Replacement of a Cis Proline Simplifies the Mechanism of Ribonuclease T1 Folding†

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ABSTRACT: The refolding of ribonuclease T1 is dominated by two major slow kinetic phases that show properties of proline isomerization reactions. We report here that the molecular origin of one of these processes is the trans → cis isomerization of the Ser54–Pro55 peptide bond, which is cis in the native protein but predominantly trans in unfolded ribonuclease T1. This is shown by a comparison of the wild type and a designed mutant protein where Ser54 and Pro55 were replaced by Gly54 and Asn55, respectively. This mutation leaves the thermal stability of the protein almost unchanged; however, in the absence of Pro55 one of the two slow phases in folding is abolished and the kinetic mechanism of refolding is dramatically simplified.

Many proteins show complex slow folding reactions, which, in part, are thought to originate from the presence of proline residues in the polypeptide chain (Kim & Baldwin, 1982; Jaenicke, 1987). After the discovery of fast- (U_F)[1] and slow-folding (U_S) species in unfolded RNase A (Garel & Baldwin, 1973), the proline hypothesis (Brandts et al., 1975) was formulated to explain some slow steps in protein folding. According to this hypothesis, slow-folding U_S species are produced by the cis/trans isomerization around X–Pro peptide bonds in denatured polypeptide chains after unfolding. These molecules thus contain incorrect proline isomers and refold slowly, because reisomerization is an intrinsically slow process with a high activation energy. The model predicted that slow processes should occur (i) in the unfolded chains and (ii) during refolding, with kinetic properties characteristic of proline isomerization as known from small model peptides. For some proteins, such slow isomerizations of the unfolded chains were indeed found (Nall et al., 1978; Schmid & Baldwin, 1978; Ridge et al., 1981; Rehage & Schmid, 1982; Goto & Hamaguchi, 1982; Osterhout & Nall, 1985; Kelley et al., 1986; Hurle & Matthews, 1987); in refolding, however, the correspondence was often poor (Nall et al., 1978; Cook et al., 1979; Schmid & Blaschek, 1981; Goto & Hamaguchi, 1982; Schmid et al., 1986). Another prediction of the proline hypothesis was that the amount of U_S molecules and the complexity of slow folding should increase with the number of proline residues. This prediction was first tested by comparing homologous proteins with different numbers of proline residues. A carp parvalbumin with a single proline showed three phases in refolding, in contrast to only two phases for related parvalbumins without proline residues (Lin & Brandts, 1978). The interpretation of these results was complicated by the strong dissimilarity of the individual folding reactions of these proteins. In the case of pancreatic RNases with 4–6 prolines, the folding kinetics were very similar and the amplitudes of slow folding were independent of the number of prolines (Krebs et al., 1983). This was explained by the assumption that not all prolines influence folding. More recently, site-directed mutagenesis was used to substitute allegedly important proline residues by other amino acids. In all cases these mutations led to major changes in both the stability and the folding kinetics of the proteins, thus complicating the molecular interpretation of the observed differences between the wild-type and the mutant proteins (Ramdas & Nall, 1986; Kelley & Richards, 1987; Wood et al., 1988; Nall et al., 1989).

Here we use RNase T1 from *Aspergillus oryzae* as a model protein to elucidate the role of specific prolines for rate-determining slow steps in folding. The refolding of this protein occurs in three major phases, a rapid one that is completed within a second and two slow processes that are completed in the minutes to hours range. The slow reactions originate from denatured forms, which are formed slowly in the unfolded state as expected from the proline hypothesis (Kiefhaber et al., 1990a). The simplest explanation of these folding results was provided by a model that assumed that the kinetic complexity originates from the isomerization of two cis proline peptide bonds (which should be predominantly trans in the unfolded polypeptide chain) (Kiefhaber et al., 1990b). RNase T1 contains four proline residues. Pro39 and Pro55 are in the cis conformation, and trans prolines occur at positions 60 and 73 (Heinemann & Saenger, 1982; Arni et al., 1987, 1988). Their locations in the folded structure are indicated in Figure 1. Our approach to elucidate the role of the cis prolines for slow refolding involves the comparison of the folding kinetics of wild-type RNase T1 with those of a mutant where one of the two cis prolines is missing.

The structural environments of Pro39 and Pro55 differ strongly in the native protein. Pro39 is in a rigid part of the polypeptide chain, it is almost inaccessible to solvent, and its neighboring residues, Tyr38 and His40, are part of the active site (Heinemann & Saenger, 1982; Arni et al., 1987, 1988). This region is conserved in all homologous eukaryotic RNases (Heinemann & Hahn, 1989). On the other hand, Pro55 is in an exposed mobile loop region with few contacts to other regions of the chain. In the closely related RNase C2 (from *Aspergillus clavatus*) the residues Ser54 and Pro55 that form the cis peptide bond in RNase T1 are replaced by Gly54 and

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[1] Abbreviations: RNase T1, Lys25 isoenzyme of ribonuclease T1 from *Aspergillus oryzae*; GdmCl, guanidinium chloride; N and U, native and unfolded protein, respectively; I, folding intermediates.

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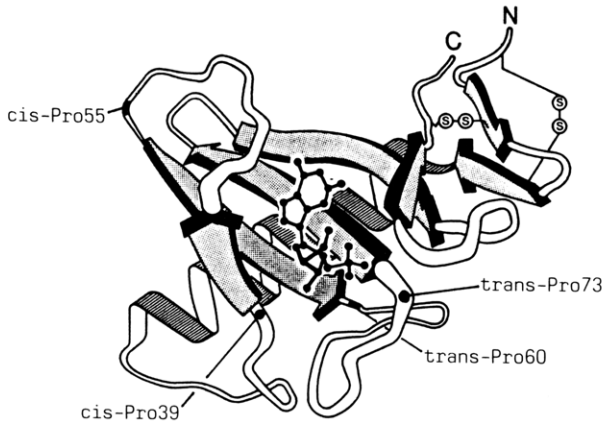


FIGURE 1: Schematic drawing of the backbone conformation of RNase T1, complexed with guanosine 2′-phosphate. The positions of the cis prolines 39 and 55, as well as the trans prolines 60 and 73, are indicated. The drawing is adapted from Heinemann and Hahn (1989).

Asn55, respectively. The flanking residues on both sides are identical in the two proteins (Heinemann & Hahn, 1989):

Position	50	55	60
RNase T1	... F S V S **S** P Y Y E W P I ...		
RNase C2	... F P V S **G** N Y Y E W P I ...		

The three-dimensional structure of RNase C2 is known. It is basically identical with that of RNase T1. The backbone folding in general, and in particular that in the 50–60 region, is strongly conserved, except for the presence of a trans Gly–Asn peptide bond at 54–55 instead of the cis Ser–Pro in RNase T1 (Polyakov et al., 1987). We therefore decided to construct a mutant of RNase T1 that contains the trans Gly54–Asn55 sequence of RNase C2 to study the influence of Pro55 on the folding mechanism. By use of this "evolutionarily guided" design we hoped to obtain a mutant protein (i) in which the cis Ser54–Pro55 bond is replaced by a normal trans peptide bond, as in RNase C2, and (ii) which is still very similar to the wild-type protein in its structure, its stability, and its folding kinetics. This strategy should allow a stringent comparison of related kinetic processes in folding and an unambiguous molecular assignment of (missing) kinetic phases to the replacement of Pro55. The results will show that the folding kinetics are indeed conserved in the mutant protein. However, a major slow reaction is missing and the folding mechanism is simplified. This indicates that isomerization of Pro55 is a major rate-determining event in the folding of RNase T1.

Materials and Methods

Materials. RNase T1 was purified 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). GdmCl (ultrapure) was from Schwarz/Mann, Orangeburg, NY. All other chemicals were from Merck, Darmstadt, West Germany.

Methods. The method of Kunkel et al. (1987) was used to construct the double mutant of RNase T1. The mutant protein was purified as was wild-type RNase T1 from the transformed *E. coli* cells.

Stability measurements were carried out by using a Gilford 2400S spectrophotometer with a 2527 thermoprogrammer. The protein concentration was 31 μM, the heating rate was 1 K/min, and the path length was 1 cm. The unfolding was reversible as judged by the coincidence of heating and subsequent cooling curve. CD spectra were measured in a JASCO J500A spectropolarimeter at protein concentrations of 31 μM in 1-mm cells (peptide CD) and 16 μM in 10-mm cells (aromatic CD). Corrected fluorescence spectra were measured in a Hitachi F4000 fluorometer. Excitation was at 268 nm (3-nm bandwidth), and protein concentrations were 0.68 μM.

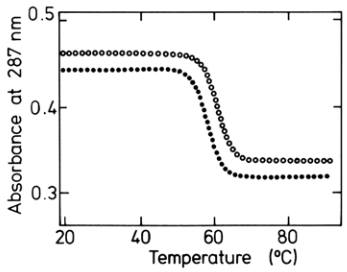


FIGURE 2: Comparison of the thermal unfolding transitions of wild-type RNase T1 (○) and the S54G,P55N mutant (●). Unfolding was monitored by the decrease in A_{287} in 0.1 M NaOAc/acetic acid, pH 5.0. The protein concentration was 31 μM; the heating rate was 1 K/min. The transition midpoints are at 61.8 °C (wild type) and 59.0 °C (mutant). The absorbance scale for the mutant protein is shifted by −0.015 to facilitate comparison.

The amount of fast-folding species was measured by an unfolding assay, as described by Kiefhaber et al. (1990b). Protein, equilibrated under unfolding conditions of 5.0 M GdmCl and 0.1 M glycine hydrochloride, pH 2.1, was exposed to variable short refolding pulses, and the amount of native protein formed after these pulses was determined by subsequent unfolding assays. The amplitude observed in this unfolding step (relative to the unfolding amplitude obtained for completely refolded protein) is a quantitative measure for the fraction of native protein present after various times of refolding. Extrapolation to zero time of refolding yields the fraction of fast-folding molecules present in the initially unfolded protein. The assays were carried out in the following way. Unfolded RNase T1 (in 5.0 M GdmCl and 0.1 M glycine hydrochloride, pH 2.1) was diluted 30-fold into 0.1 M Tris-HCl, pH 8.0, at 10 °C to initiate refolding. After various times of refolding, samples were withdrawn and diluted 4-fold in a fluorometer cell to final unfolding conditions of 5.5 M GdmCl (wild type) or 5.2 M GdmCl (mutant) at pH 1.9 and 10 °C. Unfolding was detected by the decrease in fluorescence at 320 nm after excitation at 268 nm. In each experiment, part of the protein was allowed to refold completely. Its amplitude of unfolding was set to 100%, and the results observed after short refolding pulses were related to this number. The protein concentrations were 1.4 μM in the refolding step and 0.35 μM in the unfolding assay.

Slow refolding was measured by the increase in fluorescence at 320 nm after excitation at 268 nm. Unfolded protein (14.8 μM in 5.9 M GdmCl and 0.1 M glycine hydrochloride at pH 1.7) was refolded by a 40-fold dilution with 0.1 M Tris-HCl, pH 8.0, at 10 °C in the fluorometer cell. The final protein concentration was 0.37 μM.

Results

Similar Structure and Stability of Wild-Type and Mutant Protein. The stability of RNase T1 is only slightly affected by the double mutation. The midpoint of the thermal unfolding transition is lowered by less than 3 deg, and the cooperativity appears to be unchanged (Figure 2). Assuming a two-state transition (Thomson et al., 1989), the difference in Gibbs free energy of stabilization is 0.9 kcal/mol at the transition midpoint of the wild-type protein (61.8 °C). The secondary and tertiary structures of the two forms of RNase T1 appear to be identical, as shown by the close coincidence of the CD spectra in the peptide (Figure 3a) and in the aromatic regions (Figure 3b) and of the tryptophan fluorescence

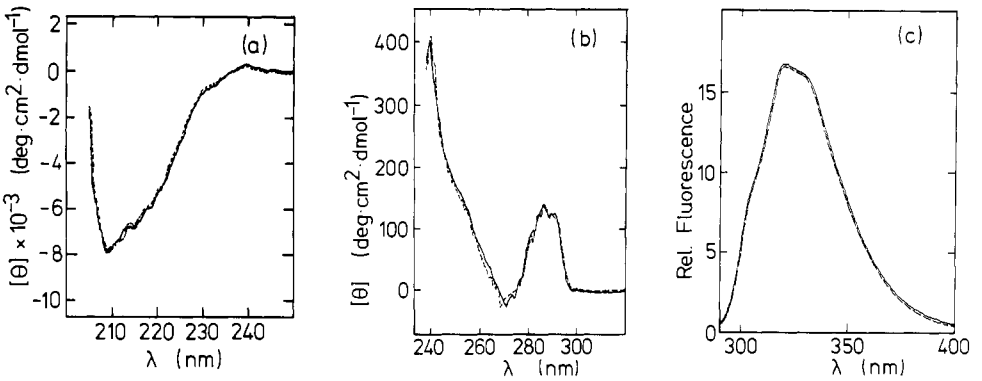


FIGURE 3: Spectroscopic characterization of wild-type RNase T1 (—) and the S54G,P55N mutant (---). The circular dichroism in the peptide region (a) and in the aromatic region (b) and the fluorescence emission (c) are shown. Solvent conditions were 0.1 M NaOAc, pH 5.0, at 25 °C.

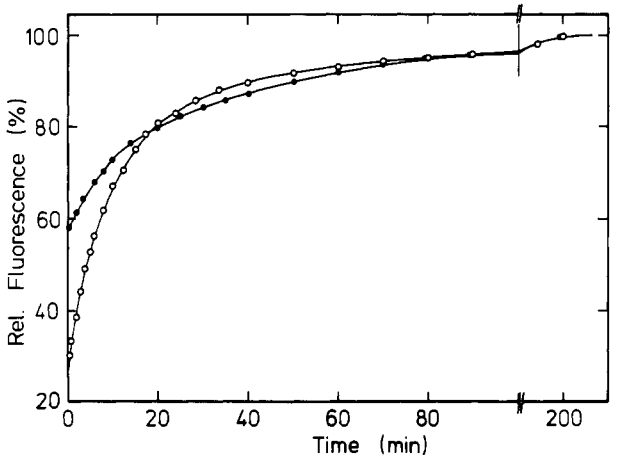


FIGURE 4: Slow refolding kinetics of wild-type RNase T1 (○) and the S54G,P55N mutant (●) in the presence of 0.15 M GdmCl and 0.1 M Tris-HCl at pH 8.0. The kinetics were analyzed as the sum of two first-order reactions with the following parameters: wild-type protein, τ_1 = 3400 s (A_1 = 0.20) and τ_2 = 350 s (A_2 = 0.42); mutant, τ_1 = 3400 s (A_1 = 0.25) and τ_2 = 550 ° (A_2 = 0.13).

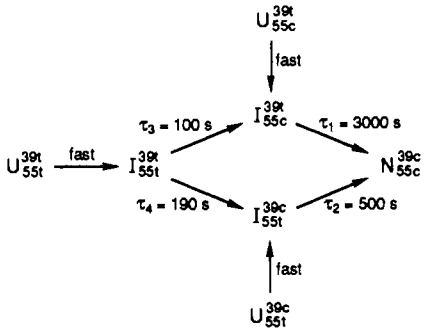
emission (Figure 3c). In addition, the mutant is similar to the wild-type protein in its activity (Grunert et al., in preparation). This close correspondence of structural and functional properties provides a necessary prerequisite for a meaningful comparison of the folding kinetics of the two forms of RNase T1.

A Major Slow Phase of Folding Is Absent in the Mutant Protein. The slow refolding of wild-type RNase T1 is characterized by the occurrence of two major phases, an intermediate phase[2] with a time constant in the 500-s range and very slow phase[2] with $\tau \approx 3000$ s (at pH 8, 10 °C) (Kiefhaber et al., 1990a). The intermediate phase appeared to be complex, with contributions from three different reactions with similar time constants. This was explained by a kinetic model, where rapid formation of partially ordered structure precedes the slow, rate-limiting steps of folding and where the kinetic competition of two proline reisomerizations determines the choice of refolding pathway (Kiefhaber et al., 1990b; cf. also Scheme Ia). The comparison of the slow folding kinetics of the wild-type and the mutant proteins (Figure 4) shows that the reactions that constitute the intermediate phase ($\tau \approx 500$ s) are almost completely missing in the folding of the mutant protein. The very slow refolding reaction persists. Its rate is unchanged, but its amplitude is increased in the absence of

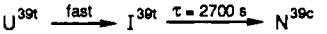
[2] The slow phases that are observed in the refolding of RNase T1 are labeled "intermediate" (faster process with time constant τ_2) and "very slow" (slower process with time constant τ_1), respectively.

Scheme I: Kinetic Models for the Slow Refolding Reactions of (a) RNase T1 and (b) the S54G,P55N Mutant under Strongly Native Conditions[a]

(a) wild type



(b) mutant



[a] U stands for unfolded species, I for intermediates of refolding, and N is the native protein. The superscript and the subscript indicate the isomeric states of prolines 39 and 55, respectively, in the correct, nativelike cis (c) and the incorrect, nonnative trans (t) isomeric state. As an example, I^{39t}_{55c} stands for an intermediate with Pro55 in the correct cis and Pro39 in the incorrect trans state. The time constants given for the individual steps refer to folding conditions of 0.15 M GdmCl, pH 8.0, at 10 °C. The values at pH 8.0 are given rather than at pH 5.0 [as in the related scheme of Kiefhaber et al. (1990)] because the folding experiments of Figures 4 and 5 were carried out at pH 8.0. The rapid refolding reaction of the molecules with correct isomers, $U^{39c}_{55c} \rightarrow N$, is not included in the two schemes.

Pro55. Consequently, isomerization of Pro55 cannot be the molecular origin for this very slow folding process. The results strongly suggest that the intermediate phase in the folding of wild-type RNase T1 is caused by the presence of Pro55. This intermediate phase is not lost completely in the folding of the mutant, but a very small reaction in this time region remains to be seen. We suggest that this reaction may originate from a minor unfolded form with an incorrect cis isomer of prolines 60 and/or 73, both of which are trans in the folded state. In the wild-type protein this reaction probably occurs as well; however, it is not resolved from the dominating reactions that are linked with the isomerization of Pro55.

The Amount of Fast-Folding Molecules Is 4-fold Increased in the Mutant Protein. The replacement of an essential proline residue should abolish a slow process in folding and consequently lead to an increase in the amount of fast-folding U_F species. This is clearly observed for the S54G,P55N mutant of RNase T1. The percentage of fast-refolding molecules increases more than 4-fold, from 3.5% in the wild-type protein to 15.5% in the mutant (Figure 5). The amounts of U_F molecules were measured by assays (Schmid, 1983) that make

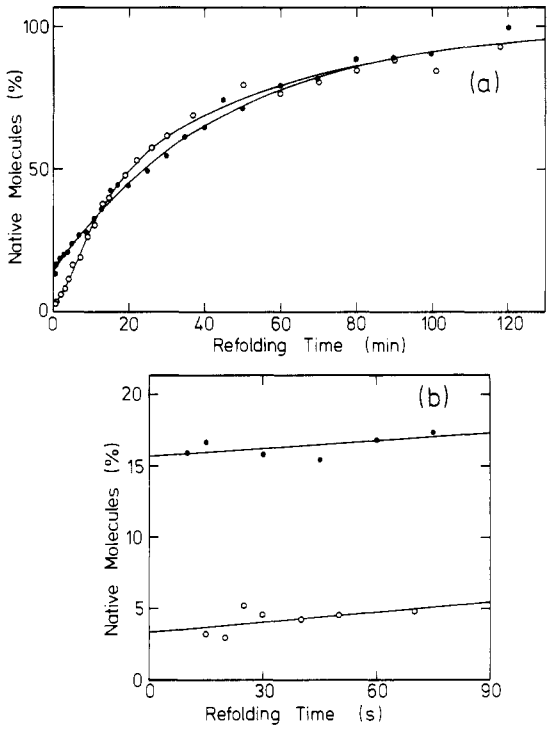


FIGURE 5: Time course of formation of native protein during refolding of wild-type RNase T1 (○) and of the S54G,P55N mutant (●) at pH 8.0 and 10 °C. (a) Entire time course. The percentage of native molecules was measured by unfolding assays as described under Methods. The unfolding assays for native molecules were carried out at pH 1.9 (0.1 M glycine hydrochloride), 10 °C in the presence of 5.5 M GdmCl (wild type) or 5.2 M GdmCl (mutant). Refolding conditions were 0.1 M Tris-HCl and 0.19 M GdmCl, pH 8.0. The theoretical curve for the wild-type protein was calculated by using the kinetic scheme (Scheme I) and the time constants given therein. It was assumed that 66% of the molecules use the upper pathway and 31% use the lower pathway (3% refold rapidly). The corresponding curve for the mutant was calculated by assuming that 15% of the molecules fold rapidly and that all slow-folding molecules (85%) regain the native state in a single slow rate-limiting step with a time constant of 2700 s (as in Scheme Ib). (b) Early time region of panel a to determine the amount of fast-folding species by extrapolation to time zero.

use of the distinctive slow unfolding kinetics of native RNase T1. Completely folded molecules unfold slowly because they are separated from unfolded or partially folded species by a high activation energy barrier (Segawa & Sugihara, 1984; Goldenberg & Creighton, 1985). After short intervals, aliquots of the refolding protein were taken and transferred to denaturing conditions, and the amplitudes of the resulting unfolding reactions were determined. They are a quantitative measure for the amount of native RNase T1 present at the time of sample transfer. As shown in Figure 5b, extrapolation to zero time of refolding gives the amount of fast-folding U_F species that was present in the mixture of unfolded species at the beginning of refolding. The slow increase of amplitudes with time (Figure 5b) is caused by the onset of the slow refolding reactions (cf. Figure 5a). The more than 4-fold increase in the amount of U_F observed after substitution of Pro55 strongly suggests that this proline exists predominantly (≈80%) as the nonnative trans isomer in the unfolded wild-type protein.

Substitution of Pro55 Simplifies the Kinetic Folding Mechanism of RNase T1. A distinctive feature of the mechanism proposed for the slow refolding of RNase T1 is a branching into two alternative pathways at the point of a major folding intermediate with two incorrect isomers, where the choice of pathway is determined by the relative rates of reisomerization of the two incorrect proline isomers. The formation of native protein on one pathway [the "upper" pathway in Kiefhaber et al. (1990b); cf. also Scheme Ia] is limited in rate by the very slow isomerization ($\tau \approx 3000$ s). On the other pathway [the "lower" pathway in Kiefhaber et al. (1990b); cf. also Scheme Ia] both involved isomerizations are similar in rate and give rise to a sigmoidal appearance of native molecules. Consequently, the slow refolding reaction of wild-type RNase T1 is best described by a complex superposition of a sigmoidal process and a very slow first-order reaction, as shown in Figure 5a. In the case of the mutant protein, the kinetics of formation of native molecules are very simple. After the fast refolding reaction that leads to 15% N within the dead time of the experiment (cf. Figure 5b), the remaining 85% N are formed in a single, very slow first-order reaction with a time constant of about 2700 s (Figure 5a). The sigmoidal phase is absent. This can be understood, since species (N, I, or U) with incorrect isomers at position 55 do not exist in the mutant. The persistance of the very slow phase in the mutant strongly suggests that the molecular origin of this process is isomerization of the conserved cis proline 39. The amplitude of this reaction is increased in the mutant protein as, unlike in the wild-type protein, the mutated molecules no longer have the choice of an alternative, more rapid refolding pathway (the lower branch of Scheme Ia). This leads to the surprising result that, although replacement of Pro55 simplifies the folding kinetics, nevertheless folding is decelerated because all U_S molecules must pass through the very slow 2700-s step upon reactivation.

Discussion

Isomerization of Pro55 Is a Rate-Limiting Step in RNase T1 Folding. The substitution of the cis Ser54–Pro55 peptide bond by a normal trans bond (Gly54–Asn55) did not change the structure and stability to a significant extent. However, it led to the disappearance of a slow kinetic phase, leaving the other refolding reactions almost unchanged. The more than 4-fold increase in the amount of fast-folding species shows that Pro55 is predominantly in the incorrect trans state in unfolded wild-type RNase T1. It is well explained by a trans/cis ratio of about 80/20 as found typically in small proline-containing peptides. A similar trans/cis ratio exists also at Pro39, as suggested by the presence of 85% U_S molecules in the mutant. These unfavorable isomer distributions lead to the presence of more than 96% slow-folding molecules in the wild-type protein.

In the mutant, the dominant wild-type species with two incorrect isomers (U_{55t}^{39t}) and the species with an incorrect Pro55 only (U_{55t}^{39c}) are no longer rate-limited in their folding by isomerization to the cis state at position 55. Consequently, all reactions originating from these species are absent, thus leading to the disappearance of the steps that constitute the intermediate phase in fluorescence and the sigmoidal appearance of native protein. The consequence is an enormous simplification of the mechanism for slow refolding. Four slow steps are required to explain the folding of wild-type U_S molecules (Scheme Ia). In the mutant, this complexity is reduced to a single slow step that limits the refolding of all U_S molecules (Scheme Ib). The two slow isomerizations that were found in unfolding and refolding of wild-type RNase T1 (Kiefhaber et al., 1990a,b) can now be assigned specifically to Pro39 and Pro55, as shown in Scheme Ia.[3]

These assignments help to elucidate molecular aspects of folding. First, extensive secondary structure can be formed rapidly at the beginning of folding (Kiefhaber et al., 1990b) with both prolines, 39 and 55, still locked in the incorrect isomeric state. This clearly rules out a directing role of the

two β-turns around 39 and 55 for the formation of secondary structure. A second remarkable feature is the rate of reisomerization of Pro39 during refolding. It is strongly dependent on the isomeric state of Pro55. In the presence of an incorrect trans Pro55, Pro39 reisomerizes with a time constant of about 190 s (cf. lower branch in Scheme Ia). However, when Pro55 isomerizes first to the native cis state (upper branch in Scheme I) or is absent (as in the mutant), then trans → cis isomerization of Pro39 is strongly decelerated and proceeds with a time constant of about 3000 s. This reaction can be accelerated by adding "structure-breaking" salts, such as GdmCl, in both wild-type and mutant RNase T1 (Kiefhaber et al., 1990b; unpublished results). This is very unusual. It suggests that the incorrect trans isomer of Pro39 is "locked" in a folded intermediate structure, which can be destabilized either by an additional incorrect isomer (at Pro55) or else by unfavorable folding conditions. This clearly indicates that, although extensive structure formation is possible in the presence of incorrect proline isomers, nevertheless such "wrong kinks" in the polypeptide chain influence the stability of intermediates and affect folding rates.

The mutant protein with a trans Gly54–Asn55 bond is almost identical with the wild-type protein in structure and stability. It is not comparable to the transient folding intermediate of the wild-type protein with a trans Ser54–Pro55 bond. This may originate from the pronounced difference in flexibility of the peptide chains: a very rigid residue (Pro) is removed, and a very flexible residue (Gly) is introduced. The consequences for the folding kinetics of a trans Ser–Pro bond and of a trans Gly–Asn bond are obviously not identical. We assume that the respective "native" conformations, i.e., a cis Ser54–Pro55 and a trans Gly54–Asn55, have a similar impact on the stability of the folded states and also on the folding kinetics that lead to these states, as is experimentally observed.

Proline Isomerization and Protein Folding. The present data on RNase T1 folding clearly demonstrate that proline isomerization is an important rate-determining process in protein folding. It is responsible for the formation of slow-folding U_S species after unfolding, and it controls the rate of crucial slow steps that are required for the attainment of the native state in refolding. Our results point to an intricate interrelationship between protein folding and proline isomerization steps. Under favorable folding conditions well-ordered intermediates are formed rapidly with essentially nativelike secondary structure and presumably some tertiary contacts (Udgaonkar & Baldwin, 1988; Roder et al., 1988). These intermediates can tolerate the presence of nonnative β-turns that are locked in by incorrect proline isomers. For later folding steps, the steric requirements become more stringent and correct prolines are required. Consequently, these late steps are limited in rate by the reisomerization of the respective prolines. The rapid formation of ordered structure affects the isomerization kinetics. In the case of RNase A, an acceleration of proline isomerization was observed in the presence of ordered structure (Cook et al., 1979; Schmid & Blaschek, 1981); for RNase T1 the opposite was found, i.e., a strong decrease in isomerization rate of Pro39 in a folding intermediate. It should be pointed out that proline isomerization is not the only slow process in protein folding. For many (in particular large) proteins, conformational steps can be much slower than proline isomerization and thereby limit folding. The role of proline isomerization for the de novo folding of nascent proteins is still unknown. The newly synthesized chains presumably contain all peptide bonds in the trans form; therefore, trans → cis isomerizations as characterized here should occur in the course of in vivo folding. Whether this process is enzymatically catalyzed (Fischer et al., 1989; Takahashi et al., 1989) in the cell remains to be elucidated.

[3] The kinetic mechanisms given in Scheme I explain essentially all the kinetic data on RNase T1 refolding. Nevertheless, these are simplifications, since they do not account for possible contributions of the two trans prolines 60 and 73 to the folding kinetics. These contributions may be small and buried under the contributions of the cis prolines 39 and 55. The minor reaction with τ = 550 s, monitored by fluorescence (Figure 4), might be an example of this kind.

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Delineation of the Functional Site of a Snake Venom Cardiotoxin: Preparation, Structure, and Function of Monoacetylated Derivatives†

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ABSTRACT: Toxin γ, a cardiotoxin from the venom of the cobra *Naja nigricollis*, was modified with acetic anhydride, and the derivatives were separated by cation-exchange and reverse-phase chromatography. Nine monoacetylated derivatives were obtained, and those modified at positions 1, 2, 12, 23, and 35 were readily identified by automated sequencing. The overall structure of toxin γ, composed of three adjacent loops (I, II, and III) rich in β-sheet, was not affected by monoacetylation as revealed by circular dichroic analysis. Trp-11, Tyr-22, and Tyr-51 fluorescence intensities were not affected by modifications at Lys-12 and Lys-35, whereas Trp-11 fluorescence intensity slightly increased when Lys-1 and Lys-23 were modified. The cytotoxic activity of toxin γ to FL cells in culture was unchanged after modification at positions 1 and 2, whereas it was 3-fold lower after modification at Lys-23 and Lys-35. The derivative modified at Lys-12 was 10-fold less active than native toxin. Using two isotoxins, we found that substitutions at positions 28, 30, 31, and 57 did not change the cytotoxic potency of toxin γ. A good correlation between cytotoxicity, lethality, and, to some extent, depolarizing activity on cultured skeletal muscle cells was found. In particular, the derivative modified at Lys-12 always had the lowest potency. Our data show that the site responsible for cytotoxicity, lethality, and depolarizing activity is not diffuse but is well localized on loop I and perhaps at the base of loop II. This site is topographically different from the AcChoR binding site of the structurally similar snake neurotoxins.

Despite extensive work, a debate still exists as to whether or not snake cardiotoxins have a specific target (Harvey, 1985; Dufton & Hider, 1988) and possess a defined functional site (Louw & Visser, 1977, 1978; Lauterwein & Wüthrich, 1978; Karlsson, 1979; Dufourcq et al., 1982; Hider & Khader, 1982; Batenburg et al., 1985). The debate is complicated by evidence suggesting structural and functional heterogeneity among these small proteins (Botes & Viljoen, 1976; Hider & Khader, 1982; Harvey, 1985; Breckenridge & Dufton, 1987; Grognet et al., 1988). To clarify this situation, we searched for the functional site of toxin γ from *Naja nigricollis*, a well-studied cardiotoxin (Tazieff-Depierre et al., 1969a,b; Boquet, 1970; Tazieff-Depierre & Trethevie, 1975; Lee & Lee, 1979; Grognet et al., 1986, 1988; Gatineau et al., 1987; Roumestand et al., 1989). The overall structure of toxin γ is similar to that of *Naja mossambica mossambica* cardiotoxin $V^{II}4$ (Rees et al., 1987) and of CTX IIb (Steinmetz et al., 1988), which consists of a small core containing four disulfide bonds from which three adjacent loops (I, II, and III) rich in β-pleated sheet protrude (Figure 1).

Previously, we reported that Trp-11 in loop I was implicated in the function of toxin γ (Gatineau et al., 1987). The role

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