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The Prolyl Isomerase SlyD Is a Highly Efficient Enzyme but Decelerates the Conformational Folding of a Client Protein

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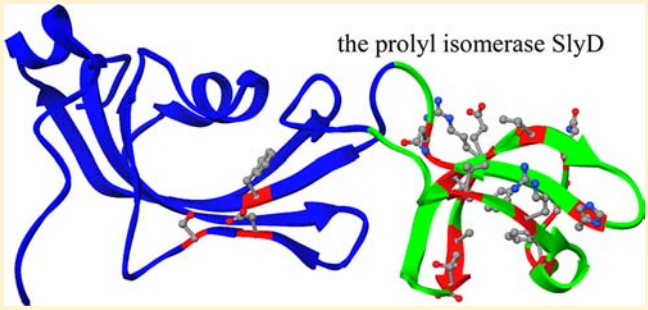
ABSTRACT: Folding enzymes often use distinct domains for the interaction with a folding protein chain and for the catalysis of intrinsically slow reactions such as prolyl *cis*/*trans* isomerization. Here, we investigated the refolding reaction of ribonuclease T1 in the presence of the prolyl isomerase SlyD from *Escherichia coli* to examine how this enzyme catalyzes the folding of molecules with an incorrect *trans* proline isomer and how it modulates the conformational folding of the molecules with the correct *cis* proline. The kinetic analysis suggests that prolyl *cis* → *trans* isomerization in the SlyD-bound state shows a rate near 100 s^{-1} and is thus more than 10^4-fold accelerated, relative to the uncatalyzed reaction. As a consequence of its fast binding and efficient catalysis, SlyD retards the conformational folding of the protein molecules with the correct *cis* isomer, because it promotes the formation of the species with the incorrect *trans* isomer. In the presence of ≥1 μM SlyD, protein molecules with *cis* and *trans* prolyl isomers refold with identical rates, because SlyD-catalyzed *cis*/*trans* equilibration is faster than conformational folding. The *cis* or *trans* state of a particular proline is thus no longer a determinant for the rate of folding.



INTRODUCTION

Conformational protein folding can be very fast, and small proteins often fold within a few milliseconds.[1−3] Folding becomes retarded when intrinsically slow reactions such as disulfide bond formation[4,5] or prolyl *cis*/*trans* isomerization[6−8] are required to reach the native state. These slow steps are catalyzed by folding enzymes such as protein disulfide isomerases[9−11] and peptidyl-prolyl *cis*/*trans* isomerases (prolyl isomerases).[12−14] Folding enzymes often use distinct sites for the binding to substrate proteins (usually called chaperone sites) and for the catalysis of the slow folding steps (at the disulfide isomerase or prolyl isomerase sites). Often, these sites are located on different domains as in the disulfide isomerases DsbC[15] or PDI[16] and in the prolyl isomerases trigger factor,[17] FkpA,[18] SurA,[19] or SlyD.[20−23]

Folding enzymes should bind to unfolded or partially folded protein chains in a generic fashion to allow interactions with a wide range of substrate proteins. Indiscriminate substrate binding, however, can interfere with the rapid conformational folding of proteins that do not form disulfide bonds or do not have incorrect prolyl isomers. Moreover, the catalysis of prolyl *cis* ⇋ *trans* equilibration might create species with incorrect prolyl isomers and thus decelerate conformational folding.

Here we used the prolyl isomerase SlyD (*sensitive-to-lysis protein D*) of *Escherichia coli* and the refolding of a variant of ribonuclease T1 (RCM-T1) to examine how the conformational folding of protein chains with correct prolyl isomers is affected by a folding enzyme. SlyD consists of a prolyl isomerase domain of the FKBP type and a chaperone domain that is inserted into a loop near the prolyl isomerase site (Figure 1A).[20,21] We find that prolyl isomerization in an unfolded protein is very fast when it is bound to SlyD. As a consequence, SlyD retards the conformational folding of protein chains with correct prolyl isomers, because it promotes the formation of species with incorrect prolyl isomers.

EXPERIMENTAL SECTION

Materials. Tris and EDTA were from Roth (Germany); isopropyl β-D-1-thiogalactopyranoside (IPTG), and kanamycin were from Gerbu (Germany); other reagents were purchased from Merck (Germany) or Sigma-Aldrich. To produce RCM-T1, (S54G, P55N)-RNase T1 was purified, reduced and carboxymethylated under denaturing conditions as described.[24] The same procedure was applied for the preparation of the reduced and carboxymethylated form of the P39A variant of RNase T1 (RCM-T1 P39A). Expression, purification, and refolding of SlyD variants were performed as described.[25] FKBP12+IF D37A/F99L was expressed as a SUMO fusion protein and purified as described.[26] Protein concentrations were determined by absorbance at 280 nm, by using absorption coefficients of 5960 $M^{-1}\cdot cm^{-1}$ for SlyD*,[27] 9530 $M^{-1}\cdot cm^{-1}$ for FKBP12,[28] and 21 020 $M^{-1}\cdot cm^{-1}$ for RNase T1.[29]

Prior to modification with Iodo-AEDANS, 100 μM of the D101C variant of SlyD* was incubated with 2 mM tris (2-carboxyethyl) phosphine in 0.1 M K-phosphate, pH 7.0, for 1 h to reduce the protein. For labeling, the reduced protein was passed over a NAP-10 column (GE Healthcare) and incubated with a 20-fold molar excess of Iodo AEDANS in 0.1 M K-phosphate, pH 7.8, for 3 h at 27 °C in the dark. The labeled protein was purified by two passages over a NAP-10 column. The extent of modification was determined by measuring the

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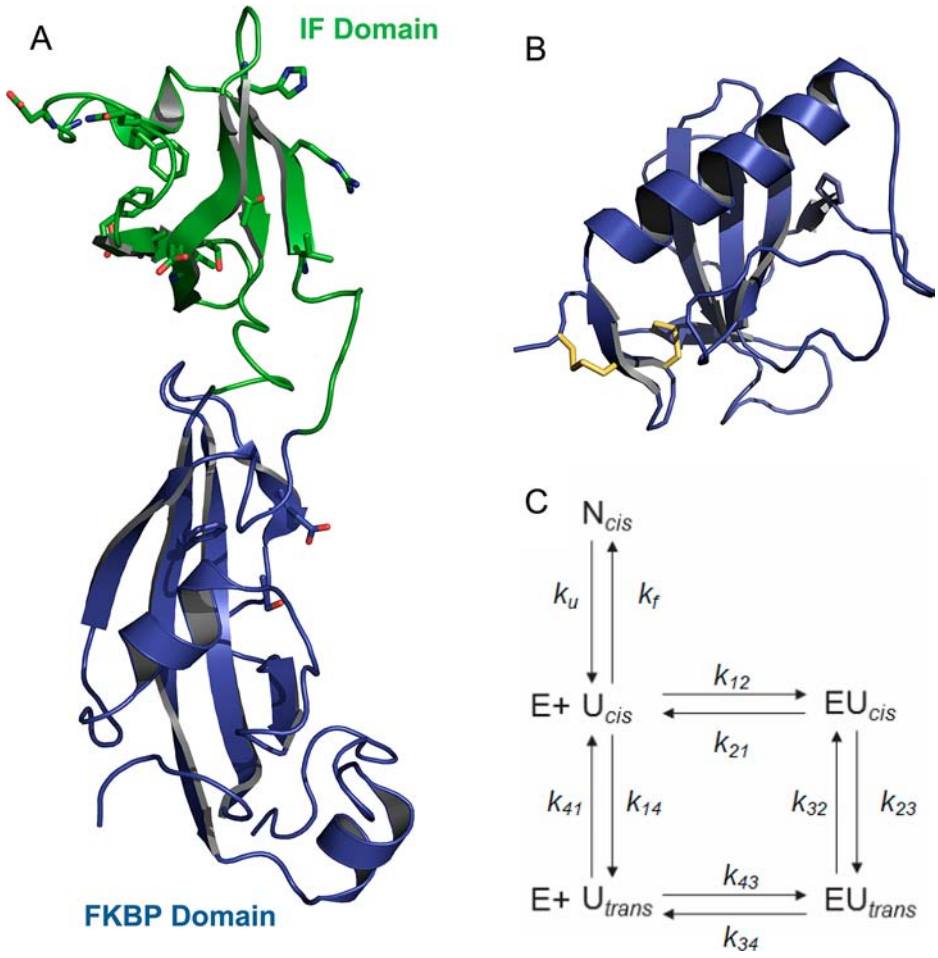

Figure 1. Three dimensional structures of: (A) SlyD* from *E. coli* (2k8i.pdb)[20] and (B) RNase T1 from *Aspergillus oryzae* (9rnt.pdb).[47] In panel A, the IF or chaperone domain is at the top (green), the prolyl isomerase domain is at the bottom (blue). Residues that are involved in substrate binding to the IF domain (V76, R78, F84, G86, D88, Q91, R95, F96, A98, E99, T100, V107, E108, T110, H116, G121, N122,) and that contribute to catalysis in the lower domain (D24, S26, F132) are shown in stick representation. These residue were obtained from NMR chemical shifts analysis of SlyD* in the presence of RCM-α-lactalbumin or RCM-T1.[20] In panel B, the two disulfide bonds of RNase T1 are shown in yellow. They are reduced and carboxymethylated in RCM-T1. Pro39 is shown in gray. It is cis in folded RCM T1. (C) Reaction scheme for the refolding of RCM-T1 in the presence of the folding enzyme SlyD*.

absorbances at 280 and 336 nm in 6.0 M GdmCl, 0.1 M K-phosphate, pH 7.5. It was usually >90%.

Kinetics of Folding of RCM-T1 after Manual Mixing. All fluorescence measurements were performed with a Jasco FP-6500 fluorescence spectrophotometer. To measure proline-limited folding, we used RCM-T1 as a substrate protein. RCM-T1 is unfolded in 0.1 M Tris/HCl, 1 mM EDTA, pH 8.0, and refolding was initiated by a jump to 2.0 M NaCl in the same buffer. The refolding of the 85% U_{trans} molecules was followed after manual mixing at 15 °C by the increase in fluorescence at 320 nm after excitation at 268 nm in the presence of 0−20 μM SlyD*.

Stopped-Flow Kinetic Measurements. The kinetics of binding between SlyD* and RCM-T1 were measured after stopped-flow mixing by using a DX.17MV sequential-mixing stopped-flow spectrometer from Applied Photophysics. The path length of the observation chamber was 2 mm and a 10 mM solution of *p*-nitroaniline in ethanol (AEDANS fluorescence) or a 10 mM solution of GMP (pH 1.5) (tryptophan fluorescence) in a 0.2 cm cell was inserted in front of the emission photomultiplier to absorb scattered light from the excitation beam. The binding kinetics were followed by fluorescence above 400 nm after excitation at 295 nm (10 nm bandwidth). The experiments were performed in 0.1 M Tris/HCl, 2.0 M NaCl, 1 mM EDTA, pH 8.0.

The fast refolding of the U_{cis} molecules of RCM-T1 was measured in stopped-flow double-mixing experiments. To produce U_{cis}, folded RCM-T1 in 0.1 M Tris/HCl, 2.0 M NaCl, 1 mM EDTA, pH 8.0, was first diluted 11-fold with buffer (0.1 M Tris/HCl, 1 mM EDTA, pH 8.0) for 30 s and then, in the second mixing, the protein was diluted 6-fold with 2.5 M NaCl in the same buffer to initiate refolding at 2.1 M NaCl. The final concentration of RCM-T1 was 0.5 μM. The kinetics were followed by fluorescence above 310 nm after excitation at 295 nm (10 nm bandwidth). Kinetic curves were measured 20 times under identical conditions, averaged and analyzed using single- or double-exponential functions. The mixing and dead time of the instrument in single and double-mixing mode was estimated using the reaction between *N*-acetyl-tryptophanamide and *N*-bromo-succinimide.[30] Numerical simulations and analysis of the kinetics were performed using Igor Pro (WaveMetrics, Inc.), GraFit 3.00 (Erithacus Software Ltd.) and Excel (Microsoft).

RESULTS

Binding of SlyD to Unfolded RCM-T1 and Catalysis of Proline-Limited Refolding. To elucidate the interrelationship between substrate binding, catalyzed prolyl isomerization, and conformational protein folding, we used SlyD* as the prolyl isomerase (Figure 1A) and RCM-T1 (Figure 1B) as the substrate protein. The loop of SlyD into which the chaperone domain is inserted is called the flap in human FKBP12, and accordingly, the chaperone domain of SlyD is called the IF

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(*insert-in-flap*) domain. SlyD* lacks the C-terminal part of SlyD (167−196), which is unstructured and rich in histidine residues.[31,32] It is absent in the SlyD proteins of several other bacteria, and its deletion did not change the folding activity of SlyD.[25,33,34]

The wild-type form of ribonuclease T1 (RNase T1) contains two disulfide bonds and two *cis* prolines (Pro39 and Pro55). It is stable, and denaturants at high concentrations are required for unfolding. Here, we employed the reduced and carboxymethylated form of the S54G/P55N variant of RNase T1 (RCM-T1).[24] It is less stable than wild-type RNase T1, and in the folded form, it contains only a single *cis* prolyl bond (Tyr38-Pro39), which simplifies its folding mechanism.[24,35−37] RCM-T1 is fully folded only in the presence of ≥2 M NaCl; in the absence of salt, it is unfolded. Refolding can thus be initiated by a jump to 2.0 M NaCl in the absence of denaturants and is accompanied by a large increase in fluorescence. Earlier, we showed by a kinetic analysis of the folding kinetics and by double-mixing experiments[24,38] that 15% of the unfolded RCM-T1 molecules (U_{cis}) contain a correct *cis* Pro39 and refold rapidly (τ = 0.7 ± 0.1 s at 15 °C, pH 8.0), whereas the remaining 85% (U_{trans}) refold slowly (τ = 530 ± 10 s) in a reaction that is limited in rate by the *trans* → *cis* isomerization at Pro39. These numbers and kinetic constants were confirmed in this work.

For designing the folding experiments in the presence of the folding enzyme SlyD, and for analyzing the results, we used the kinetic scheme in Figure 1C. It represents the simplest mechanism that links the conformational folding equilibrium ($U_{cis} \leftrightharpoons N_{cis}$) with uncatalyzed prolyl isomerization ($U_{cis} \leftrightharpoons U_{trans}$), with the binding of the two unfolded forms (U_{cis} and U_{trans}) to SlyD* and with the catalyzed prolyl isomerization in the SlyD*-bound state ($EU_{cis} \leftrightharpoons EU_{trans}$). Proline residues are unimportant for the binding of unfolded proteins to SlyD*,[25] and therefore, we assumed that the unfolded forms of RCM-T1 with a *cis* or a *trans* Pro39 bind with the same affinity to the chaperone domain of SlyD. This implies that because of the thermodynamic coupling in the closed cycle in Figure 1C, the *cis*/*trans* ratio at Pro39 is not altered by the binding to SlyD*.

To measure the binding to SlyD* uncoupled from folding, we employed the P39A variant of RCM-T1 (RCM-T1 P39A). This form is further destabilized because the *cis* state cannot be reached when Pro39 is replaced by Ala, and thus, it remains permanently unfolded, even in the presence of 2.1 M NaCl. We had used this unfolded variant previously to study substrate binding to SlyD*[34] by Förster resonance energy transfer (FRET) between the single Trp (Trp59) of RCM-T1 P39A and AEDANS-labeled SlyD* (SlyD*-AED).

RCM-T1 P39A (1 μM) bound to 1 μM SlyD*-AED in a very fast reaction, with a time constant (τ) of 24 ± 2 ms (Figure 2). When 1 μM of unfolded RCM-T1 was mixed with 1 μM SlyD*. we observed a similarly rapid binding reaction (τ = 53 ± 5 ms, Figure 2). After the increase in the first 200 ms, the FRET signal did not persist. Rather it decreased again in a slow reaction, indicating that the complex between RCM-T1 and SlyD* dissociated again, with a time constant of 40 ± 4 s. This dissociation reaction showed the same time course as the refolding of RCM-T1, which, in a parallel experiment, was monitored by the increase in protein fluorescence in the presence of an identical concentration of unlabeled SlyD* (τ = 50 ± 3 s; Figure 2). The dissociation from SlyD*-AED is thus controlled by the folding of RCM-T1, which eliminates binding to the chaperone (IF) domain of SlyD. This refolding reaction

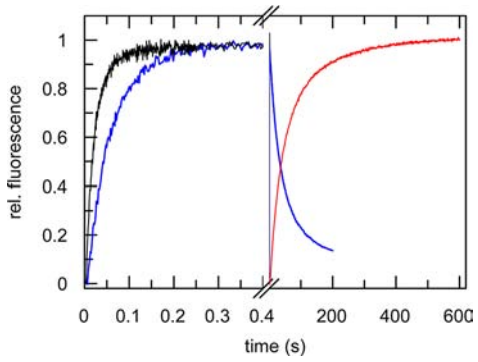

Figure 2. Time course of the interaction between 1.0 μM SlyD-AED and 1.0 μM RCM-T1 during folding (blue). It is compared with the binding of 1 μM of the permanently unfolded variant RCM-T1-P39A to 1.0 μM SlyD-AED (black), and with the refolding kinetics of 1 μM RCM-T1 in the presence of unlabeled SlyD* (red). The binding kinetics was followed by the increase in AEDANS fluorescence after excitation at 295 nm in a stopped-flow instrument, the folding kinetics by protein fluorescence at 355 nm after manual mixing. The measurements were performed in 2.1 M NaCl, 0.1 M Tris-HCl, pH 8.0, at 15 °C. The analysis gave τ = 53 ± 5 ms for binding and τ = 40 ± 4 s for folding (blue curve), and τ = 24 ± 2 ms for binding (black curve), and τ = 50 ± 3 s for folding (red curve).

is 30-fold faster than the uncatalyzed refolding of RCM-T1, because it is accelerated by SlyD*. The permanently unfolded substrate RCM-T1 P39A can be dissociated from SlyD* by dilution. This occurs with a time constant of 40 ms,[34] which is 1000-fold faster than dissociation caused by the folding of RCM-T1.

The refolding substrate protein RCM-T1 thus binds very rapidly to SlyD*, in a similar fashion as the permanently unfolded form RCM-T1 P39A that lacks Pro39, suggesting that the isomerizing proline itself and its *cis* or *trans* state (Figure 1C) are unimportant for binding. The refolding protein remains a substrate until folding is complete.

Influence of SlyD on the Conformational Folding of RCM-T1 with Correct *cis* Pro39. In the folded form of RCM-T1 (at ≥2 M NaCl), Pro39 is *cis*, but in unfolded RCM-T1, the *trans* form is favored, and at equlilibrium, only about 15% of the unfolded molecules (U_{cis}) contain a *cis* Pro39.[24,38] To increase the fraction of the fast-folding U_{cis} species, we started with folded RCM-T1, unfolded it by a dilution from 2.0 to 0.18 M NaCl and stopped unfolding after 30 s. Under these conditions, conformational unfolding (N → U_{cis}, Figure 1C) shows a time constants of 7 ± 1 s and is thus virtually complete within 30 s. The subsequent Pro39 isomerization ($U_{cis} \leftrightharpoons U_{trans}$) is slow and shows a time constant of 100 ± 10 s. After 30 s of unfolding, 60−70% of the unfolded molecules are thus still in the U_{cis} state.

To enrich the U_{cis} molecules and to examine how SlyD* influences their conformational folding, we therefore employed stopped-flow double-mixing experiments The first mixing was used to produce the U_{cis} species by unfolding RCM-T1 for 30 s at 0.18 M NaCl. The second mixing served to initiate refolding at 2.1 M NaCl. In the absence of SlyD*, a rapid refolding reaction with a time constant of 0.7 ± 0.1 s (Figure 3, trace 1) is observed. It represents the conformational $U_{cis} \leftrightharpoons N_{cis}$ folding reaction (Figure 1C). It is 800-fold faster than the refolding of the U_{trans} molecules (Figure 3, trace 5, τ = 550 ± 10 s), which is limited in rate by Pro39 isomerization ($U_{trans} \leftrightharpoons U_{cis}$, Figure 1C). In the presence of 100 nM SlyD, refolding of the U_{cis}

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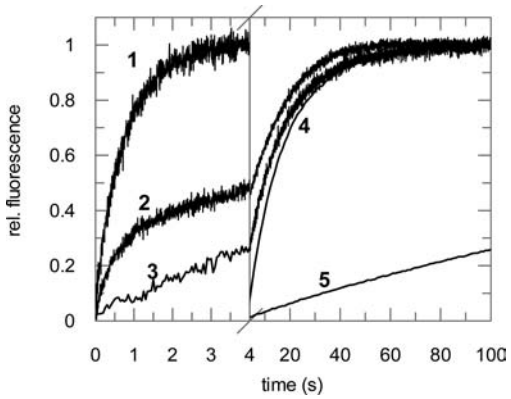

Figure 3. Fast refolding reaction ($U_{cis} \rightarrow N$) of RCM-T1 in the absence of SlyD* (trace 1) and in the presence of 0.1 μM (trace 2), or 1.0 μM SlyD* (trace 3). Traces 4 and 5 show the slow refolding reaction ($U_{trans} \rightarrow N$) in the presence of 1.0 μM SlyD* (trace 4) and in the absence of SlyD* (trace 5). U_{cis} was generated in a stopped-flow double-mixing-experiment by a 30 s unfolding pulse in 0.18 M NaCl. Refolding was initiated by dilution to 2.1 M NaCl. The reaction was followed by fluorescence above 310 nm after excitation at 295 nm. Slow refolding of U_{trans} was followed after manual mixing by fluorescence at 320 nm after excitation at 268 nm in 2.0 M NaCl. All experiments were performed with 0.5 μM RCM-T1 in 0.1 M Tris/HCl, pH 8.0, at 15 °C. The rate constants derived from the analysis of the curves are given in Figure 4A.

molecules becomes biphasic (Figure 3, trace 2). The fast phase resembles the fast refolding reaction of U_{cis} in the absence of SlyD* (trace 1) in its rate, but the amplitude is more than 2-fold decreased. The missing amplitude is accounted for by a 20-fold slower phase with a time constant of 17 s. In the presence of 1 μM SlyD (Figure 3, trace 3), the fast refolding reaction is no longer detectable, and virtually all molecules refold with τ = 17 ± 1 s.

The refolding of the slow-folding U_{trans} molecules is 30-fold accelerated when 1 μM SlyD* is present, and it also shows a time constant of 17 s (Figure 3, trace 4). Identical refolding kinetics were thus observed in the presence of 1 μM SlyD* when starting either from U_{cis} or from the 15/85 mixture of U_{cis}/U_{trans}, as present in RCM-T1 unfolded at equilibrium (Figure 3, traces 3 and 4). This vanishing of the fast refolding reaction in the presence of 1 μM SlyD* indicates that the binding of U_{cis} to SlyD* and the catalyzed *cis* ⇋ *trans* equilibration in the SlyD-bound state are much faster than the conformational folding of the U_{cis} molecules in the $U_{cis} \leftrightharpoons N_{cis}$ folding reaction (cf. Figure 1C).

Refolding Reaction Starting from U_{trans} in the Presence of SlyD*. To further examine the influence of the prolyl isomerase SlyD* on the slow proline-limited refolding ($U_{trans} \rightarrow N$) and on the direct and fast conformational folding ($U_{cis} \rightarrow N$) of RCM-T1, we performed experiments as in Figure 3 over a wide range of SlyD concentrations, from catalytic (5 nM) to superstoichiometric (10 μM) concentrations. The folding of U_{trans} was followed after manual mixing, the folding of U_{cis} after stopped-flow double mixing. The measured folding rates and amplitudes are shown in Figure 4A–C.

The refolding of the U_{trans} molecules was strongly accelerated when catalytic amounts of SlyD* were added, and 5 nM SlyD* was sufficient to enhance the folding rate 5-fold. The catalysis became less efficient with increasing SlyD* concentration, and a maximal folding rate (0.08 ± 0.01 s^{-1}, equivalent to a 50-fold increase) was reached when the SlyD* and RCM-T1 concentrations were equal (0.5 μM each). When the SlyD* concentration was further increased (up to 10 μM), the refolding rate decreased again (Figure 4A).

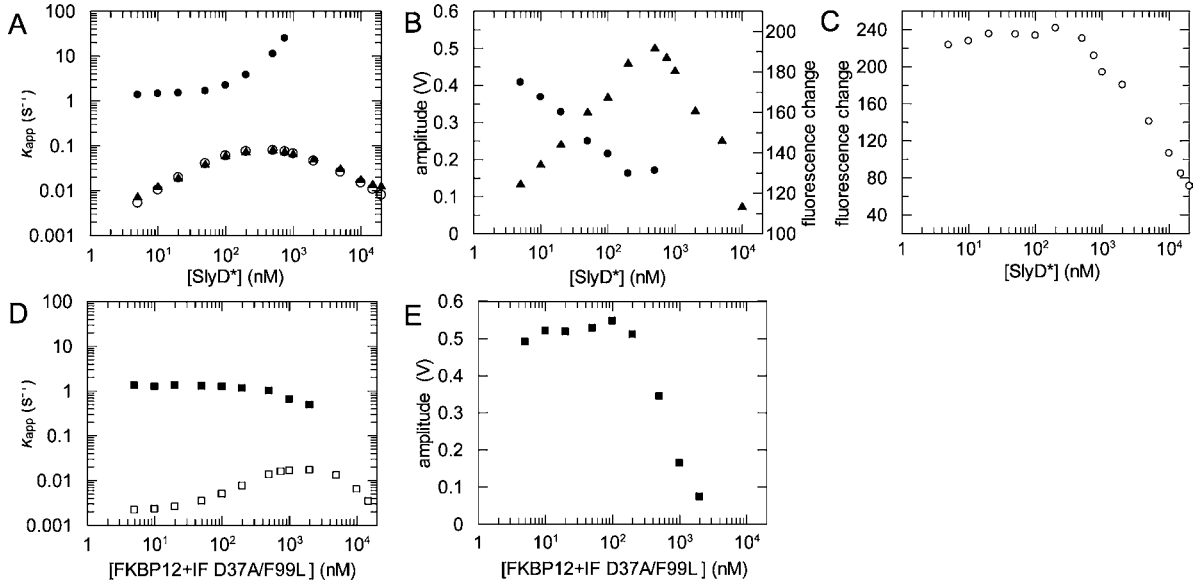

Figure 4. (A–C) Refolding of the U_{cis} and U_{trans} forms of RCM-T1 in the presence of SlyD*. (A) The observed rate constants and (B) amplitudes of (●) the fast and (▲) the slow refolding reactions of U_{cis} are shown as a function of the SlyD* concentration. U_{cis} was generated by a 30 s unfolding pulse in 0.18 M NaCl. (○) Rate (panel A) and amplitude (panel C) of the refolding reaction of U_{trans}. (■) Rate (panel D) and amplitude (panel E) of fast refolding, and (□) rate of slow refolding of the U_{cis} form in the presence of the inactivated variant FKBP12+IF D37A/F99L. U_{cis} was generated by a 30 s unfolding pulse in 0.18 M NaCl. Refolding was initiated by dilution to 2.1 M NaCl. The reaction was followed by fluorescence above 310 nm after excitation at 295 nm. Slow refolding of U_{trans} was followed after manual mixing by fluorescence at 320 nm after excitation at 268 nm in 2.1 M NaCl. All experiments were performed with 0.5 μM RCM-T1 in 0.1 M Tris/HCl, pH 8.0, at 15 °C.

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In fully unfolded RCM-T1, 85% of the molecules are in the U_{trans} form, and therefore, their SlyD*-catalyzed refolding showed a high amplitude (Figure 4C). Above 500 nM SlyD*, it started to decrease, in parallel to the transition from catalytic to stoichiometric concentrations (Figure 4C), and in the presence of about 8 μM SlyD*, the catalyzed refolding of U_{trans} had lost half of its amplitude. This suggests that, under these conditions, half of the RCM-T1 molecules can no longer fold, because they remain bound to SlyD* in an unfolded form. The fast binding equilibrium reduces the effective concentration of folding molecules, and this explains, why, at high SlyD concentration, the measured refolding rate decreased as well (Figure 4A).

Refolding Reaction Starting from U_{cis} in the Presence of SlyD*. The refolding of the U_{cis} molecules was studied in stopped-flow double mixing experiments. As before, a 30-s unfolding pulse was employed to produce about 70% U_{cis} molecules, and then their refolding was followed, also in the presence of 5 nM to 10 μM SlyD*. In the catalytic regime, the rate of fast conformational refolding ($U_{cis} \leftrightharpoons N_{cis}$) remained virtually constant (Figure 4A), because this reaction does not involve prolyl isomerization. Its amplitude, however, decreased strongly between 5 and 200 nM SlyD* (Figure 4B), and above 1 μM SlyD*, the fast refolding reaction was no longer detectable.

In a reciprocal fashion, a slow but catalyzed refolding reaction appeared that showed the same rate and the same dependence on SlyD* concentration as the refolding rate of the U_{trans} species (Figure 4A). Its amplitude increased with increasing SlyD* concentration at the expense of the amplitude of the $U_{cis} \leftrightharpoons N_{cis}$ folding reaction (Figure 4B). This is expected, because the SlyD*-catalyzed conversion of U_{cis} to U_{trans} competes with the folding of the U_{cis} molecules (Figure 1C) and thus converts fast-folding U_{cis} molecules to slow-folding U_{trans} molecules.

The folding of the U_{cis} molecules in the presence of the prolyl isomerase SlyD* thus shows the hallmarks of a kinetic competition between a monomolecular reaction ($U_{cis} \leftrightharpoons N_{cis}$ folding) and a bimolecular reaction (binding to SlyD*, followed by catalyzed $U_{cis} \leftrightharpoons U_{trans}$ isomerization, Figure 1C). The measured rate increases with SlyD* concentration because it reflects the sum of the rates of the two competing processes. The amplitude decreases because the competing process (binding to SlyD*) becomes faster with increasing SlyD* concentration. Above 0.5 μM SlyD*, the amplitude of slow, catalyzed folding decreases in a similar fashion as in Figure 4C, also because RCM-T1 molecules remain bound to SlyD*, and this binding inhibits folding.

Effect of Chaperone Binding on Conformational Protein Folding. The binding of SlyD* to U_{cis} in the E + $U_{cis} \leftrightharpoons EU_{cis}$ reaction as well as the subsequent catalysis of the $U_{cis} \leftrightharpoons U_{trans}$ isomerization in the $EU_{cis} \leftrightharpoons EU_{trans}$ reaction (Figure 1C) withdraw RCM-T1 molecules from the fast $U_{cis} \leftrightharpoons N_{cis}$ refolding path. To reveal the relative contributions of these two processes, we employed a folding enzyme with an intact chaperone domain but an inactivated prolyl isomerase domain. For SlyD*, inactivating substitutions are not known. Therefore, we employed a chimeric protein in which the chaperone (IF) domain of SlyD* is combined with the D37A/F99L variant of the well-characterized human prolyl isomerase FKBP12 (termed FKBP12+IF D37A/F99L). The substitutions D37A and F99L reduce the prolyl isomerase activity of the FKBP12 part of the chimeric protein to 3.7%.

Again, the U_{cis} species of RCM-T1 was produced by a 30-s unfolding pulse, and then, its refolding was studied in the presence of 5 nM to 10 μM of FKBP12+IF D37A/F99L (Figure 4D,E). Below 200 nM of this almost inactive prolyl isomerase, refolding is hardly affected. Both the rate and the high amplitude of the fast refolding of U_{cis} remained unchanged. The slow folding of the U_{trans} species, as measured after manual mixing, was slightly accelerated (Figure 4D,E) by FKBP12+IF D37A/F99L, but about 30-fold less efficient than in the case of SlyD*, as expected from the 30-fold decrease in prolyl isomerase activity. Above 0.5 μM FKBP12+IF D37A/F99L (in the stoichiometric regime), the measured rate of $U_{cis} \leftrightharpoons N_{cis}$ refolding decreased (Figure 4D), coupled with a loss in amplitude (Figure 4E). The strong difference between the amplitude profiles of the $U_{cis} \leftrightharpoons N_{cis}$ refolding reaction in the presence of SlyD* (Figure 4B) or the inactivated variant FKBP12+IF D37A/F99L (Figure 4E) suggests that the pronounced decrease of this amplitude in the presence of low concentrations of active SlyD* (Figure 4B) is in fact caused by the very rapid conversion of U_{cis} to U_{trans} during catalyzed $U_{cis} \leftrightharpoons U_{trans}$ isomerization in the SlyD*-bound state. This catalysis is about 30-fold less efficient in the presence of the inactivated protein FKB12+IF D37A/F99L.

When present at stoichiometric concentrations, SlyD* and FKBP12+IF D37A/F99L exert opposite effects on the measured rate of the fast refolding reaction (Figure 4A,D). This rate increased in the presence of the active prolyl isomerase and decreased in the presence of the inactivated form. This provides insight into the mechanism. When a folding reaction such as $U_{cis} \leftrightharpoons N_{cis}$ is coupled with a rapid binding equilibrium such as the association of U_{cis} with the chaperone domain of FKBP12*+IF D37A/F99L (cf. Figure 1C), then the measured refolding rate decreases because the binding equilibrium reduces the effective concentration of the reacting species (U_{cis}). An increase of the observed folding rate, as in the presence of active SlyD* (Figure 4A) is observed when the folding reaction competes with a unidirectional binding reaction. In this case, the observed rate is equal to the sum of the rate constants of the two competing reactions (as outlined above). Binding of U_{cis} to SlyD* is made irreversible apparently because the catalyzed $EU_{cis} \leftrightharpoons EU_{trans}$ isomerization after binding is very fast, faster than the rate of dissociation of U_{cis} from SlyD* (which shows a rate constant of $30 \pm 5\ s^{-1}$, ref 34).

The Rates of Catalyzed Prolyl Isomerization from Kinetic Simulation. The kinetic mechanism in Figure 1C was used to model the refolding kinetics of RCM-T1 in the presence of the folding enzyme SlyD*. The scheme in Figure 1C is characterized by 10 rate constants, and most of them are known from independent experiments. The rate constants of folding (k_u and k_f) and of prolyl isomerization in the absence of a folding enzyme (k_{14} and k_{41}) have been determined previously.[24,38] They were redetermined and confirmed in this work. The kinetics of binding of unfolded RCM-T1 to SlyD* (k_{12} and k_{21}) have been measured by using the permanently unfolded P39A variant of RCM-T1,[34] and we assume that both unfolded forms, U_{cis} and U_{trans}, bind with the same affinity and the same kinetics to SlyD* (i.e., $k_{12} = k_{43}$ and $k_{21} = k_{34}$). Thus, eight of the 10 rate constants in the scheme in Figure 1C are known from reference experiments.

The rates of catalyzed isomerization in the SlyD*-bound state of RCM-T1 are unknown. An analysis of the kinetics of catalyzed folding of RCM-T1 thus enables us to estimate the rates of catalyzed prolyl isomerization in our model protein when it is bound to the folding enzyme SlyD. Rather than

fitting curves to the observed kinetics based on the scheme in Figure 1C, we employed simulations, in which the rates of catalyzed prolyl isomerization in the SlyD*-bound state were varied. All the other rate constants were held constant at the values derived from the reference experiments (as shown in Table 1).

Table 1. Overview of the Rate Constants for the Kinetic Mechanism Shown in Figure 1C

reaction	rate constant	value	reference[a]
$U_{cis} \rightarrow U_{trans}$	k_{14}	$(1.1 \pm 0.1) \times 10^{-2}$ s^{-1}	24
$U_{trans} \rightarrow U_{cis}$	k_{41}	$(1.8 \pm 0.2) \times 10^{-3}$ s^{-1}	24
$E + U_{cis} \rightarrow EU_{cis}$	k_{12}	$(24 \pm 1) \times 10^{6}$ M^{-1} s^{-1}	34
$EU_{cis} \rightarrow U_{cis} + E$	k_{21}	25 ± 1 s^{-1}	34
$EU_{cis} \rightarrow EU_{trans}$	k_{23}	variable	
$EU_{trans} \rightarrow EU_{cis}$	k_{32}	$k_{23}/6$	
$EU_{trans} \rightarrow E + U_{trans}$	k_{34}	25 ± 1 s^{-1}	34
$E + U_{trans} \rightarrow EU_{trans}$	k_{43}	$(24 \pm 1) \times 10^{6}$ M^{-1} s^{-1}	34
$U_{cis} \rightarrow N_{cis}$	k_f	1.4 ± 0.1 s^{-1}	24
$N_{cis} \rightarrow U_{cis}$	k_u	$(9.5 \pm 0.8) \times 10^{-4}$ s^{-1}	24

[a]Source of the rate constant.

Figure 5 compares the calculated folding rates from the simulations with the experimental data. In the first set of simulations (Figure 5A), we varied the rate constant of SlyD-catalyzed *cis* → *trans* isomerization (k_{23}) from 0.1 s^{-1} to 1000 s^{-1} and calculated the rate λ of the fast folding reaction as a function of the SlyD concentration. The ratio k_{23}/k_{32} was kept constant at a value of 6 (reflecting the U_{trans}/U_{cis} ratio of 85/15 in unfolded RCM-T1[24,38]). In the absence of catalysis or when it is very poor (k_{23} = 0.1 s^{-1}), the rate of fast refolding is almost independent of the prolyl isomerase concentration, because the conformational refolding reaction ($U_{cis} \leftrightharpoons N_{cis}$) is always faster than catalyzed *cis* → *trans* isomerization in the SlyD-bound state ($EU_{cis} \leftrightharpoons EU_{trans}$). A slight retardation is observed at superstoichiometric SlyD concentrations because binding retards folding, as observed experimentally for the refolding in the presence of the inactivated enzyme FKB12+IF D37A/F99L (Figure 4D). When the rate of catalyzed *cis* → *trans* isomerization is increased in steps up to 1000 s^{-1}, a progressive increase in the apparent rate of fast refolding is observed in the calculations. The experimental rates of folding in the presence of SlyD*, as taken from Figure 4A, are best represented by the line calculated for k_{23} = 100 s^{-1} (Figure 5A). We conclude that prolyl isomerization in unfolded RNase T1 is 10 000-fold accelerated, from 0.011 s^{-1} in the absence of SlyD (Table 1) to 100 s^{-1} in the SlyD-bound state.

Figure 5B provides an alternative view of the calculated kinetics. It shows how the observed folding rate λ varies as a function of k_{23} (the rate of catalyzed *cis* → *trans* isomerization in the SlyD-bound state, Figure 1C). The comparison with the experimental λ values obtained in the presence of 0.1, 0.2, or 0.5 μM SlyD, indicates that the rate of catalyzed *cis* → *trans* isomerization (k_{23}) lies between 80 and 180 s^{-1} as suggested by the data obtained at 0.5 μM SlyD. Finally, the value of k_{23} = 100 s^{-1} was used together with the other rate constants of Table 1 to simulate the folding kinetics as a function of the SlyD* concentration. The simulated kinetics were then analyzed and the corresponding apparent rate constants compared with the experimental data (Figure 5C). The agreement between the two data sets is remarkably good. The observed rate constants

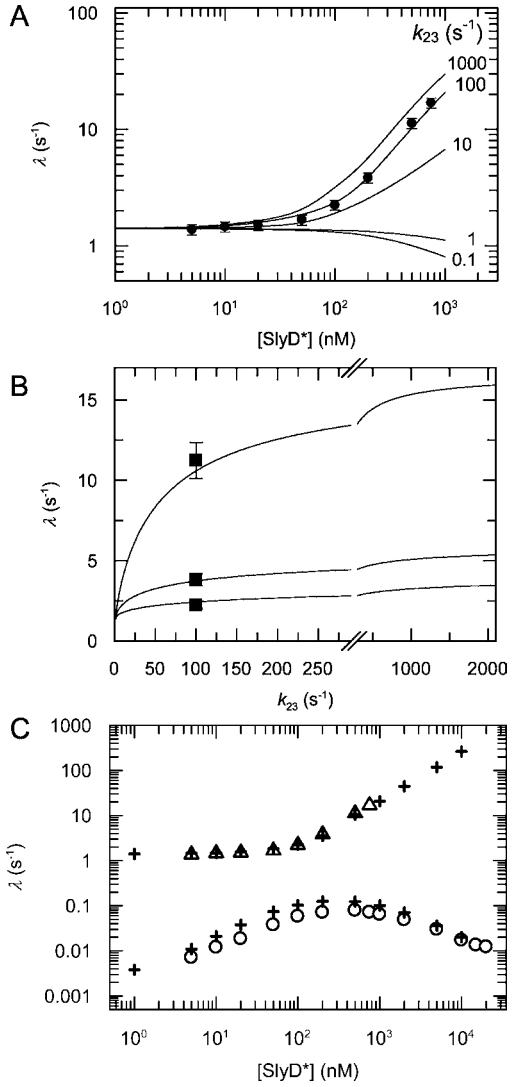

Figure 5. (A) Numerical simulations of the folding kinetics. The calculated rate of the fast refolding reaction λ is shown as a function of the SlyD* concentration. In the calculations, the rate constants as given in Table 1 were held constant, and k_{23}, the rate constant of catalyzed *cis* → *trans* isomerization was increased from 0.1 s^{-1} to 1, 10, 100, and 1000 s^{-1} (from bottom to top). The experimental data from Figure 4A are shown as circles for comparison. (B) The calculated λ values are shown as a function of the k_{23} value for 0.1, 0.2, and 0.5 μM SlyD (from bottom to top). The experimental λ values are shown as squares. (C) Comparison between the experimental rate constants for the fast phase (triangles) and the slow phase (circles) and those derived from the simulated curves ("+" symbols,) based on the mechanism in panel A, the rate constants listed in Table 1 and a k_{23} value of 100 s^{-1}.

of both the fast and the slow kinetic phase were reproduced very well in the entire range of SlyD* concentrations, which extend from catalytic (5 nM) to superstoichiometric (10 μM) concentrations.

DISCUSSION

Prolyl Isomerases Interfere with Conformational Folding. Prolyl isomerases such as SlyD use chaperone domains for a generic binding to unfolded or incompletely folded protein chains, transfer them to the active site, and catalyze the *cis* ⇋ *trans* equilibration of prolyl bonds in these

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protein chains. It is clear that folding enzymes do not convey directional information for the folding of a particular protein. Rather, by their indiscriminate binding to unfolded protein chains, they interfere with conformational folding. This is illustrated by the effects of SlyD* on the folding of the U_{cis} form of RCM-T1 with the native-like *cis* isomer at Pro39. Its fast folding is in fact retarded by the binding to the chaperone domain of SlyD and the subsequent efficient catalysis of *cis* ⇋ *trans* equilibration at Pro39, which converts most of the fast-folding U_{cis} molecules into slow-folding U_{trans} molecules, and thus the overall folding process is strongly decelerated.

Catalyzed folding is a directional process, not because folding enzymes such as SlyD favor catalysis toward the native prolyl isomer, but because those protein chains that leave the prolyl isomerase active site with a correct prolyl isomer are able to fold rapidly in an exergonic conformational folding reaction. This abolishes the affinity for SlyD, and thus, rebinding to its chaperone domain is impaired.

Catalytic Power of the Prolyl Isomerase SlyD in Protein Folding. The catalysis by SlyD* of prolyl isomerization in a bound substrate protein is highly efficient. In the presence of 1 μM SlyD*, the refolding kinetics of the transiently formed U_{cis} species of RCM-T1 (with the correct *cis* isomer of Pro39) and of the equilibrium mixture of 85% U_{trans} and 15% U_{cis} were indistinguishable, which demonstrates that the binding of unfolded molecules to SlyD* and the catalyzed *cis* ⇋ *trans* equilibration in the bound state are much faster than the conformational refolding of the U_{cis} molecules. The *cis* → *trans* isomerization in the SlyD*-bound state shows a rate of 100 s^{-1} or even higher and is thus about 10 000-fold accelerated relative to uncatalyzed isomerization. Interestingly, in single-molecule energy transfer measurements,[39] a domain opening and closing reaction was identified in SlyD, which shows also a rate of about 100 s^{-1}. It might possibly be related with catalytic turnover.

The backbone of RCM-T1 contains 103 peptide bonds, but catalysis will occur only when the Tyr38-Pro39 bond occupies the prolyl isomerase site. The intrinsic rate of catalyzed prolyl isomerization in a correctly positioned substrate might thus be higher than 100 s^{-1}, possibly between 100 and 1000 s^{-1}, as observed with short peptides for bovine FKBP12[40] and for cyclophilin.[41,42]

Intuitively, one would expect that the upper limit for the apparent rate of folding in the presence of a prolyl isomerase be determined by the rate of the conformational folding reaction of the protein molecules with correct prolyl isomers. This rate is 1.4 s^{-1} for our substrate protein RCM-T1. It is not reached because both isomerizations *cis* → *trans* and *trans* → *cis* are accelerated in the SlyD-bound state. Thus, on average, only 15% of the RCM-T1 molecules released from SlyD* contain a *cis* Pro39 and are able to refold rapidly. The maximum folding rate would thus be 0.15 × 1.4 = 0.2 s^{-1}. Experimentally, a maximal rate of 0.08 s^{-1} was observed for the folding of 0.5 μM RCM-T1 in the presence of 0.5 μM SlyD* (Figure 4A), which is 40% of the maximal rate. SlyD* is thus a very good folding enzyme. It accelerates *cis* ⇋ *trans* equilibration in the bound substrate protein so strongly that presumably folding after release becomes the major rate-determining factor. The maximally attainable folding rate is not reached, because, at high SlyD* concentrations, binding of the refolding protein to the SlyD* chaperone domain competes with catalyzed folding.

Determinants of the Catalytic Efficiency of Prolyl Isomerases in Protein Folding. A Michaelis–Menten type analysis[25] of the SlyD*-catalyzed folding of RCM-T1 gave a K_M value of 1.7 μM and a k_{cat} value of 0.7 s^{-1}. The K_M value is in the same range as the K_D for the interaction of the permanently unfolded P39A variant of RCM-T1 with SlyD* (0.5 μM)[34] and thus largely reflects substrate binding to the chaperone domain. K_M is higher than K_D presumably because some substrate molecules are lost upon transfer to the prolyl isomerase site.

The k_{cat} value for the isomerization of tetrapeptides by SlyD is about 100 s^{-1},[25,27,34] and the rate of catalyzed prolyl isomerization in RCM-T1 in the SlyD-bound state is also 100 s^{-1} or higher, which suggests that the rate of prolyl isomerization in the SlyD-bound state is similarly high for peptides and for bound protein chains. The measured k_{cat} (0.7 s^{-1}) for the SlyD-catalyzed folding of RCM-T1 is more than 100-fold lower. The overall reaction is thus not limited by the catalytic step itself. The rate of folding of RCM-T1 after the release from SlyD* (1.4 s^{-1}) might be a major determinant for the measured k_{cat} value.

As a folding enzyme, SlyD should act in a generic fashion on different substrate proteins with prolines in varying environments. This is accomplished by two factors. (i) In the SlyD-bound state, the catalysis of prolyl *cis* ⇋ *trans* isomerization is so efficient that it is no longer rate-limiting for the folding of RCM-T1, and this explains why the inherently high sequence specificity of the prolyl isomerase active site,[43] is not observed in catalyzed folding. (ii) The binding of unfolded protein chains to the chaperone domain is strong but unspecific and both binding and dissociation are fast, which allows rapid transfer to the prolyl isomerase site during catalysis and prevents inhibition by damaged proteins. Trigger factor resembles SlyD in these properties, which indicates that it uses a similar mechanism in the catalysis of proline-limited folding.

Potential Function of SlyD for Cellular Protein Folding. SlyD was discovered in *E. coli* as a host factor that is required for the lysis cycle of bacteriophage φX174 because it mediates the correct folding of the lysis protein E.[44] It also seems to be a folding factor for the export of proteins from *E. coli* via the twin-arginine translocation (TAT) pathway.[45] At present, it is unclear how the binding to unfolded proteins and the catalysis of slow folding steps contribute to the cellular function of SlyD. A mutational analysis revealed that most variants defective in phage maturation showed mutations in the prolyl isomerase domain of SlyD.[46] This suggests that the high catalytic activity of SlyD as observed here toward bound substrate proteins is important for the function of SlyD as a cellular folding factor.

■ CONCLUSION

The prolyl isomerase SlyD is a good folding enzyme. It binds rapidly to refolding proteins, and it catalyzes prolyl *cis* ⇋ *trans* isomerization in the bound state so efficiently that it is no longer rate-limiting for folding. This has, however, an adverse effect on the folding of the protein molecules with correct prolyl isomers: fast binding and prolyl *cis* ⇋ *trans* equilibration produces molecules with incorrect prolyl isomers and thus retards conformational folding.

Abbreviations. RNase T1, ribonuclease T1; RCM-T1, the reduced and carboxymethylated form of the S54G/P55N variant of ribonuclease T1; U_{cis} and U_{trans}, unfolded forms of RCM-T1 with a *cis* or *trans* Pro39, respectively; N, folded form of RCM-T1; SlyD, sensitive-to-lysis protein from *E. coli*; SlyD*, SlyD residues 1–165, followed by a hexa-His tag; IF, insert-in-flap domain of SlyD; FKBP12, 12-kDa human FK506 binding

protein, FKBP12+IF D37A/F99L, variant of FKBP12 in which the flap was replaced by the IF domain of SlyD and that harbors the mutations D37A and F99L; AEDANS, 5-(((acetylamino)-ethyl)amino)naphthalene-1-sulfonic acid; SlyD*-AED, AEDANS-labeled form of SlyD* D101C; λ, apparent rate constant of a reaction; τ, time constant ($\tau = \lambda^{-1}$); k_{ij}, microscopic rate constant of a reaction.

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Notes

The authors declare no competing financial interest.

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