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Cooperation of enzymatic and chaperone functions of trigger factor in the catalysis of protein folding

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The trigger factor of *Escherichia coli* is a prolyl isomerase and accelerates proline-limited steps in protein folding with a very high efficiency. It associates with nascent polypeptide chains at the ribosome and is thought to catalyse the folding of newly synthesized proteins. In its enzymatic mechanism the trigger factor follows the Michaelis–Menten equation. The unusually high folding activity of the trigger factor originates from its tight binding to the folding protein substrate, as reflected in the low K_m value of 0.7 μM. In contrast, the catalytic constant k_{cat} is small and shows a value of 1.3 s^{-1} at 15°C. An unfolded protein inhibits the trigger factor in a competitive fashion. The isolated catalytic domain of the trigger factor retains the full prolyl isomerase activity towards short peptides, but in a protein folding reaction its activity is 800-fold reduced and no longer inhibited by an unfolded protein. Unlike the prolyl isomerase site, the polypeptide binding site obviously extends beyond the FKBP domain. Together, this suggests that the good substrate binding, i.e. the chaperone property, of the intact trigger factor is responsible for its high efficiency as a catalyst of proline-limited protein folding.

Keywords: chaperone/enzyme kinetics/prolyl isomerase/protein folding/trigger factor

Introduction

The trigger factor is an abundant soluble protein of *Escherichia coli* with a M_r of 48 kDa. It was discovered originally by Wickner and co-workers in 1987 when they searched for cytosolic components which are involved in the export of secretory proteins (Crooke and Wickner, 1987; Lill *et al.*, 1988). They found that the trigger factor interacted with the export-competent form of a precursor protein, proOmpA, and that it bound to the large subunit of the ribosome (Crooke *et al.*, 1988).

Recently, the trigger factor was rediscovered by three groups, which used different experimental approaches to investigate early processes in cellular protein maturation. The groups of Bukau (Hesterkamp *et al.*, 1996) and Luirink (Valent *et al.*, 1995) incorporated photoactivatable chemical crosslinkers into nascent proteins and, after arresting translation, searched for proteins which possibly bind to and are crosslinked with these newly formed protein chains, while they are still associated with the ribosome. Both groups found crosslinking of presecretory and non-secretory proteins to the trigger factor.

At the same time Fischer and co-workers (Stoller *et al.*, 1995) searched for a ribosome-bound prolyl isomerase in *E.coli*. They discovered such an enzyme and identified it as the trigger factor. In its prolyl isomerase activity the trigger factor resembles the FK 506 binding proteins (FKBPs). It is, however, neither inhibited by FK 506 nor by cyclosporin A. A weak sequence homology was noted between the 165–240 region of the trigger factor and human FKBP12 (Callebaut and Mornon, 1995; Hesterkamp *et al.*, 1996). This homology is significant only for the residues that are necessary for substrate binding and activity. Proteolytic fragments of trigger factor, which encompass the putative FKBP domain (residues 132–247 and 145–251, respectively) and a recombinant form of the 148–249 fragment retained the full prolyl isomerase activity of the intact protein, when assayed with proline-containing oligopeptides (Hesterkamp and Bukau, 1996; Stoller *et al.*, 1996).

The prolyl isomerase function is thought to be important for protein folding, and initial experiments (Stoller *et al.*, 1995) showed that the trigger factor is much more effective as a folding catalyst than cyclophilin, FKBP or parvulin. These small prolyl isomerases catalyse prolyl isomerizations much better in short unstructured oligopeptides (Stein, 1993; Fischer, 1994) than in refolding protein chains (Schmid *et al.*, 1993).

To understand the basis of the high folding activity of the trigger factor, we developed a procedure to measure the Michaelis constant (K_m) and the catalytic rate constant (k_{cat}) for a catalysed folding reaction. Unlike the small substrates of other enzymes, the substrates of folding enzymes are large protein chains, which are in the process of refolding. Therefore it is difficult to elucidate the enzymatic mechanism of a folding enzyme. Experiments are restricted to a narrow range of substrate concentrations, and the initial rates of catalysed folding are not easily determined, because catalysed and uncatalysed folding as well as non-specific aggregation can occur in parallel. Because of these problems and because of their low affinity for protein substrates, it has hitherto not been possible to measure the kinetic parameters K_m and k_{cat} of cyclophilin-, FKBP- and parvulin-catalysed folding.

As in previous work (Stoller *et al.*, 1995), we use a reduced and carboxymethylated variant of ribonuclease T1 (RCM-T1) as a substrate protein to measure the enzyme kinetics of the trigger factor. This protein contains a single *cis* prolyl bond in its native state and its folding mechanism is simple and well characterized in molecular detail (Mayr *et al.*, 1996). Some 85% of all RCM-T1 molecules fold in a monophasic and reversible reaction, which is limited

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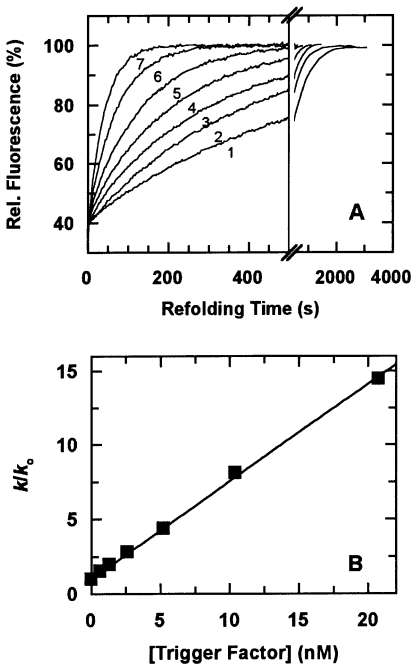

Fig. 1. Refolding kinetics of RCM-T1 in the presence of increasing concentrations of trigger factor at 15°C. (**A**) The kinetics of refolding of 0.7 μM RCM-T1, as followed by the change in fluorescence at 320 nm, are shown in the presence of 0 (trace 1), 0.7 (2), 1.3 (3), 2.6 (4), 5.2 (5), 10.5 (6) and 21 (7) nM trigger factor. (**B**) Dependence on trigger factor concentration of the rate of slow folding. The ratios of the observed rate constants in the presence, k, and in the absence, k_0, of trigger factor are shown as a function of the trigger factor concentration. A value of 1.1×10^6 M^{-1} s^{-1} is obtained for k_{cat}/K_m from the slope of the line in (B). Refolding of RCM-T1 in 0.1 M Tris–HCl, pH 8.0 was initiated by a 40-fold dilution to 2.0 M NaCl in the same buffer.

in rate by the slow *trans–cis* isomerization of the Tyr38–Pro39 prolyl peptide bond.

We find that, unlike the other prolyl isomerases, the trigger factor binds with high affinity to an unfolded protein substrate, and this chaperone-like function is the prerequisite for the excellent catalysis of folding. We also present evidence that the prolyl isomerase site and the polypeptide biding site reside on distinct domains of the trigger factor.

Results and discussion

Catalytic properties of trigger factor as a folding enzyme

The trigger factor catalyses the folding of RCM-T1 very well. The folding of 0.7 μM RCM-T1 is accelerated 9-fold when 10 nM trigger factor is added (Figure 1A), and the first-order rate constant of catalysed folding increases strongly and in a linear fashion with trigger factor concentration (Figure 1B). Its catalytic efficiency as a folding enzyme is remarkably high, and the specificity constant k_{cat}/K_m can be estimated as 1.1×10^6 s^{-1} M^{-1} from the slope in Figure 1B. This is ~100-fold higher than the k_{cat}/K_m value of FKBP12, which is a related, but small and single-domain prolyl isomerase (C.Scholz, unpublished data).

To find out why trigger factor is such an efficient folding enzyme we measured the enzyme kinetics of trigger factor-catalysed folding and determined the kinetic

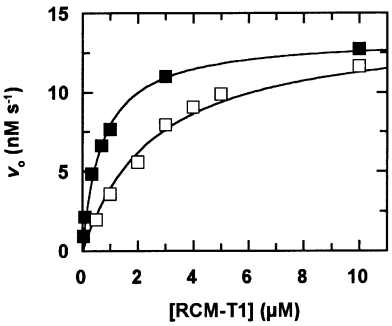

Fig. 2. Enzyme kinetics of the trigger factor-catalysed refolding of RCM-T1 in the absence (■) and in the presence (□) of 1.0 μM RCM-La at 15°C. The initial velocity of the catalysed refolding reaction at 15°C is shown as a function of the concentration of RCM-T1. The trigger factor concentration was 10.4 nM and the buffer was 0.1 M Tris–HCl, 2.0 M NaCl, pH 8.0 in all experiments. Values of $K_m = 0.7\times10^{-6}$ M and $k_{cat} = 1.3$ s^{-1} in the absence of RCM-La and of $K_m = 2.6\times10^{-6}$ M and $k_{cat} = 1.4$ s^{-1} in the presence of 1.0 μM RCM-La were obtained from the analysis of the data (as shown by the continuous lines). The initial folding rates were determined and analysed as described in Materials and methods.

parameters K_m and k_{cat} separately from a Michaelis–Menten plot. In these experiments the concentration of trigger factor was kept constant at 10.4 nM, and the initial rate of folding of the substrate protein RCM-T1 was measured between 0.1 and 10 μM RCM-T1 at pH 8.0, 15°C. Under these conditions RCM-T1 does not aggregate, and its uncatalysed folding proceeds with a half-time of 400 s. Therefore, both catalysed and uncatalysed folding occur in the presence of trigger factor and have to be accounted for in the analysis of the data. To dissect the observed refolding kinetics into the contributions from catalysed and uncatalysed folding, we used a procedure that was developed originally by Kofron *et al.* (1992) for the analysis of cyclophilin-catalysed prolyl isomerization in a tetrapeptide. In this treatment we assume that the binding equilibrium between unfolded RCM-T1 and trigger factor is rapidly established and that catalysed and uncatalysed folding occur in parallel. The contribution of uncatalysed folding was measured in control experiments at the respective concentrations of RCM-T1 in the absence of trigger factor.

The results (Figure 2) show that in its catalysis of protein folding the trigger factor is a classical enzyme. The initial rates of catalysed folding show saturation behaviour and obey the Michaelis–Menten equation. The respective analysis of the data in Figure 2 yields values of 0.7 μM for the K_m value and 1.3 s^{-1} for the catalytic rate constant k_{cat}. The ratio of these two values agrees well with the composite estimate for k_{cat}/K_m as obtained from Figure 1B, confirming that the enzyme kinetics of trigger factor-catalysed folding are adequately described by the Michaelis–Menten equation.

The kinetic constants for prolyl isomerases are difficult to determine not only for protein, but also for peptide substrates, because their binding is very weak and saturation with the oligopeptide substrates cannot be accomplished easily (Kofron *et al.*, 1991, 1992; Schmid *et al.*, 1993; Fischer, 1994). Only the catalysis by a cyclophilin (Cyp18) of the *trans–cis* prolyl isomerization in a tetrapeptide was investigated by two groups using a protease-coupled assay (Kofron *et al.*, 1991) or NMR (Kern *et al.*,

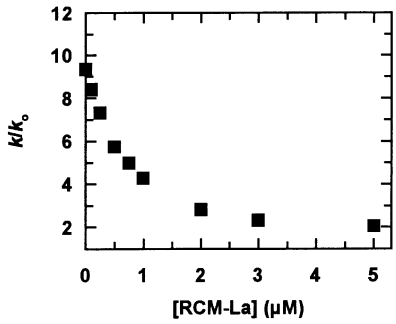

Fig. 3. Inhibition of trigger factor by unfolded RCM-La. The relative rate of catalysed refolding (k/k_0) of 0.7 μM RCM-T1 in 0.1 M Tris–HCl, 2.0 M NaCl, pH 8.0 is shown as a function of the concentration of the inhibitor RCM-La. The concentration of trigger factor was 10.4 nM; otherwise the refolding experiments were carried out as described in Figure 1.

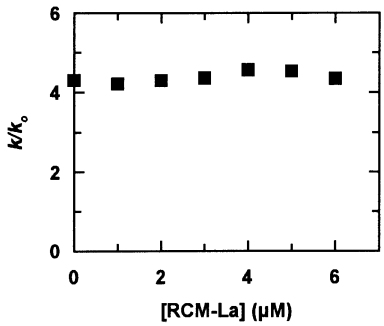

Fig. 4. Influence of unfolded RCM-La on the trigger factor-catalysed prolyl *cis–trans* isomerization in the peptide succinyl-Ala-Phe-Pro-Phe-4-nitroanilide. The relative rate of catalysed isomerization (k/k_0) of 120 μM peptide at 10°C in 0.1 M Tris–HCl, pH 8.0 is shown as a function of the concentration of RCM-La. The concentration of the trigger factor was 15 nM. The prolyl isomerase assays could not be carried out in the presence of 2 M NaCl (as the folding experiments of Figure 3) because the solubility of the assay peptide decreases with increasing salt concentration. In the presence of 1 M NaCl the prolyl isomerase activity of the trigger factor towards the peptide was not significantly decreased when 6 μM RCM-La was added. The activity assays were performed as described in Materials and methods.

1995). In the latter study, values of 220 μM and 620 s^{-1} were obtained for K_m and k_{cat}, respectively, at 10°C. A comparison of these values with the K_m and k_{cat} values observed for the trigger factor-catalysed prolyl isomerization in the folding of RCM-T1 indicates that the power of the trigger factor as a folding catalyst originates from its tight binding to the folding protein substrate, as reflected in the low K_m value. It ensures that significant binding occurs even when the substrate concentration is 1 μM or lower. With a value of ~1 s^{-1}, the turnover number k_{cat} of the trigger factor for refolding RCM-T1 is ~500-fold smaller than the k_{cat} value of cyclophilin for a tetrapeptide (Kern *et al.*, 1995). Unfortunately, the kinetic constants of the trigger factor for the catalysis of prolyl isomerization in a short peptide could not yet be measured, because in this case substrate binding seems to be very weak. The kinetic constants for the catalysis of protein folding by other prolyl isomerases (Cyp18 and FKBP12) could also not be measured, because they bind weakly to protein substrates, and because in kinetic folding experiments the protein concentration is restricted to the micromolar range.

Competitive inhibition of trigger factor by an unfolded protein

Folding enzymes should bind their protein substrates in a non-specific fashion, and, as a consequence, other unfolded proteins should interfere with this binding and inhibit the catalysis of folding. To demonstrate such an interference in trigger factor-catalysed folding, we used the reduced and carboxymethylated form of bovine α-lactalbumin (RCM-La) as a competitor. RCM-La is denatured and soluble, and, importantly, it remains unfolded under the conditions used for the catalysed folding of RCM-T1. Previously, RCM-La was shown to be a substrate protein for the chaperone GroEL (Hayer-Hartl *et al.*, 1994; Okazaki *et al.*, 1994). Indeed, RCM-La seems to compete efficiently with RCM-T1 for binding to the trigger factor. Catalysed folding is progressively decelerated when RCM-La is present at increasing concentrations (Figure 3). The inhibition follows a saturation curve, and, at high concentrations of RCM-La, the rate of the uncatalysed refolding of RCM-T1 is approached. Half-maximal inhibition of the trigger factor was observed when 0.6 μM RCM-La was present.

When the enzyme kinetics of catalysed folding are measured in the presence of 1.0 μM RCM-La, the Michaelis–Menten curve is significantly shifted to higher substrate concentrations (Figure 2), which also indicates that RCM-La competitively inhibits the folding activity of the trigger factor. A preliminary analysis of the data (as shown by the continuous line through these data in Figure 2) yields a value of 0.4 μM for the dissociation constant of the inhibitory complex between RCM-La and trigger factor. This suggests that the folding substrate RCM-T1 and the non-folding inhibitor RCM-La bind with similar affinities and compete for the same polypeptide binding site on the trigger factor. In its catalysis of folding the trigger factor could not be inhibited by the native form of α-lactalbumin with intact disulphide bonds.

Distinct sites for protein binding and prolyl isomerization

In contrast to the strong competitive inhibition of the folding activity of the trigger factor by RCM-La (cf. Figure 3), its prolyl isomerase activity towards a small substrate is not affected by the binding of this unfolded protein. When RCM-La is added in increasing concentrations to activity assays with the short peptide Suc-Ala-Phe-Pro-Phe-4-nitroanilide the prolyl isomerase activity remains virtually unchanged (Figure 4). To avoid potential cleavage and thus inactivation of the competitor RCM-La we used a newly developed variant of the prolyl isomerase assay (T.Zarnt and G.Fischer, unpublished results), which is not coupled to isomer-specific proteolysis by chymotrypsin (Fischer *et al.*, 1984). The results in Figure 4 provide good evidence that the prolyl isomerase active site of the trigger factor is separated widely enough from the polypeptide binding site so that the binding of RCM-La to this site does not interfere with the catalysis of isomerization in a small peptide.

Additional evidence for a physical separation between the protein binding site and the prolyl isomerase site comes from experiments with the isolated FKBP fragment of the trigger factor. The central FKBP domain can be excised from the intact protein and it was found to remain

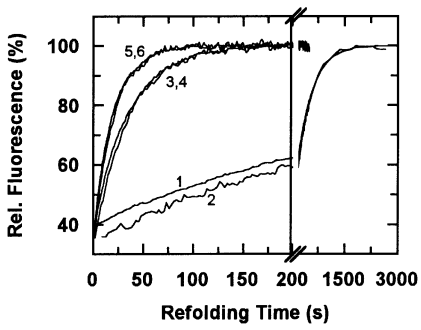

Fig. 5. Folding activity of the 140–250 FKBP fragment of trigger factor. The refolding of 0.7 μM RCM-T1 at 15°C, catalysed by 360 nM of the trigger factor fragment is shown in the absence (trace 1) and in the presence (trace 2) of 3.0 μM RCM-La. The catalysis of RCM-T1 folding by 2.0 μM FKBP12 (traces 3 and 4) and by 1.0 μM Cyp18 (traces 5 and 6) is also shown. Traces 3 and 5 were measured in the absence, and traces 4 and 6 in the presence of 3.0 μM RCM-La. All folding experiments were performed at 15°C in 0.1 M Tris–HCl, 2.0 M NaCl, pH 8.0.

fully active as a prolyl isomerase when a short tetrapeptide was used as a substrate (Hesterkamp and Bukau, 1996; Stoller *et al.*, 1996). In protein folding, however, its activity is reduced ~1000-fold and is barely detectable in our folding assays (Figure 5). Moreover, this residual activity of the FKBP domain is no longer inhibited by the unfolded protein RCM-La. It shares this insensitivity to inhibition with the small prolyl isomerases FKBP12 and Cyp18 (Figure 5), which are known to bind poorly to protein substrates (Schmid, 1993; Schmid *et al.*, 1993). The very weak catalysis of folding by the isolated FKBP domain and the lack of inhibition by unfolded protein show that the central FKBP domain is sufficient for efficient catalysis of prolyl isomerization in a peptide, but not in a folding protein.

Taken together, these results strongly suggest that the catalytic prolyl isomerase site and the high-affinity binding site for unfolded substrate proteins are located on separate domains of the intact trigger factor or require the interaction of these domains. Attempts to identify the respective protein binding sites have not yet been successful (G.Stoller and G.Fischer, unpublished results).

The strong binding of protein substrates may decelerate their dissociation from the trigger factor, and the low k_{cat} value of 1.3 s^{-1} possibly reflects a change in the rate-limiting step from bond rotation (in tetrapeptide substrates) to product dissociation (in protein substrates). Additionally, some binding events may be non-productive, when the reactive prolyl peptide bonds become positioned outside the prolyl isomerase site. Very low values of both K_m and k_{cat}, as observed here, are indeed indicative of non-productive binding of a substrate to an enzyme (Fersht, 1985).

In conclusion, we suggest that the recently discovered folding activity of the trigger factor and its polypeptide binding properties, which have been known for some time, are closely related. The high affinity towards unfolded protein chains is required for the very high efficiency of the trigger factor as a folding catalyst. In its efficient binding to unfolded proteins, the trigger factor resembles a chaperone. It should be noted, however, that the concentration of folding proteins in *E.coli* is probably in the region of 1 μM, and an enzyme which catalyses steps in protein folding should therefore show a K_m value in this concentration range. It is unknown at present whether the chaperone properties of the trigger factor are also required for additional functions, such as accepting newly synthesized protein chains at the ribosome.

Prolyl isomerizations are presumed to be late steps in protein folding (Schmid, 1992) and therefore it seems surprising that a prolyl isomerase binds very early, possibly co-translationally, to a nascent protein chain. The trigger factor might, however, remain associated with the folding chains. There is indeed evidence that the trigger factor is also bound to GroEL in a substrate-dependent manner (Kandror *et al.*, 1995). In addition, it is possible that part of the newly formed proteins can fold to completion rapidly after synthesis at or near the ribosomes and therefore do not require the DnaK or the GroE chaperone systems.

Materials and methods

Materials

α-Lactalbumin was purchased from Sigma. Recombinant forms of human cytosolic Cyp18 and FKBP12 were a gift of Boehringer Mannheim. Trigger factor and (S54G,P55N)-RNase T1 were purified as described (Stoller *et al.*, 1995, 1996; Mücke and Schmid, 1994). (S54G,P55N)-RNase T1 as well as α-lactalbumin were reduced and carboxymethylated by the procedure used for wild-type RNase T1 (Mücke and Schmid, 1994). The 148–249 fragment of the trigger factor was produced as described by Stoller *et al.* (1996).

Spectroscopic methods

For optical measurements, a Hitachi F4010 fluorescence spectrometer and a Kontron Uvikon 860 spectrophotometer were used. The concentrations of RCM-RNase T1 were determined spectrophotometrically by using an absorption coefficient of ε_{278} = 21 060 M^{-1} cm^{-1} (Takahashi *et al.*, 1970). For the trigger factor and its FKBP fragment, ε_{280} values of 15 930 M^{-1} cm^{-1} and 6970 M^{-1} cm^{-1} were calculated, respectively, by using the procedure of Gill and von Hippel (1989).

Measurements of prolyl isomerase activity

To measure the influence of RCM-La on the prolyl isomerase activity of the trigger factor a modified assay was employed. A shift in the *cis–trans* equilibrium in the assay peptide succinyl-Ala-Phe-Pro-Phe-4-nitroanilide is accompanied by a small change in the absorbance of the 4-nitroanilide moiety (T.Zarnt and G.Fischer, unpublished observation), which can be followed well at a high peptide concentration. Thus, the subsequent isomer-specific proteolysis of the peptide by chymotrypsin as in the traditional coupled assay (Fischer *et al.*, 1984) and possible unwanted cleavage of the prolyl isomerase itself or of potential inhibitors, such as RCM-La, could be avoided. For the assay the peptide substrate (60 mM) was dissolved in trifluoroethanol, containing 0.5 M LiCl (Kofron *et al.*, 1991). Under these conditions, ~50% of the peptide molecules are in the *cis* conformation. Upon dilution into aqueous buffer the *cis* content decreases to ~10%. The kinetics of the decrease in *cis* content is measured by the change in absorbance at 330 nm in a HP 8452A diode array spectrophotometer. The assays were carried out at final concentrations of 15 nM trigger factor and 120 μM peptide in 0.1 M Tris–HCl, pH 8.0 at 10°C.

Folding experiments

RCM-T1 was unfolded by incubating the protein in 0.1 M Tris–HCl, pH 8.0 at 15°C for at least 1 h. Refolding at 15°C was initiated by a 40-fold dilution of the unfolded protein to final conditions of 2.0 M NaCl and the desired concentrations of trigger factor and RCM-T1 in the same buffer. The folding reaction was followed by the increase in protein fluorescence at 320 nm (10 nm band width) after excitation at 268 nm (1.5 nm band width). The small contribution of the trigger factor to the fluorescence was subtracted from the measured values in the individual experiments. At 2.0 M NaCl slow folding was a monoexponential process and its rate constant was determined by using the program Grafit 3.0 (Erithacus Software, Staines, UK).

Enzyme kinetics of catalysed folding

In the Michaelis–Menten kinetic experiments the initial velocities of RCM-T1 folding were determined from the progress curves of folding in the presence of 10.4 nM trigger factor under the conditions described above. Measurements were carried out between 0.1 and 10 μM RCM-T1. Below 0.1 μM the signal-to-noise ratio was too low. Both uncatalysed and catalysed folding occur in these experiments. The relative contribution of uncatalysed folding increases linearly with RCM-T1 concentration, and the initial rate of catalysed folding would be progressively overestimated when determined simply from the initial slope of the progress curve of folding. Kofron *et al.* (1991) developed a method to account for both uncatalysed and enzyme-catalysed prolyl isomerization in a peptide. We used this method to analyse the catalysed folding of RCM-T1. The time-course of folding in the presence of trigger factor is described by the differential Equation (1).

$$d[U]/dt = -k_0\times[U]-k_{cat}\times[TF]\times[U]/([U] + K_m) \quad (1)$$

In Equation (1), $d[U]/dt$ is the rate of folding of the unfolded protein U, $-k_0\times[U]$ is the contribution of uncatalysed folding, and $-k_{cat}\times[TF]\times[U]/([U]+K_m)$ is the contribution of catalysed folding. k_{cat} and K_m are the catalytic rate constant and the Michaelis constant, respectively, and [TF] is the concentration of trigger factor. A non-linear least-squares fit of the observed folding kinetics to Equation (1) was performed by using the program EASY-FIT (K.Schittkowski, unpublished). In the analysis it was accounted for that only 85% of the unfolded RCM-T1 molecules contain an incorrect *trans* Pro39 (Mayr *et al.*, 1996), i.e. $[U]_0 = 0.85\times[\text{RCM-T1}]$. The slow refolding reaction, which is analysed here, originates from these molecules. The rate constant of uncatalysed folding, k_0, was measured in folding experiments in the absence of the trigger factor between 0.1 and 10 μM RCM-T1. Its value of $k_0 = 0.00175\ s^{-1}$, which was found to be independent of the concentration of RCM-T1, was used when the experimental data were fitted to Equation (1). The values for k_{cat} and K_m as obtained from this analysis were then used to calculate the initial rates of catalysed folding, v_0, at the different substrate concentrations from Equation (2). In this equation the initial value $[U]_0$ is $0.85\times[\text{RCM-T1}]$.

$$\nu_0 = k_{cat}\times[TF]\times[U]_0/([U]_0 + K_m) \quad (2)$$

The contribution $-k_0\times[U]$ from uncatalysed folding [Equation (1)] increases linearly with the concentration of RCM-T1 and dominates the observed folding kinetics at high [RCM-T1]. Therefore, data at RCM-T1 concentrations higher than 10 μM were not used for the analysis.

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