Chaperone domains convert prolyl isomerases into generic catalysts of protein folding

Roman P. Jakob[a], Gabriel Zoldák[a], Tobias Aumüller[b], and Franz X. Schmid[a,1]

[a]Laboratorium für Biochemie und Bayreuther Zentrum für Molekulare Biowissenschaften, Universität Bayreuth, D-95440 Bayreuth, Germany; and [b]Max Planck Research Unit for Enzymology of Protein Folding, D-06120 Halle/Saale, Germany

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The *cis*/*trans* isomerization of peptide bonds before proline (prolyl bonds) is a rate-limiting step in many protein folding reactions, and it is used to switch between alternate functional states of folded proteins. Several prolyl isomerases of the FK506-binding protein family, such as trigger factor, SlyD, and FkpA, contain chaperone domains and are assumed to assist protein folding in vivo. The prolyl isomerase activity of FK506-binding proteins strongly depends on the nature of residue Xaa of the Xaa-Pro bond. We confirmed this in assays with a library of tetrapeptides in which position Xaa was occupied by all 20 aa. A high sequence specificity seems inconsistent with a generic function of prolyl isomerases in protein folding. Accordingly, we constructed a library of protein variants with all 20 aa at position Xaa before a rate-limiting *cis* proline and used it to investigate the performance of trigger factor and SlyD as catalysts of proline-limited folding. The efficiencies of both prolyl isomerases were higher than in the tetrapeptide assays, and, intriguingly, this high activity was almost independent of the nature of the residue before the proline. Apparently, the almost indiscriminate binding of the chaperone domain to the refolding protein chain overrides the inherently high sequence specificity of the prolyl isomerase site. The catalytic performance of these folding enzymes is thus determined by generic substrate recognition at the chaperone domain and efficient transfer to the active site in the prolyl isomerase domain.

folding catalysis | folding helpers | folding mechanism | SlyD | trigger factor

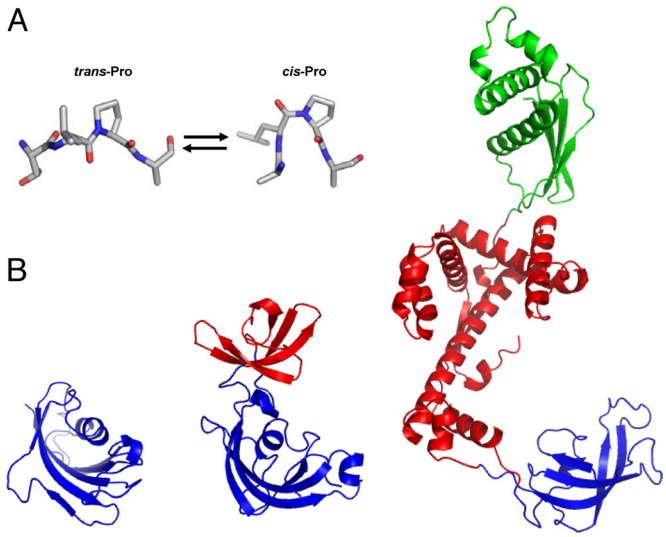

Fig. 1. Prolyl bonds and prolyl isomerases. (*A*) *Trans* and *cis* isomer of a Val-Pro peptide bond. (*B*) Backbone structures of human FKBP12 (*Left*), SlyD (*Center*), and trigger factor (*Right*). The prolyl isomerase domains are shown in blue, the chaperone domains in red, and the ribosome-binding domain of trigger factor in green. The Protein Data Bank files 1FKF for FKBP12 (58), 2k8l for *E. coli* SlyD (30), and 1W26 for *E. coli* trigger factor (25) were used to prepare the Figs. 1–3.

The *cis*/*trans* isomerization of peptide bonds before proline (Xaa-Pro or prolyl bonds; Fig. 1*A*) is an intrinsically slow reaction that depends on the nature of the amino acid Xaa (1–3). It determines the rates of many protein folding reactions (4–6), is used as a molecular switch (7–16), and is catalyzed by prolyl isomerases (17–21). Most of the prolyl isomerases that assist in cellular protein folding contain catalytic domains that are homologous to human FKBP12 (FK-506 binding protein of 12 kDa; Fig. 1*B*) and chaperone domains, which interact transiently with non-native proteins (Fig. 1*B*). The trigger factor (22–25) and SlyD [product of the *slyD* (sensitive-to lysis) gene, a cytosolic *Escherichia coli* chaperone] (26–29) belong to this family of folding enzymes. The chaperone domain of SlyD (the "insert-in-flap" or IF domain) shows a unique fold (30) and is structurally not related to other chaperones. The chaperone domain of trigger factor shows similarities with prefoldin (31)and SurA (32). FkpA (periplasmic FKBP of *E. coli*) (33–35) of prokaryotes and FKBP52 of eukaryotes (36) display similar combinations of prolyl isomerase and chaperone domains.

FKBP-type prolyl isomerases are highly specific with regard to residue Xaa before the proline (37–39). An initial characterization of human FKBP12 with various proline-containing tetrapeptides and a protease-coupled assay (17) indicated that it catalyzes isomerization at Phe-Pro approximately 1,000 fold better than at Glu-Pro (37). Prolyl peptide bonds mostly occur in coil regions or in chain reversals, often at or near the protein surface. As a consequence, prolines are often preceded by polar or charged residues (40), and thus they should be very poor substrates for the folding enzymes of the FKBP family. The sequence specificities of prolyl isomerases toward refolding protein chains are not known, as a result of the lack of a suitable assay system.

The protease-coupled peptide assay can be used only for enzymes that are resistant to proteolysis, and therefore unsuitable for most multidomain prolyl isomerases, including the trigger factor and SlyD. Rich and coworkers devised a short peptide with an Ala-Pro bond and a fluorescent amino-benzoyl (Abz) group at the amino-terminus and a quenching para-nitroanilide (pNA) group at the carboxy-terminus as a substrate for a sensitive fluorometric prolyl isomerase assay (41). Thus, coupling with a protease could be avoided. We used this principle and synthesized a library of 20 tetrapeptides of the general formula Abz-Ala-Xaa-Pro-Phe-pNA that contained all Xaa-Pro sequences to assay the substrate specificities of prolyl isomerases (42).

To characterize the sequence specificities of prolyl isomerases in refolding proteins, we established also a library of protein

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[1]To whom correspondence should be addressed. E-mail: fx.schmid@uni-bayreuth.de.

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Table 1. Catalytic efficiencies of FKBP12, FKBP12 + IF, SlyD*, and trigger factor for prolyl isomerization in the library of peptide substrates and during refolding of the N2 variants

	cis/trans isomerization in tetrapeptide†					N2 refolding‡			
Residue before Pro-Xaa	FKBP12§: k_{cat}/K_M, $mM^{-1}s^{-1}$	FKBP12: k_{cat}/K_M, $mM^{-1}s^{-1}$	FKBP12+IF: k_{cat}/K_M, $mM^{-1}s^{-1}$	SlyD*: k_{cat}/K_M, $mM^{-1}s^{-1}$	Trigger factor: k_{cat}/K_M, $mM^{-1}s^{-1}$	FKBP12: k_{cat}/K_M, $\mu M^{-1}s^{-1}$	FKBP12+IF: k_{cat}/K_M, $\mu M^{-1}s^{-1}$	SlyD*: k_{cat}/K_M, $\mu M^{-1}s^{-1}$	Trigger factor: k_{cat}/K_M, $\mu M^{-1}s^{-1}$
Ala	53 ± 3	230 ± 10	300 ± 60	230 ± 2	1,600 ± 100	0.003 ± 0.0006	5.2 ± 0.3	0.93 ± 0.05	3.6 ± 0.2
Cys	—	1,200 ± 90	1,000 ± 50	34 ± 7	2,100 ± 200	0.0039 ± 0.0005	4.3 ± 0.3	0.60 ± 0.05	2.0 ± 0.1
Asp	—	5.0 ± 0.2	28 ± 5	25 ± 1	26 ± 1	<0.0005	1.6 ± 0.1	0.33 ± 0.04	1.7 ± 0.7
Glu	0.6 ± 0.05	5.5 ± 0.5	25 ± 1	3.6 ± 0.1	30 ± 1	<0.0005	3.8 ± 0.1	0.33 ± 0.03	2.0 ± 0.1
Phe	620 ± 140	1,130 ± 30	1,200 ± 200	2,620 ± 100	3,200 ± 200	0.019 ± 0.002	7.6 ± 0.7	1.55 ± 0.09	4.4 ± 0.5
Gly	1.2 ± 1.0	190 ± 20	60 ± 6	3.0 ± 0.3	90 ± 5	0.0024 ± 0.001	2.3 ± 0.5	0.98 ± 0.06	3.4 ± 0.3
His	28 ± 10	170 ± 17	90 ± 190	380 ± 10	160 ± 30	0.0035 ± 0.0002	3.1 ± 0.2	0.84 ± 0.10	3.4 ± 0.5
Ile		770 ± 90	390 ± 10	1,510 ± 40	600 ± 50	0.0093 ± 0.001	4.0 ± 0.3	1.41 ± 0.18	4.4 ± 0.4
Lys	28 ± 5	74 ± 14	170 ± 20	330 ± 10	420 ± 40	0.0048 ± 0.0005	3.6 ± 0.2	0.62 ± 0.08	3.6 ± 0.2
Leu	640 ± 50	2,300 ± 200	1,000 ± 600	1,100 ± 100	1,000 ± 100	0.016 ± 0.002	3.5 ± 0.4	1.27 ± 0.12	4.4 ± 0.6
Met	—	630 ± 50	1,200 ± 100	1,100 ± 40	810 ± 80	0.013 ± 0.001	4.1 ± 0.2	1.19 ± 0.12	3.6 ± 0.3
Asn	—	260 ± 45	260 ± 40	90 ± 10	90 ± 30	0.0031 ± 0.0004	4.2 ± 0.2	0.57 ± 0.06	3.8 ± 0.6
Pro	—	8.6 ± 1.9	14 ± 1	1.1 ± 0.1	36 ± 1	0.0038 ± 0.001	2.3 ± 0.3	0.24 ± 0.02	1.6 ± 0.2
Gln	—	88 ± 10	74 ± 7	22 ± 2	200 ± 20	0.0068 ± 0.0005	3.4 ± 0.2	0.39 ± 0.07	3.0 ± 0.2
Arg	—	260 ± 30	130 ± 20	460 ± 10	530 ± 40	0.0044 ± 0.0003	4.8 ± 0.6	0.68 ± 0.08	3.4 ± 0.3
Ser	—	260 ± 30	160 ± 10	190 ± 10	1,400 ± 100	0.0036 ± 0.0004	3.3 ± 0.2	0.84 ± 0.04	3.1 ± 0.4
Thr	—	350 ± 50	120 ± 10	200 ± 10	1,100 ± 100	0.0036 ± 0.0007	3.7 ± 0.2	0.59 ± 0.07	2.8 ± 0.2
Val	170 ± 80	940 ± 30	450 ± 40	590 ± 100	480 ± 50	0.005 ± 0.001	3.3 ± 0.2	0.79 ± 0.09	4.6 ± 0.3
Trp	—	220 ± 20	570 ± 90	1,200 ± 40	680 ± 70	ND	ND	ND	ND
Tyr	—	770 ± 90	2,400 ± 200	2,320 ± 140	1,800 ± 100	0.011 ± 0.001	5.6 ± 0.5	1.61 ± 0.16	5.1 ± 0.6

†The k_{cat}/K_M values for the tetrapeptides were obtained in 50 mM Hepes, pH 7.8, at 15 °C in the presence of 0–60 nM prolyl isomerase by experiments as shown in Fig. S1.

‡The k_{cat}/K_M values for the refolding of N2 were obtained in 0.1 M K phosphate, 0.2 M urea, pH 7.0, at 15 °C in the presence of varying prolyl isomerase concentrations as illustrated in Fig. S2.

§Data for the peptide substrate succinyl-Ala-Xaa-Pro-Phe-pNA, as taken from Gruber et al. (54).

ND, not detected.

variants with Xaa-Pro sequences in which position Xaa was occupied by all 20 aa. As the model system, we used the N2 domain of the gene-3-protein of phage fd. In folded N2, Pro-161 is mostly in *cis* (43, 44), and, for more than 90% of all refolding N2 molecules, the *trans* → *cis* isomerization of the Asp-160-Pro-161 bond is the rate-limiting step of folding (45). By directed mutagenesis, we established a library of N2 variants in which Pro-161 is preceded by all 20 naturally occurring amino acids (46) and used it to characterize the sequence specificity of trigger factor and SlyD as catalysts of protein folding.

In the presence of a chaperone domain, the catalytic efficiency in protein folding is greatly improved and, at the same time, the high substrate specificity of the prolyl isomerase site, as observed for the peptide substrates, is almost abolished. Binding to the chaperone domains apparently overrides the inherent sequence specificity of the prolyl isomerase site and thus solves the problem of generic substrate recognition during protein folding.

Results

The Substrate Specificities of Prolyl Isomerases in the Presence of Chaperone Domains. By using a limited set of tetrapeptides in a chymotrypsin-coupled assay, Harrison and Stein discovered the high substrate specificity of FKBP12 with regard to the residue Xaa preceding proline (37, 38). Our protease-free assay with 20 peptides confirms this high specificity. FKBP12 (60 nM) accelerates the isomerization at Leu-Pro 12-fold, but leaves the isomerization rate of Glu-Pro virtually unchanged (Fig. S1*a*). From measurements of the isomerization rate as a function of the FKBP12 concentration (as in Fig. S1*c*), the catalytic efficiencies (k_{cat}/K_M) for all 20 Xaa-Pro sequences were derived (Table 1). They differ approximately 500 fold between Leu-Pro (the best) and Asp-Pro (the worst). The k_{cat}/K_M values determined with the new protease-free assay at 15 °C are approximately 5 to 10 fold higher than the values determined previously for a limited subset of peptides by the protease-coupled assay at 10 °C (37, 38). We assume that the presence of the aminobenzoyl group in our peptides slightly improves the interaction with FKBP12. However, the relative activities toward the different peptide substrates as obtained by the two assays are very similar (Table 1).

FKBP12 is a poor catalyst of prolyl isomerization in protein folding (47). Its catalytic efficiency could be strongly improved by grafting the chaperone domain of SlyD into a loop of FKBP12 near the active site to create the chimeric protein FKBP12+IF (48). The insertion of this chaperone domain left the activity and the very high specificity of the prolyl isomerase active site of FKBP12 toward Xaa-Pro in the tetrapeptide library virtually unchanged (Fig. S1 *b* and *d* and Table 1).

Next, we used the protein library of N2 variants with all amino acids before Pro-161 to examine the substrate specificity of FKBP12 in a protein folding reaction. As mentioned, FKBP12 alone is a poor catalyst of protein folding, and therefore up to 2.5 μM FKBP12 had to be used for measuring catalysis (Fig. S2*a*). Catalysis could be observed for Leu-160-N2 but not for Asp-160-N2, suggesting that the k_{cat}/K_M value is smaller than $0.5 \cdot 10^3$ $M^{-1}s^{-1}$ (Fig. S2*c*). The catalytic efficiencies of FKBP12 toward 19 variants of N2 are shown in Table 1. Fig. 2*A* compares them with the values measured for the tetrapeptide library. The activities of FKBP12 toward Xaa-Pro in the library of refolding proteins are 2 to 3 orders of magnitude lower than the corresponding values for the peptide library, but the substrate specificity is similarly high in the folding proteins and in the tetrapeptides.

The kinetic parameters change drastically when the IF domain

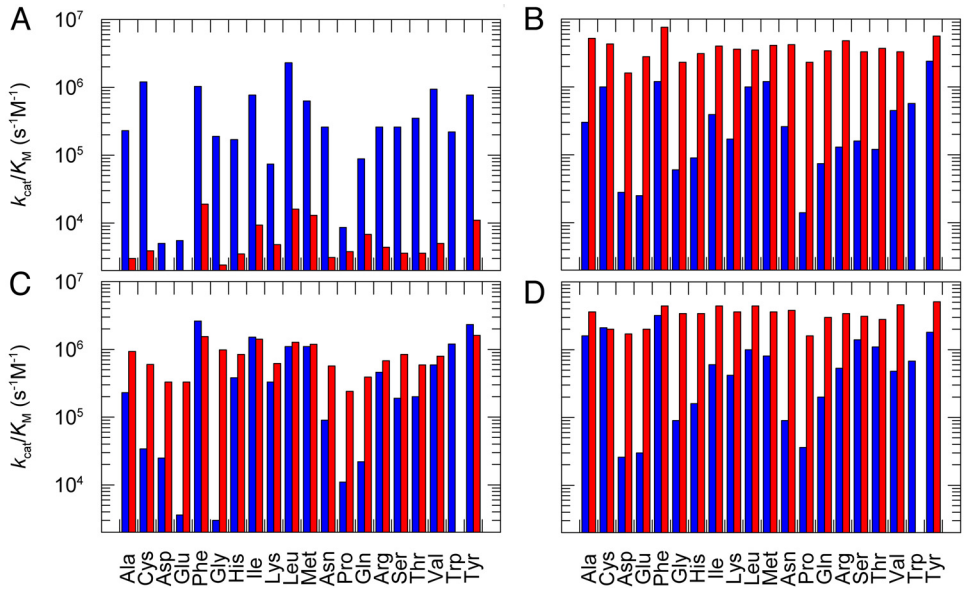

Fig. 2. Catalytic efficiencies k_{cat}/K_M of the various prolyl isomerases for the *cis/trans* isomerization of Abz-Ala-Xaa-Pro-Phe-pNa (blue bars) and for the refolding of N2-D160Xaa (red bars): (*A*) hFKBP12, (*B*) FKBP12+IF, (*C*) SlyD*, and (*D*) trigger factor. The measurements were performed as described in Table 1. The data for the tetrapeptide in *A* are taken from Zoldák G et al. (42).

with its chaperone function is inserted into FKBP12. Now, concentrations as low as 1 to 10 nM of FKBP12+IF are sufficient to strongly increase the rate of folding for all members of the N2 protein library, as shown for Leu-160-N2 and Asp-160-N2 in Fig. S2*b*. For protein variants with polar amino acids before Pro-161, such as Ser, Thr, Asn, and Arg, insertion of the IF domain increases the k_{cat}/K_M value of FKBP12 approximately 1,000 fold. Moreover, the catalytic efficiency of the FKBP domain in the protein folding assays becomes almost uniformly high for all members of the N2 library. For most N2 variants, k_{cat}/K_M values between $2{\cdot}10^6$ and $5{\cdot}10^6$ $M^{-1}s^{-1}$ are reached, demonstrating that the nature of the residue Xaa before proline becomes unimportant for the high catalytic efficiency of FKBP12 in protein folding when the IF chaperone domain is present. The data for all variants are listed in Table 1, and the activities as measured for the tetrapeptide and protein libraries are compared in Fig. 2*B*.

The chaperone domain of the chimeric protein FKBP12+IF originated from the prolyl isomerase SlyD of *E. coli*. To examine the function of the IF domain in its natural environment, we used our peptide and protein libraries to measure the substrate specificities of SlyD* as well. SlyD* is a C-terminally truncated form of SlyD that consists of the FKBP and chaperone domains. The data are shown in Fig. 2*C* and Table 1. As observed for the chimera FKBP12+IF, the activity of SlyD* toward polar residues before Pro was strongly increased when protein instead of peptide substrates were used, leading to values that approach those obtained for the hydrophobic residues (Fig. 2*C*).

Trigger factor is located at the bacterial ribosome, close to the exit site for nascent protein chains, and presumably involved in de novo protein folding. It consists of a ribosome-binding domain, a prolyl isomerase domain of the FKBP type, and a chaperone domain (Fig. 1*B*), and it catalyzes proline-limited protein folding reactions in vitro very well (23, 49, 50). Like the other FKBPs, trigger factor shows a high substrate specificity toward Xaa-Pro bonds in tetrapeptides (Table 1) and, in its activity profile (Fig. 2*D*), it strongly resembles FKBP12+IF (Fig. 2*B*). In the protein folding assays with the library of N2 variants, the activity of trigger factor is very high and almost independent of the nature of residue Xaa before proline. This is exemplified by the less than threefold difference in the k_{cat}/K_M values for the Leu-160 and Asp-160 variants of N2 (Fig. S2*F*) and the uniform heights of the red bars in Fig. 2*D*.

Enzyme Kinetics of Catalyzed Protein Folding. k_{cat}/K_M is a composite value that characterizes the efficiency of an enzyme at low substrate concentration. To obtain the kinetic parameters k_{cat} and K_M separately, we measured the enzyme kinetics of catalyzed folding. In these experiments, the initial rate of folding was determined as a function of the substrate protein concentration. Catalyzed and uncatalyzed folding occur in the presence of a folding catalyst and were accounted for in the analysis by using a procedure originally developed for prolyl isomerization in peptides (51) and adapted for catalyzed protein folding (48, 49). The range of substrate concentrations is restricted to less than 10 μM in these experiments, because the uncatalyzed reaction dominates at high substrate concentration.

We measured the enzyme kinetics of catalyzed folding for 3 representative members of our library of N2 variants: the Ala-160 variant with the "default" amino acid alanine, the Asp-160 variant with a charged residue, and the Leu-160 variant with a hydrophobic residue before the proline. As the enzymes we used FKBP12+IF, SlyD* and trigger factor. The corresponding Michaelis-Menten plots are shown in Fig. 3. The scatter in the data is considerable because uncatalyzed folding contributes. Nevertheless, the initial rates of catalyzed folding show saturation behavior in all cases and apparently obey the Michaelis-Menten equation. The kinetic constants k_{cat} and K_M derived from this analysis for the 3 prolyl isomerases and the 3 substrate proteins (Table 2) are remarkably similar. The k_{cat} values range between 2 s^{-1} and 4 s^{-1}, and the K_M values between 0.6 μM and 3.8 μM. For all 3 enzymes, the K_M values for the Ala-160 and Leu-160 variants are similar, those for the Asp-160 variant are approximately twofold larger. The k_{cat}/K_M values calculated from the individual parameters agree well with the composite k_{cat}/K_M values determined from experiments at increasing enzyme concentrations (as in Fig. S2). This confirms that folding catalyzed by the various prolyl isomerases in this study can be analyzed by using the Michaelis-Menten equation.

For FKBP12 without a chaperone domain, a saturation behavior as in Fig. 3 could not be observed in the range of substrate

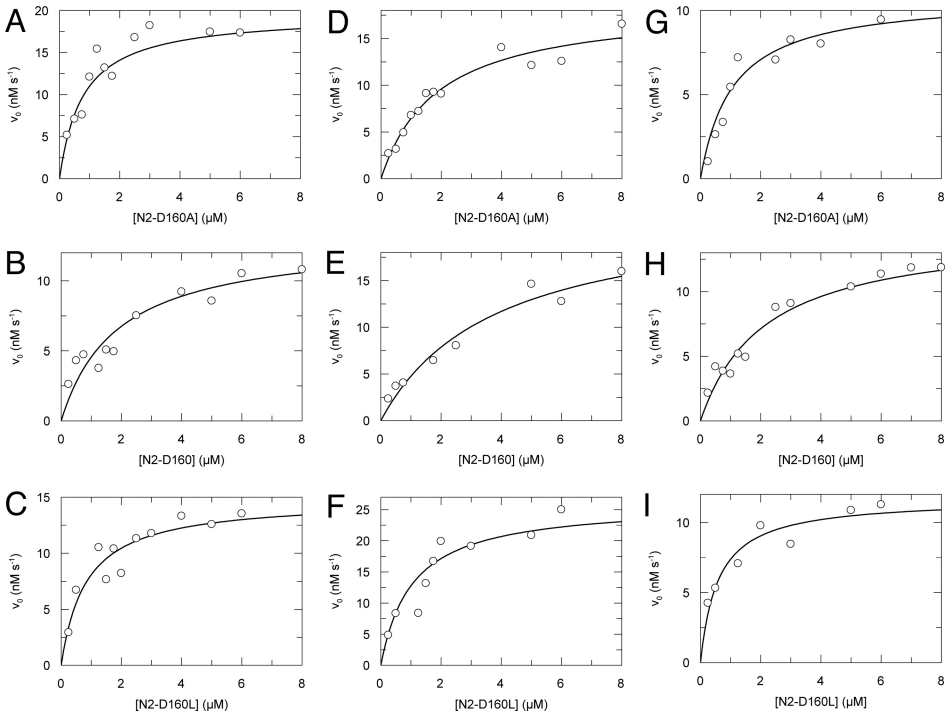

Fig. 3. Michaelis-Menten kinetics of the refolding of Ala-160-N2 (*A*, *D*, and *G*), Asp-160-N2 (*B*, *E*, and *H*), and Leu-160-N2 (*C*, *F*, and *I*) catalyzed by FKBP12+IF (*A–C*), SlyD* (*D–F*), and trigger factor (*G–I*). The initial velocities of catalyzed refolding at 15 °C are shown as a function of the concentration of N2. The concentrations were 5 nM for FKBP12+IF and trigger factor and 10 nM for SlyD*, and the buffer consisted of 0.2 M urea and 0.1 M K phosphate (pH 7.0). The values for K_M and k_{cat} are listed in Table 2. The initial folding rates were determined and analyzed as described in *Materials and Methods*.

concentrations that are accessible for measuring the catalyzed folding of N2 (Fig. S3), which indicates that the substrate affinity of FKBP12 for folding protein chains is very low. Kinetic constants could also not be determined for the catalysis of prolyl isomerization of the tetrapeptides of our library, because of their limited solubility.

Discussion

In the absence of a chaperone domain, prolyl isomerases of the FKBP family bind both peptide and protein substrates with a low affinity, but a very high specificity with regard to the amino acid Xaa before the proline. Catalysis is as much as 1,000 fold more efficient when the proline is preceded by a hydrophobic residue instead of a negatively charged residue. This high local sequence specificity is abolished when proline-limited protein folding reactions are catalyzed by prolyl isomerases that contain a chaperone domain for substrate binding. These enzymes show K_M values for catalyzed folding in the range of approximately 1 μM, and the catalytic efficiency is independent of the local sequence context of the prolyl bond to be isomerized.

Apparently, these are generic effects. The chaperone domains of SlyD and trigger factor are structurally unrelated (Fig. 1*B*), yet they change the catalytic properties of the corresponding prolyl isomerase domains in the same fashion. Remarkably, the arti-

Table 2. Enzyme kinetic parameters for the catalysis of N2-D160X refolding by FKBP12+IF1, SlyD*, and trigger factor

		Michaelis-Menten experiments[‡]		
N2 variant	N2 refolding[†]: k_{cat}/K_M, (M^{-1} s^{-1})	Turnover no.: k_{cat}, s^{-1}	Michaelis constant: K_M, μM	Composite value: k_{cat}/K_M, $M^{-1}s^{-1}$
FKBP12 + IF1				
Ala	5.2×10^6	3.9 ± 0.5	0.8 ± 0.2	5.0×10^6
Asp	1.6×10^6	2.6 ± 0.3	1.9 ± 0.6	1.4×10^6
Leu	3.5×10^6	2.9 ± 0.4	0.8 ± 0.3	3.5×10^6
SlyD*				
Ala	0.93×10^6	1.9 ± 0.2	1.9 ± 0.3	0.98×10^6
Asp	0.33×10^6	2.2 ± 0.4	3.8 ± 0.9	0.57×10^6
Leu	1.27×10^6	2.7 ± 0.3	1.8 ± 0.5	1.5×10^6
Trigger factor				
Ala	3.6×10^6	2.2 ± 0.2	1.1 ± 0.2	2.1×10^6
Asp	1.7×10^6	2.9 ± 0.2	2.1 ± 0.4	1.4×10^6
Leu	4.4×10^6	2.3 ± 0.6	0.6 ± 0.2	4.0×10^6

[†]The experiments were performed as described in Table 1.
[‡]The experiments were performed as described in Fig. 3.

ficial introduction of the chaperone domain of SlyD into human FKBP12 created a folding enzyme that is virtually indistinguishable from trigger factor with regard to substrate specificity and catalytic efficiency.

The dissociation constants of the complexes formed between trigger factor or SlyD and non-native proteins, the K_M values for catalyzed folding, and the K_I values for their inhibition by permanently unfolded proteins are all in the range of 1 μM (49, 50). This suggests that a well balanced affinity is important for the proper function of the chaperone domains of folding enzymes. Too low an affinity would compromise substrate recognition, and too high an affinity would interfere with substrate transfer to the prolyl isomerase active site.

The turnover numbers of catalyzed folding obtained for the different prolyl isomerases in this study are also very similar (2–4 s^{-1}) and agree well with the value of 1.3 s^{-1} determined previously for the catalysis of folding of ribonuclease T1 by trigger factor (49). For Leu-Pro isomerization in a tetrapeptide, FKBP12 shows a k_{cat} value of 600 s^{-1} (and a K_M of 0.5 mM at 5 °C) (39). In the catalysis of proline-limited folding, the turnover is thus decelerated by almost 3 orders of magnitude. The very low k_{cat} values are unlikely to reflect the rate of the catalyzed *cis*/*trans* isomerization in the protein substrates. We suggest that the k_{cat} value is related with the dissociation of the substrate protein from the chaperone binding site and its transfer to the prolyl isomerase site. Permanently unfolded substrate proteins dissociate from the chaperone domain of trigger factor with a rate of approximately 8 s^{-1} (52). k_{cat} is approximately fivefold lower, probably because dissociation from the chaperone site does not always lead to productive substrate transfer to the prolyl isomerase site.

Thus, a simple model emerges for the mechanism of action of prolyl isomerases in protein folding. Initially, the chaperone domain binds to non-native protein substrates with an adjusted micromolar affinity. The binding equilibrium is highly dynamic, which allows intramolecular transfer to the prolyl isomerase site, as well as clearance of the binding site from misfolded proteins if necessary. The kinetic parameters of catalyzed folding, k_{cat} and K_M, are both determined by the chaperone domain of these folding enzymes. K_M reflects its affinity for the refolding protein and k_{cat} the efficiency of transfer to the prolyl isomerase site. The actual catalysis of isomerization is several orders of magnitude faster than substrate delivery, and therefore the inherently high sequence specificity of the prolyl isomerase site does not affect the overall catalytic efficiency in protein folding. The chaperone domains thus convey these prolyl isomerases with a very broad specificity as required for folding enzymes that must act on a broad range of substrate proteins either co- or post-translationally.

Protein disulfide isomerases also show separate chaperone domains for substrate binding and catalytic domains for disulfide exchange (53–56). It is possible that they use similar principles in the catalysis of oxidative protein folding.

Materials and Methods

Expression and Purification of Variants of the N2 Domain and the Prolyl Isomerases. As the WT protein the isolated N2 [residues 102–205 of mature G3P, extended by $(His)_6$] domain with the mutation Q129H was used. The mutated variants of N2 were expressed and purified as described (45, 46). Recombinant FKBP12, SlyD* and trigger factor were produced and purified as described (57).

Peptide Assay for Prolyl Isomerases. The prolyl isomerase activities of the proteins were measured by a protease-free assay (42). For the assay, the peptide substrates Abz-Ala-Xaa-Pro-Phe-pNa (3 mM) were dissolved in anhydrous trifluoroethanol containing 0.55 M LiCl. Under these conditions, approximately 50% of the peptide molecules are in the *cis* conformation. Upon 600-fold dilution into aqueous buffer, the *cis* content decreases to approximately 10%. The kinetics of the decrease in *cis* content was measured by the change in fluorescence at 416 nm after excitation at 316 nm in a Jasco FP-6500 fluorescence spectrophotometer. The assays were performed in 50 mM Hepes/NaOH, (pH 7.8) at 15 °C. Under these conditions, the *cis*-to-*trans* isomerization of the prolyl bond was a mono-exponential process.

Folding Experiments. For the folding experiments, a Hitachi F4010 fluorescence spectrometer was used. N2 variants were unfolded by incubating the protein in 0.1 M K phosphate, pH 7.0, and 5.0 M urea at 15 °C. Refolding at 15 °C was initiated by a 25-fold dilution to a final concentration of 0.33 μM in 0.2 M urea, 0.1 M K phosphate, pH 7.0, and the desired concentration of the prolyl isomerase. The folding reaction was followed by the increase in protein fluorescence at 340 nm (10 nm band width) after excitation at 280 nm; 5 nm band width). Under these conditions, N2 refolding was a mono-exponential process and, in all folding experiments, the small contribution of the prolyl isomerases to the fluorescence was subtracted from the measured values. When the concentration of N2 is much smaller than the K_M value of catalyzed folding, the apparent rate constant k_{app} of catalyzed folding is equal to $k_{uncat} + k_{cat}\cdot[E]_0/K_M$, where k_{uncat} and k_{cat} are the rates of uncatalyzed and catalyzed folding, respectively, and $[E]_0$ is the prolyl isomerase concentration. k_{cat}/K_M is thus determined from the slope when k_{app} is plotted as a function of $[E]_0$. The rate constants of folding were determined by using GraFit 3.0 (Erithacus Software).

Enzyme Kinetics of Catalyzed Folding. In the Michaelis-Menten kinetic experiments, the initial velocities of N2 folding were determined from the progress curves of folding in the presence of 5 nM FKBP12+IF or trigger factor or 10 nM SlyD* under the conditions described in the preceding paragraph. Measurements were carried out between 0.2 and 10 μM N2. At less than 0.2 μM, the signal-to-noise ratio was too low. Both uncatalyzed and catalyzed folding occur in these experiments. The relative contribution of uncatalyzed folding increases linearly with N2 concentration, and the initial rate of catalyzed folding would be progressively overestimated when determined simply from the initial slope of the progress curve of folding. Kofron et al. (51) developed a method to account for both uncatalyzed and enzyme-catalyzed prolyl isomerization in a peptide. We used this method and analyzed the data as described (49, 57).

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