Folding Mechanism of Ribonuclease T1 in the Absence of the Disulfide Bonds†

Matthias Mücke and Franz X. Schmid*

Laboratorium für Biochemie, Universität Bayreuth, D-95440 Bayreuth, Germany

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ABSTRACT: In the absence of its two disulfide bonds, ribonuclease T1 can exist in a native-like folded conformation when ≥2 M NaCl is present. We measured the kinetics of unfolding and refolding of two reduced and carboxymethylated variants of ribonuclease T1 with one cis proline (the Ser54Gly/Pro55Asn variant) and with two cis prolines (the wild-type protein) as a function of the NaCl concentration. Single and double mixing techniques were used. Analysis of the kinetic results demonstrates that the two cis prolyl bonds at Pro39 and Pro55 remain cis in the folded state after the reduction and carboxymethylation of the disulfide bonds. Folded molecules with trans isomers could not be found. The substitution of cis-Pro55 influences the proline-limited folding reaction, and the analysis of the changes in the folding kinetics shows that the trans → cis isomerizations of both prolines are slow and are rate-determining steps for the refolding of ribonuclease T1 in the presence as well as in the absence of the disulfide bonds. The direct folding reaction of protein chains with correct prolyl isomers is also affected by the Ser54Gly/Pro55Asn mutation. The rate of refolding is decreased, whereas the rate of unfolding is almost unaffected. The kinetic analysis points to two main consequences of the Ser54Gly/Pro55Asn mutation for the stability and the folding mechanism of RNase T1. It is moderately destabilizing, because the deletion of a conformationally restricted residue (Pro55 → Asn) and the insertion of a flexible residue (Ser54 → Gly) both tend to increase the entropy of the unfolded state. The cis ⇌ trans isomerization of Pro55 is abolished, however, leading to a decrease in the entropy of the unfolded protein. These two entropic contributions seem to partially compensate each other, and the net change in free energy as a consequence of the Ser54Gly/Pro55Asn double mutation is very small.

Ribonuclease T1 (RNase T1)[1] is a small single-domain protein of 104 amino acids (M_r = 11 085) with two disulfide bonds, which are close to each other in the folded protein (Figure 1) (Heinemann & Saenger, 1982; Martinez-Oyanedel et al., 1991). The Cys2−Cys10 bond connects locally two successive β-strands; it is about 90% accessible to solvent and can be selectively reduced by dithiothreitol (Pace & Creighton, 1986; Pace et al., 1988). The Cys6−Cys103 disulfide links the two ends of the protein chain and is inaccessible to solvent. In folded RNase T1 there are two cis prolyl peptide bonds, Tyr38-Pro39 and Ser54-Pro55; both are distant from the disulfide bonds in the native protein (cf. Figure 1).

The unfolding and refolding of RNase T1 molecules with intact disulfides are strongly influenced by the isomerizations of Pro39[2] and Pro55. Results obtained for the wild-type protein (Kiefhaber et al., 1990a,b) and for variants with substitutions of Pro39 or Pro55 (Kiefhaber et al., 1990c;

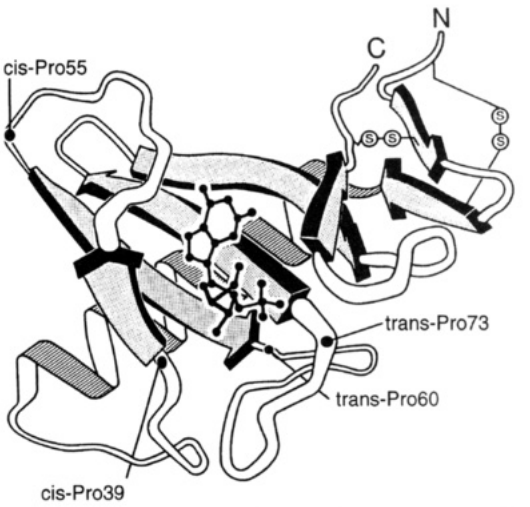

FIGURE 1: Schematic drawing of the backbone structure of RNase T1, complexed with guanosine 2′-phosphate. The two disulfide bonds connect Cys2 with Cys10 and Cys6 with Cys103. (Drawing courtesy of U. Heinemann, Berlin.)

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* To whom correspondence should be addressed. Tel: ++49 921 553660; Fax: ++49 921 553661.

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[1] Abbreviations: RNase T1, ribonuclease T1; wt-T1, wild-type form of RNase T1; (-Pro55)-T1, the S54G/P55N variant of ribonuclease T1 with the substitutions Ser54 → Gly and Pro55 → Asn; 2SS, form with both disulfides intact; RCM, form in which both disulfide bonds are reduced and the cysteine residues carboxymethylated; N, native protein; U^{39c}_{55c}, U^{39t}_{55c}, U^{39c}_{55t}, and U^{39t}_{55t}, unfolded forms of RCM-wt-T1 with Pro55 and Pro39 in the cis (c) or trans (t) conformations; U^{39c} and U^{39t}, unfolded forms of RCM-(-Pro55)-T1 with Pro39 in the cis or trans state, respectively [U^{39c}_{55c} and U^{39c} are also denoted as U_F (for fast-folding species), and the other species, as U_S (for slow-folding species)]; GpC, guanylyl(3′−5′)cytidine; GdmCl, guanidinium chloride; DTE, dithioerythritol; λ_i, measured or macroscopic rate constant of a reaction; τ, time constant of a reaction ($=\lambda^{-1}$); k_i, microscopic rate constant.

[2] To facilitate reading, we use the expressions "direct folding" for folding reactions that do not involve prolyl isomerizations, such as the N ⇌ U_F reaction, "proline-limited folding" for folding reactions that are limited in rate by prolyl isomerization, "cis-ProX" for a prolyl residue that is preceded by a cis peptide bond in the native protein, "isomerization of ProX" to denote the cis/trans isomerization of the peptide bond preceding ProX, and "strongly native" to describe refolding conditions under which the native protein has a high stability.

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Mayr et al., 1993a,b; Mayr & Schmid, 1993b) suggest that in the denatured state 80−90% of both Pro39 and Pro55 are in the incorrect trans conformation. The isomerizations of these two prolines lead to an equilibrium mixture of four different unfolded species. Two to four percent of all molecules contain correct cis isomers at both Pro39 and Pro55 (U_{39c}^{55c}) and fold rapidly. In addition, three slow-folding species are present: two with one incorrect proline isomer each (U_{39c}^{55t} and U_{39t}^{55c}) and another, dominant species (about 70% of all molecules) with two incorrect prolines (U_{39t}^{55t}). In the course of refolding, all slow-folding species regain most of their secondary structure and part of the tertiary contacts rapidly in the time range of milliseconds when folding is carried out under strongly native conditions, where the stability of folded RNase T1 is high (Kiefhaber et al., 1992c; Mullins et al., 1993). The subsequent slow steps are limited in rate by the trans → cis reisomerizations of Pro39 and Pro55. These isomerizations are coupled with further folding and are accompanied by changes in absorbance and fluorescence. Interestingly, the trans → cis isomerization of Pro39 is strongly retarded when partially-folded structure with an incorrect trans Pro39 is formed early in refolding (Kiefhaber et al., 1992a).

To infer the role of the two disulfide bonds for the folding mechanism, we investigate here the folding kinetics of RNase T1 in the absence of the disulfide bonds. In particular, we ask whether Pro39 and Pro55, which are cis in the native, disulfide-bonded protein, remain in the cis conformation after breaking of the disulfide bonds and whether the trans → cis isomerizations of these prolines are still the rate-determining steps of folding. The disulfide bonds of RNase T1 can be broken by reduction and carboxymethylation of the thiols (Oobatake et al., 1979; Pace et al., 1988). As a consequence, the protein unfolds (at 15 °C and pH 8), but reversible refolding to a native-like folded state can be induced by adding 2 M NaCl. In previous experiments we have shown that unfolding and NaCl-induced refolding are slow reactions that are catalyzed by prolyl isomerase, indicating that prolyl isomerizations do indeed contribute to the folding of RNase T1 even when the disulfide bonds are broken (Mücke & Schmid, 1992).

To map out the individual contributions of Pro39 and Pro55, we measure the stability and the kinetics of unfolding and refolding of the RCM forms of the wild-type protein with two cis prolines and of the S54G/P55N variant [(-Pro55)-T1] with a single cis proline (at position 39) as a function of the NaCl concentration. The comparative analysis of the kinetic data demonstrates that Pro39 and Pro55 remain in the native-like cis conformation even when the disulfide bonds are broken and that the trans → cis isomerizations of both prolines determine the rate of refolding in the presence as well as in the absence of the disulfide bonds.

MATERIALS AND METHODS

Materials. Wild-type RNase T1 and the S54G/P55N variant were purified as described (Mayr & Schmid, 1993a) from Escherichia coli cells transformed with a plasmid carrying a chemically synthesized gene which was cloned and expressed in E. coli as described (Quaas et al., 1988). Cytosolic peptidyl-prolyl cis/trans isomerase from E. coli was a gift from N. Takahashi (TONEN Corp., Nishi-Tsurugaoka, Japan) and assayed as described by Fischer et al. (1989). The relative activity of PPI is expressed as peptide accleration units (PU/mL). The numerical value of the activity is given by $k/k_0 - 1$, where k_0 is the rate constant of the uncatalyzed peptide isomerization and k is the rate of peptide isomerization in the presence of PPI (Mücke & Schmid, 1992). GdmCl (ultrapure) and urea (ultrapure) were from Schwarz/Mann (Orangeburg, NY), and dithioerythritol (DTE), iodoacetate, and the assay peptide for prolyl isomerase, Suc-Ala-Ala-Pro-Phe-4-nitroanilide, were from Sigma (St. Louis, MO). Chymotrypsin A4 was from Boehringer-Mannheim (Mannheim, Germany). Guanylyl(3′−5′)cytidine (GpC) was from Pharma Waldhof (Düsseldorf, Germany). All other chemicals were from Merck (Darmstadt, Germany). The reduced and carboxymethylated forms of wild-type and (-Pro55)-T1 were produced as described by Mücke & Schmid (1994a).

Spectroscopic Methods. The concentrations of RCM-wt-T1 and RCM-(-Pro55)-T1 were determined spectrophotometrically using the absorption coefficient $A_{278}^{0.1\%} = 1.9$ of the native wild-type protein (Takahashi et al., 1970; Yu et al., 1994). For optical measurements a JASCO J-600A spectropolarimeter, a Hitachi F4010 fluorescence spectrometer, a Kontron Uvikon 860, and a Hewlett-Packard A8652 spectophotometer were used.

Assay of RNase T1 Activity. The enzymatic activity of the various forms of RNase T1 was measured by the hydrolysis of the dinucleotide GpC in 0.1 M Tris-HCl and 2 mM EDTA, pH 8.0, 15 °C, in the presence and in the absence of 2.5 M NaCl. The GpC concentration was 50 μM, and the protein concentration varied from 2 to 200 nM. The reactions were monitored by the change in absorbance at 280 nm (Grunert et al., 1991). The initial slopes are proportional to the enzymatic activity.

NaCl- and Urea-Induced Folding Transitions. RCM-wt-T1 and RCM-(-Pro55)-T1 (0.7 μM) were incubated at 15 °C in the presence of 0.1 M Tris-HCl, pH 8.0, and varying concentrations of NaCl and urea for at least 12 h. The fluorescence of the samples was measured at 320 nm (10 nm band width) after excitation at 268 nm (1.5 nm band width). The folding transitions were analyzed by assuming a two-state transition between the folded (N) and the unfolded (U) conformations. A nonlinear least-squares fit (Santoro & Bolen, 1988) of the experimental data was used to obtain the Gibbs free energy of unfolding ΔG_D as a function of the urea concentration for the two forms.

CD Spectroscopy. CD spectra were recorded with 20 nm/min and a time constant of 1 s in 0.1-cm (far-UV) and 1-cm (near-UV) thermostated cuvettes at 15 °C. The band width was 1 nm. The protein concentrations were 10 μM in the far-UV region and 20 μM in the near-UV region. The spectra were measured 10 times, averaged, and corrected for the contributions of the respective solvents. To measure the refolding kinetics of RCM-(-Pro55)-T1, the change in the CD signal at 212 nm was followed as a function of the refolding time after a 15-fold dilution of the unfolded protein (in 0.05 M Tris-HCl, 0.1 M sodium acetate, pH 8.0, 25 °C) to give final conditions of 0.1 M Tris-HCl and 2.6 M NaCl, 15 °C. The protein concentration was 10 μM.

Unfolding and Refolding Kinetics. Unfolding and refolding kinetics were typically initiated by a 40-fold dilution of native or unfolded protein to the appropriate final concentration of NaCl. The dead time of mixing was about 2 s. The kinetics were followed by the change in fluorescence at 320

nm (10 nm band width) after excitation at 268 nm (1.5 nm band width). All kinetic experiments were carried out in 0.1 M Tris-HCl, pH 8.0 at 15 °C. The final protein concentration was 0.7 μM. To measure the direct refolding reaction of RCM-wt-T1 within the transition region, a double-mixing technique was used (Kiefhaber & Schmid, 1992) to produce the U_{39c}^{55c} species. Native protein in 2.8 M NaCl, 0.05 M Tris-HCl and 0.05 M sodium acetate, pH 8.0, 15 °C, was rapidly unfolded by a 14-fold dilution to 2.0 M GdmCl, 0.2 M NaCl, and 0.1 M Tris-HCl, pH 8.0, 15 °C. After 15 s, refolding was initiated by a 10-fold dilution to 0.1 M Tris-HCl, pH 8.0, 15 °C, and various concentrations of NaCl. The final protein concentration was 0.4 μM. The observed kinetic curves were analyzed as a sum of exponential functions by using the program GraFit 3.0 (Erithacus Software, Staines, U.K.).

Prolyl Isomerizations in Unfolded RCM-wt-T1 and Unfolded RCM-(-Pro55)-T1. The prolyl isomerizations were measured by a double-mixing technique (Brandts et al., 1975; Schmid, 1986). To produce the folded state of the two RCM forms, they were first incubated in 0.05 M Tris-HCl, 0.05 M sodium actetate, and 2.8 M NaCl, pH 8.0, 15 °C, for 2 h. Rapid unfolding was then induced by a 14-fold dilution to 0.1 M Tris-HCl, 2.0 M urea, and 0.2 M NaCl, pH 8.0, 15 °C. After various times of unfolding and prolyl isomerization aliquots were withdrawn and diluted 10-fold in the fluorimeter cell to give refolding conditions of 0.1 M Tris-HCl, 1.6 M NaCl, and 0.2 M urea, pH 8.0, 15 °C, and a final protein concentration of 0.45 μM. The resulting slow refolding reactions were followed by the increase in fluorescence at 320 nm (excitation at 268 nm). Under these conditions slow refolding was a first-order reaction in each case with time constants of 2030 s for RCM-wt-T1 and 560 s for RCM-(-Pro55)-T1. The amplitudes of these reactions were then plotted as a function of the unfolding time. They reflect the time course of formation of the U_S species.

Analysis of the Folding Kinetics of RCM-(-Pro55)-T1 in the Transition Region. Biphasic kinetics were observed in unfolding and in refolding experiments carried out in the transition region. The measured kinetic parameters consisted of two measured rate constants, λ_1 and λ_2, which were identical in unfolding and in refolding experiments under the same final conditions, and two sets of reduced amplitudes a_1^R and a_2^R in refolding and a_1^U and a_2^U in unfolding. The reduced amplitudes are given as fractions of the total change in fluorescence between the native and the unfolded state, as defined by Kiefhaber et al. (1992b). We used the relations derived by Mücke and Schmid (1994a) to calculate the four microscopic rate constants k_{12}, k_{21}, k_{23}, and k_{32} from the measured kinetic parameters λ_1, λ_2, a_1^R, a_2^R, a_1^U, and a_2^U and the measured equilibrium constant of unfolding K_{eq}. These relations are based on the three-species mechanism in eq 1 and its kinetic analysis by Ikai and Tanford (1973), Hagerman (1977) and Kiefhaber et al. (1992b).

$$U^{39t} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} U^{39c} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} N \qquad (1)$$

Analysis of the Folding Kinetics of RCM-wt-T1 in the Transition Region. The kinetic analysis of the folding mechanism of a protein with two cis prolines was discussed by Kiefhaber et al. (1992b), and we follow their notation and analysis here. RCM-wt-T1 can form four different unfolded species, U_{55t}^{39t}, U_{55c}^{39t}, U_{55t}^{39c}, and U_{55c}^{39c}, and the native species N (cf. eq 2). When the two prolyl isomerizations occur with the same rate constants (k_{12} and k_{21}), then the folding mechanism can be simplified and described by the linear mechanism in eq 3.

$$\begin{array}{ccccc} U_{55c}^{39t} & \rightleftharpoons & U_{55c}^{39c} & \rightleftharpoons & N \\ \uparrow\downarrow & & \uparrow\downarrow & & \\ U_{55t}^{39t} & \rightleftharpoons & U_{55t}^{39c} & & \end{array} \qquad (2)$$

$$U_{55t}^{39t} \underset{k_{21}}{\overset{2k_{12}}{\rightleftharpoons}} (U_{55c}^{39t} + U_{55t}^{39c}) \underset{2k_{21}}{\overset{k_{12}}{\rightleftharpoons}} U_{55c}^{39c} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} N \qquad (3)$$

A rigorous analysis of the folding kinetics of RCM-wt-T1 to determine the microscopic rate constants of isomerization (k_{12} and k_{21}) and of folding (k_{23} and k_{32}) in eq 3 from the measured rate constants and amplitudes was not possible, because only two macroscopic rate constants (λ_1 and λ_3) could be determined at all NaCl concentrations. Therefore, a stepwise analysis was carried out, based on the following assumptions. (i) The rate constants for the two isomerizations of Pro39 and Pro55 are identical with the values observed for Pro39 alone in RCM-(-Pro55)-T1, $k_{12} = 1.8 \times 10^{-3}$ s^{-1} and $k_{21} = 1.1 \times 10^{-2}$ s^{-1}. (ii) The fast- and the slow-folding reactions are well separated, and therefore, λ_3 is equal to the sum of k_{23} and k_{32} (eq 4). (iii) The logarithms of the rate constants of refolding (ln k_{23}) and of unfolding (ln k_{32}) depend linearly on NaCl concentration (eqs 5a,b).

$$\lambda_3 = k_{23} + k_{32} \qquad (4)$$

$$\ln k_{23} = \ln k_{23}^{\,0} + m_{23}[\mathrm{NaCl}] \qquad (5a)$$

$$\ln k_{32} = \ln k_{32}^{\,0} + m_{32}[\mathrm{NaCl}] \qquad (5b)$$

In eqs 5a,b, $k_{23}^{\,0}$ and $k_{32}^{\,0}$ are the values for k_{23} and k_{32} in the absence of NaCl, and m_{23} and m_{32} (=d ln k_{ij}/d [NaCl]) reflect the dependences of ln k_{23} and ln k_{32} on NaCl concentration, respectively. The combination of eqs 4 and 5a,b gives eq 6, which was used to determine $k_{23}^{\,0}$, $k_{32}^{\,0}$, m_{23}, and m_{32} from the experimental values of λ_3 by a nonlinear least-squares fit.

$$\lambda_3 = k_{23}^{\,0} \exp(m_{23}[\mathrm{NaCl}]) + k_{32}^{\,0} \exp(m_{32}[\mathrm{NaCl}]) \qquad (6)$$

The constant values for k_{12} and k_{21} and the NaCl-dependent values for k_{23} and k_{32}, derived from eq 6 were used to calculate the equilibrium constant K_{eq} by using eq 7 and the macroscopic rate constants λ_i by solving the characteristic equation (eqs 8a–d) as outlined by Kiefhaber et al. (1992b). These values are then compared with the experimental values for K_{eq}, λ_1, and λ_3.

$$K_{eq} = \frac{[U_{55c}^{39c}] + [U_{55c}^{39t}] + [U_{55t}^{39c}] + [U_{55t}^{39t}]}{[N]} = \frac{k_{32}}{k_{23}}\left[\frac{(k_{12} + k_{21})^2}{k_{12}^{\,2}}\right] \qquad (7)$$

$$f(\lambda) = \lambda^4 + A_3\lambda^3 + A_2\lambda^2 + A_1\lambda = 0 \qquad (8a)$$

$$A_3 = -(3k_{12} + 3k_{21} + k_{23} + k_{32}) \qquad (8b)$$

$$A_2 = 3k_{32}(k_{12} + k_{21}) + k_{23}(3k_{12} + k_{21}) + 2(k_{12} + k_{21})^2 \quad (8c)$$

$$A_1 = -[2k_{32}(k_{12} + k_{21})^2 + 2k_{12}{}^2k_{23}] \quad (8d)$$

RESULTS

Structure and Activity of the RCM Forms of RNase T1 with One and Two Cis Prolines. The disulfide bonds of the variants of RNase T1 with two cis prolines (the wild-type protein) and with a single cis proline [the (-Pro55) form] were broken by reduction and subsequent carboxymethylation (Pace et al., 1988). The resulting species, RCM-wt-T1 and RCM-(-Pro55)-T1, are unfolded in 0.1 M Tris-HCl, pH 8.0 at 15 °C. A transition to a native-like conformation can be induced, however, by adding ≥2 M NaCl, as shown by the comparison of the CD spectra in the amide and in the aromatic regions in the presence and in the absence of 2.5 M NaCl (Figure 2A, B). In 2.5 M NaCl both RCM forms show spectra in the amide region which resemble in shape the CD spectra of the forms with intact disulfide bonds, although the CD is slightly reduced. A similar spectrum was observed previously by Oobatake et al. (1979) for reduced RNase T1 under different conditions. In the aromatic region the CD is virtually unaltered by breaking of the disulfide bonds (Figure 2B); a small decrease in intensity is found only for a band near 285 nm. Interestingly, the changes in the aromatic CD spectrum were much smaller after the breaking of both disulfide bonds than after the breaking of only the Cys2−Cys10 bond [cf. Figure 2 of this paper and Figure 2 of Mayr et al. (1994a)]. The signs and intensities of the CD bands of disulfides depend strongly on their dihedral angles (Neubert & Carmack, 1974). In native RNase T1 this angle is positive (+78°) for the Cys2−Cys10 disulfide bond, but negative (−83°) for the Cys6−Cys103 disulfide bond (U. Heinemann, personal communication), and therefore, their contributions to the CD probably compensate each other. In summary, the CD spectra in Figure 2 demonstrate that in the absence of NaCl the two RCM forms are largely unfolded and virtually devoid of residual secondary structure. In the presence of 2.5 M NaCl they are in a folded conformation and display native-like secondary and tertiary structure.

The RCM forms show 1−2% of the activity of RNase T1 with intact disulfides under the same experimental conditions. This activity does not result from residual unmodified protein with intact disulfides and is only observed in the presence of 2.5 M NaCl. The activity of RNase T1 with intact disulfides is decreased to 40% when 2.5 M NaCl is added. An incubation of wild-type RNase T1 with iodoacetic acid did not lead to a significant deactivation, suggesting that the observed decrease in activity after reduction and carboxymethylation is not caused by a chemical modification of catalytic residues.

Stability of the RCM Forms as a Function of NaCl and Urea. The tryptophan fluorescence of the RCM forms of RNase T1 decreases strongly upon unfolding (Pace et al., 1988; Mücke & Schmid, 1992). The NaCl-induced refolding transitions of RCM-wt-T1 and of RCM-(-Pro55)-T1 as measured by fluorescence (Figure 3A) are very similar. RCM-(-Pro55)-T1 is slightly less stable than RCM-wt-T1, and the midpoints of the folding transitions are at 1.1 and at 0.8 M NaCl, respectively.

Unfolding can also be induced by adding urea to solutions of the two proteins that contain a constant concentration of

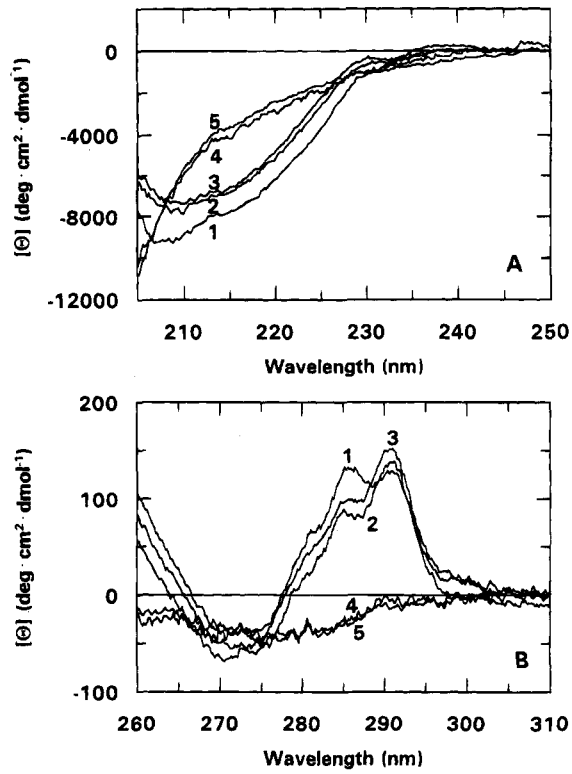

FIGURE 2: CD spectra of the various forms of RNase T1. All measurements were made in 0.1 M Tris-HCl, pH 8.0 at 15 °C. The buffer for the folded forms of RCM-wt-T1 and RCM-(Pro55)-T1 additionally contained 2.5 M NaCl. The spectra in the far-UV region (A) were recorded at a protein concentration of 10 μM in 0.1-cm cells, the spectra in the near-UV region (B) at 20 μM protein in 1.0-cm cells. (1) Folded 2SS-(-Pro55)-T1, (2) folded RCM-wt-T1, (3) folded RCM-(-Pro55)-T1, (4) unfolded RCM-wt-T1, (5) unfolded RCM-(-Pro55)-T1. Folded 2SS-wt-T1 shows the same spectra as 2SS-(-Pro55)-T1 (data not shown).

2.5 M NaCl. The respective transitions (Figure 3B) are reversible as well, and again RCM-(-Pro55)-T1 is found to be slightly less stable than RCM-wt-T1. Tentative two-state analyses of these transitions yield free energies of stabilization in the presence of 2.5 M NaCl and 0 M urea of −10.9 kJ/mol for RCM-wt-T1 and −10.2 kJ/mol for RCM-(-Pro55)-T1. When the disulfide bonds were left intact, these two forms of RNase T1 differed by about 3.6 kJ/mol in stability [at 60 °C; cf. Kiefhaber et al. (1990c)].

Apparently, tertiary and secondary structure break down cooperatively, and there is no evidence for partially folded intermediates in the NaCl-induced folding transitions of the RCM proteins. CD spectra of RCM-(-Pro55)-T1 at different NaCl concentrations, ranging from 0 to 3 M, show an isodichroic point near 207 nm (Figure 4A), and unfolding transitions monitored by CD at 202 nm and at 215 nm match the fluorescence-detected transition within experimental error (Figure 4B). The two-state character of the folding transition of RCM-(-Pro55)-T1 was confirmed by using unfolding assays to measure the concentration of native molecules as a function of the NaCl concentration (Mücke & Schmid, 1994b).

Unfolding and Refolding Kinetics of RCM-wt-T1 and RCM-(-Pro55)-T1. Unfolding experiments (Figure 5A) were carried out at the end of the transition (cf. Figure 3A) by diluting the folded proteins from 2.6 to 0.4 M NaCl. The measured kinetics were biphasic for both proteins and accounted for the entire decrease in fluorescence as observed in the equilibrium unfolding transitions. The relative

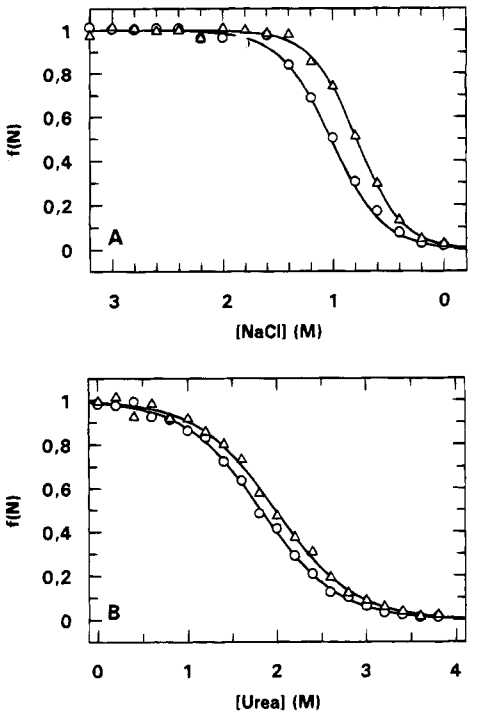

FIGURE 3: Folding transitions of (Δ) RCM-wt-T1 and (○) RCM-(-Pro55)-T1. The transitions were followed at pH 8.0, 15 °C, by measuring the fluorescence at 320 nm after excitation at 268 nm. The fraction of native protein is shown as a function of (A) decreasing NaCl concentration and (B) increasing urea concentration. The observed transitions were analyzed on the basis of a two-state model, and a linear dependence of the base lines in the pre- and posttransitional regions was assumed to determine the fraction of native protein f(N). The lines represent best fits of the data by using the procedure of Santoro and Bolen (1988). The proteins were incubated for at least 12 h in 0.1 M Tris-HCl, pH 8.0, 15 °C, in the presence of the indicated concentrations of NaCl or of 2.5 M NaCl and the indicated concentrations of urea.

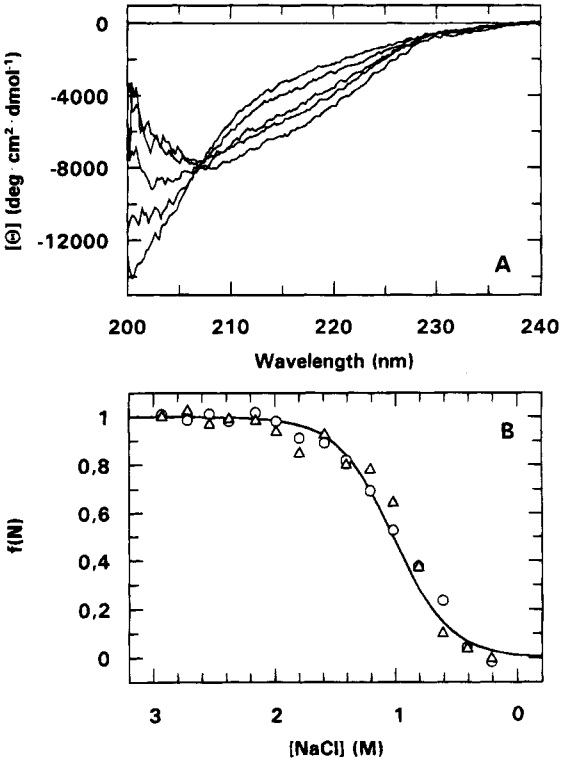

FIGURE 4: Folding transition of RCM-(-Pro55)-T1 monitored by amide CD. (A) CD spectra in 0.1 M Tris-HCl, pH 8.0, 15 °C, in the presence of 0, 0.6, 1.0, 1.4, and 3.0 M NaCl (from top to bottom at 220 nm and from bottom to top at 205 nm). The spectra were measured as described in Figure 2. (B) NaCl-induced folding transition. The experimental conditions were the same as in panel A. The normalized changes in ellipticity at 202 nm (○) and at 215 nm (Δ) are shown as a function of decreasing NaCl concentration. The fluorescence-detected transition (taken from Figure 2A) is shown as a continuous line for comparison.

amplitudes of the two phases were, however, strongly different for the two proteins. Unfolding of RCM-wt-T1 was dominated by the slow reaction (τ = 340 s, 88% amplitude), and the amplitude of the fast reaction (τ = 7 s) was only 12%. In the unfolding of RCM-(-Pro55)-T1 the amplitude of the slow reaction (τ = 125 s) was only 30%, and the fast reaction (τ = 11 s) had the major amplitude (70%).

The kinetics of refolding were measured after a dilution of the two RCM forms from 0 to 2.0 M NaCl (Figure 5B). Under these conditions refolding of RCM-wt-T1 was dominated by a slow reaction (τ = 1700 s, 98% amplitude), and the fast reaction (τ = 9 s) had a minor amplitude of only 2%. For RCM-(-Pro55)-T1 the slow refolding reaction was 3-fold faster (τ = 600 s) and the fast reaction was complete within the dead time of manual mixing (3 s). It could be measured, however, within the NaCl-induced transition (see Figures 9 and 11). These differences in the kinetics of unfolding and refolding suggest that the isomerization of Pro55 [which is absent in RCM-(-Pro55)-T1] contributes significantly to the unfolding and refolding kinetics of the RCM wild-type protein.

Both wild-type RNase T1 and the (-Pro55) variant form native-like secondary structure very rapidly during refolding when the disulfide bonds are intact and a native-like amide CD signal is regained in less than 15 ms (Kiefhaber et al., 1990c, 1992a; Mullins et al., 1993). This is not observed when the disulfide bonds are broken. During the refolding of RCM-(-Pro55)-T1 (Figure 6) only about 30% of the change in CD at 212 nm occurs in the dead time of the experiment, and this change originates predominantly from the refolding of the fast-folding molecules. The major change in CD at 212 nm (about 70%) occurs in the slow phase of folding. Protein chains with incorrect prolyl isomers apparently cannot form extensive native-like secondary structure prior to the rate-limiting step, because the stabilities of folded or partially folded molecules are strongly reduced when the disulfide bonds are broken.

Catalysis of Unfolding and Refolding of RCM-(-Pro55)-T1 by Prolyl Isomerase. Both unfolding and refolding of RCM-wt-T1 are catalyzed by prolyl isomerase (Mücke & Schmid, 1992), indicating that the slow folding of RNase T1 is limited by prolyl isomerization even when the disulfide bonds are broken. It was not possible, however, in these experiments, to relate this catalysis with a specific cis proline of the wild-type protein. RCM-(-Pro55)-T1 has only a single cis proline (at position 39), and its slow refolding reaction is also accelerated by prolyl isomerase (Figure 7A). This shows that the trans → cis isomerization of Pro39 is the rate-limiting step of refolding of RCM-(-Pro55)-T1. In unfolding, the cis ⇌ trans equilibration of Pro39 is also catalyzed (Figure 7B). As expected, the fast N ⇌ U_F unfolding reaction is not influenced by prolyl isomerase, and thus Pro39 isomerization approaches the fast unfolding reaction in rate when prolyl isomerase is present at high concentration.

Prolyl Isomerizations in the Unfolded State. The slow-folding species of RNase T1 are produced by prolyl cis/trans isomerizations in the denatured protein chains after the N

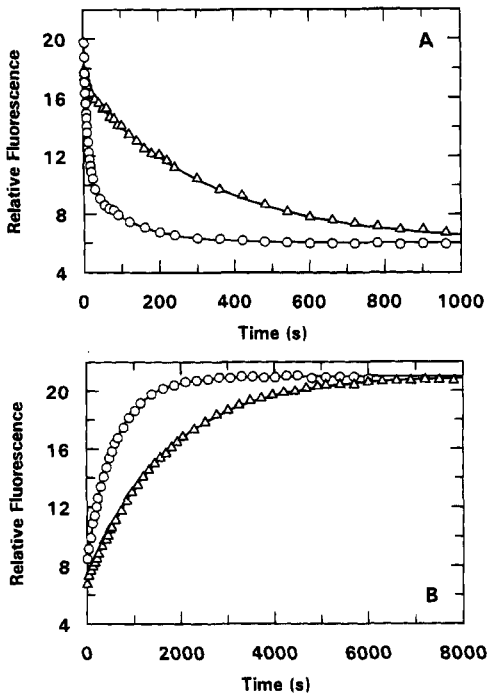

FIGURE 5: (A) Unfolding kinetics and (B) refolding kinetics of (Δ) RCM-wt-T1 and (○) RCM-(-Pro55)-T1. Folding and unfolding were measured by the change in fluorescence at 320 nm after excitation at 268 nm at a protein concentration of 0.7 μM. (A) For the unfolding reaction the protein was preincubated in 0.1 M Tris-HCl and 2.6 M NaCl, pH 8.0, 15 °C, overnight. Unfolding was initiated by a 40-fold dilution to final conditions of 0.1 M Tris-HCl and 0.4 M NaCl, pH 8.0, 15 °C. The unfolding kinetics for RCM-wt-T1 are taken from Mücke and Schmid (1992). For comparison the fluorescence values of the unfolded proteins were adjusted to give a final value of 6. The observed kinetics are well described by a sum of two exponential functions. For RCM-wt-T1 the slow phase has a time constant of 340 s and a reduced amplitude of 88%, and the fast phase has a time constant of 7 s and a reduced amplitude of 12%. For RCM-(-Pro55)-T1 the respective values are 125 s (30%) and 11 s (70%). (B) For the refolding reactions the proteins were unfolded in 0.1 M Tris-HCl, pH 7.8 at 25 °C, overnight. Refolding was initiated by a 40-fold dilution to final conditions of 0.1 M Tris-HCl and 2.0 M NaCl, pH 8.0, 15 °C. The refolding of RCM-wt-T1 follows a biexponential time course with time constants of 1700 s (98% amplitude) and 9 s (2% amplitude). The refolding of RCM-(-Pro55)-T1 is monophasic with a time constant of 600 s. The fast refolding reaction is complete in the dead time of mixing (3 s).

→ U_F unfolding reaction has occurred. Their formation can be followed by a double-mixing technique, where the amplitudes of the slow refolding reactions in the second step are measured as a function of the duration of unfolding in the first step (Brandts et al., 1975; Schmid, 1986).

The formation of the U_S species of RCM-wt-T1 is shown in Figure 8 and compared with the data for RCM-(-Pro55)-T1, which had been measured earlier (Mücke & Schmid, 1994a). Both proteins were rapidly unfolded in 2.0 M urea and 0.2 M NaCl, at pH 8.0, 15 °C, and the refolding assays were carried out in 1.6 M NaCl and 0.2 M urea. Under the unfolding conditions the N → U_F reactions of the two proteins are complete within the first 5 s of unfolding. The rate of formation of U_S molecules is different for the two proteins. When both cis prolines are present (in RCM-wt-T1), it is about 2-fold higher than when only cis-Pro39 is present [in RCM-(-Pro55)-T1]. This result can be understood on the basis of the kinetic schemes in eqs 1−3. The rate of formation of U_S from U_F is faster when two independent isomerizations lead away from U_F (eq 2) instead of only one

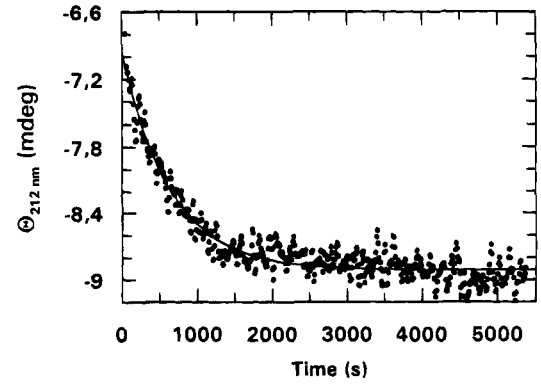

FIGURE 6: Slow formation of secondary structure during the refolding of RCM-(-Pro55)-T1. The change in the CD signal at 212 nm is shown as a function of the refolding time. The unfolded protein (in 0.05 M Tris-HCl and 0.1 M sodium acetate, pH 8.0, 25 °C) was refolded by a 15-fold dilution to give final conditions of 0.1 M Tris-HCl and 2.6 M NaCl, pH 8.0, 15 °C. The final protein concentration was 10 μM. The signal of the native protein was set as 100%. The change of the CD signal is well described by a single first-order reaction with a time constant of 620 s.

(eq 1). These results suggest strongly that both Pro39 and Pro55 are cis in folded RNase T1 when the disulfide bonds are absent, that they isomerize in the unfolded protein, and that an isomerization of either proline is sufficient to create a U_S species.

Unfolding and Refolding in the NaCl-Induced Transitions. The comprehensive analysis of the kinetics of both unfolding and refolding in the transition region is the best method to determine the number of prolines that are involved in folding, because the observed kinetics differ in a characteristic manner when one or two prolyl isomerizations are coupled with the N ⇌ U_F unfolding reaction (Kiefhaber et al., 1992b). Partially-folded intermediates are usually not populated in the transition region, and therefore, the three- and five-species mechanisms in eqs 1 and 2 can be used to determine the microscopic rate constants k_{ij} of folding and isomerization from the experimentally measured rate constants λ_i and amplitudes a_i.

The simpler case is provided by RCM-(-Pro55)-T1 which follows the three-species mechanism in eq 1. Unfolding and refolding are reversible reactions, and the final fluorescence values observed in the kinetic experiments coincide with the NaCl-induced equilibrium transition (Figure 9A). The kinetics are well approximated by a sum of two exponential functions throughout the transition region, and identical values were found for the measured rate constants λ_1 and λ_2 in unfolding and in refolding experiments under identical conditions (Figure 9B). The respective amplitudes a_1 and a_2 are shown in Figure 9C for refolding and in Figure 9D for unfolding. λ_1 and λ_2 are separated by more than a factor of 10 under all conditions. This factor is about 1000 in the native baseline region (above 2 M NaCl) (Figure 9B), and λ_1 becomes equal to $k_{12} = 1.7 \times 10^{-3}$ s^{-1} (cf. eq 1). In the transition region (1.6−0.2 M NaCl) λ_1 increases about 7-fold and reaches a limiting value of 1.27×10^{-2} s^{-1} near 0 M NaCl. Under these conditions λ_1 is equal to $k_{12} + k_{21}$. The dependence on NaCl of the measured rate constant of fast folding λ_2 shows a V-shaped profile with a minimum at the midpoint of the N ⇌ U^{39c} transition (0.55 M NaCl), where $k_{23} = k_{32}$.

In refolding the slow reaction has the major amplitude (Figure 9C), because U^{39t} dominates in unfolded RCM-(-Pro55)-T1. In unfolding the amplitude of the fast reaction

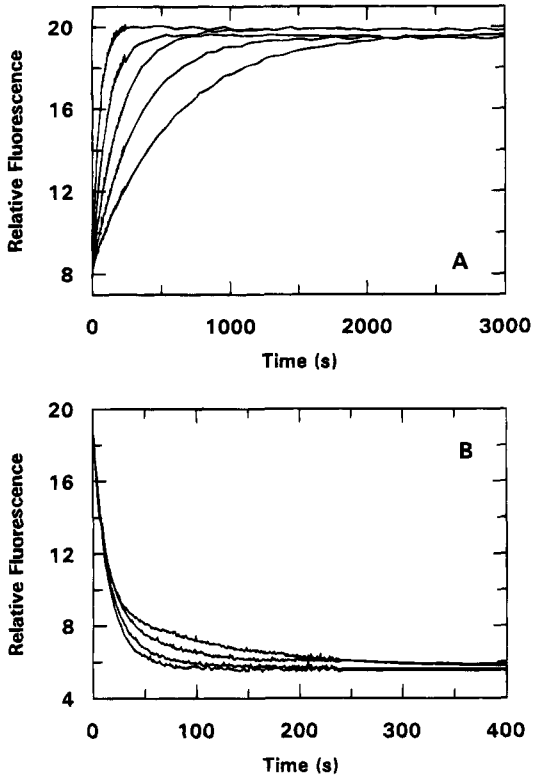

FIGURE 7: Catalysis of (A) refolding and (B) unfolding of RCM-(-Pro55)-T1 by prolyl isomerase. The change in fluorescence at 320 nm (excitation at 268 nm) is shown as a function of the time of refolding. (A) Refolding was initiated by a 40-fold dilution of unfolded RCM-(-Pro55)-T1 (in 0.1 M Tris-HCl, pH 8.0, 15 °C) to give final conditions for folding of 0.7 μM protein in 2.0 M NaCl and 0.1 M Tris/HCl, pH 8.0, 15 °C. The refolding kinetics in the presence of (from bottom to top) 0, 30, 60, 120, and 300 PU/ml prolyl isomerase are shown. The time constants of refolding were (from bottom to top) 570 s, 310 s, 200 s, 100 s, and 52 s. (B) Unfolding was initiated by a 40-fold dilution of folded protein (in 0.1 M Tris-HCl, pH 8.0, 15 °C and 2.6 M NaCl) to final conditions of 0.7 μM protein in 0.1 M Tris-HCl, pH 8.0, 15 °C and 0.4 M NaCl. The kinetics of unfolding in the presence of (from top to bottom) 0, 50, 110, and 270 PU/ml prolyl isomerase are shown. The time constants of unfolding were (from top to bottom) 120 s (28%) and 11 s (72%); 49 s (31%) and 11 s (69%); 37 s (29%) and 12 s (71%); 21 s (56%) and 9 s (44%).

a_2 increases strongly at the expense of the amplitude of the slow reaction a_1 with decreasing concentration of NaCl (Figure 9D), and near 0 M NaCl a_1 approaches zero. This is expected, because the ordered structure is lost in the N $\rightarrow$ U^{39c} unfolding reaction, and the $U^{39c} \rightleftharpoons U^{39t}$ equilibration is accompanied by a change in fluorescence only when it is coupled with further unfolding in the N $\rightleftharpoons$ U^{39c} reaction as in the transition region. In the region of the unfolded base line the $U^{39c} \rightleftharpoons U^{39t}$ isomerization can therefore be measured only by slow refolding assays as in Figure 8.

Kinetic Analysis of Folding of RCM-(-Pro55)-T1. The kinetics of a linear three-species mechanism as in eq 1 were discussed by Ikai and Tanford (1973), Hagerman and Baldwin (1976), Hagerman (1977), and Kiefhaber et al. (1992b), and analytical expressions were derived to calculate the four microscopic rate constants (k_{12}, k_{21}, k_{23}, and k_{32}) from the measured amplitudes (a_1^R, a_2^R, a_1^U and a_2^U) and rate constants (λ_1 and λ_2) and from the equilibrium constant K_{eq}. In the transition region the unfolding and the refolding kinetics and K_{eq} could be measured, yielding seven experimental parameters (two apparent rate constants, four amplitude values, and K_{eq}) to determine the four microscopic rate

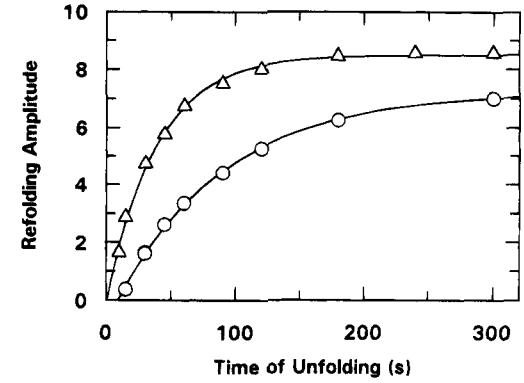

FIGURE 8: Time course of formation of slow refolding molecules of (Δ) RCM-wt-T1 and (○) RCM-(-Pro55)-T1 after rapid denaturation. The amplitudes of the slow refolding reactions are shown as a function of the time of incubation under unfolding conditions. The two RCM forms were preincubated in 0.05 M Tris-HCl, 0.05 M sodium acetate, and 2.8 M NaCl, pH 8.0, 15 °C, to produce the folded state. Rapid unfolding was then induced by a 14-fold dilution to 0.1 M Tris-HCl, 2.0 M urea, and 0.2 M NaCl, pH 8.0, 15 °C. After various times of unfolding aliquots were withdrawn and diluted 10-fold to give refolding conditions of 0.1 M Tris-HCl, 1.6 M NaCl, and 0.2 M urea, pH 8.0, 15 °C. The final protein concentration was 0.45 μM. The slow refolding reactions were monitored by the change in fluorescence at 320 nm (excitation at 268 nm). The time constants of slow refolding under the assay conditions were 2030 s and 560 s for RCM-wt-T1 and RCM-(-Pro55)-T1, respectively. The time constants for the formation of U_S are 38 s for RCM-wt-T1 and 84 s for RCM-(-Pro55)-T1. The corresponding fits are shown by the continuous lines. The data for RCM-(-Pro55)-T1 are taken from Mücke and Schmid (1994a).

constants of eq 1. We employed the method described by Mücke and Schmid (1994a) to determine these microscopic rate constants as a function of the NaCl concentration by using as experimental values the equilibrium constant K_{eq} and the measured rate constants λ_1 and λ_2 in combination either with the amplitude of the slow unfolding reaction (a_1^U) or with the amplitude of the slow refolding reaction (a_1^R). The results are shown in Figure 10. To a first approximation the rate constants of Pro39 isomerization in the $U^{39c} \rightleftharpoons U^{39t}$ reaction (k_{12} and k_{21}) are independent of the NaCl concentration and show values of $k_{12} = 1.8 \times 10^{-3}$ s^{-1} and $k_{21} = 1.1 \times 10^{-2}$ s^{-1}. They agree well with the rate constants for the $U^{39c} \rightleftharpoons U^{39t}$ equilibration in the presence of 2.0 M urea as derived from the double-mixing experiments in Figure 8. For the N $\rightleftharpoons$ U^{39c} folding transition ln k_{23} and ln k_{32} depend linearly on the concentration of NaCl, as is often observed in denaturant-induced transitions (Tanford, 1968; Matthews, 1987; Jackson & Fersht, 1991). The rate constant of refolding k_{23} increases 9-fold and the rate of unfolding k_{32} decreases 13-fold when the NaCl concentration is increased by 1 M (Figure 10). The calculated microscopic rate constants in Figure 10 reproduce not only the dependence on NaCl of the measured values for λ_1 and λ_2 (Figure 9B) and of the amplitudes of the slow phases a_1^U and a_1^R, which were used for the calculations, but also the amplitudes of the fast phases a_2^U and a_2^R (Figure 9C,D). This good agreement suggests that the folding kinetics of RCM-(-Pro55)-T1 in the region of the NaCl transition can be modeled by the simple three-state mechanism in eq 1 and that the microscopic rate constants in Figure 10 describe the kinetics of folding and isomerization very well.

The microscopic rate constants of unfolding and refolding k_{23} and k_{32} become equal at the midpoint of the N $\rightleftharpoons$ U^{39c} folding transition, and the lines for ln k_{23} and ln k_{32} in Figure 10 intersect each other. This point is reached at 0.55 M

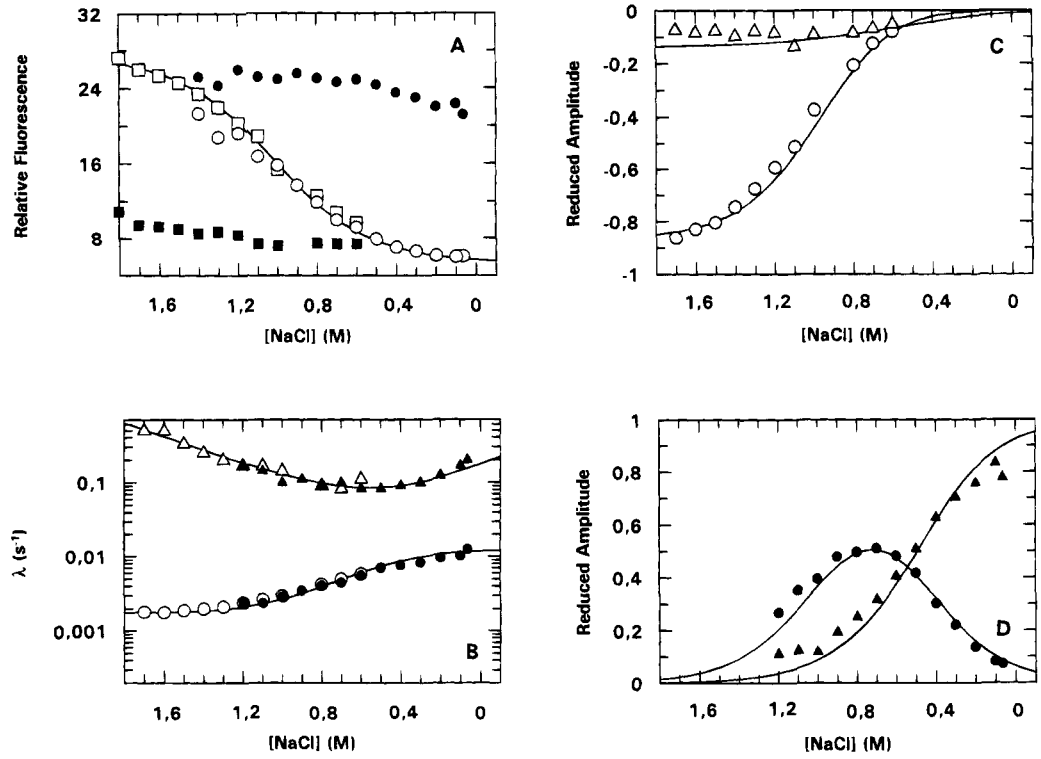

FIGURE 9: Kinetic analysis of the folding reactions of RCM-(-Pro55)-T1 within the NaCl-induced folding transition. The final conditions for all experiments were 0.1 M Tris-HCl, pH 8.0, 15 °C, and the indicated concentration of NaCl. Unfolding and refolding kinetics were followed by the change in fluorescence at 320 nm after excitation at 268 nm. Reactions were started by a 40-fold dilution to the final conditions. The protein concentration was 0.7 μM. For the unfolding experiments the protein was preincubated in 0.1 M Tris-HCl and 2.6 M NaCl, pH 8.0, 15 °C, to produce the folded state. For the refolding experiments the protein was first unfolded in 0.1 M Tris-HCl, pH 8.0, 25 °C. (A) The initial and the final values of the kinetics are shown as a function of the NaCl concentration. The curve shows the fit for the equilibrium folding transition (taken from Figure 2A). (■) Initial and (□) final values of the refolding reaction; (●) initial and (○) final values of the unfolding reaction. (B) Measured rate constants λ_1 and λ_2 for the unfolding and refolding reactions as a function of the NaCl concentration. (○) λ_1 and (△) λ_2 of refolding; (●) λ_1 and (▲) λ_2 of unfolding. (C) Reduced amplitudes (○) of the slow and (△) of the fast refolding reaction. (D) Reduced amplitudes (●) of the slow and (▲) of the fast unfolding reaction. The continuous lines in panels B–D were calculated by using the best fits for the microscopic rate constants shown in Figure 10 and the method described by Mücke and Schmid (1994a).

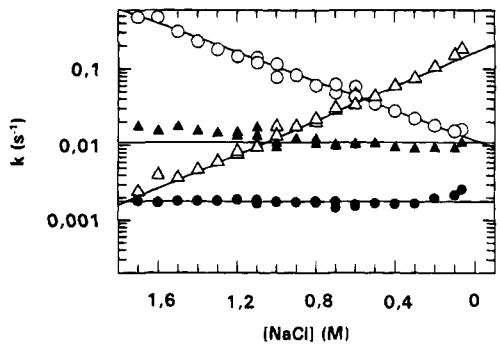

FIGURE 10: Analysis of the kinetic data in Figure 9 based on the mechanism in eq 1, as described by Mücke and Schmid (1994a). The dependences of (●) k_{12}, (▲) k_{21}, (○) k_{23}, and (△) k_{32} on the NaCl concentration are shown. The lines represent constant values of 1.8×10^{-3} s^{-1} and 1.1×10^{-2} s^{-1} for k_{12} and k_{21}, respectively. The lines drawn for k_{23} and k_{32} follow the equations $\ln k_{23} = -4.44 + (2.2\ M^{-1})[NaCl]$ and $\ln k_{32} = -1.80 - (2.58\ M^{-1})[NaCl]$.

NaCl, and the respective value for k_{23} ($=k_{32}$) = 4.0×10^{-2} s^{-1}. This value is remarkably similar to the value of k_{23} ($=k_{32}$) = 3.0×10^{-2} s^{-1} observed in the urea-induced unfolding transition of this protein (Mücke & Schmid, 1994a).

Folding Kinetics of RCM-wt-T1. Folding and unfolding of RCM-wt-T1 are also reversible reactions, and the final fluorescence values observed in the kinetic experiments follow the equilibrium transition (Figure 11A). The slow folding reaction of RCM-wt-T1 had a large amplitude in both unfolding and refolding experiments, and its apparent rate constant λ_1 could be determined in single-mixing experiments with high accuracy. It is shown in Figure 11B as a function of the NaCl concentration. Only 2–4% of fast folding U_{55c}^{39c} molecules are present in unfolded RCM-wt-T1 (cf. eq 2). Therefore, the amplitude of the fastest reaction (phase 3) is extremely small, and λ_3 could not be measured in these experiments. A transient very high concentration of U_{55c}^{39c} molecules could be produced, however, by a short 15 s unfolding pulse in 2.0 M GdmCl and 0.2 M NaCl, pH 8.0. Under these unfolding conditions the $N \rightarrow U_{55c}^{39c}$ reaction (eq 2) is complete within 3 s and the subsequent prolyl isomerizations are slow (τ = 40 s). Therefore, about 70% of all molecules are in the U_{55c}^{39c} state after this unfolding pulse, and the rate of fast refolding λ_3 of these molecules could be measured with good accuracy as a function of the NaCl concentration in the second step of the experiment (Figure 11B). The two measured rate constants λ_1 and λ_3 are well separated throughout the NaCl-induced folding transition. Above 1 M NaCl the fast reaction is more than 1000-fold faster than the slow reaction, and even in the transition region they differ by more than 20-fold.

Kinetic Analysis of Folding of RCM-wt-T1. For RCM-wt-T1 two prolyl isomerizations are coupled with folding and the scheme with five species in eq 2 is the simplest model for the folding mechanism. Even when the two isomerizations in the unfolded protein are identical in rate (eq 3), three

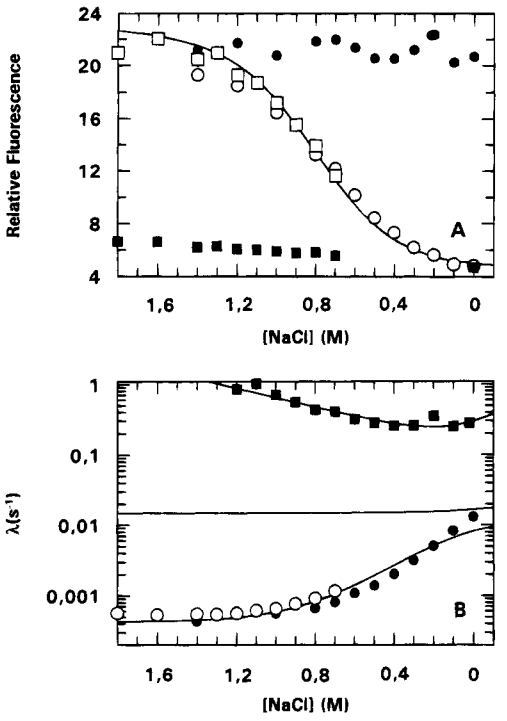

FIGURE 11: Kinetic analysis of the folding reactions of RCM-wt-T1 within the NaCl-induced folding transition. For the determination of λ_1 the same experimental conditions were used as for RCM-(-Pro55)-T1. They are described in the legend to Figure 9. (A) Initial and final values of the kinetics as a function of the NaCl concentration. The continuous line shows the fit for the equilibrium folding transition, (taken from Figure 2A). (■) Initial and (□) final values of the refolding reaction; (●) initial and (○) final values of the unfolding reaction. (B) Measured rate constants λ_1 and λ_3 for the unfolding and refolding reactions as a function of the NaCl concentration. (●) λ_1 of unfolding; (○) λ_1 of refolding; (■) λ_3 of refolding measured after 15 s of unfolding. For the determination of λ_3 within the transition region a double-mixing technique was applied. Native protein (in 2.8 M NaCl, pH 8.0, 15 °C) was rapidly unfolded by a 14-fold dilution to 2.0 M GdmCl and 0.2 M NaCl, pH 8.0, 15 °C, for 15 s to produce the species U_{55c}^{39c}. Refolding was then started by a 10-fold dilution to pH 8.0, 15 °C, and concentrations of NaCl ranging from 0.02 to 1.2 M. The final protein concentration was 0.4 μM. All samples contained a residual concentration of 0.2 M GdmCl, which leaves the stability of RNase T1 almost unaffected, because stabilizing and destabilizing effects cancel each other (Mayr & Schmid, 1993c). The lines in panel B represent the calculated values for λ_1, λ_2, and λ_3 (from bottom to top). They were calculated on the basis of eqs 8a-d by using NaCl-independent values of $k_{12} = 1.8 \times 10^{-3}$ s^{-1} and $k_{21} = 1.1 \times 10^{-2}$ s^{-1} and the NaCl-dependent values for k_{23} and k_{32} calculated from the equations ln $k_{23} = -2.07 + (1.60\ M^{-1})$[NaCl] and ln $k_{32} = -1.79 - (4.96\ M^{-1})$[NaCl].

kinetic phases should be observable in unfolding and in refolding with three apparent rate constants $\lambda_1-\lambda_3$ and three amplitudes a_1-a_3 as experimental parameters to determine the microscopic rate constants in eq 3. Unfortunately, the amplitude of phase 2 was virtually zero under many conditions [cf. Kiefhaber et al. (1992b)], and phase 3 could be observed only in the double-mixing experiments. As a consequence, the number of experimental parameters is not sufficient for a rigorous analysis of the kinetic data. Instead, a tentative analysis of the unfolding and refolding kinetics of RCM-wt-T1 is carried out, which is based on the observed rate constants λ_1 and λ_3 only.

The strong separation in rate between the fast and the slow reaction greatly facilitates this analysis, because then the N ⇌ U_F transition can be treated as an independent two-state reaction. λ_3 is equal to the sum of the microscopic rate constants k_{23} and k_{32} in this case (eq 4) and does not depend on the rate constants of prolyl isomerization k_{12} and k_{21} (Kiefhaber et al., 1992b). An excellent fit of the experimental data for λ_3 in Figure 11B is obtained by assuming a linear dependence on [NaCl] of ln k_{23} and ln k_{32} according to eqs 5a and 5b. These fits yield values of $k_{23}^0 = 0.13$ s^{-1}, $k_{32}^0 = 0.17$ s^{-1}, $m_{23} = 1.60$ M^{-1}, and $m_{32} = -4.96$ M^{-1}. k_{23} and k_{32} show identical values of 0.12 s^{-1} at 0.3 M NaCl, which is the midpoint of the N ⇌ U_{55c}^{39c} transition.

The microscopic rate constants k_{12} and k_{21} of the isomerizations of Pro39 and Pro55 in the unfolded protein could not be determined in a similar fashion from the dependence on NaCl concentration of the observed rate constant λ_1. Instead, in a first approximation, we assumed that the isomerizations of the two prolines are identical with the isomerization of Pro39 in unfolded RCM-(-Pro55)-T1 and show the same microscopic rate constants $k_{12} = 1.8 \times 10^{-3}$ s^{-1} and $k_{21} = 1.1 \times 10^{-2}$ s^{-1}, independent of the NaCl concentration (cf. Figure 10).

The microscopic rate constants of folding and isomerization and the equilibrium constant of folding K_{eq} are related by eq 7. We have therefore used eq 7 and the four microscopic rate constants k_{12}, k_{21}, k_{23}, and k_{32} as determined above to calculate K_{eq} as a function of the NaCl concentration. These calculated values are identical with the values for K_{eq} that result from the two-state analysis of the experimental equilibrium transition in Figure 3A. This coincidence provides strong support for the validity of the approximations that were used to estimate the microscopic rate constants.

The set of microscopic rate constants has also been used to recalculate the macroscopic rate constants λ_1, λ_2, and λ_3 as a function of NaCl concentration on the basis of the mechanism in eq 3 by solving the characteristic equation (eqs 8a–d). The calculated profile for λ_3 fits the experimental data in Figure 11B very well, which indicates that the separate analysis of the fast refolding reaction on the basis of eq 6 povides the correct values for k_{23} and k_{32}. The calculated profile for λ_1 in Figure 11B coincides also reasonably well with the experimental values for λ_1 as a function of the NaCl concentration. Experimental and calculated values differ slightly at low NaCl concentrations, because phase 2 contributes to the measured kinetics. Phase 2 has an extremely small amplitude under almost all conditions, except in unfolding experiments near the end of the transition [0–0.2 M NaCl, cf. Kiefhaber et al. (1992b)]. Under these conditions λ_1 and λ_2 become very similar, and the experimentally measured rate constants of the slow reaction approach the profile for λ_2 at 0 M NaCl (cf. Figure 11B).

The dependence on NaCl concentration of the measured rate of slow folding in Figure 11B provides a diagnostic tool to evaluate the effect of cis proline mutations on the folding kinetics. When two prolyl isomerizations instead of one are coupled with folding (cf. eq 2), then U_S species are produced by two independent isomerizations and therefore the rate of their formation after unfolding should increase. On the other hand, in the refolding direction two isomerizations must occur instead of one before folding (U_{55c}^{39c} → N) can proceed, and therefore, slow refolding should be decelerated. As a consequence, in the presence of an additional cis proline λ_1 should increase under unfolding conditions, but decrease under refolding conditions. These simple considerations can

be applied here, because the rate of folding ($N \rightleftharpoons U_{55c}^{39c}$) and the rates of isomerization of Pro39 and Pro55 are well separated. A comparison of the data for RCM-wt-T1 (Figure 11B) with the data for RCM-(-Pro55)-T1 (Figure 9B) shows that the dependences on NaCl of λ_1 differ strongly indeed. The value of λ_1 of RCM-wt-T1 in the native base line of Figure 11B (5.0×10^{-4} s^{-1} at 1.6 M NaCl) is 3.5-fold smaller, and the value in the unfolded base-line region (1.8×10^{-2} s^{-1} at 0 M NaCl) is 2-fold larger than the corresponding λ_1 values of RCM-(-Pro55)-T1 in Figure 9B. As a consequence λ_1 of RCM-wt-T1 varies about 50-fold in the transition region, whereas λ_1 of RCM-(-Pro55)-T1 varies only about 7-fold, as expected when the isomerization of Pro55 contributes to the folding kinetics of RCM-wt-T1.

The assumption that Pro39 and Pro55 show identical kinetics of isomerization in unfolded RNase T1 is an oversimplification and may also be responsible for the small deviation between the experimental and the calculated values for λ_1 in Figure 11B. The two proline residues are flanked by different amino acids (Tyr38-Pro39-His40 and Ser54-Pro55-Tyr56), and therefore, they may differ in the kinetics of isomerization. Nevertheless, the kinetic analysis of the folding transition of RCM-wt-T1 suggests strongly that both Pro39 and Pro55 remain cis in the folded protein when the disulfide bonds are broken and that the isomerizations of both are slow and rate-limiting steps in unfolding and in refolding.

DISCUSSION

Cis Conformations of Pro39 and Pro55 in RNase T1 with Broken Disulfide Bonds. Native RNase T1 with intact disulfide bonds has two cis peptide bonds at Pro39 and at Pro55, as shown by X-ray crystallography (Heinemann & Saenger, 1982; Martinez-Oyanedel et al., 1991). Both these prolines remain cis after breaking the two disulfide bonds, provided that the folded conformation is stabilized by adding NaCl. We draw this conclusion not from structural data but from the analysis of the unfolding and refolding kinetics of variants of RNase T1 with one or two cis prolines and with the disulfide bonds either intact or broken. In the absence of the disulfide bonds, folded forms with a trans-Pro39 or a trans-Pro55 could not be detected.

Nature of the NaCl-Induced Folding Transition of the RCM Forms. The RCM forms of RNase T1 are in an unfolded state in the absence of denaturants, such as urea or GdmCl. They are thus interesting models to study the denatured state of a protein under refolding conditions and to examine whether a conformation of the molten globule type (Kuwajima, 1989; Ptitsyn, 1992) can be attained. The analysis by circular dichroism (Figures 2 and 4) indicates that little if any ordered secondary structure is present and that RNase T1 is unfolded at pH 8.0, 15 °C, when the disulfide bonds are broken. A native-like secondary structure can be induced in the RCM molecules, however, by adding NaCl. This salt-induced folding transition is cooperative and shows properties of a two-state process. The transition curves monitored by fluorescence and by CD are identical within experimental error, and the CD spectra at various concentrations of NaCl are well represented by linear combinations of the spectra for the native and unfolded proteins. In addition, the decrease in the fraction of native molecules parallels the fractional changes in the spectral properties (Mücke & Schmid, 1994b)

A folded state with a native-like CD spectrum in the aromatic region is attained at ≥2 M NaCl, not a molten globule as in the salt-induced transitions of several other proteins (Goto et al., 1990a,b; Hagihara et al., 1993; Hamada et al., 1993). The folded and the unfolded states of the RCM forms of RNase T1 interconvert slowly in the NaCl-induced transition, unlike intermediates of the molten-globule type, which are usually in rapid equilibrium with the unfolded protein (Kuwajima, 1989). Apparently, a molten-globule conformation cannot easily be induced in RNase T1, neither by removing two strong tertiary interactions, nor by adding salt to an unfolded form of the protein. In the first case the protein unfolds, and in the second case a native-like conformation is attained. These properties distinguish RNase T1 from several other small proteins, such as α-lactalbumin (Kuwajima, 1977; Ikeguchi et al., 1986a,b), apo-myoglobin (Griko et al., 1988; Hughson et al., 1990; Barrick & Baldwin, 1993a,b), staphylococcal nuclease (Shortle & Meeker, 1989; Dill & Shortle, 1991; Flanagan et al., 1993), or cytochrome *c* (Goto et al., 1990a,b).

Role of Pro39 and Pro55 for Folding in the Absence of the Disulfide Bonds. To dissect the roles of cis-Pro39 and cis-Pro55 for the folding mechanism of RNase T1 in the absence of the disulfide bonds, we compared the unfolding and refolding kinetics of RCM-wt-T1, which contains both cis prolines, and of RCM-(-Pro55)-T1, which contains only cis-Pro39. The role of this proline was characterized by a rigorous kinetic analysis of the NaCl-induced folding transition of RCM-(-Pro55)-T1. This analysis was feasible, because folding was reversible, because the two kinetic phases could be detected under all conditions, and because the amplitudes and apparent rate constants could be measured in both the unfolding and the refolding direction. A complete set of macroscopic kinetic parameters (λ_1, λ_2, a_1^R, a_2^R, a_1^U, and a_2^U) could thus be obtained, and their dependence on NaCl concentration could be modeled by a set of four microscopic rate constants (k_{12}, k_{21}, k_{23}, and k_{32}). The NaCl-dependent folding kinetics of RCM-(-Pro55)-T1 are well explained by the three-species mechanism in eq 1. After the rapid $N \rightarrow U^{39c}$ unfolding reaction U^{39c} is slowly converted to U^{39t} by the isomerization of Pro39 until an equilibrium distribution of about 15% U^{39c} and 85% U^{39t} is reached. The individual rate constants $k_{12} = 1.7 \times 10^{-3}$ s^{-1} for the trans → cis and $k_{21} = 1.1 \times 10^{-2}$ s^{-1} for the cis → trans isomerization of Pro39 are virtually independent of the NaCl concentration and agree well with the corresponding values observed in the urea-induced unfolding transitions of RCM-(-Pro55)-T1 and of 2SS-(-Pro55)-T1 (Mücke & Schmid, 1994a). This indicates that the covalent cross-linking of the unfolded RNase T1 molecules by the disulfide bonds does not affect the equilibrium constant and the rate constants of the Pro39 cis ⇌ trans isomerization.

Such favorable conditions for the kinetic analysis as in this case are rarely met in experimental studies of protein folding. Often the fast phases of refolding lose amplitude in the transition because the concentrations of fast refolding species are small and because the rates of the fast and the slow phases approach each other (Hagerman & Baldwin, 1976; Hagerman, 1977).

The replacement of the other cis proline (Pro39) by alanine strongly destabilized the protein (Mayr et al., 1993b) and changed the folding mechanism, because the 38−39 peptide bond remains in the cis conformation after the Pro39Ala substitution (Mayr et al., 1994b). A novel very slow reaction,

the trans → cis isomerization of the Tyr38-Ala39 bond, occurs in the refolding of this variant and masks the isomerization of Pro55 (Mayr & Schmid, 1993b; Odefey et al., unpublished results). Therefore, the kinetic approach of using a RCM variant with a single cis proline only at position 55 to elucidate its role for the folding mechanism could not be used. Instead, we inferred the role of Pro55 from the comparison of the unfolding and the refolding kinetics of the RCM forms of the wild-type protein and the Pro55 variant. The presence of the second cis proline (in RCM-wt-T1) led to characteristic changes in the folding kinetics: the amplitude of the fast folding reaction was strongly decreased, and the rate of the slow reaction was increased in unfolding, but decreased in refolding. The kinetic mechanisms in eqs 1 and 2 predict such changes when the additional proline is cis and when its trans → cis isomerization is a slow step of folding. Therefore, we propose that the isomerizations of both Pro39 and Pro55 are involved in the slow-folding reactions of RNase T1 when the disulfide bonds are broken. The results also confirm our earlier conclusion (Kiefhaber et al., 1990c) that after the replacement of cis Pro55 the Gly54-Asn55 peptide bond is trans in the folded protein with intact disulfide bonds.

The direct N ⇌ U_F folding reactions of both RCM forms remain significantly faster than the proline-limited U_F ⇌ U_S isomerizations under all conditions (Figures 9B and 11B). This is in contrast to the folding of RNase T1 with intact disulfide bonds, where the direct folding reaction becomes much slower than the proline-limited reactions near the midpoint of the folding transition (Kiefhaber & Schmid, 1992). As discussed previously (Mücke & Schmid, 1994a), this suggests that the presence of the disulfide bonds may interfere unfavorably with the chain folding of RNase T1.

Influence of the S54G/P55N Mutation on the Stability of RNase T1. The substitution of a cis proline by a residue that is trans in the folded molecules can affect protein stability in several ways. First, it eliminates a slow isomerization in the unfolded molecules and thus stabilizes the native state by $\Delta\Delta G_D = RT \ln K_{ct}$ (where K_{ct} is the equilibrium constant [cis]/[trans] for the substituted proline in the unfolded wild-type protein). In addition, the amino acid substitutions that were introduced to replace the cis proline can change the stabilities of both the native and the unfolded states of the protein.

For both RCM-wt-T1 and RCM-(-Pro55)-T1 the direct folding reaction (N ⇌ U_F) and the prolyl isomerization (U_F ⇌ U_S) differ strongly in rate throughout the transition region, and therefore, the consequences of the substitution of cis-Pro55 for the actual unfolding reaction (N ⇌ U_F) and for the prolyl isomerization (U_F ⇌ U_S) could be analyzed separately. The effects on the N ⇌ U_F unfolding reaction were evaluated by comparing the profiles for λ_2 in Figure 9B and λ_3 in Figure 11B. These profiles reach minima at the midpoints of the respective N ⇌ U_F transitions, where $k_{23} = k_{32}$, and thus report on the change in stability of N relative to U_F caused by the S54G/P55N mutation. The minimum for λ_2 in the folding kinetics of RCM-(-Pro55)-T1 is at 0.55 M NaCl (Figure 9B); the minimum for λ_3 in the folding of RCM-wt-T1 (Figure 11B) is at 0.1 M NaCl. This suggests that the S54G/P55N mutation destabilizes the N form of RCM-wt-T1 by about 5 kJ/mol relative to U_F.

The difference in overall stability between RCM-wt-T1 and RCM-(-Pro55)-T1 is, however, much smaller: the midpoints of the N ⇌ (U_F + U_S) transitions in Figure 3 differ only by 0.2 M NaCl, which is equivalent to a decrease in ΔG_D of about 2 kJ/mol. Apparently, the unfavorable effect of the S54G/P55N mutation on the N ⇌ U_F equilibrium is in part compensated by the elimination of Pro55 isomerization in the unfolded protein. This results in a favorable contribution and increases the stability by about 3 kJ/mol, leading to a net change in ΔG_D of only about 2 kJ/mol. A stabilizing contribution of 3 kJ/mol is indeed expected when the cis/trans equilibrium at Pro55 in the unfolded wild-type protein is 20/80.

The comparison of the profiles for λ_2 in Figure 9B and for λ_3 in Figure 11B indicates that the decrease in the stability of N relative to U_F after the S54G/P55N mutation originates almost entirely from a decrease in the rate of refolding and not from an increase in the rate of unfolding. An interpretation of these changes based on the two-state model and the approach of Matthews and co-workers (Matthews, 1987; Jennings et al., 1991) would indicate that the S54G/P55N mutation changed the Gibbs free energy of the unfolded state U_F relative to the transition state of folding and not the free energy of the native state relative to the transition state.

Taken together, the S54G/P55N mutation seems to affect primarily the entropy of the unfolded state of RNase T1. The deletion of a prolyl cis/trans isomerization in the unfolded protein should decrease its entropy, and thus increase the overall stability. On the other hand, the replacement of a conformationally restricted residue (Pro55) and the introduction of a less restricted residue (Gly54) should increase the entropy ot the unfolded state (Matthews et al., 1987) and thus destabilize N relative to U_F. This interpretation is certainly an oversimplification, because it does not account for effects of the S54G/P55N mutation on the energy of the folded protein.

Concluding Remarks. The stability of RNase T1 is strongly reduced when the disulfide bonds are broken, but still both Pro39 and Pro55 are in the cis conformation. Folded molecules with trans isomers could not be found. This indicates that trans prolines at these positions would destabilize folded RCM-RNase T1 more strongly than the steric strain exerted by the respective cis prolyl bonds. It should be noted that the unfavorable contributions of the strained conformations at Pro39 and at Pro55 to the free energy of denaturation can be determined by measuring the cis ⇌ trans equilibrations at these positions in the unfolded state (cf. eq 2).

A destabilization of the folded state by an alternative prolyl isomer is not always observed. It is strongly dependent on the structural context. At Pro43 of calbindin the cis/trans equilibrium is not changed during folding (Chazin et al., 1989; Svensson et al., 1992). In this case the chain region around Pro43 appears to be uncoupled from the cooperative folding process, and folding energy is not used to change its cis/trans equilibrium. A coexistence of cis and trans forms is also observed for Pro117 of staphylococcal nuclease. In this case the cis form is slightly favored over the trans (Hynes et al., 1994).

The two disulfide bonds are extremely important for the stability of native RNase T1. They do not, however, determine the folding mechanism and the structure of the folded protein. A native-like conformation is also attained in the absence of the disulfide bonds under strongly native conditions. The two-state character of the equilibrium transition is conserved, and the basic mechanism of folding is unchanged. The two trans → cis isomerizations of Pro39

and Pro55 are still the rate-determining steps of folding, but partially folded intermediates with incorrect prolyl isomers are not observed. In particular, the stability of folding intermediates with an incorrect trans-Pro39 is low in the absence of the disulfide bonds. This is favorable for folding, because the trans → cis isomerization of Pro39 is retarded in these intermediates.

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