptophan residues in folded proteins determines their fluorescence lifetimes. Excited Trp 59 of RNase T1 is known to decay with two lifetimes in unfolded as well as in native wild-type RNase T1 at pH 8 and 20 °C, but with a single lifetime in the native protein at pH 5 and 20 °C. At lower temperatures (10 °C), only a single lifetime can be detected in native RNase T1 both at pH 8 and pH 5. The two fluorescence lifetimes at pH 8 and 20 °C were suggested to originate from the existence of two conformations of the indole ring of Trp 59 (Chen et al., 1987). Only a single conformation is apparently available for Trp 59 at pH 5 and at lower temperatures. This is probably due to the higher stability of the folded state under these conditions (Kiefhaber et al., 1990d). In (S54G,P55N)-RNase T1 also, both at pH 8 and at pH 5 at 10 °C only a single fluorescence lifetime can be resolved in the native state (Table 2).

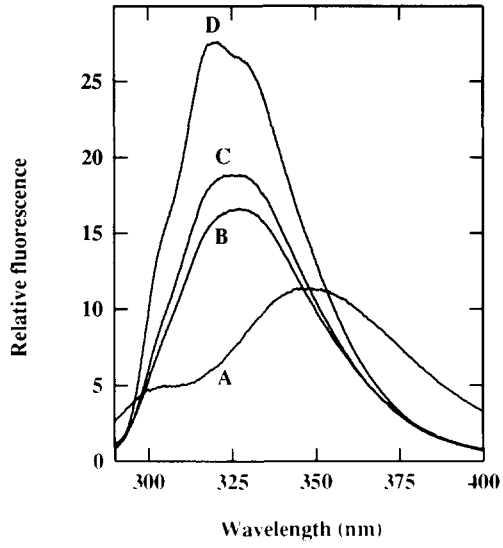


Fig. 4. Fluorescence spectra of (S54G,P55N)-RNase T1 in the unfolded state (A), of refolding molecules 20 s (B) and 10 min (C) after initiation of refolding, and of the completely refolded protein (D) at pH 5 and 10 °C. Refolding was initiated by diluting unfolded molecules (in 6.0 M GdmCl, 0.1 M Na acetate, pH 5.0) 40-fold with 0.1 M Na acetate, pH 5.0 at 10 °C. After the indicated time intervals fluorescence emission spectra were recorded with a scan speed of 60 nm/min. Excitation was at 268 nm. All spectra were corrected for the respective buffer. The spectrum of the unfolded protein was recorded in 6.0 M GdmCl, 0.1 M Na acetate, pH 5.0 at 10 °C. The protein concentration was 0.38 μM.

Because the conversion of the intermediate to the native protein during the folding process is very slow in (S54G,P55N)-RNase T1 at 10 °C ($\tau \approx 6{,}500$ s) we were able to measure the fluorescence lifetimes of Trp 59 in the folding intermediate. Table 2 shows that two lifetimes of 3.54 ± 0.08 ns (amplitude = 89%) and 1.37 ± 0.29 ns are found for the intermediate at pH 5 in contrast to the single lifetime of the completely refolded protein. These lifetimes are different from the two lifetimes of the denatured protein both in their values and in their amplitudes (Table 2). They are, however, similar to the two lifetimes of 3.78 ns (amplitude = 89%) and 1.5 ns that are found in the native native state of the wild-type protein at pH 8 and 20 °C (Chen et al., 1987). The existence of two fluorescence lifetimes of Trp 59 at the stage of the intermediate may indicate that the structure of the intermediate is not as rigid as the native structure and thus the fluorophore can exist in two different conformations.

The accessibility of tryptophan residues in a protein can be estimated from fluorescence quenching experiments. We used quenching by acrylamide to compare the accessibility of Trp 59 in the unfolded state, in the folding intermediate, and in the native protein. The decrease in the fluorescence lifetime with increasing concentrations

Table 2. *Comparison of the fluorescence properties of the folding intermediate with native and unfolded (S54G,P55N)-RNase T1*[a]

Species	Conditions	τ_1 (ns)[b]	τ_2 (ns)[b]	$\langle\tau\rangle$ (ns)[b]	f_1	k_q (M^{-1} s^{-1})[c]
NATA	pH 7	2.9 ± 0.1[d]	Single exponential		1.00	$70 \cdot 10^8$[e]
NATA	pH 7, 6 M GdmCl	2.9 ± 0.1[d]	Single exponential		1.00	n.d.
N	pH 5	4.08 ± 0.06	Single exponential		1.00	$0.88 \cdot 10^8$
N	pH 8	4.03 ± 0.01	Single exponential		1.00	$0.68 \cdot 10^8$
U	pH 5, 6 M GdmCl	4.64 ± 0.04	1.02 ± 0.04	3.55 ± 0.01	0.80	$10.05 \cdot 10^8$
I (45 s)	pH 5	3.54 ± 0.08	137 ± 0.29	n.d.	0.89	n.d.
I (10 min)	pH 5	3.66 ± 0.14	1.73 ± 0.47	3.32 ± 0.01	0.87	$0.84 \cdot 10^8$
Refolded	pH 5	4.14 ± 0.03	Single exponential		1.00	$0.69 \cdot 10^8$
I (45 s)	pH 8	4.21 ± 0.13	1.87 ± 0.42	3.75 ± 0.02	0.88	$1.01 \cdot 10^8$
Refolded	pH 8	3.94 ± 0.01	Single exponential		1.00	$0.59 \cdot 10^8$

[a] Refolding was initiated at 10 °C by 40-fold dilution of unfolded protein (in 6 M GdmCl, 0.1 M NaAc, pH 5.0) into the indicated refolding conditions. The protein concentration was 90 μM.

[b] τ_1 and τ_2 denote the observed fluorescence lifetimes; $\langle\tau\rangle$ is the mean lifetime for the case that more than one fluorescence lifetime is detected.

[c] Under conditions where two fluorescence lifetimes are detected the mean lifetime ($\langle\tau\rangle$) is used to calculate the quenching constant; n.d., not determined.

[d] From Grinvald and Steinberg (1976).

[e] From Eftink and Ghiron (1976).

of the quencher was measured. This dynamic method is preferable, because, in contrast to static determinations of fluorescence intensities, the results depend only on quencher concentration, not on protein concentration. Figure 5 shows the respective Stern–Vollmer plots for the native protein, the denatured protein, and for the folding intermediate after 10 min of refolding at pH 5.0 and 10 °C. Because the second, shorter lifetime of the folding intermediate shows only a very small amplitude, mean lifetimes were used to calculate the Stern–Vollmer constants (K_{SV}) for the folding intermediate; this also facilitates comparison of the lifetimes of the intermediate with those of the native protein. Obviously, the accessibility to the quencher of Trp 59 is already drastically reduced in the intermediate with a Stern–Vollmer constant of 0.28 M^{-1} compared to 3.47 M^{-1} for the unfolded molecules. The Stern–Vollmer constant of the intermediate and of the refolded protein are identical. According to Equation 1, the respective quenching constants are $10.05 \cdot 10^8$ M^{-1} s^{-1} for the unfolded protein, $0.85 \cdot 10^8$ M^{-1} s^{-1} for the folding intermediate, and $0.69 \cdot 10^8$ M^{-1} s^{-1} for the native protein (Table 2). This means that the accessibility of Trp 59 for acrylamide is only slightly different in the folding intermediate and in the native state, but it is largely reduced in both states compared to the unfolded state. Similar results were obtained when folding was performed at pH 8 and 10 °C (Table 2).

Kinetics of formation of the folding intermediate

Because the recovery of the amide CD signal was complete within the dead-time of manual mixing (20 s), the stopped-flow technique was applied to monitor the rapid formation of the folding intermediate. Figure 6 shows the change in the CD signal at 225 nm in the first few seconds after the initiation of refolding at pH 5 and 25 °C. Obviously, the entire CD signal that was observed after 20 s in the manual mixing experiments (Fig. 4A) was already recovered within the dead-time of the stopped-flow experiments (15 ms). The same results were obtained when refolding was performed at pH 8 and 25 °C.

Additionally, part of the fluorescence change between native and unfolded (S54G,P55N)-RNase T1 occurs very rapidly in the time course of refolding. About 60% of the fluorescence signal is recovered in the slow refolding phases, whereas the remaining 40% is regained in the dead time of mixing (1 s with the use of a cuvette with an integrated magnetic stirrer) both at 10 °C and at 25 °C (Table 1). Because the U_F species comprises about 17% of the unfolded molecules, the rapid step on the pathway of the U_S molecules causes a change in fluorescence at 320 nm of about 30%. This indicates that major changes in fluorescence that are associated with the formation of the intermediate also occur in the time range of milliseconds.

Discussion

We have simplified the problem of investigating the structural properties of folding intermediates of RNase T1 with incorrect prolyl isomers by investigating a variant, (S54G,P55N)-RNase T1, which contains only one instead of two cis prolyl peptide bonds. In this variant the number of folding intermediates is greatly reduced compared to the wild-type protein, facilitating the interpretation of

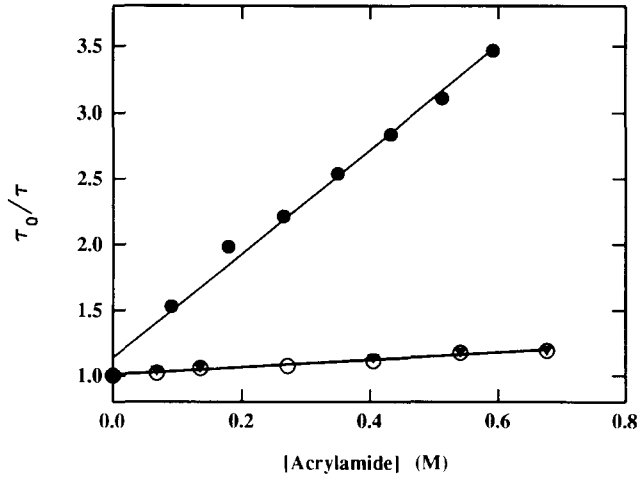


Fig. 5. Stern-Vollmer plots for quenching of the tryptophan fluorescence of (S54G,P55N)-RNase T1 by acrylamide. Quenching of the unfolded protein (●), of refolding molecules after 10 min of refolding (▼), and of completely refolded molecules (○) in 0.1 M Na acetate, pH 5.0 at 10 °C are displayed. The lifetimes of the denatured protein were measured in 6.0 M GdmCl, 0.1 M Na acetate, pH 5.0 at 10 °C. Refolding was initiated by 40-fold dilution of unfolded protein (in 6.0 M GdmCl, 0.1 M Na acetate, pH 5.0) into 0.1 M Na acetate, pH 5.0 at 10 °C. In the case of the unfolded protein and of the refolding molecules, where two lifetimes were observed, the mean lifetimes were used to derive τ_0/τ. Analysis of the data according to Equation 1 gives Stern-Vollmer constants (K_{SV}) of 3.57 M^{-1} for the unfolded protein and of 0.28 M^{-1} for both the refolding solution after 10 min of refolding and for the renatured protein. The protein concentration was 90 μM.

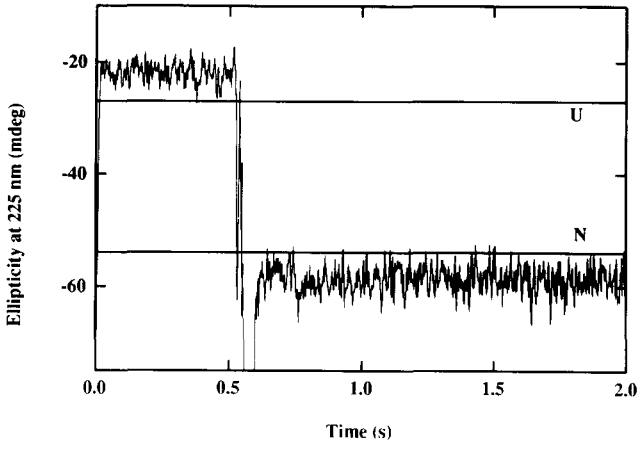


Fig. 6. Stopped-flow measurement of the recovery of the far UV CD signal during refolding of (S54G,P55N)-RNase T1 in 0.1 M Na acetate, 0.15 M GdmCl, pH 5.0 at 25 °C. In the stopped-flow device syringe A was filled with 0.1 M Na acetate, pH 5.0 and syringe B contained denatured (S54G,P55N)-RNase T1 (in 6.0 M GdmCl, 0.1 M glycine at pH 2.0). In a first step 800 μL from syringe A were injected into the optical cell and the signal of the buffer solution was monitored. After 500 ms 20 μL of the unfolded protein (syringe B) were mixed with 780 mL of the buffer solution (syringe A) to initiate refolding. The delay time from the actual mixing of the two solutions to the appearance of the CD signal was 15 ms. The sampling interval was 2 ms and the filtering time constant was 1 ms. The average of 20 successive kinetics is shown. The protein concentration was 90 μM.

the experimental results (Kiefhaber et al., 1990a; Scheme I). In refolding about 83% of unfolded (S54G,P55N)-RNase T1 molecules regain the native state slowly (U_S molecules), whereas the remaining 17% can refold within a few milliseconds (U_F molecules). Slow refolding of (S54G,P55N)-RNase T1 is largely determined by unfolded molecules with a single incorrect prolyl isomer (presumably a trans Pro 39). About 85% of all U_S molecules appear to refold on a single pathway that involves the very rapid formation of a partially folded intermediate with an incorrect Pro 39. The secondary structure of this intermediate is formed within the first 15 ms of refolding and is too fast to detect by the use of stopped-flow mixing techniques. This indicates that the relaxation time of its formation is in the time range of a few milliseconds or faster. The remaining 15% of the U_S molecules form the same intermediate in a slow reaction (τ = 170 s in unfolding assays and 340 s in fluorescence at pH 5.0 and 10 °C) that involves prolyl isomerization and is catalyzed by peptidyl-prolyl cis-trans isomerase (cf. Table 1). In addition to an incorrect Pro 39, these molecules probably have one of the two trans prolyl peptide bonds (at Pro 60 and Pro 73) in the nonnative conformation. These molecules, with additional wrong prolyl isomers, can apparently also form some kind of ordered structure rapidly, since the CD signal in the far UV region does not change during the formation of the intermediate in the slow phase. The structure of the intermediate is, however, stabilized after these prolyl bonds have re-isomerized to their native trans state.

The conversion of the intermediate to native RNase T1 is very slow (τ = 6,500 s at pH 5 and 10 °C) and is probably limited in rate by the trans to cis isomerization of the Tyr 38-Pro 39 peptide bond (cf. Scheme Ib and Kiefhaber et al., 1992). Because the folding intermediate is formed rapidly and is converted very slowly into the native state, it persists for an extended time. This facilitates its characterization. The intermediate has several properties that are reminiscent of a molten globule or collapsed intermediate; its tertiary structure is, however, more extensive than that expected for a molten globular state.

The native-like peptide CD of the folding intermediate indicates that at least the single extended α-helix (Ser 13-Asp 29; Kinemage 2) of RNase T1 is already present very early in folding. The transient occurrence of a peptide CD signal that is more negative than the CD of the native protein is probably related to dichroic properties of aromatic residues, which show positive CD bands in folded proteins around 225 nm (Adler, 1974; Khan et al., 1989). These positive bands are apparently not formed until the rate-limiting, slowest step of folding. Although the folding intermediate and the native structure show similar CD spectra in the region above 280 nm, significant differences exist around 270 nm and 235 nm. These results indicate that some ordering around aromatic residues of

RNase T1 has already occurred at the stage of the intermediate. During the slow formation of native protein, however, structural changes in the environment of aromatic residues seem to take place, giving rise to new CD bands. An assignment of the CD bands to individual residues is not possible, because RNase T1 contains a high number of aromatic residues (1 Trp, 9 Tyr, and 4 Phe out of 104 amino acids).

When the intermediate is formed the fluorescence emission maximum of the single Trp residue is shifted from 349 nm (in the denatured state) to 325 nm. The fine structure of the emission spectrum with shoulders near 307 nm and 328 nm is, however, not apparent until the intermediate is slowly converted to the native protein in the rate-limiting re-isomerization of Pro 39. Structured emission spectra for Trp fluorescence are very rarely observed in proteins and indicate an extremely hydrophobic environment of the fluorophore (Lakowicz, 1983). This is consistent with the molecular environment of Trp 59, which is completely buried in the hydrophobic core of native RNase T1. In the folding intermediate, Trp 59 shows two fluorescence lifetimes in contrast to a single lifetime in native (S54G,P55N)-RNase T1. The two lifetimes of Trp 59 in the folding intermediate are, however, different from the two lifetimes observed in the denatured state. The observation of two fluorescence lifetimes has earlier been reported for wild-type RNase T1 under slightly destabilizing conditions like high temperature, high pH, or in the presence of increased concentrations of GdmCl (Chen et al., 1987; Gryczynski et al., 1988) and probably originates from two distinct conformations of Trp 59 (Chen et al., 1987). Presumably, Trp 59 of (S54G,P55N)-RNase T1 can also exist in at least two distinct conformations at the stage of the folding intermediate. Upon conversion to the native state the tryptophan residue seems to become immobilized and only a single fluorescence lifetime is observed.

These findings, together with the low accessibility of Trp 59 for quenching molecules, suggest that a hydrophobic core and defined tertiary contacts already exist at the stage of the folding intermediate. This is in agreement with our earlier findings that Trp 59 interacts with the incorrect Tyr 38–Pro 39 peptide bond at the stage of the folding intermediate and thereby decreases its rate of isomerization (Kiefhaber et al., 1992). The native-like organization of the environment of Trp 59, however, is not achieved until the completion of the final step of folding, when the very slow trans to cis isomerization of the prolyl peptide bond has occurred. This is not surprising, since Trp 59 and Pro 39 are in close contact within the hydrophobic core of the native structure (Kinemage 2). Consequently, the tertiary interactions in the hydrophobic core are optimized in the rate-limiting isomerization of proline 39. It leads to the increase in fluorescence intensity, the recovery of the fine structure of the fluorescence spectrum with a maximum at 320 nm, and shoulders at 307 and 328 nm, and to the disappearance of the second, minor fluorescence lifetime of Trp 59.

In addition to the recovery of most of the structural elements, the folding intermediate of RNase T1 exhibits about 40% of the RNase activity of the native protein. The active site of RNase T1 comprises several residues (His 40, Glu 58, His 92), which are part of the major antiparallel β-sheet structure (Kinemages 1, 2). This indicates that at least part of this structure is already organized in a native-like way in the intermediate. This is a significant finding, since sheet structures are more difficult to determine by CD spectroscopy than helices, particularly when the accessible wavelength range is limited by the strong absorbance of denaturants in residual concentrations.

Another indicator of the high structural stability of the folding intermediate of (S54G,P55N)-RNase T1 is its slow-unfolding kinetics at high concentrations of GdmCl (τ = 15 s at 2.8 M GdmCl, pH 5, 10 °C). This is in contrast to equilibrium intermediates of the molten globule type, which are typically in rapid equilibrium with the unfolded state.

In summary, the intermediate that forms rapidly during slow refolding of (S54G,P55N)-RNase T1 shows more extensive native-like properties than expected for an intermediate of the molten globule type. Apparently, under favorable conditions incorrect prolyl isomers do not arrest chain folding at the stage of a collapsed intermediate without specific tertiary contacts. Evidently, if a molten globule forms initially during the folding of RNase T1, it is very short lived and folding then advances very rapidly to an extensively structured intermediate that accumulates prior to the rate-limiting re-isomerization of the incorrect prolyl isomer. The presence of such an incorrect isomer strongly decreases the rate of renaturation from the time range of milliseconds to that of hours. It does, however, allow extensive chain folding to occur until a form is reached that resembles the native state in several properties. These findings agree with models of protein folding in which native-like structure is formed early in folding and the rate-limiting step of folding involves structurally subtle but kinetically slow rearrangements. Extensive structure formation in the presence of nonnative prolyl isomers has previously been predicted by Levitt (1981) on the basis of conformational energy calculations and has been observed in the folding of ribonuclease A (Cook et al., 1979). In the case of RNase A, however, the formation of a native-like structure is slowed down drastically by the presence of nonnative prolyl isomers (Schmid & Blaschek, 1981; Krebs et al., 1985).

Our results imply that proteins can form compact folding intermediates rapidly that have definite secondary and tertiary structure elements and also partial catalytic activity, even in the presence of wrong prolyl isomers. Under favorable conditions, i.e., under conditions, where the native state is very stable, most of the structure forma-

tion can occur in these fast-folding steps, which are probably comparable to the folding reactions of the U_F molecules that have all prolyl peptide bonds in their native conformation. In contrast to the folding of the U_F molecules, which yields native protein, the fast-folding reactions of the U_S molecules lead to transient-folding intermediates.

Materials and methods

(S54G,P55N)-RNase T1 was a gift from U. Hahn, Berlin. 2'GMP and GpC were purchased from Sigma (St. Louis, Missouri). GdmCl, ultrapure was from Schwarz/Mann (Orangeburg, New York). All other chemicals were from Merck (Darmstadt, Germany).

Fluorescence spectra and fluorescence kinetics were recorded on a Hitachi F4010 fluorimeter, equipped with a magnetic stirrer. Absorbance was measured in a Kontron Uvikon 860 spectrophotometer. For CD measurements a Jasco J600 spectropolarimeter was used. Stopped-flow CD measurements were performed with an SFM3 stopped-flow mixing device from Bio-Logic connected to a Jobin-Yvon CD6 spectrodichrograph. In all measurements the cuvettes were thermostatted at the indicated temperature. The temperature was checked before and after each experiment.

Refolding kinetics were performed by 40-fold dilution of denatured RNase T1 (in 6.0 M GdmCl, 0.1 M Na acetate, pH 5) with 0.1 M Na acetate/HAc, pH 5.0 or with 0.1 M Tris/HCl, pH 8.0 at the indicated temperatures. The activity of RNase T1 was measured by the increase in absorbance at 257 nm upon hydrolysis of the dinucleotide GpC. The enzyme was diluted 40-fold into a GpC solution (in 10 mM Tris/HCl, 2mM EDTA pH 8.0 or in 10 mM Na acetate, 2 mM EDTA, pH 5.0, at 10 °C). The concentration of GpC was adjusted such that the absorbance at 257 nm was 0.8 in a 1-cm cuvette. To prevent further refolding of RNase T1 in the activity assay, 5 μM trypsin was added to the GpC solution (Kiefhaber et al., 1990b). The increase in absorbance upon GpC hydrolysis was measured for 5 min and the value of ΔA_{257}/min was used as a measure for the enzymatic activity.

Unfolding assays for the native protein and for the transiently formed intermediate were performed as described previously (Kiefhaber et al., 1990b,c). Unfolded (S54G,P55N)-RNase T1 (in 5.0 M GdmCl, 0.1 M Na acetate, pH 5.0) was diluted 40-fold with 0.1 M Na acetate, pH 5.0 or into 0.1 M Tris/HCl, pH 8.0 at 10 °C to initiate refolding. After various times of refolding (t_i) aliquots of the refolding solution were transferred to conditions where either the folding intermediate (at 2.8 M GdmCl, pH 5.0, 10 °C) or the native protein (at 5.2 M GdmCl, 0.1 M glycine pH 2.0, 10 °C) could be unfolded. The amplitude of the respective unfolding reaction served as a measure for the amount of intermediate or native protein that was present in the refolding solution at the time t_i, respectively. The protein concentration was 3.2 μM in the refolding step.

The GdmCl induced unfolding transition of (S54G, P55N)-RNase T1 was measured in 0.1 M Na acetate, pH 5.0 at 10 °C. After the equilibrium was attained (24 h) the fluorescence of the samples was determined at 320 nm (10-nm band width) after excitation at the isosbestic point at 268 nm (1.5-nm band width). The GdmCl concentrations were determined by measuring the refraction of the samples (Pace, 1986). A nonlinear least-squares fit according to Santoro and Bolan (1988) was used to obtain the m-value and $\Delta G(H_2O)$ of the transition. All kinetics and transition curves were analyzed using the programs Kinfit and Grafit.

Fluorescence lifetime measurements

A fully automatic phase fluorimeter was used for the detection of fluorescence lifetimes (Clays et al., 1989). The excitation source consisted of a mode-locked cavity-dumped dye laser and a frequency doubler to obtain light with a wavelength of 295 nm. The output is a train of pulses of about 20 ps width and a frequency of 0.4 MHz. Fourier analysis shows that such a periodic function contains the basic frequency of 0.4 MHz and all its harmonics up to several hundreds of GHz. The emission part consists of a monochromator and a fast photomultiplier (sample: Philips, UV-sensitive, model XP2020; reference: RCA, model 1P28). The gain of the photomultiplier is modulated with a chosen harmonic plus a small increment of 700 Hz. The output of the photomultiplier is the product of the periodic fluorescence signal and the modulation function. Out of this product the 700-Hz term is filtered and phase measurements are performed at this low frequency (cross-correlation). The frequency synthesizers used to drive the mode locker and to modulate the photomultiplier gain are coupled to the same crystal to improve the phase stability. With this method, phase-shift data can be read out within a short measurement time (14 min) and data can be collected at many modulation frequencies.

Detailed statistical techniques were used in the data analysis of phase fluorimetry. Mean standard deviation and the percentage in the interval [−2, +2] of the weighted residuals are compared with the predicted values of 0, 1, and 95.5, respectively. An additional measure for the quality of the fit, the so-called Q-value, is also calculated. The autocorrelation function of the weighted residuals and a plot of these residuals as a function of the calculated phase shift are used in data analysis as additional visual tests. Data reduction was performed on a Micro VAX 2000 minicomputer using a nonlinear least-squares algorithm (Bevington, 1969).

Phase measurements were performed at 50 different frequencies between 1 MHz and 280 MHz. Five independent phase measurements were carried out to allow the

calculation of the standard deviation at each frequency. The excitation wavelength was fixed at 295 nm; the emission wavelength was set to 330 nm. To get accurate information we collected data from two or more measurements under the same conditions. Decisions on the number of exponentials that are necessary to fit the data were based mainly on the reduced χ^2-value and on the autocorrelation of the residuals.

Quenching experiments were carried out in the presence of different concentrations of acrylamide. The mean lifetimes were used for the analysis of the quenching of the fluorescence lifetimes. This can be justified, because the two lifetimes have very similar values and they both originate from the single tryptophan residue (Trp 59) in RNase T1. The quenching data were analyzed in terms of the Stern–Vollmer equation (Lakowicz, 1983):

$$\tau_0/\tau = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q], \qquad (1)$$

where τ_0 and τ are the fluorescence lifetimes in the absence of quencher and at a given concentration of quencher, respectively, K_{SV} is the Stern–Vollmer constant, k_q is the second-order rate constant for the collisional quenching of the fluorophore with the quencher, and $[Q]$ is the concentration of quencher.

Acknowledgments

We thank R.L. Baldwin, G. Böhm, A.V. Chakrabartty, M. Goldberg, D.V. Laurents, and L. Mayr for fruitful discussion and comments on the manuscript. We are indebted to U. Hahn and O. Landt for supplying us with RNase T1. This research was supported by grants from the Deutsche Forschungsgemeinschaft (Schm 444 /9-1 and Ki 437/1-1) and from the Fonds der Chemischen Industrie (to T.K. and F.X.S.).

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