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Folding of RNase T1 Is Decelerated by a Specific Tertiary Contact in a Folding Intermediate

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ABSTRACT **The replacement of tryptophan 59 of ribonuclease T1 by a tyrosine residue does not change the stability of the protein. However, it leads to a strong acceleration of a major, proline-limited reaction that is unusually slow in the refolding of the wild-type protein. The distribution of fast- and slow-folding species and the kinetic mechanism of slow folding are not changed by the mutation. Trp-59 is in close contact to Pro-39 in native RNase T1 and probably also in an intermediate that forms rapidly during folding. We suggest that this specific interaction interferes with the *trans* → *cis* reisomerization of the Tyr-38–Pro-39 bond at the stage of a native-like folding intermediate. The steric hindrance is abolished either by changing Trp-59 to a less bulky residue, such as tyrosine, or, by a destabilization of folding intermediates at increased concentrations of denaturant. Under such conditions folding of the wild-type protein and of the W59Y variant no longer differ. These results provide strong support for the proposal that *trans* → *cis* isomerization of Pro-39 is responsible for the major, very slow refolding reaction of RNase T1. They also indicate that specific tertiary interactions in folding intermediates do exist, but do not necessarily facilitate folding. They can have adverse effects and decelerate rate-limiting steps by trapping partially folded structures.**

Key words: folding mutant, proline isomerization, folding kinetics

INTRODUCTION

Folding of RNase T1 is a complex kinetic process that involves several fast and slow reactions.[1,2] Part of this heterogeneity originates from the simultaneous presence of fast-folding (U_F) and slow-folding (U_S) molecules in the unfolded state. About 4% of all unfolded molecules (the U_F species) regain the native structure in the milliseconds time range. The slow folding molecules (U_S, 96%) fold in a sequential manner. The rapid formation of partially folded structure is followed by several slow steps that determine the formation of native RNase T1. These reactions show time constants in the 250 sec range (termed the "intermediate" phase) and in the 7,000 sec range (the "very slow" phase) at pH 5 and 10°C. The intermediate phase is heterogeneous with contributions from a sequential and a single first-order reaction. The very slow phase appears to be a homogeneous first-order process. In a kinetic model for the slow folding of RNase T1 (Scheme I) we have assumed that after the rapid formation of partially folded intermediates, *trans* → *cis* isomerizations of two X-Pro peptide bonds become rate-limiting for the final slow steps. The kinetic properties of these reactions are compatible with the proline model[1] and, moreover, all observed slow kinetic phases are catalyzed by the enzyme peptidyl-prolyl *cis–trans* isomerase.[1–4]

RNase T1 contains four prolyl peptide bonds, two of which (at Pro-60 and at Pro-73) are *trans*, whereas the other two (at Pro-39 and at Pro-55) are *cis*.[5] In the kinetic model of Scheme I, we have speculated that the two *cis* prolines 39 and 55 could be responsible for most of the slow steps of RNase T1 folding. This hypothesis was supported by the folding properties of a mutant protein in which one of the *cis* X-Pro bonds, Ser-54–Pro-55, was replaced by a "normal" peptide bond, Gly-54–Asn-55. In this variant the intermediate sequential refolding phase is missing while the slowest phase remains essentially unchanged.[6] At the same time, the amount of U_F molecules increases 4-fold from 4 to 16%. Such an increase is expected when the replaced proline is predominantly in the incorrect (*trans*) state in the unfolded wild-type protein. The conclusions from these results were 2-fold. (1) The intermediate phase

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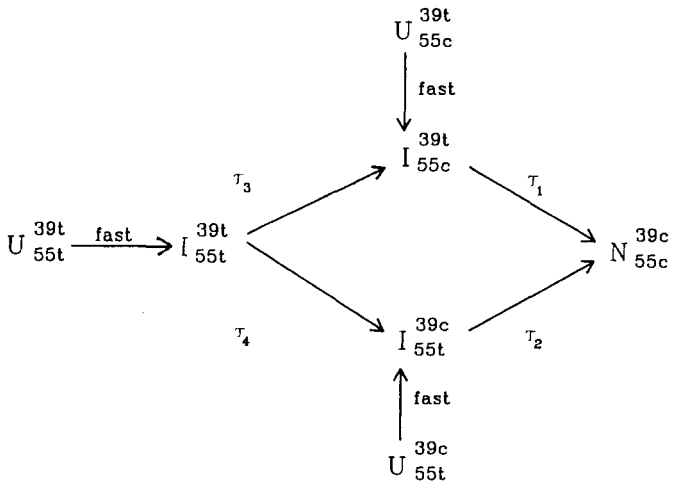
Abbreviations: RNase T1, Lys-25-isoenzyme of ribonuclease T1 from *Aspergillus oryzae;* W59Y RNase T1, ribonuclease T1 with tryptophan 59 replaced by tyrosine; RNase A, ribonuclease A; PPIase, peptidyl-prolyl *cis–trans* isomerase; GpC, guanylyl(3′→5′)cytidine; GdmCl, guanidinium chloride; NaAc, sodium acetate; N and U, native and unfolded protein, respectively; I, folding intermediate; U_S and U_F, slow and fast folding molecules, respectively.



Scheme I. Kinetic model for the slow refolding reactions of RNase T1 under strongly native conditions. U is the unfolded molecule, I are intermediates, and N the native protein. The superscript and the subscript denote the isomeric states of Pro-39 and Pro-55, respectively, in the native-like *cis* (c) and in the non-native *trans* (t) state. The relaxation times for wild-type RNAse T1 are τ_1 = 3,000 sec, τ_2 = 500 sec, τ_3 = 100 sec, τ_4 = 190 sec at pH 8.0, 10°C and τ_1 = 7,000 sec, τ_2 = 250 sec, τ_3 = 170 sec, τ_4 = 400 sec at pH 5.0, 10°C. The scheme is adapted from ref. 6.

of RNase T1 folding is related to the isomerization of Pro-55. (2) The very slow proline isomerization that is still present in the variant occurs in more than 80% of all molecules. Such large amplitudes are expected for the isomerization of prolines that are *cis* in the native protein, but predominantly *trans* in the unfolded polypeptide. The *trans* state is generally favored in the absence of ordered structure.[7]

The very slow refolding reaction of RNase T1 is unexpectedly slow compared to other proline-limited refolding kinetics[8–11] and also compared to the isomerization of the respective proline in the unfolded state, as measured by the "double jump" technique.[1] A tentative explanation has been that the incorrect isomer of this Xaa–Pro bond becomes "trapped" in a native-like intermediate and is thus sterically hindered from reaching the native isomer. Accordingly, addition of structure breaking salts, such as urea or GdmCl in nondenaturing concentrations, accelerates the very slow folding reaction.[2]

We speculated that *trans* → *cis* isomerization of the second *cis* Pro bond in RNase T1 (Tyr-38–Pro-39) might be rate-limiting for the very slow refolding phase. Attempts to obtain mutant forms of RNase T1 with a substitution at position 39 have failed so far. Pro-39 is conserved in all ribonucleases homologous to RNase T1 and may be important for the stability of the protein.

In an alternative approach, we tried to influence this very slow step by a mutation that leaves the Try-38–Pro-39 bond intact, but which is adjacent to Pro-39 in the native protein. In such an attempt, Trp-59 was replaced by a tyrosine residue (W59Y variant), since Trp-59 makes van der Waals contacts with Pro-39 (Fig. 1). Fluorescence measurements in combination with molecular dynamics studies had indicated that the fluorescence properties of Trp-59 are strongly influenced by the interaction with Pro-39.[12,13] Additionally, Waki et al. have shown that in chemically synthesized RNase T1, Trp-59 can be replaced by tyrosine without loss in activity.[14]

We report here that wild-type RNase T1 and the W59Y variant are similar in stability and folding. However, the major very slow refolding phase is strongly accelerated in the W59Y variant while the general mechanism of folding remains unchanged. This suggests that a specific tertiary contact of Trp-59, presumably with Pro-39, already exists in a folding intermediate and blocks the reisomerization of Pro-39. This hindrance can be relieved either by destabilizing the intermediate or by replacing Trp-59 by a smaller amino acid.

MATERIALS AND METHODS

Wild-type RNase T1 was purified from *Escherichia coli* cells that were transformed with a plasmid carrying a chemically synthesized gene, which was cloned and expressed as described elsewhere.[15] The W59Y variant was constructed according to the method of Kunkel et al.[16] and the protein was purified from the transformed cells using the procedure described for the wild-type protein.[15] GpC was from Sigma, St. Louis, MO. GdmCl ultrapure was from Schwarz/Mann, Orangeburg, NY. PPIase from pig kidney was a gift from Dr. Kurt Lang.

For spectroscopic measurements a Hitachi F-4010 fluorescence spectrophotometer with integrated stirrer and a Kontron Uvikon 860 spectrophotometer were used. Both photometers were equipped with thermostatable cell holders. CD measurements were performed in a Jasco J-600 spectropolarimeter. CD-spectra were recorded with 20 nm/min and a time

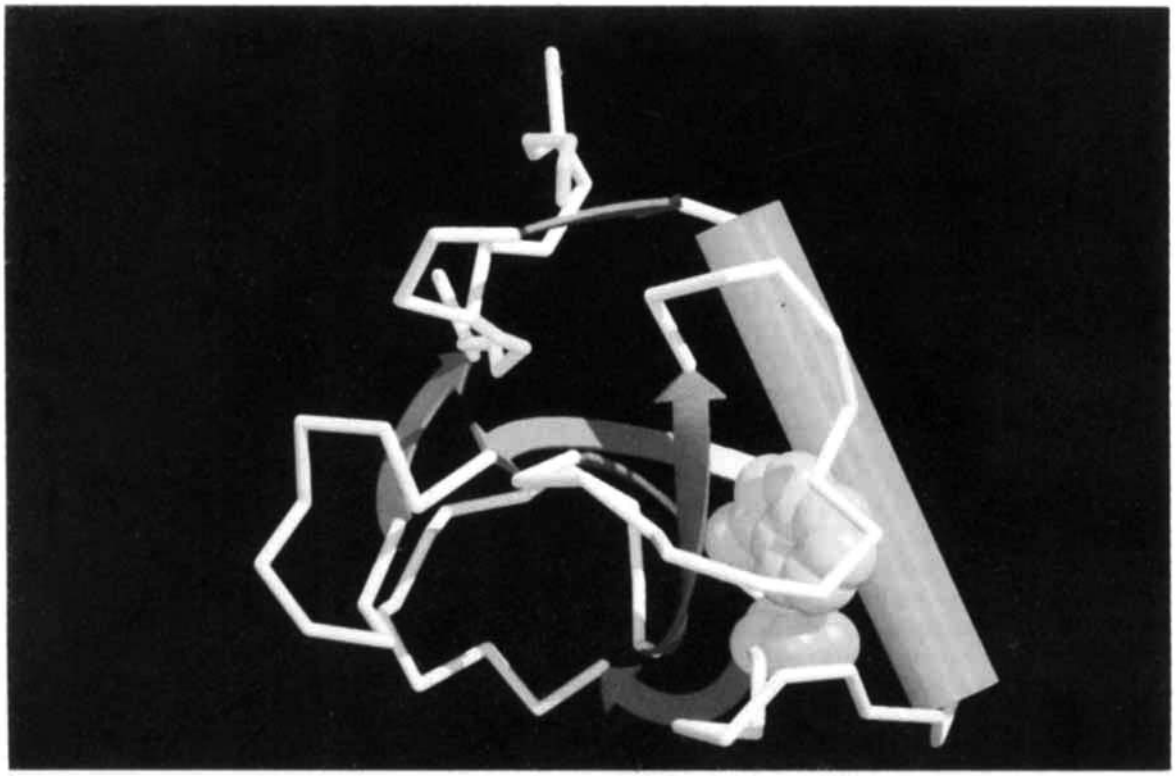


Fig. 1. Location of Trp59 (dark) and Pro-39 (white) in the three-dimensional structure of RNase T1.

constant of 2 sec in 1-mm thermostatable cuvettes. The band width was set to 1 nm. Spectra of the native and of the unfolded protein were accumulated 8 times. All spectra were corrected for the contributions of the respective buffer.

Thermal unfolding curves were measured in a Gilford 2400S spectrophotometer with a 2527 thermoprogrammer. Protein concentrations were 61 μM for the W59Y variant and 87 μM for the wild-type protein. The heating rate was 1 K/min and the path length was 1 cm. The thermal transition was measured by the change in absorbance at 287 nm. Unfolding was reversible as judged by the coincidence of heating and subsequent cooling transitions.

GdmCl-induced equilibrium unfolding transitions were measured in 0.1 M NaAc buffer containing 0–7 M GdmCl at pH 5.0 and 25°C. GdmCl concentrations were determined by measuring the refraction of the samples.[17] After equilibrium was attained (30 hr) the fluorescence was determined at 303 nm (5 nm band width) after excitation at 268 nm (5 nm band width) in 1-cm cells. In analogous experiments, the transitions were monitored by the change in absorbance at 287 nm and by the change in amide CD at 222 nm. In absorbance measurements a 1-cm cell and in CD measurements a 0.5-cm thermostatable cuvette was used. The band width of the CD-spectropolarimeter was set to 1 nm. Protein concentrations were 2.5 μM in fluorescence and 9.85 μM in absorbance and CD measurements. A nonlinear least-squares fit of the experimental data to Eq. (1) was used to obtain the m-values and $\Delta G°(H_2O)$ for each transition.[18]

$$Y_{obs} = [(Y_N + m_N[D]) + (Y_U + m_U[D]) \times \exp-(\Delta G^0{}_{N-U}/RT + m_G [D]/RT)]/[1 + \exp-(\Delta G^0{}_{N-U}/RT + m_G[D]/RT)]. \quad (1)$$

Y_{obs} is the observed experimental value, Y_N and Y_U are the values for the native and unfolded protein at zero denaturant, m_N and m_U are the slopes of the pre- and posttransitional baseline, respectively, $\Delta G^0{}_{N-U}$ is the difference in Gibbs free energy between native and unfolded protein, and $m_G = d\Delta G^0{}_{N-U}/d[\text{GdmCl}]$.

Refolding kinetics were initiated by a 40-fold dilution of unfolded RNase T1 (kept in 6.0 M GdmCl, 0.1 M NaAc, pH 5.0) with the appropriate refolding solution in a 1-cm spectrophotometer cell. The dead time of mixing was about 1 sec with the use of a magnetic stirrer below the optical cell. The kinetics of refolding were monitored by the change in fluorescence at 320 nm (wild-type) or 303 nm (W59Y variant) after excitation at the isosbestic point (268 nm), or by the increase in absorbance at 287 nm. Buffers employed were 0.1 M NaAc at pH 5.0 and 0.1 M Tris-HCl at pH 8.0.

The activity of RNase T1 was measured by the

increase in absorbance at 257 nm upon hydrolysis of the dinucleotide GpC. RNase T1 samples were 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 1-cm cell. The resulting increase in absorbance was measured for 5 min. The value of ΔA_{257}/min served as a measure of the activity of the enzyme.

Unfolding assays were performed as described by Kiefhaber et al.[1] Completely unfolded W59Y RNase T1 (in 6.0 M GdmCl, 0.1 M glycine-HCl buffer at pH 1.8) was diluted 30-fold with 0.1 M Tris-HCl pH 8.0 at 10°C. After various times of refolding (t_i) aliquots were withdrawn from the refolding solution and diluted 10-fold to give final conditions of 5.4 M GdmCl, 0.1 M glycine-HCl, pH 2.0 at 10°C. Under these conditions native RNase T1 unfolds with a relaxation time of 65 sec, while the unfolding of intermediates is complete within the dead time of mixing. The observed amplitude of unfolding is a measure for the amount of native molecules that are present after t_i. Aliquots of the sample were allowed to refold completely (at least 4 hr) and the corresponding amplitude of unfolding was set as 100%.

Figure 1 was created using the coordinate file 1RNT[5] and the program Raster3D on a Silicon Graphics 4D/70 workstation.

RESULTS

Stability of the W59Y Variant

The replacement of Trp-59 by a tyrosine residue left the stability of RNase T1 almost unchanged. The thermal unfolding transitions (Fig. 2A) display midpoints of 61.8 ± 0.5°C for the wild-type protein and 60.6 ± 0.5°C for the W59Y variant.

The unfolding transitions induced by GdmCl were determined by various methods. Absorbance at 287 nm and tyrosine fluorescence at 303 nm were used as probes for changes in tertiary structure, while the amide CD was used to monitor changes in secondary structure. The coincidence of the respective transition curves (Fig. 2B) suggests that they can be described by the two-state approximation.[19,20] The transition midpoint of 3.55 ± 0.05 M GdmCl for the variant is very close to the value of 3.70 ± 0.05 M GdmCl for wild-type RNase T1.[1] Linear extrapolation of the data to zero GdmCl[18,20,21] gives a $\Delta G°$ of 50 ± 5 kJ/mol for the W59Y variant compared to 45 ± 5 kJ/mol for the wild-type protein under the same conditions.[1] Apparently, the replacement of Trp-59 by tyrosine has only minor effects on the stability of RNase T1 and does not change the two-state character of the equilibrium unfolding transition.[23]

Slow Folding Kinetics

The slow folding kinetics of RNase T1 and the W59Y variant are compared in Figure 3. Since in

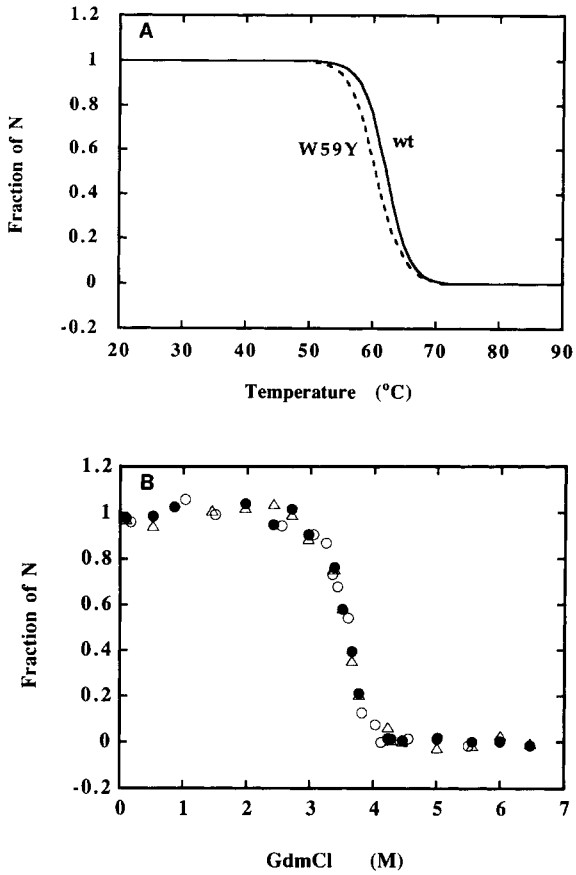


Fig. 2. **(A)** Thermal unfolding curves of wild-type (——) and W59Y (– – –) RNase T1 in 0.1 M NaAc at pH 5.0. Unfolding was monitored by the decrease in absorbance at 287 nm. Protein concentrations were 87 μM for the wild-type and 61 μM for the mutant protein; The heating rate was 1 K/min. The midpoints of transition are 61.8°C for wild-type and 60.6°C for W59Y RNase T1. **(B)** GdmCl-induced unfolding curve of the W59Y variant in 0.1 M NaAc, pH 5.0 measured at 25°C by fluorescence at 303 nm (○), by absorbance at 287 nm (●), and by CD at 222 nm (△). Protein concentrations were 2.5 μM in fluorescence and 9.85 μM in absorbance and CD measurements. Analysis of the transition curve gives a $[GdmCl]_{1/2}$ of 3.55±0.05 M GdmCl and an *m*-value of −14.3±1.0 (kJ/mol)/M.

the variant the single tryptophan residue has been changed to tyrosine, its refolding was followed by the decrease in tyrosine fluorescence at 303 nm, while renaturation of the wild-type protein was monitored by the increase in tryptophan fluorescence at 320 nm. Under identical conditions (pH 5.0, 10°C) the folding of the W59Y variant is much faster than folding of the wild-type protein (Fig. 3). The very slow refolding phase of the wild-type protein is not observed in the mutated form, while the intermediate phase is still present with relaxation times of 330 and 125 sec under the given conditions. Similar results were obtained under other refolding conditions and by using different probes to monitor renaturation (Table I). In no case could a refolding phase slower than 450 sec at 10°C be detected for the W59Y variant. The disappearance of the very slow

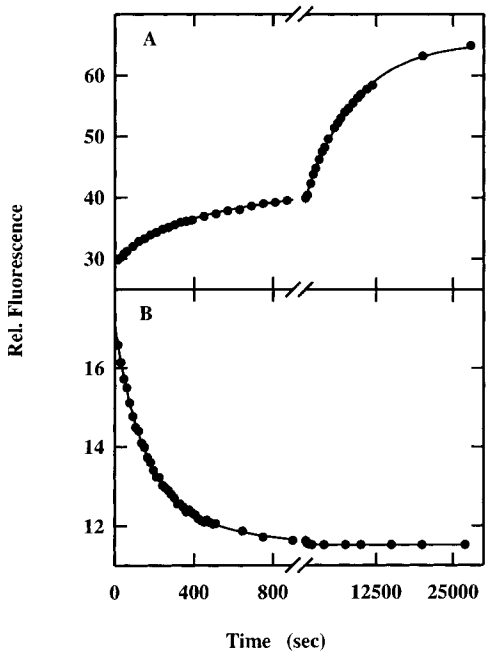


Fig. 3. Refolding of wild-type **(A)** and W59Y **(B)** RNase T1 in the presence of 0.1 M NaAc and 0.15 M GdmCl at pH 5.0 and 10°C. Refolding was monitored by the change in fluorescence at 320 nm (wild-type) or 303 nm (W59Y variant) after excitation at 268 nm. Protein concentrations were 0.36 μM for wild-type and 1.35 μM for the mutated protein.

phase in the folding of the W59Y variant is not caused by the loss of Trp-59 as a reporter group for this step. It is also absent when refolding is monitored by the regain of enzymatic activity (Table I). It cannot be ruled out, however, that catalytically active folding intermediates with the same spectroscopic properties as the native protein are formed in the folding of the W59Y variant and that the very slow reaction still exists but cannot be detected.

Formation of Native Molecules

Unfolding assays carried out after various time intervals of refolding are the most sensitive probe for the formation of native protein during folding.[1,2,6,24] These assays are based on the observation that native molecules are separated from unfolded or partially folded chains by a high barrier of activation energy,[25,26] and therefore unfold much slower than any partially folded intermediates. The kinetics of formation of native protein as determined by this technique are compared in Figure 4 for the wild-type protein and the W59Y variant. This comparison reveals several aspects that aid in the understanding of the molecular effects of the W59Y mutation on the folding mechanism: (1) The very slow phase is definitely absent in the refolding of the W59Y variant and renaturation is complete within 35 min under the given conditions (pH 8.0, 10°C). In contrast, renaturation of the wild-type protein takes at least 240 min. (2) The amount of fast folding molecules, U_F, is not affected by the mutation. In both cases, about 4% native molecules are regained rapidly. Apparently, the W59Y mutation does not change the equilibrium distribution of fast- and slow-folding species in the unfolded chain. (3) Similar to the wild-type protein, the regain of native molecules in the folding of the W59Y variant shows an initial lag phase. This originates from the consecutive reisomerization of two incorrect proline isomers on the two alternative folding pathways of RNase T1 (cf. Scheme I). This similarity is to be expected, since both *cis* prolines (Pro-39 and Pro-55) are present in the wild-type protein and the W59Y variant.

An analysis of the kinetics in Figure 4 for the W59Y variant according to the mechanism in Scheme I gives time constants of about 450 sec for the two final isomerization steps (reactions 1 and 2 of the mechanism in Scheme I). This indicates that the very slow step (reaction 1) is accelerated about 10-fold in the W59Y variant under these conditions.

GdmCl Dependence of the Slow Refolding Kinetics

Unlike most other protein folding reactions, the very slow phase of wild-type RNase T1 folding increases in rate with increasing residual concentrations of GdmCl. This unusual property was suggested to originate from a loosening or destabilization of rapidly formed structure in a folding intermediate that prevents Pro-39 from reisomerization.[2] Figure 5 shows that this behavior is no longer observed in the W59Y variant. The slow refolding kinetics of wild-type RNase T1 and of the W59Y variant are compared at 0.15 and 1.9 M GdmCl. In the presence of 0.15 M GdmCl the folding kinetics are strongly different, since (as shown in Table I) the very slow phase is absent in the variant (Fig. 5A). In contrast the folding kinetics of both RNase T1 species become almost identical when the residual concentration of denaturant is raised to 1.9 M (Fig. 5B). As pointed out, the very slow folding reaction of the wild-type protein becomes faster under these unfavorable conditions whereas the folding of the W59Y variant is strongly decelerated when the concentration of GdmCl is increased, similar to the folding of other proteins.[9,10] This indicates that the replacement of Trp-59 by a tyrosine residue affects the folding kinetics only under conditions where the rates of the final slow steps of folding are determined by the rapid formation of partially folded intermediates. Under unfavorable conditions the folding kinetics are barely influenced by the mutation.

Rapid Structure Formation

One possible reason for the "normalization" of the very slow refolding phase in the variant could be the inability to form partially folded intermediates

TABLE I. Comparison of the Refolding Kinetics of W59 Y and Wild-Type RNase T1*

Experimental conditions	Probe‡	W59Y mutant†				Wild-type					
		τ_2 (sec)	τ_3 (sec)	A_2§	A_3	τ_1 (sec)	τ_2 (sec)	τ_3 (sec)	A_1	A_2	A_3
0.1 M NaAc, pH 5.0, 25°C	Fl	54	—	1.0	—	1000	70	—	0.42	0.35	—
0.1 M NaAc, pH 5.0, 25°C	Abs	50	—	0.50	—	900	80	—	0.18	0.45	—
0.1 M NaAc, pH 5.0, 10°C	Fl	330	125	0.62	0.38	7400	230	—	0.47	0.13	—
0.1 M NaAc, pH 5.0, 10°C	Abs	170	—	0.35	—	7500	360	—	0.34	0.16	—
0.1 M Tris/HCl, pH 8.0, 10°C	Fl	370	220	0.59	0.41	3600	730	290	0.16	0.23	0.23
0.1 M Tris/HCl, pH 8.0, 10°C	Abs	325	—	0.43	—	2500	400	—	0.22	0.36	—
0.1 M Tris/HCl, pH 8.0, 10°C	Act	450	—	0.65	—	2500	300	—	0.68	0.22	—

*Refolding was initiated by 40-fold dilution of denatured RNase T1 (in 6.0 M GdmCl, 0.1 M NaAc, pH 5.0) into the given refolding buffers. In all measurements the residual GdmCl concentration was 0.15 M. Error limits are ±5% for the amplitudes and ±10% for the relaxation times. The intermediate phase comprises of at least two different steps with similar relaxation times (cf. Scheme I). These steps can be resolved only under certain experimental conditions.[1,2]

†The symbol τ_1 is not used in the analysis of the folding kinetics of the W59Y variant to point out that the τ_1-phase of the wild-type protein is absent in the folding of the variant.

‡Fl, fluorescence measurements at 303 nm (W59Y variant) or at 320 nm (wild-type) after excitation at 268 nm. Abs, absorbance measurements at 287 nm. Act, activity measurements as described under Materials and Methods.

§Amplitudes are given as fractions of the total change in fluorescence or absorbance as observed in the equilibrium unfolding transitions. In the activity measurements the amplitude is the fraction of activity that is regained in the respective phase. In the case of the fluorescence measurements with the mutated protein the amplitudes are given as the fraction of the total amplitude of the slow steps, since the total change in fluorescence that occurs is larger than the change observed in the corresponding equilibrium unfolding transitions. This phenomenon is currently under investigation.

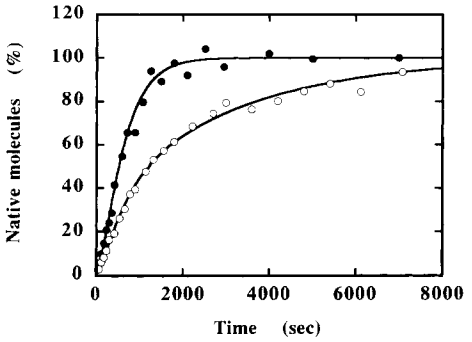


Fig. 4. Formation of native molecules in the time course of refolding of wild-type (○) and W59Y (●) RNase T1 at pH 8.0 and 10°C. The percentage of native molecules was determined by unfolding assays as described in Materials and Methods. Refolding conditions were 0.1 M Tris-HCl, 0.2 M GdmCl, pH 8.0. The theoretical curves were calculated by using the kinetic scheme (Scheme I) and the relaxation times given therein. The curve for the W59Y variant was calculated with relaxation times of 200 sec for τ_3 and τ_4 and 450 sec for τ_1 and τ_2. It was assumed that 4% of the molecules refold rapidly (U_F).

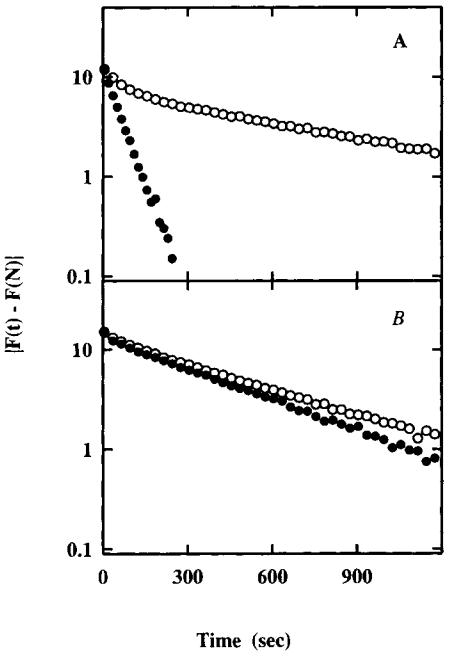


Fig. 5. Semilogarithmic plot of the refolding of wild-type (○) and W59Y (●) RNase T1 in 0.1 M NaAc, pH 5.0 at 25°C. The residual concentrations of GdmCl were 0.15 M **(A)** and 1.9 M **(B)**. Refolding was monitored by the change in fluorescence at 320 nm (wild-type) or 303 nm (W59Y variant) after excitation at 268 nm. Protein concentrations were 0.47 μM (wild-type) and 2.53 μM (W59Y variant).

under strongly native conditions. To investigate, whether rapid structure formation is still possible in the W59Y variant, a CD spectrum was recorded 20 sec after dilution of unfolded protein into native conditions. At this time only about 5% of all molecules have attained the native state (cf. Fig. 4); however, the CD spectrum, as in wild-type RNase T1,[2] already closely resembles that of the native enzyme (Fig. 6). This clearly indicates that the acceleration of the very slow refolding phase in the W59Y variant is not caused by a general destabilizing effect of this replacement on an early intermediate.

Refolding in the Presence of PPIase

All slow steps in the folding of RNase T1 are catalyzed by PPIase.[4] This was a valuable result to identify these steps as proline isomerizations.[1,2] All isomerizations in the 200 to 500 sec time range that constitute the intermediate phase were accelerated very efficiently by PPIase, whereas catalysis of the very slow phase was poor. Here we use the catalysis

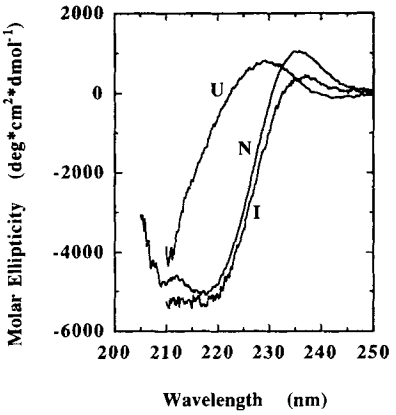


Fig. 6. CD spectra of native (N) and unfolded (U) W59Y variant and a folding intermediate (I) which is formed rapidly after initiation of refolding. RNase T1 W59Y was refolded in 0.1 M NaAc, 0.15 M GdmCl at pH 5.0 and 10°C. After 20 sec of refolding the spectrum of the intermediate was recorded. The spectrum of the native protein was recorded after refolding was complete (35 min). The protein concentration was 41.9 μM.

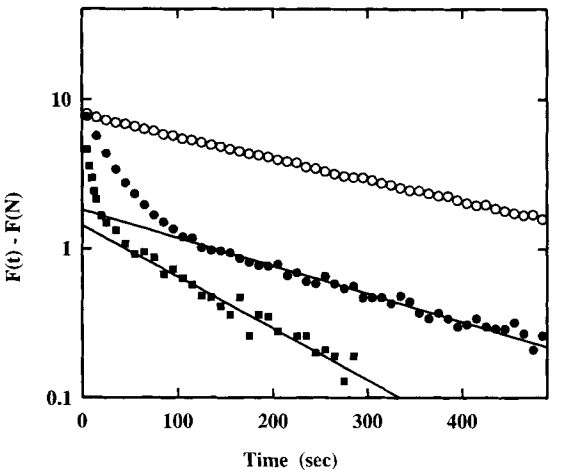


Fig. 7. Semilogarithmic plot of the refolding of W59Y RNase T1 in the presence of 0 μM (○), 0.28 μM (●), and 1.4 μM (■) PPIase in 0.1 M Tris-HCl, 0.15 M GdmCl at pH 8.0 and 10°C. Concentration of RNase T1 was 2.0 μM. Refolding was detected by the decrease in fluorescence at 303 nm after excitation at 268 nm.

by PPIase to address the question whether the isomerization that creates the very slow phase is really abolished in the folding of the W59Y variant. Alternatively, it might still be present, but cannot be resolved from the other isomerizations since it is no longer decelerated as in the folding of the wild-type protein. The results in Figure 7 demonstrate that this reaction does indeed still occur in the folding of the variant. As reported before for the wild-type protein, part of the slow refolding of the W59Y variant is strongly accelerated, while catalysis of the other part is very poor. Consequently, in the presence of increasing concentrations of PPIase, the folding kinetics of the W59Y variant clearly become biphasic. About 70% of slow folding is catalyzed very efficiently and comparable to the intermediate phase in wild-type RNase T1. The remaining 30% are only marginally accelerated in their refolding, as it was also shown for the very slow phase in the wild-type protein. This confirms that the isomerization that gives rise to the very slow refolding phase is still present in the W59Y variant, but its rate is "normalized" by the replacement of the tryptophan residue. Therefore, it is not easily distinguished from the isomerization reactions in the "intermediate" phase unless PPIase is added.

DISCUSSION

The replacement of Trp-59 by Tyr does not significantly influence the stability of RNase T1. The kinetic analysis of the W59Y variant, however, surprisingly revealed that its folding is much faster compared to that of the wild-type protein. This phenomenon is due to the loss of the major very slow phase and it is found with all probes that were used to monitor folding. The slow steps in the folding of RNase T1 are all limited in rate by prolyl isomerization reactions.[1,2,6] Therefore, the mutation of a nonproline residue, such as Trp-59, should not lead to the disappearance of a slow reaction or to a redistribution of fast and slow folding species. Indeed, several features suggest that the folding mechanism (Scheme I) is unchanged: (1) The relative amount of fast folding molecules is not affected in the variant (cf. Fig. 4). (2) The appearance of native molecules in the time course of refolding exhibits a lag phase that is caused by the sequential steps on both the upper and the lower refolding pathway in Scheme I. (3) Intermediates with a high content of secondary structure are formed rapidly in the folding of both wild-type and W59Y variant (cf. Fig. 6). (4) The presence of PPIase leads to a clear separation of the slow folding reactions of the W59Y variant into two distinct kinetic phases. One step is well accelerated by the enzyme, and thus resembles the intermediate phase of the wild-type protein. The other phase is only poorly catalyzed and corresponds to the very slow reaction of the wild-type protein. Apparently, this particular reaction is still present in the W59Y variant; but it is strongly accelerated and coincides with the intermediate phase under most conditions.

The nature of the rate-limiting step of the slowest folding phase of the wild-type protein as well as the W59Y variant is the reisomerization of an incorrect proline isomer at the stage of a well-ordered folding intermediate. We have suggested earlier that specifically the *trans* → *cis* reisomerization of the Tyr-38–Pro-39 bond is involved and that this reaction is unusually slow, since the rapid structure formation hinders the reisomerization. The present results strongly support this assignment. Pro-39 and Trp-59 are in close contact in the native protein (Fig. 1) and

presumably also in the rapidly formed intermediate. This close packing leads to a strain and retards the isomerization of Pro-39. The strain is relieved when the structure of the folding intermediate is destabilized. This explains the acceleration of the very slow folding phase of the wild-type protein with increasing GdmCl concentrations. In the mutated protein this reaction is no longer retarded, since, after replacement of Trp-59 by a less bulky tyrosine residue, more space is available for Pro-39 to reisomerize. Trp-59 exerts its "obstructive" effect on refolding only under strongly native conditions, where ordered structure is formed rapidly. As a consequence, the slow folding kinetics of the Trp-59 and the Tyr-59 variants of RNase T1 become virtually identical above 1.7 M GdmCl.

An alternative explanation, in which the effect of the W59Y mutation is mediated by a general destabilization of early intermediates, appears to be less likely, since rapid formation of secondary structure is observed in the folding of the wild-type and the mutant proteins (cf. Fig. 6). Minor differences in the stabilities of these early intermediates of the two forms of RNase T1 could exist, but are not detectable in our experiments.

We cannot completely exclude at present that the altered folding of the W59Y variant is mediated not by a long-range effect on Pro-39 isomerization, but by a local influence on Pro-60, which is adjacent to Trp-59 in sequence. In that case, the "very slow" isomerization that occurs in more than 80% of all unfolded molecules should originate from Pro-60 and not from Pro-39. This appears less likely, since Pro-60, which is *trans* in the native protein, would be required to isomerize to an 80:20 equilibrium mixture of *cis:trans* isomers after unfolding. Such a dominance of the *cis* isomer in the absence of ordered structure is not expected.[7] In addition, the most likely effect of varying the residue Xaa of a Xaa–Pro bond is a shift in the *cis/trans* ratio.[27] This should change the equilibrium distribution of fast and slow folding species and affect the refolding kinetics under any conditions. In the W59Y variant the U_F/U_S ratio remains constant and the kinetics are changed only under conditions where rapid structure formation is possible. Taken together, our results do not support a model in which the isomerization of Pro-60 causes the very slow phase of RNase T1 folding and thus mediates the effect of the W59Y mutation.

The W59Y protein is a "conditional folding mutant" of RNase T1. The difference in stability ($\Delta G°$) between the native and the unfolded state is not affected by the replacement. However, a major folding reaction is significantly accelerated in the mutant under certain conditions. Since the kinetic mechanism of folding[2,6] and the three-dimensional structure[5] of RNase T1 are known, we are able to explain the molecular role of position 59 for the folding of RNase T1. It interacts with Pro-39 in a folding intermediate and large amino acids, such as tryptophan at position 59 hinder the *trans* → *cis* isomerization of the Tyr-38–Pro-39 bond in the final step of refolding. In this respect, it is then the wild-type protein (the W59 variant) that shows a "defect" in folding.

CONCLUSION

The kinetic results obtained with the W59Y variant of RNase T1 bear several implications for the folding of this protein and for protein folding in general. They support the kinetic model (Scheme I) in which the isomerizations of the two *cis* prolines 39 and 55 determine the slow steps of refolding. The strong deceleration of folding by an incorrect *trans* isomer of Pro-39 is only observed in molecules in which the other proline (Pro-55) has already reverted to the native *cis* state (I^{39t}_{55c}). It is not found at the stage of the intermediate I^{39t}_{55t} with two incorrect prolines. Apparently the block of Pro-39 isomerization can be relieved not only by the W59Y mutation or by increasing the denaturant concentration, but also by a decreased stability of the intermediate due to a second "wrong kink" at Ser-54–Pro-55 in the refolding polypeptide.

In addition to a native-like secondary structure the intermediate I^{39t}_{55c} with an incorrect Pro-39 displays at least one specific tertiary interaction between positions 39 and 59 of the chain. This contact affects the rate of the final step of folding. The structures of early folding intermediates have been examined by a combination of amide proton labeling techniques with high-resolution NMR for RNase A,[28,29] cytochrome *c*,[30] and barnase.[31] In all these cases some specific tertiary contacts were formed very early during folding.

Structure formation and proline isomerization steps are mutually interdependent during refolding. This is now evident for RNase T1. Rapid folding decelerates reisomerization of Pro-39 by formation of "premature" native-like structure that involves an interaction of Pro-39 with Trp-59. Whether *trans* → *cis* reisomerization of Pro-39 requires extensive unfolding or only a local reorganization can not be judged from our experiments. A different effect of intermediates on the rate of folding was observed in the case of RNase A. In this case, proline reisomerization was accelerated in a rapidly formed native-like folded intermediate.[32–34]

Our results indicate that partially folded intermediates are not always of advantage for folding, since proteins may become trapped in an incorrect structure which represents a local minimum on the energy scale and is separated from the final, native state by a high activation energy. A fairly low stability of intermediate structures may be an important factor to warrant correct and rapid folding.[35]

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