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Prolyl Isomerases Show Low Sequence Specificity toward the Residue Following the Proline

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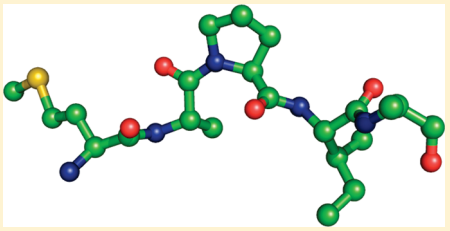
Supporting Information

ABSTRACT: Prolyl isomerases catalyze the *cis*/*trans* isomerization of peptide bonds preceding proline. Previously, we had determined the specificity toward the residue before the proline for cyclophilin-, FKBP-, and parvulin-type prolyl isomerases by using proline-containing oligopeptides and refolding proteins as model substrates. Here, we report the specificities of members of these three prolyl isomerase families for the residue following the proline, again in short peptide and in refolding protein chains. Human cyclophilin 18 and parvulin 10 from *Escherichia coli* show high activity, but low specificity, with respect to the residue following the proline. Human FKBP12 prefers hydrophobic residues at this position in the peptide assays and shows a very low activity in the protein folding assays. This activity was strongly improved, and the sequence specificity was virtually eliminated after the insertion of a chaperone domain into the prolyl isomerase domain of human FKBP12.



The *cis*/*trans* isomerization of a peptidyl−prolyl bond (prolyl isomerization, Figure 1a) is intrinsically slow because the peptide bond has partial double-bond character. Prolyl isomerizations are rate-limiting steps in protein folding,[1−3] and they are employed as biological switches.[4−11] Prolyl isomerases catalyze these reactions, and they are grouped into three families: the cyclophilins, the FK-506 binding proteins (FKBPs), and the parvulins (Figure 1b−d).[12−14]

The first prolyl isomerase was discovered using the tetrapeptide succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (Suc-Ala-Ala-Pro-Phe-pNA) and chymotrypsin in a protease-coupled assay,[15] which is still used as the standard assay.[16−19] It can only be employed for prolyl isomerases that are protease-resistant. To avoid the coupling with proteolysis, several variants of the peptide assay were developed. Rich and co-workers[20,21] introduced tetrapeptide substrates that are labeled N-terminally with a 2-aminobenzoyl (Abz) residue and C-terminally with either a nitrophenylalanine residue or a 4-nitrobenzyl moiety. The extent of quenching of the Abz fluorescence was found to be sensitive to the *cis* or *trans* state of the Ala−Pro bond in the tetrapeptide. In combination with a solvent jump from anhydrous LiCl in trifluoroethanol (TFE) to aqueous buffer, this was exploited to develop a protease-free prolyl isomerase assay.[20,22]

Zoldák et al.[23] synthesized a library of 20 tetrapeptides of the general formula aminobenzoyl-Ala-Xaa-Pro-Phe-4-nitroanilide (Abz-Ala-Xaa-Pro-Phe-pNA), in which the position Xaa before the proline was occupied by all 20 proteinogenic amino acids. This peptide library was used in combination with the protease-free fluorimetric assay to determine the activities and substrate specificities of human members of the three prolyl isomerase families: cyclophilin 18 (hCyp18), FKBP12 (hFKBP12), and parvulin 14 (hPar14).

With regard to the amino acid after the proline, the specificities of prolyl isomerases are virtually unknown. In the protease-coupled assay, the residue after the proline must comply with the preference of the protease, which cleaves the bond between this residue and the pNA moiety. Chymotrypsin and a Phe-pNA after the proline are used in the standard assay. In particular cases, assay peptides with Lys or Ala after the Pro were used in combination with other proteases such as trypsin or subtilisin.[24,25]

To determine the specificities of prolyl isomerases with respect to the residue following the proline, we synthesized nine representative Abz-Ala-Ala-Pro-Xaa-Ala-pNA pentapeptides with a variety of residues at the position Xaa after the proline for use in the protease-free prolyl isomerase assay. To examine the specificities of the prolyl isomerases for the residue after the proline in a refolding protein, we also produced a panel of protein variants in which the position Xaa after a rate-limiting proline was occupied by the same nine residues as in the assay peptides. For these experiments we used the N2 domain of the gene-3-protein of phage fd.[26,27]

MATERIALS AND METHODS

Protein Expression and Purification. Site-directed mutagenesis, expression, and purification of the isolated N2′ domain (residues 102−205 of the gene-3-protein of phage fd, extended

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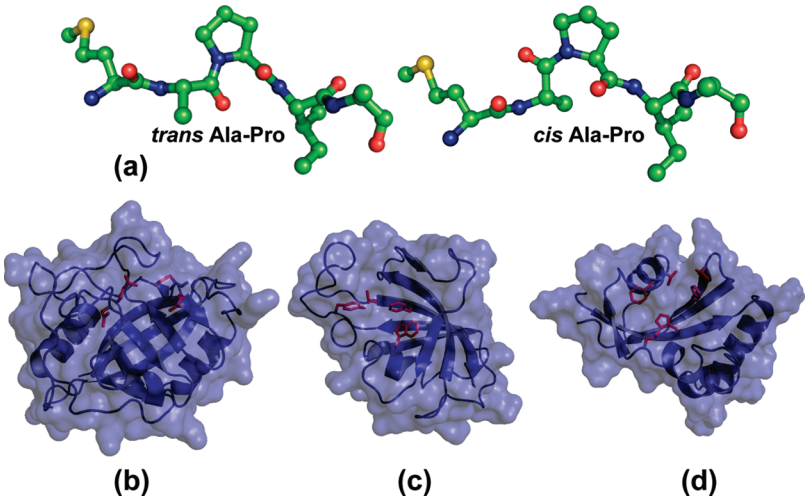

Figure 1. (a) A proline-containing peptide in the *trans* and the *cis* conformation. The two structures represent the M88-Ala89-Pro90-I91-A92 fragment of the HIV-1 CA protein as taken from the crystal structure of the complex between HIV-1 CA and hCyp18 (PDB accession code, 1M9X).[42] (b–d) Structures of (b) hCyp18 (PDB accession code, 1M9X),[42] (c) hFKBP12 (PDB accession code, 1FKF),[46] and (d) *Ec*Par10 (PDB accession code, 1JNT).[47] Side chains of residues of the active sites (hCyp18: His54, Arg55, Asn102, Gln111; hFKBP12: Trp59, Tyr82, Leu97, Phe99; *Ec*Par10: His8, Asn49, Val58, Phe61, T79, H84) are shown in stick representation. The figure was prepared by using Pymol.[48]

by His_6), additionally carrying the stabilizing substitution Q129H, were performed as described previously.[26,28] The genes for the prolyl isomerases hFKBP12, hFKBP12+IF, and *Ec*Par10 were cloned into pET11a as His_6-Smt3 (SUMO protein from *Saccharomyces cerevisiae*) fusion proteins.[29] *E. coli* BL21(DE3) Δ*slyD* cells carrying the vector with the desired gene were grown at 37 °C in double yeast tryptone medium. At an OD_{600} of 0.6, protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactoside. Four hours after induction, cells were harvested by centrifugation (10 min at 5000*g*), resuspended in 50 mM Tris/HCl, pH^{RT} 7.9, 50 mM NaCl, 10 mM EDTA, and lysed using a microfluidizer. After centrifugation the supernatant containing the soluble fusion protein was applied to a 5-ml Ni-NTA column (Qiagen, Hilden, Germany), washed with 25 mL of column buffer (50 mM Tris/HCl pH^{RT} 7.9, 100 mM KCl) and eluted with 15 mL of column buffer containing 250 mM imidazole. The Smt3 fusion protein was cleaved with the human SUMO protease His_6-Senp2[30] (1 mg of protease per 150 mg of fusion protein) at 4 °C overnight while dialyzing against column buffer. The His_6-Smt3 fragment and the His_6-Senp2 protease were removed by a second Ni-NTA chromatography. The soluble prolyl isomerases were in the flow-through. Gel filtration on a 16/60 Superdex 75 prep grade column (GE Healthcare, Uppsala, Sweden) with 100 mM potassium phosphate pH 7.5 showed that all proteins were purified in a monomeric form. hCyp18 was isolated as described.[31,32]

Peptide Synthesis. The 3-nitrotyrosyl-labeled peptide amides were synthesized and purified as described.[33] All pNA peptides were produced as the 3-nitrotyrosyl-labeled peptide amides on a 2-chlorotrityl resin by fragment condensation. After detachment of the peptide fragments from the resin by treatment with 33% 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) in dichloromethane (DCM), the Abz-peptide fragments were assembled by coupling with Ala-pNA or Phe-pNA and benzotriazole-1-yloxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP)/*N*-methylmorpholine in *N*,*N*-dimethylformamide. After synthesis, the solvent was evaporated and the peptides were extracted with water/ethyl acetate. The crude products were then purified by preparative RP-HPLC essentially as described[33] on an Abimed system (Langenfeld, Germany) with a column Interchrom Modulo-Cart Strategy 5, C18-2, 250-10 (Interchim, Montlucon, France) using a water/acetonitrile gradient containing 0.1% trifluoroacetic acid in the solvents. The purified peptides were lyophylized.

Equilibrium Unfolding Transitions of the N2′ Variants. Urea-induced unfolding transitions of the N2′ variants were measured in 100 mM potassium phosphate, pH 7.0 at 15 °C as described.[28] The fluorescence of 1 μM N2′ at 340 nm (bandwidth 10 nm) was followed after excitation at 280 nm (bandwidth 5 nm) in 1 cm cells in a Hitachi F4010 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The transitions were analyzed as described.[28]

Thermal transitions were monitored by the change in circular dichroism (CD) at 222 nm using a Jasco J-600A spectropolarimeter (Jasco, Tokyo, Japan) equipped with a PTC 348 WI Peltier element. Four μM protein in 100 mM potassium phosphate, pH 7.0 was used. For the N2′ variants with Leu, Phe, or Arg after Pro161, the protein concentration was reduced to 1 μM to avoid aggregation during unfolding. A heating rate of 1 °C/min, a bandwidth of 1 nm, and 10 mm path length were used. Under these conditions, unfolding of the N2′ variants was >80% reversible. The analysis was performed as described.[28]

Unfolding and Refolding Kinetics. The unfolding and refolding kinetics of the N2′ variants were measured essentially as described.[26,28] They were followed by the change in fluorescence above 320 nm after excitation at 280 nm (bandwidth 10 nm) by using a DX.17MV stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, UK) at 15 °C in 100 mM potassium phosphate, pH 7.0, and varying concentrations of urea. Native or denatured (in 5.0 M urea) N2′ variants were diluted 11-fold to a final protein concentration of 0.5 μM. Kinetics were measured at least eight times under identical conditions and averaged.

The *cis*/*trans* ratios at Pro161 of the N2′ variants were determined in stopped-flow double mixing experiments at 15 °C. In the first step 33 μM native N2′ (in 100 mM potassium phosphate, pH 7.0) was diluted 11-fold with 100 mM glycine, pH 1.8, to unfold the protein. At the final pH of 2.0 conformational

unfolding is complete within 0.01 s.[26] In the second step, refolding was initiated after 0.1 s by a further 6-fold dilution to final conditions of 100 mM potassium phosphate, pH 7.0. Under these conditions, refolding is dominated by the fast folding reaction ($U_c \rightarrow N_c$). It was followed by fluorescence as described above. During the 0.1 s unfolding pulse, the prolyl *cis*/*trans* equilibrium remains essentially unchanged, and therefore the amplitude of the fast refolding reaction is a measure for the fraction of N2′ molecules with a *cis* Pro161 in the native protein. The kinetic measurements were performed at least 15 times, averaged, and analyzed as described.[26] The content of molecules with a *cis* Pro161 in the unfolded state was determined from the relative amplitudes of the slow ($U_t \rightarrow N_t$) and the fast ($U_c \rightarrow N_c$) conformational refolding reactions at 0.5 M urea, as described.[26]

The proline-limited folding reactions of the N2′ variants were measured as a function of the urea concentration in 100 mM potassium phosphate, pH 7.0 at 15 °C, after manual mixing in a Hitachi F 4010 fluorescence spectrophotometer in 1 cm cells as described.[26,28]

Protease Free Prolyl Isomerase Assay. The prolyl isomerase assays were essentially performed as described by Zoldák et al.[23] at 15 °C, by using either a Hitachi F4010 (excitation bandwidth 5 nm, emission bandwidth 10 nm) or a Jasco FP 6500 (excitation bandwidth 3 nm, emission bandwidth 5 nm) fluorescence spectrophotometer.

The pNA and NO_2-Tyr peptides were dissolved in anhydrous 0.55 M LiCl/TFE to obtain stock solutions with peptide concentrations of 750 μM. The *cis*/*trans* isomerization was initiated by dilution of a small sample of the peptide stock solution with aqueous buffer (100 mM potassium phosphate, 1 mM EDTA, pH 7.0) in a 1 cm cell to a final concentration of 3 μM peptide in the absence or the presence of a prolyl isomerase. For the pNA peptides, fluorescence was detected at 416 nm after excitation at 316 nm. To increase the signal-to-noise ratio of the NO_2-Tyr peptides, the assay was performed at pH 6.0 and fluorescence was measured at 425 nm (excitation at 316 nm).

For the protein folding assay, the different variants of N2′ were unfolded in 5.0 M urea for at least 2 h at 15 °C. Refolding was initiated by dilution of small protein samples to final conditions of 0.33 μM N2′, 100 mM potassium phosphate, 0.2 M urea, pH 7.0, in the absence or the presence of a prolyl isomerase. The increase in fluorescence upon *trans* → *cis* isomerization at Pro161 during refolding of N2′ was detected at 340 nm after excitation at 280 nm.

The apparent rate constant of prolyl isomerization k_{app} was obtained by fitting a monoexponential function to the time course of the fluorescence change. The measured k_{app} values obey the relation $k_{app} = k_0 + [E]k_{cat}/K_M$, where k_0 is the rate constant of the uncatalyzed reaction, [E] is the enzyme concentration in the cuvette, and k_{cat} and K_M are the turnover number and the Michaelis constant, respectively. The apparent rate constants were plotted as a function of the enzyme concentration, and the catalytic efficiency k_{cat}/K_M was calculated from the slope. In these experiments, the enzyme concentrations were typically varied between 0 and 200 nM. Only in the folding experiments with hFKBP12, the concentration range was extended up to 4 μM because the catalytic efficiency of hFKBP12 was very low in this assay.

RESULTS

Procedures for Assaying Prolyl Isomerase Activity. In the panel of assay peptides (Abz-Ala-Ala-Pro-Xaa-Ala-pNA), an Ala was placed before Pro, and the variable position Xaa after Pro was occupied by a charged (Asp, Glu, Lys, Arg), a polar (Ser), an aliphatic (Ala, Val, Leu), or an aromatic (Phe) residue. All these peptides could be used for the protease-free assay as described by Zoldák et al.[23] In this assay, the peptides are dissolved in anhydrous LiCl/TFE to increase the fraction of the *cis* isomer. The *cis*/*trans* re-equilibration is initiated by dilution with aqueous buffer in the presence of a prolyl isomerase and followed by the increase in Abz fluorescence.

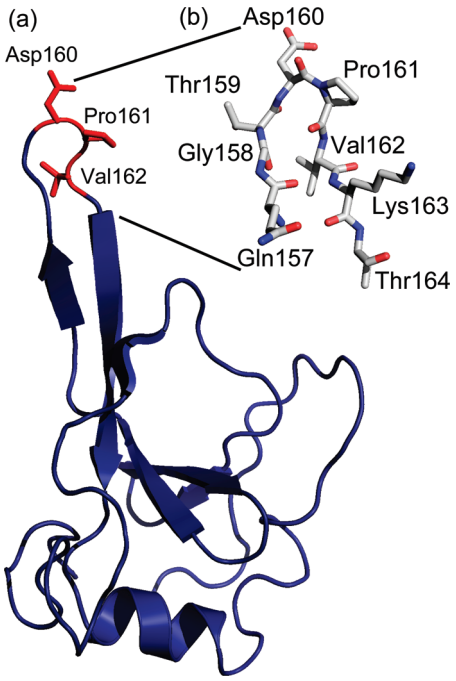

Figure 2. (a) Tertiary structure of the N2 domain of the gene-3-protein. The side chains of Asp160, Pro161, and Val162 are shown in stick representation. (b) Enlarged view of the loop region from Gln157 to Thr164. The figure was prepared by using PyMol[48] and the crystal structure of G3P (PDB accession code 1G3P).[36]

To examine how variations of the amino acid after the proline affect the catalysis of prolyl isomerization during a protein folding reaction, we used the N2′ domain of the gene-3-protein of phage fd as the model protein (Figure 2a). N2′ is a variant of N2, which carries the stabilizing substitution Q129H and a His_6 tag at the amino terminus.[28] Pro161 of N2′ resides at the tip of a loop that connects two β strands (Figure 2b), and in the crystal structure, Pro161 adopts the *cis* conformation.[34,35] In solution, the *cis*/*trans* ratio in the unfolded form is 10/90, and in the folded form it is 90/10.[26,28] For about 80% of all refolding N2′ molecules, the *trans* → *cis* isomerization of the Asp160-Pro161 bond is thus the final rate-limiting step of folding.[28] By directed mutagenesis, we produced a set of nine variants of N2′, in which Pro161 is followed by the same residues as in the labeled peptides. In these variants, Pro161 of N2′ was preceded by Ala, as in the peptides, not by an Asp, as in wild-type N2′. The N2′ variants are designated by a three-letter code that reflects the sequence around Pro161. In 5.0 M urea, N2′ is fully unfolded and 7% of all molecules show a *cis* Pro161.[28] During refolding, the *cis* content increases to 85% in the course of a proline-limited folding reaction. The concomitant increase in protein fluorescence is used to monitor this folding reaction in the absence and in the presence of prolyl isomerases.

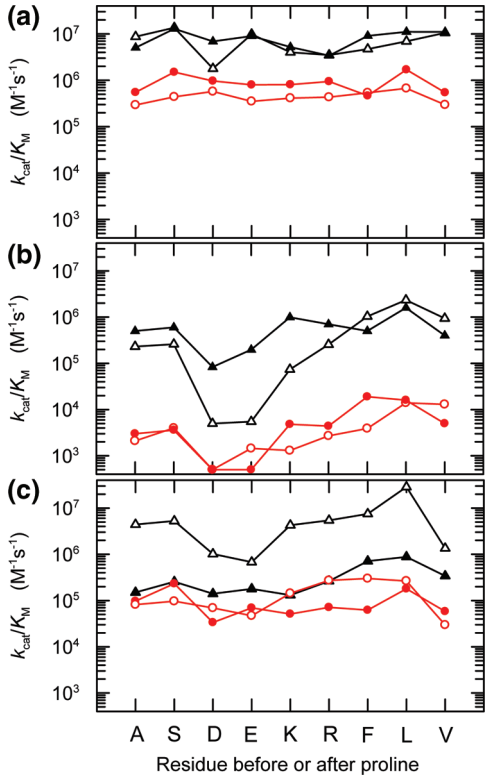

Figure 3. Variation of the catalytic efficiencies of (a) hCyp18, (b) hFKBP12, and (c) *Ec*Par10 as a function of the amino acid before the proline (open symbols) and after the proline (filled symbols). The k_{cat}/K_M values were derived from assays with the oligopeptides Abz-Ala-Xaa-Pro-Phe-pNA and Abz-Ala-Ala-Pro-Xaa-Ala-pNA (black) after a solvent jump from 0.55 M LiCl/TFE to 100 mM potassium phosphate, 1 mM EDTA, pH 7.0 at 15 °C or from protein folding assays (red) with the variants of the N2′ domain in 100 mM potassium phosphate, 0.2 M urea, pH 7.0. The peptide data for the residue before the proline for hCyp18 and hFKBP12 are taken from Zoldák et al.,[23] and the data for the protein folding assay with N2′ concerning the position before proline for hFKBP12 are from Jakob et al.[27] The numerical k_{cat}/K_M values are shown in Table S3 (Supporting Information). The precision of the k_{cat}/K_m values is about ±10% and reflected by the size of the symbols.

The stabilities of the nine N2′ variants with amino acid substitutions after Pro161 were assessed by measuring thermal as well as urea-induced unfolding transitions. All of them were stably folded proteins. The variant with Ala on either side of Pro161 (APA) shows a T_M value of 38.3 °C and a $[urea]_M$ value of 2.40 M. Most of the substitutions after Pro161 changed the T_M value by less than ±2 °C and $[urea]_M$ by less than ±0.1 M (Supporting Information Table S1). Only the variants with negatively charged residues after Pro161 were more strongly destabilized with T_M values of 33.2 °C (APD) and 35.3 °C (APE). At 15 °C, where the prolyl isomerase activities were determined, all N2′ variants were stably folded.

The folding kinetics of the N2′ variants were measured as a function of the urea concentration, and the *cis*/*trans* ratios at Pro161 in the folded and the unfolded forms were determined by stopped-flow double-mixing experiments as described[26,28] (Supporting Information Table S2). For most variants, the *cis* content in the unfolded protein was close to 10% as in wild-type N2,[28] and in the folded form it was between 75 and 90% (Supporting Information Table S2), also similar to the values obtained for the wild-type protein and for the variants with substitutions at the position before Pro161.[26] Divergent values were observed for the variants with Asp after Pro161. In this case, the *cis* content was increased in the unfolded form and decreased in the folded form, in agreement with the reduced stability of this variant. It indicates that this negatively charged side chain after Pro161 is not well accommodated in the native conformation of the *cis*-Pro161 containing loop of N2′. In the wild-type protein, this position is occupied by a valine.

Specificity of Human Cyclophilin 18. hCyp18 displayed a high k_{cat}/K_M value (about 10^7 M^{-1} s^{-1}) in the protease-coupled assay with the Suc-Ala-Ala-Pro-Phe-pNA tetrapeptide and chymotrypsin.[15,17] The efficient catalysis by hCyp18 originates from a very high turnover number,[37] which is combined with a rather low substrate affinity. With a panel of 20 Abz-Ala-Xaa-Pro-Phe-pNA peptides, in which Pro was preceded by all 20 proteinogenic amino acids, k_{cat}/K_M values were obtained that differed less than 10-fold for 19 of the 20 amino acids (a low k_{cat}/K_M of 1.4×10^4 M^{-1} s^{-1} was observed only for the peptide with the Pro-Pro sequence).[23]

For the nine peptides with varying residues after the proline, hCyp18 showed also very high k_{cat}/K_M values, and they differed only 4-fold (between 3.4×10^6 M^{-1} s^{-1} for Pro-Arg and 13×10^6 M^{-1} s^{-1} for Pro-Ser, Figure 3a). Corresponding amino acid substitutions before and after the proline thus had very similar, if not identical, effects. Apparently, cyclophilin combines a high catalytic efficiency with a very low substrate specificity toward the residues at these positions.

hCyp18 also catalyzed the prolyl isomerization during the refolding of N2′ very well. For the nine variants with different amino acids after the proline, the k_{cat}/K_M values were in the range of 10^6 M^{-1} s^{-1} and varied no more than 4-fold (Figure 3a). The role of the amino acid before the proline for the catalytic efficiency of hCyp18 in protein folding had not been investigated before.[27] Therefore, we used a second set of variants of N2′, in which the residue before Pro161 was replaced by the same nine residues as in the set of variants with variations after the proline. Here the k_{cat}/K_M values differed only 3-fold between the individual variants. In the catalysis of the proline-limited refolding of N2′, hCyp18 is thus highly active and shows low sequence specificity toward the amino acids before and after the proline (Figure 3a), as in the experiments with the proline-containing peptides.

Specificity of Human FKBP12. Prolyl isomerases of the FKBP family are less active than the cyclophilins but show a much higher specificity with regard to the amino acid before the proline. They strongly prefer hydrophobic residues over negatively charged ones. Thus, hFKBP12 shows a 500-fold higher activity toward Leu-Pro than toward Asp-Pro or Glu-Pro in tetrapeptide substrates (Figure 3b).

At the position following the proline, hFKBP12 disfavors negatively charged residues as well, but the activity toward Pro-Leu is only about 20-fold higher than the activity toward Pro-Asp. Apart from Asp, hFKBP12 is rather tolerant toward the chemical nature of the residue after the proline (Figure 3b). Interestingly, hFKBP12 prefers positively charged residues at this position relative to Phe or Val.

hFKBP12 is a very poor catalyst of protein folding reactions. The activities measured for our panel of N2′ variants with nine different amino acids after Pro161 are about 100-fold lower than those obtained with hCyp18 and also about 100-fold lower than those measured with the peptides (Figure 3b). The rank order is,

however, similar. The highest activity in the protein folding assays was observed when, in the substrate N2′, Pro161 was followed by Phe, and the catalysis at Pro-Asp was too weak to be measured reliably. The effects of the amino acid variations after the proline resemble those measured for the set of N2′ variants with the variations before the proline (Figure 3b).

Specificity of Parvulin. The prolyl isomerase activity of human parvulin (hPar14) is very low[18] and difficult to measure. hPar14 shows a preference for Arg−Pro and Lys−Pro bonds, but even for peptides with these sequences the activity is 100-fold and 1000-fold lower than the corresponding activities of hFKBP12 and hCyp18.[23] For our panel of peptides, we could detect activities only for those with Leu or Phe after the proline. The corresponding k_{cat}/K_M values were 0.4×10^3 and 0.6×10^3 M^{-1} s^{-1}, respectively. This indicates that very low catalytic activity toward tetrapeptides is an intrinsic property of hPar14, and it cannot be increased by sequence variations before or after the proline.

To determine the sequence specificity of a member of the parvulin family, we employed parvulin 10 from *E. coli* (*Ec*Par10). This parvulin showed a high catalytic activity in the protease-coupled assay with a moderate preference for nonpolar residues before the proline.[18,39] Here we used the two panels of Abz peptides with nine different residues before or after the proline (Abz-Ala-Xaa-Pro-Phe-pNA and Abz-Ala-Ala-Pro-Xaa-Ala-pNA) in the protease-free assay to determine the local sequence specificity of *Ec*Par10 (Figure 3c). In its specificity profile toward the residue before the proline, *Ec*Par10 resembled hFKBP12 and preferred nonpolar residues such as Leu ($k_{cat}/K_M = 29 \times 10^6$ M^{-1} s^{-1}) to negatively charged residue, such as Glu ($k_{cat}/K_M = 0.68 \times 10^6$ M^{-1} s^{-1}) (Figure 3c). This preference was, however, less pronounced for *Ec*Par10 (about 40-fold) than for hFKBP12 (about 500-fold, Figure 3b).

The selectivity of *Ec*Par10 toward the residue after the proline was very low. Nonpolar residues were still preferred over charged or polar ones, but the preference for Pro-Leu was only 5-fold higher than for Pro-Glu. Unlike in the cases of hCyp18 and hFKBP12, the peptides with variations before the proline were significantly better substrates than those with the variations after the proline (Figure 3c), which seems surprising. In the panel of peptides with the variations before the proline, the proline is separated from the pNA moiety at the C-terminus by a single residue (Phe), and in the panel with the variations after the proline two residues (Xaa and Ala) separate it from pNA. As a consequence, the pNA moiety occupies a different position when the peptides are bound to the active sites of the prolyl isomerases. This seems to be important for *Ec*Par10 but not for hCyp18 and hFKBP12.

In the assays with the variants of the N2 domain, the sequence specificity was low toward the residue before as well as after the proline (Figure 3c). For the 18 variants of N2′, the observed differences in activity were 7-fold or less. Before the proline, we still noted a slight preference for Leu and Phe, and Glu was slightly disfavored. For the position after the proline, the highest activity was noted for Ser and the lowest for Asp, but as outlined, the difference was only 7-fold.

An Alternative Set of Peptide Substrates. The results obtained with *Ec*Par10 (Figure 3c) indicate that the location of the pNA group might influence the prolyl isomerase activity toward the peptides with sequence variations after the proline. To examine this, we synthesized a second set of peptides (Abz-Ala-Ala-Pro-Xaa-NO_2-Tyr-NH_2), in which the quenching nitrophenyl group was part of the last amino acid (3-nitrotyrosine), and thus closer to the residue Xaa. The carboxyl group was protected by amidation. In this set of peptides, the variable position after the proline was occupied by the same nine residues as in the set of pNA peptides. All NO_2-Tyr peptides could be used for the protease-free assay. The signal-to-noise ratio was slightly lower than for the pNA peptides. The rates of the uncatalyzed isomerizations were similar for the corresponding peptides in the two series (Table S4).

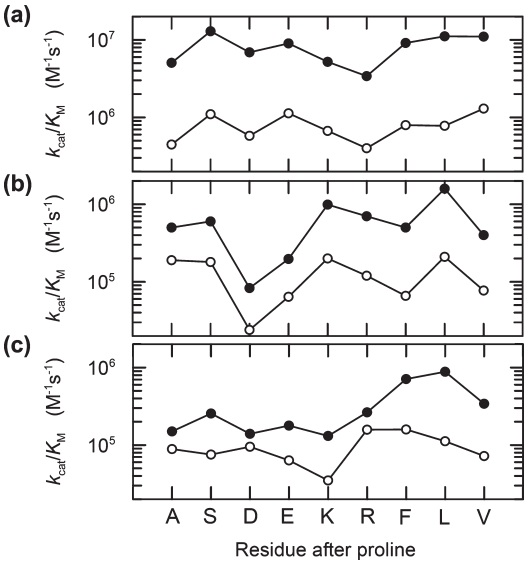

Figure 4. Influence of the position of the nitrophenyl group on the catalytic activities of (a) hCyp18, (b) hFKBP12, and (c) *Ec*Par10 as a function of the amino acid after the proline. Closed symbols: catalytic efficiencies obtained with the series of Abz-Ala-Ala-Pro-Xaa-Ala-pNA peptides. Open symbols: catalytic efficiencies obtained with the series of Abz-Ala-Ala-Pro-Xaa-NO_2-Tyr peptides. The experiments were performed at 15 °C in the presence of varying concentrations of prolyl isomerases in 100 mM potassium phosphate, 1 mM EDTA, pH 7.0 for the pNA peptides and pH 6.0 for the NO_2-Tyr peptides. The numerical k_{cat}/K_M values are shown in Table S3 (Supporting Information). The precision of the k_{cat}/K_m values is about ±10% and reflected by the size of the symbols.

For all three prolyl isomerases, lower k_{cat}/K_M values were found for the peptides of the NO_2-Tyr series (Figure 4). The difference between the two sets of peptides was most pronounced for hCyp18 (Figure 4a). The k_{cat}/K_M values varied, however, in a very similar fashion with the nature of the amino acid after the proline in the two series of peptides. This is evident in particular for hCyp18 and hFKBP12. For *Ec*Par10, the differences between pairs of peptides from the two series were small in general but enhanced when Pro was followed by residues with aromatic or aliphatic side chains (Figure 4c).

All prolyl isomerases were assayed at peptide concentrations that are much smaller than K_M. The measured activities are thus sensitive to differences in both substrate affinity and turnover number. In the peptides of the NO_2-Tyr series, the nitrophenyl chromophore is separated from the residue Xaa after the proline by a single amide bond and in the pNA series by two amide bonds. Thus, the pNA peptides probably represent a continuous protein chain slightly better than the NO_2-Tyr peptides, which might explain the differences in the activities measured with the two panels of peptides. Again, the results for *Ec*Par10 indicate that structural differences in the substrate at two or three positions after the proline affect its specificity.

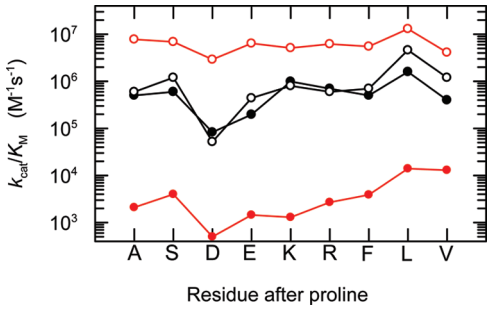

Figure 5. Catalytic efficiencies of hFKBP12 (closed symbols) and of hFKBP12+IF (open symbols) as a function of the amino acid after the proline. Black, k_{cat}/K_M values derived from assays with oligopeptides; red, k_{cat}/K_M values derived from the protein folding assays with the N2′ variants measured at 15 °C, pH 7.0 as described in Figure 3. The numerical k_{cat}/K_M values are shown in Table S3 (Supporting Information). The precision of the k_{cat}/K_m values is about ±10% and reflected by the size of the symbols.

Effect of a Chaperone Domain on the Specificity of Human FKBP12. Presumably, hFKBP12 is a very poor catalyst of protein folding because its affinity for refolding protein chains is very low. In fact, its catalytic efficiency could be increased by more than 2 orders of magnitude by inserting the chaperone domain of SlyD into the flap, which is a loop near the active site of hFKBP12.[27,38] SlyD is a homologue of hFKBP12 in *E. coli*, which naturally carries this chaperone domain. Since it is inserted into the flap, it is called the "insert-in-flap" or IF domain. Accordingly, the form of hFKBP12 with this chaperone domain is called hFKBP12+IF.

The activity of the FKBP moiety toward the set of peptides is not significantly affected by the chaperone (IF) domain (Figure 5), which confirms that inserting this domain near the prolyl isomerase site does not change its activity or sequence specificity (Figure 5). The efficiency for the catalysis of folding of N2′ is, however, strongly enhanced. For most of the N2′ variants with different residues after Pro161, the catalytic efficiency increased by more than 1000-fold after the insertion of the chaperone domain (Figure 5). The most dramatic increase was observed for the variant, in which Pro161 is followed by Asp. The catalytic efficiency is below the detection limit (at about 5×10^2 M^{-1} s^{-1}) in the absence of the chaperone domain (hFKBP12), but in its presence (hFKBP12+IF), it increased to 2.9×10^6 M^{-1} s^{-1}. Similarly high k_{cat}/K_M values were observed for all N2′ variants (Figure 5), indicating that the high activity observed in the presence of the chaperone domain is virtually independent of the nature of the residue after the proline. This complements our previous work, which had shown that the chaperone domain also abolished the very high specificity of hFKBP12 for the residue before the proline.[27] It provides further support for the suggestion that prolyl isomerases with chaperone domains show a very high activity that is determined by the strong binding of protein substrates to the chaperone domain. This binding is independent of the local sequence around the proline and therefore overrides the inherently high sequence specificity of the prolyl isomerase active site of hFKBP12.

DISCUSSION

The single-domain prolyl isomerases hCyp18 and hFKBP12 differ in the specificity toward the residue before the proline. hCyp18 shows an indiscriminate, but very high catalytic efficiency with values near 10^7 M^{-1} s^{-1}, when probed with proline-containing oligopeptides.[15,17,23] hFKBP12 is less active but highly specific for the residue before the proline.[17,23,40,41] Now, similar results were obtained with respect to the residue after the proline. The generally very high catalytic efficiency of hCyp18 was almost independent of the nature of the residue after the proline, probably because the residue after the proline is exposed to the solvent, as in the complex between hCyp18 and the HIV capsid protein.[42]

hFKBP12 shows similar activity profiles for the residues before and after the proline, but the preference for hydrophobic over negatively charged residues is much less pronounced for the position after the proline. High-resolution structures of FKBP12 with peptide or protein substrates are not available, and therefore the orientation of the peptide chain at the FKBP12 active site is not known.

The strong difference in sequence specificity between hCyp18 and hFKBP12, as observed for the oligopeptides, probably explains why they differ so strongly in their efficiency as catalysts of proline-limited protein folding reactions. hCyp18 with its shallow active site[42] and almost indiscriminate substrate specificity can bind to proline-containing segments in refolding protein chains with a similar affinity as it binds to oligopeptides. Together with the very high k_{cat} value,[37,43,44] this leads to the observed very high k_{cat}/K_M values near 10^6 M^{-1} s^{-1}. For hFKBP12, the nature and the orientation of the side chains before and after the proline are apparently very important for productive binding. In refolding proteins, proline-containing segments often are partially ordered already, and therefore binding to the active site is compromised. This explains the poor performance of hFKBP12 as a catalyst of prolyl isomerization in a refolding protein. Its efficiency in protein folding is high, however, when the FKBP domain is linked with a chaperone domain that secures good and indiscriminate substrate binding.[27,38] In this case, productive substrate transfer to the prolyl isomerase site presumably becomes rate-limiting for catalysis.[45]

*Ec*Par10, our representative from the third family of prolyl isomerases, shows catalytic efficiencies and specificities that are intermediate between hCyp18 and hFKBP12. As hCyp18, it shows a very high overall activity with k_{cat}/K_M values as high as 3×10^7 M^{-1} s^{-1}. In its sequence specificity, it resembles hFKBP12, with a preference for nonpolar residues before and after the proline. However, this preference is much less pronounced than for hFKBP12. This intermediate position of *Ec*Par10 between hCyp18 and hFKBP12 is also reflected in its efficiency as a catalyst of proline-limited protein folding. The sequence specificity of *Ec*Par10 for the residues before and after the proline is low, and it catalyzes the folding of our model protein N2′ with an at least 10-fold higher efficiency than hFKBP12, but an about 10-fold lower efficiency when compared with hCyp18.

ASSOCIATED CONTENT

Supporting Information. Four tables with supporting data on the stabilities and the folding kinetics of the N2′ variants and the numerical k_{cat}/K_M values for all prolyl isomerases, as measured in the peptide and protein folding assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

hCyp18, human cyclophilin 18; hFKBP12, human FK506 binding protein 12; hPar14, human parvulin 14; *Ec*Par10, parvulin 10 from *Escherichia coli*; SlyD, sensitive-to-lysis protein of *E. coli*; hFKBP12+IF, hFKBP12 carrying the *insert-in-flap* (IF) domain of SlyD; Abz, 2-aminobenzoyl; pNA, *p*-nitroanilin; NO_2-Tyr, 3-nitrotyrosine; TFE, trifluoroethanol; Xaa, amino acid; CD, circular dichroism; k_{ct} and k_{tc}, microscopic rate constant for *cis* → *trans* and *trans* → *cis* isomerization reactions, respectively; K_{tc}, equilibrium constant for *cis*/*trans* isomerization; λ, macroscopic rate constant; *cis*-Pro, *cis* isomer of the Xaa−Pro peptide bond; N2′, N2 domain (residues 102−205) of the gene-3-protein of phage fd extended at the N terminus by His_6 with the substitution Q129H; U_t and U_c, N_t and N_c, unfolded (U) and folded (N) forms of N2′ with *trans* or *cis* Pro161; k_{cat}, turnover number; K_M, Michaelis constant.

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