Enzymatic Catalysis of Prolyl Isomerization in an Unfolding Protein†

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ABSTRACT: Prolyl isomerases are able to accelerate slow steps in protein refolding that are limited in rate by cis/trans isomerizations of Xaa–Pro peptide bonds. We show here that prolyl isomerizations in the course of protein unfolding are also well catalyzed. To demonstrate catalysis we use cytoplasmic prolyl isomerase from *Escherichia coli* as the enzyme and reduced and carboxymethylated ribonuclease T1 as the substrate. This form of ribonuclease T1 without disulfide bonds is nativelike folded only in the presence of moderate concentrations of NaCl. Unfolding can be induced by reducing the NaCl concentration at ambient temperature and in the absence of denaturants. Under these conditions prolyl isomerase retains its activity and it catalyzes prolyl cis/trans isomerization in the unfolding protein. Under identical conditions within the NaCl-induced transition unfolding and refolding are catalyzed with equal efficiency. The stability of the protein and thus the final distribution of unfolded and folded molecules attained at equilibrium is unchanged in the presence of prolyl isomerase. These results demonstrate that prolyl isomerase functions in protein folding as an enzyme and catalyzes prolyl isomerization in either direction.

Peptidyl prolyl cis/trans isomerases (PPIs)[1] are ubiquitous enzymes that occur in virtually all cell types and subcellular compartments (reviewed by Fischer & Schmid, 1990; Schmid, 1991; Schreiber, 1991; Lorimer, 1992; Stein, 1992; and Schmid et al., 1992). They catalyze the cis ⇌ trans isomerization of peptide bonds preceding proline (Xaa–Pro) in peptides and proteins. Other enzymatic activities have not yet been found (Fischer et al., 1984; Lang et al., 1987; Lin et al., 1988; Harrison & Stein, 1990; Kofron et al., 1991). Two unrelated classes of prolyl isomerases are known: the cyclophilins and the FK 506 binding proteins (FKBPs). Their enzymatic activities are strongly inhibited by the immunosuppressants cyclosporin A (CsA) (Fischer et al., 1989; Schönbrunner et al., 1991) and FK 506 (Siekierka et al., 1989; Harding et al., 1989; Tropschug et al., 1990), respectively. PPI of the cyclophilin type was discovered independently by virtue of its prolyl isomerase activity (Fischer et al., 1984) and of its high affinity for CsA (Handschumacher et al., 1984). The biological functions of prolyl isomerases are still unclear. Also, it is not known at present whether the immunosuppressive effects of CsA and FK 506 are mediated by the known cyclophilins and FKBPs and whether the prolyl isomerase activity is involved in these functions (Schreiber, 1991; Flanagan et al., 1991; Liu et al., 1991).

Cis peptide bonds not involving proline are extremely rare in folded proteins (Stewart et al., 1990; MacArthur & Thornton, 1991; Herzberg & Moult, 1991) since the trans state is strongly preferred energetically (Jorgensen & Gao, 1988). For peptide bonds preceding proline (Xaa–Pro), however, the difference in free energy between the cis and trans conformers is small and the trans state is only slightly favored. Trans/cis ratios near 90/10 are usually found for prolyl bonds in oligopeptides (Grathwohl & Wüthrich, 1976a,b, 1981). In folded proteins of known three-dimensional structure about 6% of all Xaa–Pro bonds are in the cis conformation (Stewart et al., 1990; MacArthur & Thornton, 1991). The isomerization between the two conformers is a slow reaction since it involves the rotation about the peptide C–N bond which has partial double-bond character. Prolyl peptide bond isomerizations are involved in many protein folding reactions and frequently determine the overall rate of folding (Brandts et al., 1975; Schmid & Baldwin, 1978; Cook et al., 1979; Goto & Hamaguchi, 1982a,b; Kelley & Stellwagen, 1984; Ramdas et al., 1986; Schmid et al., 1986; Kelley & Richards, 1987; White et al., 1987; Wood et al., 1988; Lang & Schmid, 1990; Kiefhaber et al., 1990a,b; Kuwajima et al., 1991; Jackson & Fersht, 1991). Prolyl bonds that are cis in the native protein are particularly important, since the incorrect trans state predominates usually in the unfolded protein in vitro and presumably also after biosynthesis in the cell. Prolyl isomerases can catalyze slow folding reactions that involve prolyl peptide bond isomerizations (Lang et al., 1987; Lin et al., 1988; Kiefhaber et al., 1990b; Schönbrunner et al., 1991; Jackson & Fersht, 1991). The efficiency of catalysis is strongly variable. It depends on the location of the prolines in the polypeptide chains as well as on the folding conditions. The extent of exposure of the Xaa–Pro bonds in the refolding protein chain is probably an important factor for the catalysis by PPI.

When native proteins are rapidly denatured (in the $N \rightarrow U_F$ step, cf. eq 1), unfolded polypeptide chains are formed initially with the prolyl peptide bonds still in the native-like conformation. These molecules (U_F) can usually refold rapidly. The U_F molecules then slowly interconvert with the slow-refolding U_S^i species by virtue of one or more Xaa–Pro isomerizations (the $U_F \rightleftharpoons U_S^i$ reactions) and ultimately a mixture of fast-folding (U_F) and slow-folding (U_S^i) molecules (cf. eq 1) is attained. The relative concentrations of U_F and U_S^i at equilibrium are determined by the number of prolyl bonds, by their isomeric state in the folded protein, and by the cis/trans equilibria in the denatured protein.

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[1] Abbreviations: PPI, peptidyl prolyl cis/trans isomerase; RNase T1, ribonuclease T1; RCM-RNase T1, ribonuclease T1 with reduced disulfide bonds and the thiols carboxymethylated; FKBP, FK 506 binding protein; CsA, cyclosporin A; GdmCl, guanidinium chloride; DTE, dithioerythritol; N, native state of a protein; U_F and U_S, fast- and slow-folding forms of an unfolded protein, respectively; PU, peptide acceleration unit, a measure for the PPI activity (explained in the Materials and Methods section).

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$$N \overset{\text{fast}}{\rightleftharpoons} U_F \overset{\text{slow}}{\rightleftharpoons} U_S^i \quad (1)$$

PPI can catalyze slow proline-limited steps in the refolding of the U_S^i molecules. Here we ask whether this enzyme can accelerate prolyl isomerizations in denatured proteins as well. If they are true enzymes, prolyl isomerases should be able to catalyze the reaction in either direction, depending solely on the folding or unfolding conditions and on the stability of the substrate protein.

The experimental verification of this prediction turned out to be very difficult, since the unfolding of a protein is usually induced by exposure to increased temperature or to denaturants, such as urea or guanidinium chloride (GdmCl), at high concentrations. Unfortunately, the thermal stability of the known prolyl isomerases is low, and they are markedly inactivated by urea or GdmCl at concentrations higher than 0.5 M. To maintain PPI in an active conformation in such experiments, a substrate protein with a marginal stability would be desirable that could be unfolded at low temperature and ideally in the absence of chemical denaturants.

The reduced and carboxymethylated form of ribonuclease T1 (RCM-RNase T1) is a protein with a marginal stability only, and it is suitable to investigate the function of PPI in protein unfolding. Native RNase T1 has two disulfide bonds (Cys2–Cys10 and Cys6–Cys103) that contribute strongly to its conformational stability (Oobatake et al., 1979a,b; Pace et al., 1988; Pace, 1990). These two bonds are broken in RCM-RNase T1 and consequently this form of RNase T1 is unfolded under "native conditions" such as at pH 8 and 15 °C. Similar to RNase T1 with intact disulfides, RCM-RNase T1 is strongly stabilized by addition of NaCl and is converted to a folded, enzymatically active form in the presence of 2 M NaCl (Pace et al., 1988). Folding or unfolding of this variant of RNase T1 can thus be induced simply by an increase or a decrease, respectively, of the concentration of NaCl. Chemical denaturants such as urea or GdmCl can be omitted. The folding mechanism of RNase T1 with intact disulfides is well studied. Two prolyl isomerizations occur after unfolding in the denatured molecules and lead to several slow refolding reactions, which are all catalyzed by PPI (Kiefhaber et al., 1990a,b; Schönbrunner et al., 1991). The cis peptide bonds at Pro39 and at Pro55 are thought to be of particular importance for these slow folding processes (Kiefhaber et al., 1990c, 1992a).

Our results with RCM-RNase T1 show that in the absence of disulfide bonds the slow refolding reactions are also limited in rate by prolyl isomerizations and are well catalyzed by PPI. Additionally, when this variant of RNase T1 is unfolded by diluting out the NaCl, a catalysis of prolyl isomerization by PPI during unfolding is indeed observed.

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). The purified protein was a generous gift from L. Mayr (Bayreuth). Cytosolic PPI from *E. coli* was supplied by N. Takahashi (Tonen Inc.) and isolated as described (Hayano et al., 1991). The k_{cat}/K_M value for this PPI preparation was 6×10^6 M^{-1} s^{-1}. GdmCl was from Schwarz/Mann, Orangeburg, NY. Dithioerythritol (DTE), iodoacetate, and the assay peptide for PPI, Suc-Ala-Ala-Pro-Phe-4-nitroanilide, were from Sigma, St. Louis, MO. Chymotrypsin A was from Boehringer Mannheim, Germany. All other chemicals were from Merck, Darmstadt, Germany.

Spectroscopic Methods. RNase T1 and RCM-RNase T1 concentrations were determined spectrophotometrically assuming an absorption of 1.9 at 278 nm for a 1 mg/mL solution (Takahashi et al., 1970). For optical measurements a Jasco J-600A spectropolarimeter, a Hitachi F4010 fluorescence spectrometer, and a Kontron Uvikon 860 spectrophotometer were used.

Reduction and Carboxymethylation of RNase T1. Typically, 0.24 μmol of RNase T1 was dissolved in 275 μL of 0.2 M Tris/HCl, pH 8.7, containing 7.0 M GdmCl and 2 mM EDTA. The protein was reduced by adding 30 μL of a 0.2 M DTE solution (in 0.2 M Tris/HCl, 7.0 M GdmCl, and 2 mM EDTA) to give a final concentration of 20 mM DTE and incubating at 25 °C for 2 h. Reduction was carried out under nitrogen. The reduced protein was subsequently carboxymethylated by adding 60 μL of 0.6 M iodoacetate solution in 0.2 M Tris/HCl, pH 7.5, and incubating for 5 min in the dark. The reaction was stopped by adding 100 μL of 0.5 M reduced glutathione in 0.2 M Tris/HCl, pH 7.5. The modified protein was immediately separated from the reagents by gel filtration over a 1 × 16 cm Sephadex G-25 column, equilibrated with 0.1 M sodium acetate, pH 5.0. The resulting 4-fold carboxymethylated protein migrated as a single band in native polyacrylamide gel electrophoresis (Pace & Creighton, 1986) and as a single peak when chromatographed over an analytical MonoQ anion-exchange column. The assay for free thiols with Ellman's reagent (Habeeb, 1972) was negative. The protein was stored in solution at 4 °C.

PPI Activity Assays. PPI activities were measured by using the chromogenic peptide succinyl-Ala-Ala-Pro-Phe-4-nitroanilide in a coupled assay with chymotrypsin that was developed by Fischer et al. (1984). The assay conditions were as described by Fischer et al. (1989). Generally, PPI activities were determined at the same temperatures and solvent conditions as used in the folding and unfolding experiments. From 0 to 3.5 M NaCl the activity of *E. coli* PPI was found to be almost independent of NaCl concentration. The relative activity of PPI is expressed as peptide acceleration units (PU) per milliliter. 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. As an example, 1 PU/mL PPI leads to a 2-fold increase in isomerization rate.

NaCl-Induced Folding Transitions. RCM-RNase T1 (0.5 μM) was incubated at 15 °C in the presence of 0.1 M Tris/HCl, pH 8.0, and varying concentrations of NaCl for at least 5 h. The fluorescence of the samples was measured at 320 nm (10-nm bandwidth) after excitation at 268 nm (1.5-nm bandwidth). The folding transition was 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 to eq 2 was used to obtain ΔG(2 M NaCl) for RCM-RNase T1.

$$F_{obs} = [(F_N + m_N[D]) + (F_U + m_U[D]) \exp[-(\Delta G^\circ_{N-U}/RT + m_G[D]/RT)]]/[1 + \exp[-(\Delta G^\circ_{N-U}/RT + m_G[D]/RT)]] \quad (2)$$

F_{obs} is the measured fluorescence, F_N and F_U are the values for the native and the unfolded protein at zero denaturant, [D] is the denaturant concentration, and m_N and m_U are the slopes of the pre- and posttransitional baseline, respectively. ΔG°_{N-U} is the difference in Gibbs free energy between native and unfolded protein, and $m_G = d\Delta G^\circ_{N-U}/d$ [GdmCl].

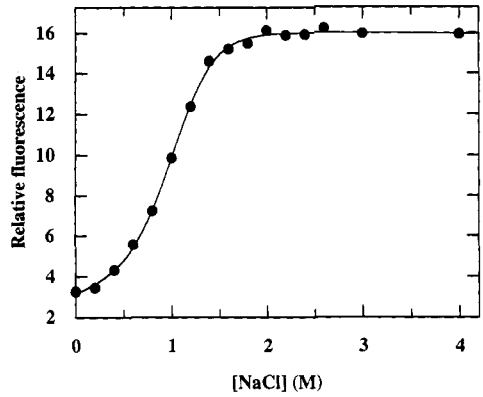

FIGURE 1: Folding transition of RCM-RNase T1 induced by increasing the concentration of NaCl at pH 8.0, 15 °C. The increase in protein fluorescence at 320 nm after excitation at 268 nm is given as a function of the NaCl concentration. The final conditions were 0.5 μM RCM-RNase T1 in 0.1 M Tris/HCl and the indicated NaCl concentration. The line represents an analysis of the data according to the two-state model by using the method of Santoro and Bolen (1988). The midpoint of the transition is at 1.0 M NaCl.

CD Spectra. CD spectra were recorded at 20 nm/min with a time constant of 1 s in 0.1-cm thermostatable cuvettes at 15 °C. The bandwidth was 1 nm. The protein concentrations were 4.5–4.8 μM and the spectra were 20-fold accumulated. All spectra were corrected for the contributions of the respective buffers.

Unfolding and Refolding Kinetics. Folding kinetics were typically initiated by a 40–48-fold dilution of unfolded or folded RCM-RNase T1 to the appropriate final concentration of NaCl. The dead time of mixing was about 2 s with the use of a magnetic stirrer below the optical cell. The kinetics were followed by the change in fluorescence at 320 nm (10-nm bandwidth) after excitation at 268 nm (1.5-nm bandwidth). All kinetic experiments were carried out in 0.1 M Tris/HCl, pH 8.0, at 15 °C.

RESULTS

Stability of Reduced and Carboxymethylated RNase T1. The reduction and subsequent modification of the two disulfide bonds leads to a strong decrease in the stability of RNase T1. About 85% of the RCM-RNase T1 molecules are in a folded state at the pH of its optimum stability (pH 5.0) at 12.5 °C and in the presence of 0.25 M NaCl. The remaining 15% are already unfolded (Pace et al., 1988). At pH 8.0 the stability of RCM-RNase T1 is further reduced and the protein is largely unfolded when NaCl is absent. A nativelike folded conformation can be induced, however, by increasing the concentration of NaCl. The observed folding transition (Figure 1) shows a midpoint near 1.0 M NaCl and RCM-RNase T1 is in an ordered conformation above 2.0 M NaCl. There is no evidence for partially folded intermediates in the transition region. By assuming a simple two-state transition, a linear relationship between ln K and the NaCl concentration is observed. We therefore use the linear extrapolation model and the method of Santoro and Bolen (1988) to obtain a free energy of stabilization of −10.5 kJ/mol at 2.0 M NaCl for RCM-RNase T1. The linear model was developed to describe denaturant-induced unfolding transitions. By using this model here, we do not imply that denaturant-induced unfolding and NaCl-induced folding share a common mechanism.

The folded form of RCM-RNase T1 resembles the native protein with intact disulfides. The CD spectra of the two forms in the amide region (Figure 2) are very similar. The

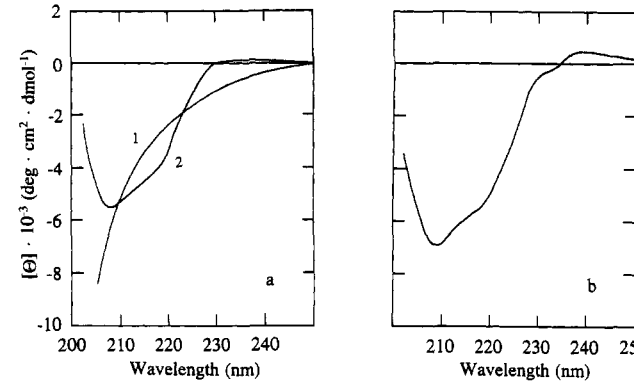

FIGURE 2: Circular dichroism spectra of RCM-RNase T1 and RNase T1. (a) CD spectra of RCM-RNase T1 in the presence of 2.0 M NaCl (spectrum 2) and in the absence of NaCl (spectrum 1). (b) CD spectrum of RNase T1 in the presence of 2.0 M NaCl. All measurements were carried out at 15 °C in 0.1 M Tris/HCl, pH 8.0, in 0.1-cm cells. The protein concentrations were 4.5–4.8 μM.

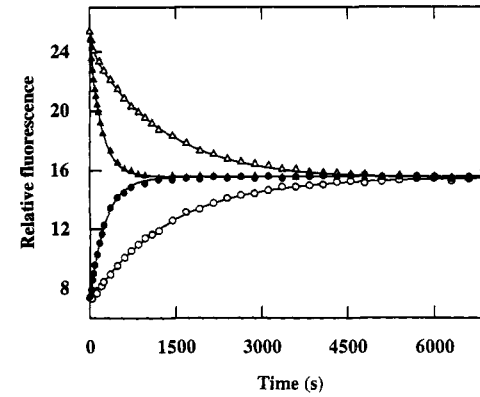

FIGURE 3: Unfolding (△,▲) and refolding (○,●) kinetics of RCM-RNase T1. All experiments were carried out in the presence of 0.8 M NaCl, pH 8.0, at 15 °C. The changes in protein fluorescence at 320 nm (excitation at 268 nm) are shown as a function of time. In the unfolding experiments 28 μM folded RCM-RNase T1 (in 2.4 M NaCl and 0.1 M sodium acetate, pH 5.0, 10 °C) was unfolded by a 40-fold dilution to final conditions of 0.8 M NaCl and 0.1 M Tris/HCl, pH 8.0, 15 °C, in the absence (△) and in the presence (▲) of 90 PU/mL PPI. In the refolding experiments 28 μM unfolded RCM-RNase T1 (in 0.1 M Tris/HCl, pH 7.8, 25 °C) was refolded by a 40-fold dilution to the same final conditions in the absence (○) and in the presence (●) of 90 PU/mL PPI. Unfolding in the absence of PPI (△) was approximated by the sum of two exponential curves with time constants of 1270 and 28 s and relative amplitudes of 86% and 14%, respectively. Refolding in the absence of PPI (○) was approximated by a single-exponential curve with a time constant of 1370 s. In the presence of PPI the time constant for refolding (●) decreased to 270 s and for unfolding (▲) to 220 s. These approximations are represented by the continuous lines.

intensity of the bands is, however, lower in the case of RCM-RNase T1, and small differences are found around 240 nm. We do not know whether they originate from the replacement of the disulfide bonds. The same result was obtained for reduced RNase T1, which lacks the carboxymethyl groups (Oobatake et al., 1979a,b). A nativelike conformation is also suggested by the high enzymatic activity of RCM-RNase T1 in the presence of NaCl (Pace et al., 1988). The CD spectrum of RCM-RNase T1 obtained in the absence of NaCl (Figure 2a) resembles the spectrum of thermally unfolded RNase T1 (Oobatake et al., 1979b).

Unfolding and Refolding Kinetics of RCM-RNase T1. Unfolding and refolding of RCM-RNase T1 are reversible processes. This is illustrated by the kinetic experiments shown in Figure 3. Native protein (kept at 2.4 M NaCl) and unfolded protein (kept at 0 M NaCl) were both diluted to identical

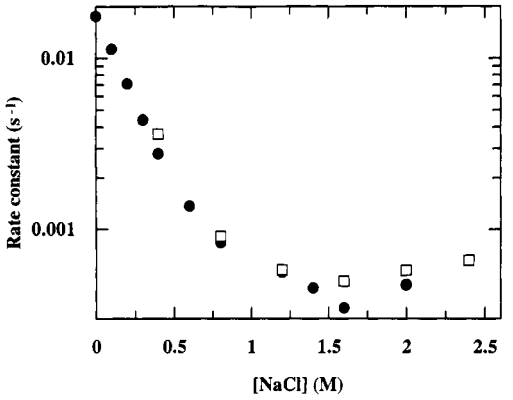

FIGURE 4: Unfolding and refolding kinetics of RCM-RNase T1 as a function of the NaCl concentration. The logarithms of the measured rate constants of refolding (□) and of unfolding (●) are shown as a function of the concentration of NaCl in 0.1 M Tris/HCl, pH 8.0, at 15 °C. The protein concentration was 0.7 μM. In the unfolding experiments 33 μM folded RCM-RNase T1 (in 2.25 M NaCl and 0.1 M sodium acetate pH 5.0, 10 °C) was diluted 48-fold to the indicated concentrations of NaCl and 0.1 M Tris/HCl, pH 8.0, 15 °C. In the refolding experiments 33 μM unfolded RCM-RNase T1 (in 0.1 M Tris/HCl, pH 7.8, 25 °C) was diluted 48-fold to the final NaCl concentrations.

final conditions of 0.8 M NaCl (pH 8.0, 15 °C), which is slightly below the midpoint of the unfolding transition (cf. Figure 1). The observed unfolding and refolding kinetics (Figure 3) are slow and governed by virtually identical time constants of 1270 s for unfolding and 1370 s for refolding. In unfolding, additionally a minor fast reaction is monitored with a time constant of 28 s and an amplitude of 15%. This phase does not show a measurable amplitude in the refolding kinetics. The final fluorescence values reached in the two experiments are identical. The rate of the major slow folding reaction displays a minimum within the transition region (near 1.4 M NaCl) and the measured folding and unfolding rates increase when either the native or the unfolded baselines are approached (Figure 4). Such a V-shaped dependence of the folding rate on the conditions with a minimum in the transition region as in Figure 4 is frequently found in protein folding, when the concentrations of denaturants, such as urea or GdmCl, are varied (Tanford, 1968; Segawa & Sugihara, 1984; Matthews, 1987; Kuwajima, 1989). The kinetic results indicate that the NaCl-dependent refolding and unfolding of RCM-RNase T1 are dominated by a slow, reversible reaction. In unfolding a fast reaction is also detected with a minor amplitude within the transition. This reaction gains rapidly in amplitude under more strongly unfolding conditions (i.e., when the NaCl concentration is reduced) and it reflects primarily the $N \rightleftharpoons U_F$ unfolding reaction. An increase in amplitude for this reaction is expected from the kinetic analysis of the three-state mechanism in eq 1 (Hagerman & Baldwin, 1976; Hagerman, 1977; Kiefhaber et al., 1992b). The fast reaction could not be detected in the refolding kinetics.

Catalysis of Slow Refolding by PPI. The slow refolding reaction of RCM-RNase T1 is catalyzed by PPI (Figure 5, top). A 40-fold dilution of unfolded protein (kept in the absence of NaCl) to a final concentration of 2.0 M NaCl (at 15 °C, pH 8.0) was employed to initiate the reaction. Uncatalyzed refolding is slow under these conditions and is well approximated by a single-exponential phase with a time constant of 1800 s. The corresponding amplitude accounts for virtually the entire change in fluorescence as expected from the equilibrium unfolding transition (Figure 1). If an additional fast refolding reaction occurs within the dead time of mixing (2 s), then its amplitude should not exceed 5%.

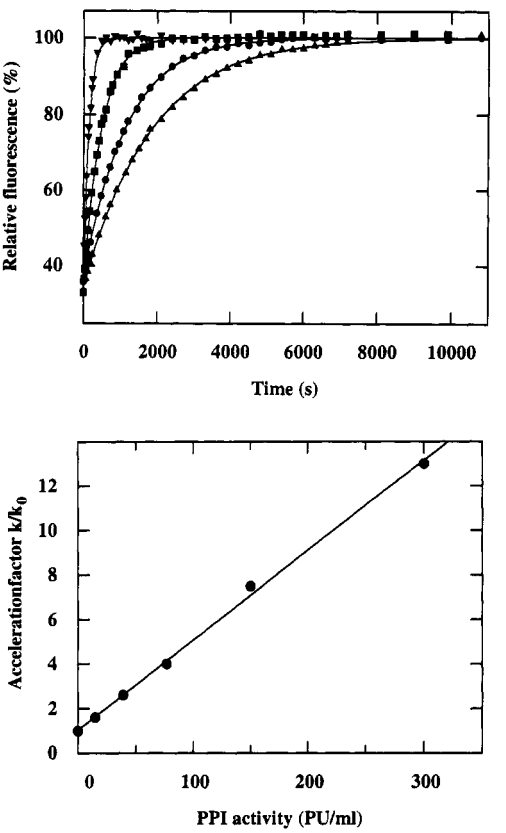

FIGURE 5: (Top) Catalysis of refolding of RCM-RNase T1 by prolyl isomerase. The change in fluorescence at 320 nm (excitation at 268 nm) is shown as a function of the time of refolding. The fluorescence of the folded protein is set as 100%. Refolding was initiated by a 40-fold dilution of unfolded RCM-RNase T1 (in 0.1 M Tris/HCl, pH 7.8, 25 °C) to give final conditions for folding of 0.55 μM RCM-RNase T1 in 2.0 M NaCl and 0.1 M Tris/HCl, pH 8.0, at 15 °C. The refolding solution contained (▲) 0, (●) 15, (■) 77, and (▼) 300 PU/mL PPI. The resulting time constants for refolding are (▲) 1820 s, (●) 1130 s, (■) 460 s, and (▼) 140 s. (Bottom) Relative increase of the apparent rate of refolding as a function of the PPI activity. The ratios of the observed rate constants ($k = \tau^{-1}$) in the presence (k) and in the absence (k_0) of PPI are shown.

Fast-folding molecules (3.5%) are indeed present in unfolded RNase T1 with intact disulfides (Kiefhaber et al., 1990a). The slow refolding of RCM-RNase T1 is accelerated when PPI is added to the refolding solution and the measured rate constant increases in a linear fashion with PPI concentration (Figure 5, bottom). A 13-fold increase in rate is observed in the presence of 300 PU/mL PPI activity. This PPI activity would accelerate the isomerization of the Ala–Pro bond in the assay peptide Suc-Ala-Ala-Pro-Phe-4 nitroanilide by a factor of 300. We do not find evidence for complexity in the folding kinetics of RCM-RNase T1 under the employed conditions. This suggests that the isomerization of a single prolyl residue largely controls slow refolding, or alternatively, that several prolyl residues reisomerize with similar rates in the presence as well as in the absence of PPI.

Catalysis of Prolyl Isomerization in Unfolding RCM-RNase T1. In unfolding experiments prolyl isomerizations are thought to be rate-determining for the equilibration reactions of fast and slow refolding species, U_F and U_S^i (cf. eq 1). These equilibrations occur in the unfolded molecules after the actual unfolding reaction, $N \rightarrow U_F$, has occurred. They are difficult to detect and to measure, since, in most proteins, they are not correlated with measurable changes in the physical properties of the unfolded molecules. The $U_F \rightleftharpoons U_S$ reaction can, however, be followed easily within the transition region, because it is kinetically coupled with

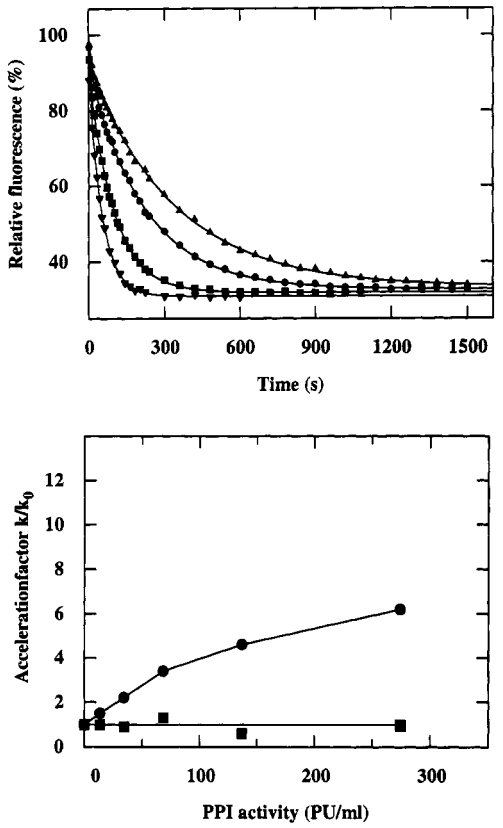

FIGURE 6: (Top) Catalysis of unfolding of RCM-RNase T1 by prolyl isomerase. The change in fluorescence at 320 nm (excitation at 268 nm) is shown as a function of the time of unfolding. The fluorescence of the folded protein is set as 100%. Unfolding was initiated by a 48-fold dilution of folded RCM-RNase T1 (in 2.25 M NaCl and 0.1 M sodium acetate, pH 5.0, 10 °C) to give final conditions for unfolding of 0.7 μM RCM-RNase T1 in 0.4 M NaCl and 0.1 M Tris/HCl, pH 8.0, at 15 °C. The refolding solution contained (▲) 0, (●) 14, (■) 70, and (▼) 275 PU/mL of PPI. The resulting time constants for the slow phase of unfolding are (▲) 340 s (88%), (●) 220 s (87%), (■) 100 s (89%), and (▼) 54 s (92%). The corresponding relative amplitudes are given in parentheses. (Bottom) Variation of the apparent rate constants of the fast (■) and the slow (●) phases of unfolding as a function of the PPI activity. The ratios of the observed rate constants ($k = \tau^{-1}$) in the presence (k) and in the absence (k_0) of PPI are shown.

unfolding. In this region, N is still favored over U_F in the N $\rightleftharpoons$ U_F equilibrium and the amplitude of the fast unfolding reaction is thus very small. In the subsequent slow $U_F \rightleftharpoons U_S$ equilibrium, however, the U_S state is strongly favored over U_F in many proteins and also in RNase T1. The two reactions are kinetically coupled (eq 1), and hence the slow attainment of the $U_F \rightleftharpoons U_S$ equilibrium in RCM-RNase T1 leads concomitantly to significant additional unfolding of N. It is therefore correlated with a large decrease in fluorescence.

We made use of this kinetic coupling here to measure the slow phase of RCM-RNase T1 unfolding and to investigate the influence of PPI on its rate. The experiments were carried out in the presence of 0.4 M NaCl. Under these conditions about 90% of all RCM-RNase T1 molecules are unfolded at equilibrium (Figure 1) and the kinetic coupling between unfolding and isomerization is strong. The observed unfolding kinetics are shown in Figure 6 (top). In the absence of PPI, unfolding is slow and can be approximated by a biphasic reaction with a minor fast phase (τ = 8 s, 12% relative amplitude) and a dominant slow phase (τ = 340 s, 88% relative amplitude). PPI does not affect the time constant and the amplitude of the fast phase (Figure 6, bottom). The slow phase, however, is accelerated by PPI. A 3.4-fold increase in rate is observed in the presence of 70 PU/mL PPI (Figure 6, bottom).

The dependence of the rate constant for the slow phase of unfolding on the concentration of PPI is not linear (Figure 6, bottom). The apparent catalytic efficiency of PPI decreases at high enzyme concentrations. The reason for this behavior is not clear at present. It should be noted, however, that the rate of the slow isomerization approaches the rate of the fast unfolding reaction when the concentration of PPI is increased. Under these conditions the observed rate constant for slow unfolding becomes a complex function of all microscopic rate constants and can no longer be assigned exclusively to the $U_F \rightleftharpoons U_S$ reaction. A related phenomenon was observed in the refolding of the constant fragment of the immunoglobulin light chain. In the presence of high concentrations of PPI the measured rate constants of the slow and fast phases of refolding approached each other and an apparent decrease in the efficiency of PPI was also found (Lang et al., 1987).

Catalysis of Folding and Unfolding under Identical Conditions. The reversibility of RCM-RNase T1 folding allows refolding and unfolding experiments to be carried out under identical final conditions in the transition region as shown in Figure 3. When unfolding of previously folded molecules and refolding of previously denatured molecules are performed at the same final concentrations of 0.8 M NaCl and when identical concentrations of 90 PU/mL PPI are present, both reactions are equally well accelerated by factors of 5.1 and 5.8, respectively. Furthermore, the same final fluorescence values are observed in the presence and in the absence of PPI, in unfolding as well as in refolding (Figure 3). This shows clearly that PPI has an effect only on the rates, not on the extent, of folding. The equilibrium between the folded and the unfolded form of RCM-RNase T1 is not affected by PPI.

DISCUSSION

In previous investigations of the folding mechanism of RNase T1 (Kiefhaber et al., 1991a–c, 1992a) the two disulfide bonds were intact in the denatured and in the native state. The folding of these cross-linked molecules was found to be dominated by two slow trans → cis isomerizations, presumably at Pro39 and Pro55. Both were catalyzed by PPI, albeit with differing efficiency. After the reductive cleavage of the disulfides and the modification of the four sulfhydryl groups by carboxymethylation, the resulting RCM-RNase T1 can be maintained in a folded and probably nativelike structure in the presence of moderate concentrations of NaCl (this work; Oobatake et al., 1979b; Pace et al., 1988). Therefore folding could be studied in the absence of disulfide bonds. The cleavage of the single disulfide bond of the variable domain of the immunoglobulin light chain also lowered the stability of this protein fragment but did not lead to a complete loss of the folded structure (Goto & Hamaguchi, 1981, 1982b).

As found for intact RNase T1, the refolding of RCM-RNase T1 is reversible and is dominated by one or more slow prolyl isomerizations, which occur in the time range of hours at 10–15 °C. This molecular assignment could be readily made, since the entire slow refolding reaction of RCM-RNase T1 was catalyzed by PPI (cf. Figure 5). This enzyme is a valuable tool to characterize the molecular nature of slow steps in protein folding. It should be noted, however, that only a "positive" answer is meaningful. A lack of catalysis could be due to a rate-limiting step other than prolyl isomerization, or alternatively, the prolyl residues might not be accessible for PPI in the refolding protein.

The slow folding of RCM-RNase T1 is well approximated by a single phase, whereas slow folding in the presence of the disulfides is at least biphasic (Kiefhaber et al., 1990a,b). Nevertheless, the time ranges required for slow folding of RNase T1 in the presence and in the absence of the disulfide bonds are quite similar. The monophasic slow refolding of RCM-RNase T1 could originate from a single prolyl isomerization only or, alternatively, from several prolines that isomerize with similar rates.

Because of its marginal stability RCM-RNase T1 could be unfolded and refolded in the absence of denaturants simply by varying the concentration of NaCl in the solution at 15 °C and pH 8. PPI is enzymatically active under all these conditions and thus we could use RCM-RNase T1 to demonstrate that PPI catalyzes prolyl isomerization not only in the course of protein refolding but also during unfolding in the slow $U_F \rightleftharpoons U_S$ reaction of unfolded RCM-RNase T1.

Within the transition region PPI catalyzes the attainment of the equilibrium mixture of folded and unfolded molecules with the same efficiency in unfolding experiments that start from initially folded protein and in refolding experiments that start from initially unfolded protein. Identical final fluorescence values were attained in all these experiments, in the absence and in the presence of PPI. These are significant results. They demonstrate that PPI shows two important enzymatic properties. First, it catalyzes prolyl isomerization steps in protein folding in either direction, and second, the actual direction and the final state are determined not by the enzyme but by the difference in Gibbs free energy associated with the coupled folding and isomerization reactions. This difference in energy depends only on the structure and the stability of the substrate protein under the given folding conditions. Under favorable solvent conditions partially folded structure is formed rapidly, then the prolyl isomerization occurs, and the nativelike isomeric state (either cis or trans) is stabilized by rapid further folding to the native state. In the transition region, the attainment of the folding equilibrium is accelerated, and finally, under unfolding conditions PPI catalyzes the rapid equilibration of cis and trans prolyl isomers after the ordered structure has collapsed in the $N \rightarrow U_F$ unfolding reaction. This activity of PPI is normally not detectable, since the conditions that are required for the in vitro unfolding of most proteins lead to rapid inactivation and unfolding of prolyl isomerases.

Similar enzymatic properties were found for protein disulfide isomerase, the other catalyst of slow steps of folding. This enzyme can catalyze the oxidative formation of disulfide bonds, their reductive cleavage, or the reshuffling of existing disulfides. The direction depends solely on the structural properties of the substrate protein and on the redox potential of the solvent (Freedman, 1984). Neither prolyl isomerase nor protein disulfide isomerase carries specific information regarding the final product of the folding process. The catalytic efficiencies of these two enzymes are probably not identical for all disulfide bonds to be formed and for all prolyl bonds that are isomerized, respectively, in a refolding polypeptide chain. It is conceivable that the rank order of the formation of the disulfide bonds and of the reisomerization of the incorrect prolyl bonds could possibly be changed in the presence of these catalysts. In such a way, protein disulfide isomerase and prolyl isomerase could possibly affect the folding pathway of a protein.

PPI accelerates with high efficiency the isomerization of Xaa–Pro peptide bonds in oligopeptides, in unfolded protein chains, and in partially folded intermediates during slow folding. Prolyl isomerization can also occur in folded proteins, such as in calbindin (Kördel et al., 1990a) and in staphylococcal nuclease (Evans et al., 1987). Positive evidence for a catalysis of such reactions by PPI in folded proteins is, however, still lacking (Kördel et al., 1990). Whether catalysis of prolyl isomerization in unfolded proteins is important for protein folding or unfolding in the cell is not known at present. Unfolded RCM-RNase T1 might be a good model for denatured or not yet folded proteins in the cell, since it can be produced in the absence of denaturants and at ambient temperature. These conditions resemble cellular conditions more closely than urea or GdmCl at high concentrations.

The cellular functions of the PPI activity of cyclophilins and FK 506 binding proteins and the strong inhibition by the respective immunosuppressants cyclosporin A and FK 506 are still largely unclear. There is first evidence that the NinaA gene product of *Drosophila*, a member of the cyclophilin family, is required for the correct folding and transport of rhodopsin in the endoplasmic reticulum (Stamnes et al., 1991; Colley et al., 1991) and that CsA retards the cellular maturation of collagen (Steinmann et al., 1991) and transferrin but not of serum albumin or α_1-antitrypsin (Lodish & Kong, 1991). Possibly, not all proteins are dependent on catalysis of prolyl isomerization in their cellular folding. The ubiquitous occurrence of different families of prolyl isomerases together with their high catalytic efficiency suggests that the enzymatic catalysis of cis/trans isomerizations of prolyl peptide bonds may not be restricted to protein folding reactions but could also be involved in other cellular processes. Catalysis of prolyl isomerization has now been demonstrated for oligopeptides, for unfolded polypeptide chains, and for refolding proteins. It is still unknown whether in addition prolyl isomerizations in folded proteins are used as conformational switches and whether such reactions are catalyzed by prolyl isomerases.

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