Peptidyl Prolyl *cis*/*trans*-Isomerases: Comparative Reactivities of Cyclophilins, FK506-Binding Proteins, and Parvulins with Fluorinated Oligopeptide and Protein Substrates

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ABSTRACT: Peptidyl prolyl *cis*/*trans*-isomerases catalyze the *cis*−*trans* isomerization of prolyl bonds in oligopeptides and various folding states of proteins. The proline residue in PPIase substrates at the P1′ subsite, which follows the isomerizing peptide bond, appears to be the common recognition element for all subfamilies of this enzyme class. The molecular principles that govern substrate specificity at the P1′ subsite were analyzed using 4-fluoroproline-containing tetrapeptide 4-nitroanilides and barstar Cys40Ala/Cys82Ala/Pro27Ala/Pro48→4-fluoroproline quadruple variants. Generally, PPIase catalysis demonstrated stereospecificity for monofluoro substitutions at the 4-position of the pyrrolidine ring. However, the replacement of hydrogens with fluoro atoms did not impair productive interactions for the majority of PPIase−substrate complexes. Comparison of specificity constants for oligopeptide and protein substrates revealed striking differences in the 4-fluoroproline substituent effects between members of the PPIase families. Introduction of 4(*R*)-fluoroproline resulted in an oligopeptide substrate completely resistant to catalytic effects of FKBP-like PPIases. By contrast, the 4(*R*)-fluoroproline barstar variant demonstrated only slightly reduced or even better catalytic susceptibility when compared to the parent barstar Cys40Ala/Cys82Ala/Pro27Ala/Pro48 substrate. On the other hand, Suc-Ala-Ser-4(*S*)-FPro-Phe-pNA exhibits a discriminating specificity toward the prototypic parvulin, the *Escherichia coli* Par10. The *E. coli* trigger factor, in the extreme, catalyzes Cys40Ala/Cys82Ala/Pro27Ala/4-F_2Pro48 with a more than 20-fold higher efficiency when compared to the proline-containing congener. These findings support the combined subsite concept for PPIase catalysis in which the positioning of a substrate in the active cleft must activate a still unknown number of remote subsites in the transition state of the reaction. The number of critical subsites was shown to vary between the PPIase families.

Proline-directed peptide bond *cis*/*trans*-isomerases, designated as peptidyl prolyl *cis*/*trans*-isomerases (PPIases,[1] EC 5.2.1.8), are ubiquitously distributed enzymes evolved to accelerate the interconversion between prolyl bond isomers[2] of proline-containing polypeptide chains (*1*). Conformational heterogeneity involving prolyl bonds enables polypeptide chains to react isomer-specifically in bioreactions (*2*). Importantly, catalysis by PPIases enables polypeptides to kinetically escape isomer-specific reaction steps. Thus, PPIase involvement consequent from its enzymatic activity was seen in many cellular processes (*3*).

Three different families, cyclophilins, FK506-binding proteins, and parvulins, constitute the enzyme class of PPIases. These families are unrelated in their amino acid sequences and their susceptibility to druglike inhibitors such as FK506, rapamycin, and cyclosporin A.

Of much current interest in the field of natural substrates of PPIases is the recent discovery of the well-defined catalytic specificity for the pSer(pThr)-Pro substrate signature sequence of the cancer-relevant PPIase Pin1 (*4*−*7*). No other known human PPIase shares its P1 subsite specificity [following the nomenclature of Schechter and Berger (*8*)].

While there is much detailed knowledge about the involvement of Pin1-catalyzed steps in cellular signaling, bioreactions involving the participation of a particular FKBP or cyclophilin are difficult to detect. In most cases, the many members of these PPIase families are coexpressed in a given mammalian cell and are thought to exhibit similarly broad substrate specificity, making their distinct biological roles difficult to understand. Knowledge of differential substrate

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[1] Abbreviations: 4-FPro, 4-fluoroproline; 4-F_2Pro, 4-difluoroproline; Cyp, cyclophilin; FKBP, FK506-binding protein; Par, parvulin; PPIase, peptidyl prolyl *cis*/*trans*-isomerase; TF, trigger factor.

[2] The term prolyl bond used throughout the paper indicates the peptide bond preceding proline in an amino acid sequence, and prolyl isomerization indicates the *cis*−*trans* isomerization of the peptide bond preceding proline.

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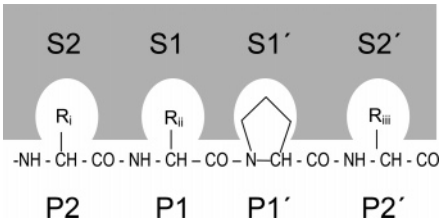

FIGURE 1: Nomenclature for binding of a peptide or protein substrate to a PPIase according to the Schechter and Berger nomenclature for binding of a peptide substrate to a peptidase (*8*). The PPIase is represented as the shaded area. R_i-R_{iii} are the side chains of amino acids. P2, P1, P1′, and P2′ are the amino acid residues. S2, S1, S1′, and S2′ are the corresponding subsites on the enzyme.

specificities is fundamentally important in unraveling the functional role for PPIase−protein interactions in cells. Thus, oligopeptides have been utilized to determine the nature of subsite specificity of prototypic PPIases that varied in the P1 subsite. By convention following Schechter and Berger (*8*), the substrate amino acid residues are called P and the subsites on the PPIase that interact with the substrate are called S. The amino acid residues on the N-terminal site of the prolyl bond, which is targeted by PPIase catalysis, are numbered P1, P2, etc.; residues C-terminal of the prolyl bond are numbered P1′, P2′, etc. (Figure 1). Cyclophilins appear as rather promiscuous catalysts with these substrates. Succinylated tetrapeptide 4-nitroanilides reveal the limited influence of the P1 side chain on k_{cat}/K_m values for Cyp18 as well as other members of the cyclophilin family (*9*−*11*). On the other hand, the binding cleft of Cyp18 accommodates nine residues of HIV-1 capsid-derived polypeptide substrates that are all located within interactive distances (*12*−*14*). Consequently, the few specificity constants measured with tetrapeptides varying in P2 position reveal considerable effects of this subsite (*10*). In contrast, catalytic efficiencies for FKBPs and the prototypic *Escherichia coli* parvulin (Par10) were shown to be higher by ~3 orders of magnitude for peptides containing leucine or phenylalanine instead of amino acids with small or charged side chains in the P1 position (*9*, *15*, *16*). This preference was attributed to an increase in k_{cat} rather than to more avid substrate binding (*17*).

The proline residue in the P1′ position is thought to determine the primary specificity of PPIases and appears to be crucial for activation of the catalytic machinery, but quantitative data are lacking (*18*, *19*). In fact, PPIases with the exception of the *E. coli* trigger factor catalytic domain neither catalyzed prolyl isomerization in nor exhibited active site affinity for oligopeptides having D-proline at this position (*20*). Furthermore, azetidine-2-carboxylic acid, thiazolidine-4-carboxylic acid, and thiazolidine-2-carboxylic acid are the only substitutions allowed for Cyp18 substrates among oligopeptides with P1′ site variations such as ring size and replacement of ring carbons with oxygen or sulfur (*21*). Due to combined effects exerted by the proline analogues, the question of whether steric restrictions, changed hydrophobicity, or electronic contributions mediated the inertness of most substitutions at the P1′ position to Cyp18 remained open.

In contrast, structural investigations of PPIase−inhibitor complexes show the adaptation of the P1′-directed binding pocket to residues other than proline. In the Cyp18−cyclosporine A complex, the side chain of MeVal11 occupies the cyclophilin S1′ pocket (*22*). The acyl−pipecoloyl amide bond of FK506 was suggested to serve as an analogue for the prolyl bond of a substrate of FKBP (*23*).

Thus, in some respects, PPIases might utilize structural features of transition state binding of substrates reminiscent of protease catalysis, including important interactions more peripheral than P1 and P1′ subsites in influencing productive binding. As in the protease field, the differential P1′−S1′ interaction capability of members of PPIase families proved to be essential for designing isoenzyme-specific substrates. It is likely that more information is required to differentiate between a cumulative model in which PPIases combine with substrates at multiple subsites and another model in which an isolated subsite is responsible for productive interaction. This knowledge will play a decisive role in identifying cellular substrates of a particular PPIase.

As a common strategy, fluorine-containing substrates are used as mechanistic probes to elucidate mechanisms of productive substrate binding. Because of its favorable stereoelectronic properties, the fluorine atom forms an effective bioisostere for hydrogen. The introduction of a fluorinated amino acid into a definite position of a substrate peptide or protein will allow us to probe the importance of individual interactions of a specific subsite in the enzyme activity of PPIases.

Our approach to the P1′ substrate specificity of PPIases was to compare catalytic parameters of proteins and oligopeptide substrates simultaneously. This was accomplished by incorporating the 4(*S*)-fluoroproline [4(*S*)-FPro], 4(*R*)-fluoroproline [4(*R*)-FPro], and 4-difluoroproline (4-F_2Pro) analogues at sequence position Pro48 of the barstar Cys40Ala/Cys82Ala/Pro27Ala triple variant by the method of selective pressure incorporation during heterologous expression in *E. coli* (*24*). This substitution has substantial inductive effects on the *cis*−*trans* isomerization of the prolyl bond without being disturbed by severe steric restrictions. Oligopeptides containing Cγ-monosubstituted fluoroprolines of known stereochemistry were expected to produce kinetic data sensitive to the spatial requirements of the S1′ contacting site in the enzyme. Since the rate of refolding of the unfolded barstar Cys40Ala/Cys82Ala/Pro27Ala triple variant is limited by the *trans* → *cis* isomerization of the Tyr47−Pro48 bond, PPIase catalysis can be easily quantified on the basis of a polypeptide substrate and can be compared with that of standard tetrapeptide substrates containing similar P1′ substitutions.

Despite the small size of the fluorine atom chosen to be a less disruptive substitution, members of the PPIase families each responded in kinetics to the 4-fluoroproline mimics introduced at the P1′ position of substrates. In addition, the responses were shown to be different for 4-fluoroproline-containing tetrapeptide 4-nitroanilides and unfolded 4-FPro48 barstar quadruple variants in that the monofluorinated protein substrates were less discriminating among the enzymes.

MATERIALS AND METHODS

Protein Expression and Purification. The gene encoding barstar Cys40Ala/Cys82Ala/Pro27Ala was subcloned into the plasmid pKK-223 for expression under control of the *tac* promotor. The protein was overexpressed in *E. coli* JM109 cells as inclusion bodies. The production of the recombinant

4-FPro48 variants by the method of selective pressure incorporation was performed according to established procedures (*24*). The isolation and purification of the barstar Cys40Ala/Cys82Ala/Pro27Ala and the 4-FPro48 variants were performed as previously described (*25*). The inhibitory activity of the barstar proteins on barnase activity was checked qualitatively according to the procedure described elsewhere (*26*). All 4-FPro48 variants are able to inhibit the RNA cleaving activity of barnase. The recombinant proteins consist of 90 amino acids containing the first methionine. The molecular mass of the barstar proteins was determined by electrospray mass spectrometry to ensure the correct incorporation of the proline analogue.

The PPIases were prepared as recombinant proteins in *E. coli* and purified as described previously (*27*−*33*).

Peptide Synthesis. The soluble Suc-Ala-Ser-Pro-Phe-pNA peptide and its respective 4(*R*)-FPro-, 4(*S*)-FPro-, and 4-F_2-Pro-containing derivatives were synthesized on a Syro II multiple-peptide synthesizer (MultiSynTech) using Fmoc chemistry SPPS protocols and PyBOP/HOBT as the activation reagent. Peptides were purified by preparative reverse phase HPLC. The purity of all compounds was checked by HPLC and by capillary electrophoresis. Peptide identity was confirmed by electrospray mass spectrometry. ESI-MS: Suc-Ala-Ser-Pro-Phe-pNA calcd 640.5 $(M + H)^+$, found 641.7; Suc-Ala-Ser-4(*R*)-FPro-Phe-pNA calcd 658.5 $(M + H)^+$, found 659.3; Suc-Ala-Ser-4(*S*)-FPro-Phe-pNA calcd 658.5 $(M + H)^+$, found 659.3; Suc-Ala-Ser-4-F_2Pro-Phe-pNA calcd 676.5 $(M + H)^+$, found 677.3.

Prolyl Isomerization of Oligopeptides. The kinetics of the *cis* → *trans* isomerization of 4-fluoroproline-containing peptide 4-nitroanilides of the structure Suc-Ala-Ser-FPro-Phe-pNA as well as of standard peptides of the structure Suc-Ala-Xaa-Pro-Phe-pNA (Xaa is Phe or Ala) were measured by using the protease-coupled assay as described by Fischer et al. (*34*). Measurements were carried out in 35 mM HEPES buffer (pH 7.8) at 10 °C using a Hewlett-Packard 8452 diode array UV−vis spectrophotometer. For isomer-specific proteolytic cleavage, chymotrypsin or trypsin was used at a final concentration of 0.83 mg/mL (400 units/mg, Merck) or 50 μg/mL (40 units/mg, Merck), respectively. Stock solutions of oligopeptides were prepared in DMSO at a concentration of 10 mg/mL. The assay was started by adding 3 μL of the peptide to the reaction mixture in a final volume of 1.2 mL, resulting in a final peptide concentration of 25 μg/mL.

The influence of different PPIases on the *cis* → *trans* isomerization of the peptides was measured by performing the assay both in the absence and in the presence of the enzymes. Under the conditions of the experiments ($[S] \ll K_M$), the rate of enzyme-catalyzed isomerization can be described by the first-order rate equation $v = k_{obs}[cis]$; $k_{obs} = k_0 + k_{enz}$, and $k_{obs} = k_0 + k_{cat}/K_M[E_0]$, where [*cis*] is the time-dependent concentration of the *cis* isomer, k_0 is the rate constant of the uncatalyzed *cis* → *trans* isomerization, and k_{obs} is the observed first-order rate constant in the case of PPIase catalysis.

The PPIase activities in urea for protease-sensitive PPIases (SlyD and Par10) were determined by the protease-free method according to the method of Janowski et al. (*35*). Briefly, Suc-Ala-Phe-Pro-Phe-pNA was dissolved in a 5.5 M LiCl/ anhydrous TFE mixture, giving a stock concentration of 20 mg/mL. The assessment of the *cis*−*trans* isomerization was initiated by addition of a substrate solution to a mixture of 35 mM HEPES (pH 7.8), 2 μM BSA, and PPIase at the desired concentration. Data analysis was performed by single-exponential nonlinear regression using the SigmaPlot Scientific Graphic System (Jandel Corp.).

The substrate properties of 4-fluoroproline-containing peptide 4-nitroanilides toward SlyD were not accessible, because of their instability in the assay. In addition, 4-fluoroproline-containing peptides are not suited to the protease-free assay.

Equilibrium Unfolding Measurements of Barstar Cys40Ala/Cys82Ala/Pro27Ala and Barstar Quadruple Variants. Urea unfolding studies on the barstar proteins were monitored by far-UV circular dichroism. CD spectra of the proteins at a concentration of 2−3 μM in 50 mM sodium phosphate buffer (pH 8.0) were recorded on an Aviv (Lakewood, NJ) model 62A DS circular dichroism spectrometer using cuvettes with an optical path length of 10 mm for far-UV CD measurements. The temperature was set at 20 °C. Spectra were acquired at a scan speed of 0.5 s/nm, an accumulation number of 10, and a slit width of 1 mm. The ellipticity at 228 or 230 nm was used for monitoring denaturant unfolding.

Influence of PPIases on the Slow Refolding Kinetics of Barstar Cys40Ala/Cys82Ala/Pro27Ala and Barstar Quadruple Variants. Unfolded barstar Cys40Ala/Cys82Ala/Pro27Ala was produced by incubation of the protein in 4 M urea, and unfolded (4-F_2Pro48) and 4(*R*)-FPro48 barstar Cys40Ala/Cys82Ala/Pro27Ala variants were obtained by incubation of the protein in 5 M urea and 4(*S*)-FPro48 barstar Cys40Ala/Cys82Ala/Pro27Ala in 6 M urea, respectively. Incubation was performed in 50 mM sodium phosphate (pH 8.0) for 1 h. Refolding was induced by diluting the unfolded protein in 50 mM sodium phosphate (pH 8.0) to a final protein concentration of 3 μM in 1.6 M urea or 2.5 M urea. The temperature was set at 25 °C. The reaction was monitored by the change in the intrinsic fluorescence at 320 nm after excitation at 280 nm on a FluoroMax-2 instrument (ISA Jobin-Yvon-Spex Instruments S. A., Inc.) using 4 and 10 mm cuvettes. Each value represents the mean of three determinations.

The influence of different PPIases on the refolding of barstar Cys40Ala/Cys82Ala/Pro27Ala and the 4-FPro48 variants was measured by the change in the rate constant of the *trans* → *cis* isomerization both in the presence and in the absence of the respective enzymes. The final concentration of PPIases was 2 μM. Enzyme concentrations were determined by using the known molar absorption coefficients at 280 nm. The catalytic efficiencies of the PPIases used in this study were determined by either the protease-coupled assay (*h*Cyp18, *h*FKBP12, *E. coli* TF) or the protease-free assay (SlyD, *E. coli* Par10) as described here. Suc-Ala-Phe-Pro-Phe-pNA was used for all PPIases investigated except for Cyp18. In this assay, the favorable catalytic efficiency of Suc-Ala-Ala-Pro-Phe-pNA toward Cyp18 was utilized.

The effect of the denaturant on the catalytic efficiency of the PPIases was determined by conducting the assays in the presence of appropriate concentrations of urea. The logarithm of the catalytic activity of the enzymes is found to be linearly dependent on the urea concentration according to the equation $\ln(k_{cat}/K_M) = \ln(k_{cat}/K_M)^0 - m[\text{urea}]$, where k_{cat}/K_M is the catalytic efficiency at a given denaturant concentration,

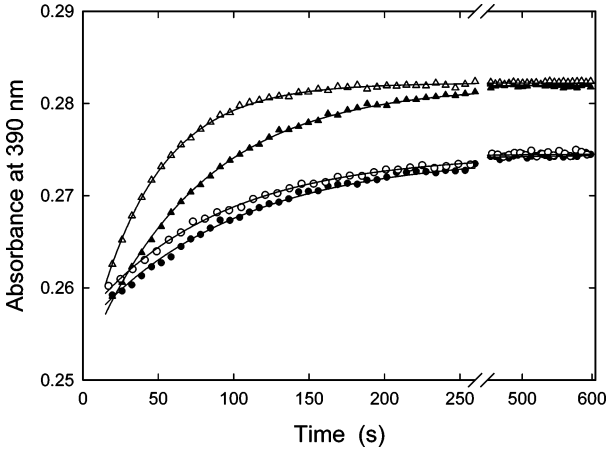

FIGURE 2: Progress curves for the α-chymotrypsin-mediated hydrolysis of Suc-Ala-Ser-4(*S*)-FPro-Phe-pNA in the absence (▲) and presence of 11 nM *h*Cyp18 (△), and hydrolysis of Suc-Ala-Ser-4(*R*)-FPro-Phe-pNA in the absence (●) and presence of 11 nM Cyp18 (○). Data were collected using 35 mM HEPES buffer (pH 7.8) at 10 °C in the presence of 0.83 mg/mL chymotrypsin. The lines show the curve fits for a single-exponential reaction. Suc-Ala-Ser-4(*S*)-FPro-Phe-pNA: (▲) no Cyp18, $\tau = 74$ s; (△) 11 nM Cyp18, $\tau = 42$ s. Suc-Ala-Ser-4(*R*)-FPro-Phe-pNA: (●) no Cyp18, $\tau = 99$ s; (○) 11 nM Cyp18, $\tau = 88$ s.

$(k_{cat}/K_M)^0$ is the catalytic efficiency in water, and *m* is the slope of the logarithmic plot. The catalytic efficiency of the different enzymes was considered by extrapolation to a denaturant concentration of 0 M. The catalytic efficiencies of the PPIases with respect to the barstar proteins were measured at 1.6 M urea and extrapolated to 0 M urea using the slopes *m* determined from the dependence of the enzymatic activity on the respective peptide substrates at different urea concentrations (*25*).

RESULTS

Fluorinated P1′ Residues in Oligopeptides. A series of N-succinylated tetrapeptide 4-nitroanilides of the general sequence Suc-Ala-Ser-Xaa-Phe-pNA, where Xaa is Pro, 4(*S*)-FPro, or 4(*R*)-FPro, has been used to map the S1 subsite of human Cyp18 (*h*Cyp18), human FKBP12 (*h*FKBP12), *E. coli* trigger factor (*E. coli* TF), human Pin1 (*h*Pin1), *E. coli* Par10 (*E. coli* Par10), and human Par14 (*h*Par14). To quantitatively determine the k_{cat}/K_m values, rate constants of spontaneous isomerization and the ratio of *cis*/*trans* isomers for the imidic peptide bond of Suc-Ala-Ser-Xaa-Phe-pNA were measured using isomer-specific proteolysis. This method provides the distinct advantage of simultaneous determination of the *cis*/*trans* ratio and the rate constants of *cis* → *trans* isomerization (*36*) and could be easily adapted for the measurement of oligopeptides containing 4-fluorinated proline residues in the P1′ position (Figure 2). Each analogue was characterized for the spontaneous interconversion dynamics in aqueous solution and the PPIase-catalyzed isomerization. The rate constants of spontaneous isomerization were found to be affected by 4-fluoroproline substitutions. Fluorine confers a small magnitude increase in the rate constants of the forward and reverse isomerization. The *cis*/*trans* isomer ratios were nearly unchanged with the exception of that with the 4(*S*)-FPro substitution (Table 1). Replacement of proline with 4(*S*)-FPro induces a markedly increased *cis* content of 22%, which is caused by a 2-fold faster *trans* to *cis* interconversion. The *cis* content of the 4-F_2Pro peptide is identical to that of the parent Pro compound, because isomerization rates simultaneously increase in both directions. Consequently, the high spontaneous rate constant of *cis* to *trans* isomerization of Suc-Ala-Ser-4-F_2Pro-Phe-pNA prevented a reliable assessment of PPIase catalysis and thus has been omitted from Table 2.

Under the experimental conditions that were used, first-order rate constants of the PPIase-catalyzed *cis* to *trans* interconversion can be converted to k_{cat}/K_m values using protein concentration so that they are identical to the concentration of active enzyme (Figure 2) (*34*). Among the PPIases that were investigated, *h*Par14 and *h*Pin1 at concentrations of up to 1.25 μM and 150 nM, respectively, failed to induce catalytic rate enhancements in the proline and 4-monosubstituted fluoroproline derivatives summarized in Table 2. *h*Cyp18 expressed the highest catalytic activity toward all three peptide derivatives; the introduction of 4-fluoroproline leads to decreases in the catalytic efficiency in the following order: Pro > 4(*S*)-FPro > 4(*R*)-FPro (Table 2). When Cyp18 catalysis was considered, 4(*S*)-FPro- and 4(*R*)-FPro-derived substrates showed 24.3 and 3.1% of the k_{cat}/K_m values, respectively, of that of the parent Pro peptide. Similarly, very low catalytic efficacy must be assumed for the 4(*R*)-FPro-containing peptide with *h*FKBP12 and *E. coli* TF, because a rate enhancement could not be detected at enzyme concentrations of 800 and 400 nM, respectively. Nevertheless, *E. coli* TF catalyzed the 4(*S*)-FPro peptide with ∼15% of the catalytic efficiency obtained for the parent Pro peptide. *h*FKBP12 tolerates the introduction of 4(*S*)-FPro best. In contrast to Cyp18 and FKBPs, *E. coli* Par10 does not tolerate the 4(*S*)-FPro substitution well and its catalytic efficiency decreases below the detection limit, whereas the 4(*R*)-FPro peptide is tolerated relatively well. It exhibits ∼30% of the catalytic efficiency of the Pro peptide.

Fluorinated P1′ Residues in Barstar Quadruple Variants. Next, we analyzed the rates of spontaneous *trans* to *cis* isomerization of the Tyr47−Pro48 bond in the barstar Cys40Ala/Cys82Ala/Pro27Ala variant and its variants containing the 4-FPro48 replacements. It has already been shown that the slowest kinetic phase in refolding of the urea-denatured barstar variant originates from *trans* to *cis* isomerization of the Tyr47−Pro48 peptide bond that is in a *cis* conformation in the native state but is converted into the *trans* isomer in the unfolded chain. The rate constant of the prolyl isomerization-dependent kinetic phase was separated from the main folding phase by ∼3 orders of magnitude (*25*, *37*). Consequently, isomerization rates in the presence and absence of PPIases were measured by a solvent jump from the unfolded state at high urea concentrations to urea concentrations in the pretransition region of the equilibrium unfolding curves (Figure 3). Spontaneous rates of isomerization proved to be independent of the urea concentration. Qualitatively, the 4-FPro48 substitutions affect spontaneous isomerizations in the nativelike folding intermediate of the barstar variants parallel to those observed for the 4-FPro-derived tetrapeptides in that only monosubstitution of fluorine in the 4(*S*) configuration increased the rate constant of *trans* to *cis* isomerization (Table 3). A fluorine substitution in 4(*R*) stereochemistry behaved as an inert hydrogen replacement for both 4(*R*)-FPro- and 4-F_2Pro-derived moieties.

Table 1: Parameters of the *cis*−*trans* Isomerization of Proline- and Fluoroproline-Containing Compounds[a]

chemical compound	% *cis*	K	$k_{cis\rightarrow trans}$ ($\times 10^{-3}$ s^{-1})	$k_{trans\rightarrow cis}$ ($\times 10^{-3}$ s^{-1})
Suc-Ala-Ser-Pro-Phe-pNA	15.4 ± 0.2[c]	5.5[d]	8.3 ± 0.1[e]	1.5[f]
Suc-Ala-Ser-4(*R*)-FPro-Phe-pNA	11.0 ± 0.2[c]	8.1[d]	12.2 ± 0.3[e]	1.5[f]
Suc-Ala-Ser-4(*S*)-FPro-Phe-pNA	22.0 ± 1.0[c]	3.6[d]	11.3 ± 1.3[e]	3.2[f]
Suc-Ala-Ser-4-F_2Pro-Phe-pNA	15.6 ± 0.4[c]	5.4[d]	30.9 ± 2.1[e]	5.7[f]
NAc-Pro-OMe[b]	15.1	5.6	1.39	0.25
NAc-4(*R*)-FPro-OMe[b]	10.7	8.3	3.23	0.39
NAc-4(*S*)-FPro-OMe[b]	27.7	2.6	1.83	0.69
NAc-4-F_2Pro-OMe[b]	20.8	3.8	4.75	1.25

[a] Values resulting from five independent measurements are represented as the mean ± the standard deviation. [b] Parameters were calculated from thermodynamic data measured by NMR (*24*). [c] Measured at 10 °C at substrate concentrations of 25 μg/mL. [d] The equilibrium constant is defined as $K = [trans]/[cis]$, where [*trans*] and [*cis*] are the concentrations of the *trans* and *cis* conformers at equilibrium, respectively. [e] Determined by fitting the progress curve measured in the protease-coupled assay to a single-exponential first-order reaction. [f] Calculated based on the equilibrium constant $K = [trans]/[cis] = k_{cis\rightarrow trans}/k_{trans\rightarrow cis}$, and the rate constant $k_{cis\rightarrow trans}$.

Table 2: Catalytic Efficiency of PPIases on the *cis* → *trans* Isomerization of Proline- and 4-Fluoroproline-Containing Peptides[a]

	k_{cat}/K_M (mM^{-1} s^{-1})			
compound	*h*Cyp18	*h*FKBP12	*E. coli* TF	*E. coli* Par10
Suc-Ala-Ser-Pro-Phe-pNA	3550 ± 420	19 ± 2	78 ± 9	368 ± 73
Suc-Ala-Ser-4(*R*)-FPro-Phe-pNA	111 ± 13	≪10	≪10	112 ± 19
Suc-Ala-Ser-4(*S*)-FPro-Phe-pNA	865 ± 110	26 ± 1	11 ± 1	≪10

[a] Rate constants of the *cis* → *trans* isomerization were determined using the protease-coupled assay both in the absence and in the presence of the PPIase. The peptide was dissolved in DMSO to a concentration of 10 mg/mL. The reaction was started by adding 3 μL of the stock peptide solution to 1.2 mL of 35 mM HEPES buffer (pH 7.8) containing protease (0.83 mg/mL chymotrypsin or 50 μg/mL trypsin) giving a final peptide concentration of 25 μg/mL. The temperature was maintained at 10 °C. The progress curves were fitted to single-exponential first-order kinetics. Values resulting from five independent measurements are represented as the mean ± the standard deviation.

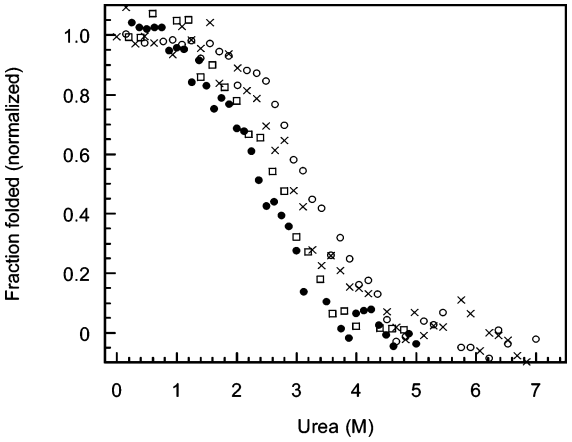

FIGURE 3: Urea unfolding transition of barstar Cys40Ala/Cys82Ala/Pro27Ala triple variant (□) (*25*), its barstar 4(*R*)-FPro48 variant (●), the barstar 4(*S*)-FPro48 variant (○), and the barstar 4-F_2Pro48 variant (×) monitored by far-UV circular dichroism at 228 nm [4(*R*)-FPro48 variant] or 230 nm [4(*S*)-FPro48 and 4-F_2Pro48 variants]. The protein concentration was 2 μM in 50 mM sodium phosphate buffer (pH 8.0). The temperature was set at 20 °C.

The rate acceleration of the slow kinetic phases of 4-FPro48 barstar Cys40Ala/Cys82Ala/Pro27Ala quadruple variants (abbreviated as 4-FPro48 barstar variants) by catalytic amounts of five different PPIases was examined (Figure 4 and Table 3). The list of PPIases included *E. coli* SlyD, a nickel ion-dependent PPIase of the FKBP type known to exhibit remarkable catalytic efficiency toward protein substrates (*38*). The refolding reaction was performed in a final urea denaturant concentration of 1.6−2.5 M. *h*Par14 and *h*Pin1 could not be assayed, because no enzyme activity was detectable at 1.6 M urea toward standard peptide substrates. The $(k_{cat}/K_m)^0$ values found by extrapolation of measured k_{cat}/K_m values to zero urea concentration were calculated according to the method of Golbik et al. (*25*). The enzyme-specific correction factors *m*, though their contribution to the k_{cat}/K_m values of the PPIase-catalyzed refolding of the barstar variants is small, had to be taken into account to include urea-mediated inhibition of PPIase activity in the catalyzed refolding (*25*). The magnitude of *m* resulted from the slope of the linear semilogarithmic plot of the PPIase activity as a function of urea concentration (Table 4).

Unlike the 4-FPro-derived tetrapeptides, which were for the most part less reactive in PPIase catalysis than the parent Pro peptide, 4-FPro48 barstar variants were found to be up to 20-fold more susceptible to PPIase catalysis. Of the many enzyme−substrate combinations that were investigated, the 4-F_2Pro48 barstar variant acting on *E. coli* TF exhibited the highest catalytic activity toward 4-FPro48 barstar variants (Table 3). Unexpectedly, the 4-F_2Pro48 barstar variant proved to be completely inactive when probed with *h*FKBP12, *E. coli* SlyD, and *E. coli* Par10. Furthermore, four of five enzymes differentiate between the 4(*R*)-FPro48 and the 4(*S*)-FPro48 barstar variant with the 4(*S*) stereoisomer at higher catalytic efficacy. *E. coli* Par10 represents the exception with an ~2-fold higher k_{cat}/K_m value for the 4(*R*)-FPro48 barstar variant. Thus, when compared to those of other PPIases, *E. coli* Par10 catalysis displayed an inverse correlation with 4-FPro stereochemistry in both the refolding of barstar variants and the tetrapeptide-based assay.

DISCUSSION

The kinetic experiments described here allow, for the first time, determinations of rate constants of the spontaneous and PPIase-catalyzed *cis* to *trans* and *trans* to *cis* isomerization of 4-fluoroprolyl bonds C- and N-terminally linked to other peptide units. Whereas the influence of 4-fluoroproline residues on spontaneous isomerization rates was found to be rather limited, PPIase-catalyzed isomerization is much

Table 3: Catalytic Activity of Peptidyl Prolyl *cis*/*trans*-Isomerases on Prolyl *trans* → *cis* Isomerization of Barstar Cys40Ala/Cys82Ala/Pro27Ala and L4-FPro48 Variants

		$(k_{cat}/K_M)^0$ (mM^{-1} s^{-1})[b]									
barstar Cys40Ala/Cys82Ala/Pro27Ala Pro48	$k_{trans\rightarrow cis}$[a] (s^{-1})	*h*Cyp18		*h*FKBP12		*E. coli* TF		*E. coli* SlyD		*E. coli* Par10	
Pro[c]	0.0084 ± 0.0003	200.4	100%	14.2	100%	3.5	100%	1.2	100%	45.4	100%
4(*R*)-FPro48[d]	0.0073 ± 0.0003	6.1	3%	13.3	94%	7.9	225%	5.1	420%	40.8	90%
4(*S*)-FPro48[e]	0.0296 ± 0.0017	14.5	7%	19.9	141%	31.2	887%	19.1	1575%	28.4	62%
4-F_2Pro48[d]	0.0301 ± 0.0025	16.3	8%	0	0%	79.2	2250%	0	0%	0	0%

[a] The isomerization rate constant $k_{trans\rightarrow cis}$ of the Tyr47−Pro48 or Tyr47−4-FPro48 bond, respectively, in the barstar Cys40Ala/Cys82Ala/Pro27Ala variants was determined in refolding experiments at 25 °C. All experiments were performed in 50 mM sodium phosphate buffer (pH 8.0). [b] The $(k_{cat}/K_M)^0$ values (mM^{-1} s^{-1}) have been calculated by extrapolation of the measured k_{cat}/K_M values to 0 M urea. The standard deviation of the measurements of the kinetic constants was not larger than 10%. [c] Unfolding in 4 M urea, and refolding in 1.6 M urea. [d] Unfolding in 5 M urea, and refolding in 1.6 M urea. [e] Unfolding in 6 M urea, and refolding in 2.5 M urea.

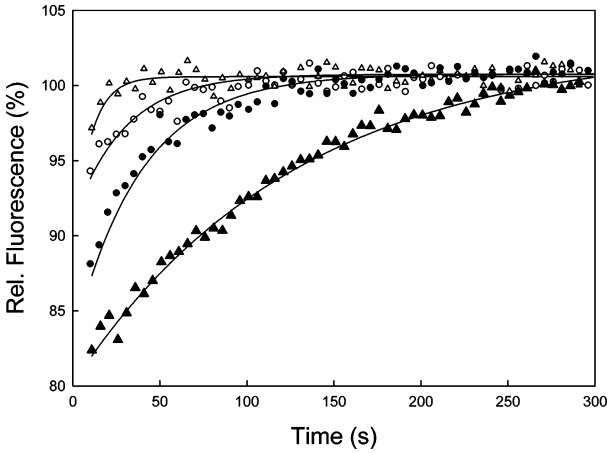

FIGURE 4: Influence of *h*Cyp18 on the proline-limited refolding phase of barstar Cys40Ala/Cys82Ala/Pro27Ala and its 4-F_2Pro48 variant. Progress curves for the slow refolding phase of barstar Cys40Ala/Cys82Ala/Pro27Ala in the absence (▲) and presence of a micromolar level of *h*Cyp18 (△) and of the barstar Cys40Ala/Cys82Ala/Pro27Ala 4-F_2Pro48 variant in the absence (●) and presence of a micromolar level of hCyp18 (○). Refolding experiments were started from 4 M urea-unfolded barstar Cys40Ala/Cys82Ala/Pro27Ala and 5 M urea-unfolded barstar Cys40Ala/Cys82Ala/Pro27Ala 4-F_2Pro48 variant in 50 mM sodium phosphate buffer (pH 8.0) at 25 °C. A value of 100 was assigned to the fluorescence of the native protein in each case. The lines show the curve fits for a single-exponential reaction. Barstar Cys40Ala/Cys82Ala/Pro27Ala: (▲) no Cyp18, $\tau = 125$ s; (△) 2 μM Cyp18, $\tau = 10$ s. Barstar Cys40Ala/Cys82Ala/Pro27Ala 4-F_2Pro48 variant: (●) no Cyp18, $\tau = 35$ s; (○) 2 μM Cyp18, $\tau = 29$ s.

more sensitive. In fact, several PPIases did not catalyze substrates covering a single fluorine atom at the P1′ subsite at all.

Although fluorinated prolines have been used extensively to evaluate the interplay among pyrrolidine ring conformation, torsional angles, and *cis*/*trans* isomer stability in collagen mimics, kinetic data of triple helix formation suggesting a conformational effect underlying fluorine substitutions are lacking (*39*−*43*). A major goal of our enzymatic study was to investigate whether sterically less disruptive substitutions at the P1′ subsite, which is naturally occupied by proline, affect PPIase catalysis in peptide and protein substrates differently. By allowing fluorine to substitute for hydrogen in the proline ring at the P1 position of substrates, as was realized by the 4(*S*)-FPro, 4(*R*)-FPro, and 4-F_2Pro analogues, we still assumed the contribution of the altered P1′ subsite to the catalyzed prolyl isomerization to be multifactorial. As major influences, fluorine substitutions on carbon atoms lead to altered electronegativity (*44*) and increased hydrophobicity (*45*).

Table 4: Urea Dependence of the Specific Activity of PPIases toward Oligopeptide Substrates

PPIase	slope of the logarithmic plot[a]	*m*[e]
*h*Cyp18	−0.575[b,d]	0.92
*h*FKBP12	−0.556[b]	0.89
E. coli TF	−1.106[b]	1.77
E. coli SlyD	−0.988[c]	1.58
E. coli Par10	−1.062[c]	1.70

[a] The enzymatic activity of the different PPIases was determined at different urea concentrations. The enzymes were first incubated in 35 mM HEPES (pH 7.8) at 25 °C for 10 min. An aliquot of the incubation solution was added to the assay mixture containing the same concentration of urea in 35 mM HEPES (pH 7.8) and 0.83 mg/mL protease (protease-coupled test). [b] The assay mixture contained 2 μM BSA (protease-free test). [c] The assay mixture was incubated at 10 °C for 5 min. The reaction was started by adding 2−3 μL of the peptide substrate Suc-Ala-Phe-Pro-Phe-pNA. In the protease-coupled method, the substrate was dissolved in DMSO, giving a stock concentration of 10 mg/mL. In the protease-free method, the substrate was dissolved in a 5.5 M LiCl/TFE mixture giving a stock concentration of 20 mg/mL. Enzyme concentrations used were 4.57 nM [*h*Cyp18 (*25*)], 25 nM (*h*FKBP12), 15 nM (*E. coli* TF), 220 nM (*E. coli* SlyD), and 10 nM (*E. coli* Par10). [d] Suc-Ala-Ala-Pro-Phe-pNA was used as the substrate. [e] $\ln(k_{cat}/K_M) = \ln(k_{cat}/K_M)^0 - m[\text{urea}]$.

Notably, a complete data set for spontaneous isomerization rates is inevitably required to determine enzymatic parameters of PPIase-catalyzed reactions. Thus, rates of spontaneous forward and backward *cis*−*trans* isomerization in the 4-fluoroproline-derived tetrapeptides had to be determined. They differ uniformly from those observed in the respective N-acetylated 4-fluoroproline methyl ester reported previously (*24*, *46*) by a factor of 4−6 in favor of the faster rates for the tetrapeptides (Table 2). This factor is similar to that observed for the parent proline compounds. A similar comparison for the rates of *trans* to *cis* isomerization in tetrapeptides with barstar variants gave a 5−9-fold higher rate constant for the protein, indicating a rather small degree of fluorine-specific variability. For those compounds for which the comparisons have been made, both the residues flanking the prolyl bond and the reaction temperature contribute to the different isomerization rates summarized in Tables 1 and 3. However, within the series of proline mimics, these factors remained constant.

These data reveal that the effects thought to be potentially important for the interaction between 4-fluorine atoms and the prolyl C−N bond order, such as electronegativity (*24*, *46*), stereoelectronic contributions (*47*), ring puckering (*48*),

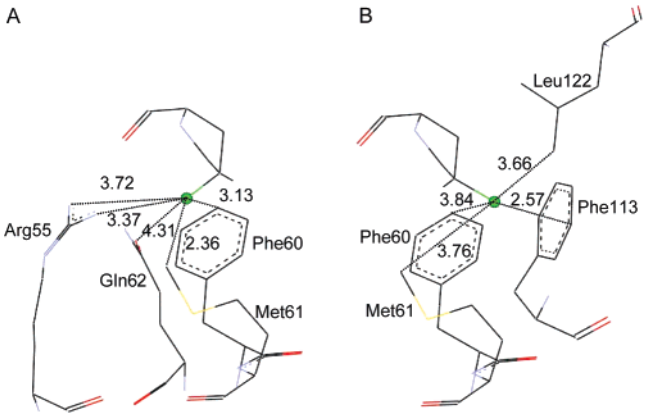

FIGURE 5: Schematic presentation of distances of 4-FPro substrates bound in the acitve site of *h*Cyp18 to all amino acid residues within 4.5 Å. The fluorine of (A) 4(*S*)-fluoroproline and (B) 4(*R*)-fluoroproline of an oligopeptide substrate is shown as a green sphere. The figure was generated using WebLab ViewerPro based on the *h*Cyp18 structure in complex with Suc-Ala-Ala-Pro-Phe-pNA (PDB entry 1RMH) (*58*).

and conformational constraints (*49*), are not markedly dependent on the nature of functional groups surrounding a prolyl bond. Thus, these findings are consistent with the notion that 4-fluoroproline methyl esters represent a useful model for dissecting the relative importance of single components of substituent effects of fluorine-bearing proline mimics in polypeptides (*24*, *46*).

On the basis of these results, a comparative reactivity scale of the enzyme-catalyzed acceleration of prolyl isomerization of 4-fluoroproline substrates with a changed P1′ subsite is available and has added significantly to the database of substrate specificity of PPIases. Cyclophilins, FKBPs, and parvulins represent protein families currently known to be associated with PPIase activity, whose primary physiological functions, cellular substrates, and mechanisms of catalysis are still not well understood. The fact that k_{cat}/K_m values for PPIase-catalyzed prolyl isomerizations were mostly decreased by fluorine substitution at position 4 of P1′ proline in tetrapeptides but do not respond similarly in corresponding barstar variants indicates that there are additional forces around the 4-position of proline affecting the transition state of isomerization in polypeptide substrates exclusively. Stereospecific positioning of the fluorine substituent relative to the active site surface seems to be very important in adjusting the fit of the substrates within the active site cleft of the enzymes (Figure 5). The 4(*S*)-FPro substitution confers catalytic susceptibility to substrates significantly more than the 4(*R*)-diastereomer throughout the enzyme families regardless of whether the enzymes reacted with tetrapeptides or proteins. A single exception was *E. coli* Par10, which shows the inverse stereochemical preference in that it tolerates the 4(*R*)-FPro substitution better than the 4(*S*)-FPro substitution (Tables 2 and 3). Interestingly, superimposition of the active site residues of *h*Cyp18 and the human parvulin *h*Pin1 revealed that two residues of the *h*Cyp18 active site, Phe60 and His126, exhibit mirror symmetry with respect to *h*Pin1 residues His157 and Phe125 (*50*). This mirror symmetry of respective residues His9 and Phe52 was also observed in *E. coli* Par10 (*51*). We assume that these two residues are involved in the correct spatial positioning of the proline ring as a prerequisite for catalysis. If the P1′ substitution is situated in tetrapeptides, FKBPs showed the most severe stereospecific discrimination in that no catalysis was observed for the 4(*R*)-FPro peptide. The 4(*S*)-FPro-mediated inertness of the peptide substrate toward *E. coli* Par10 is an example of the reversed situation. Perhaps the most striking aspect of this comparison is the considerable activity of the barstar variants for those diastereomeric 4-FPro replacements conferring inertness to tetrapeptides. In these cases, the power of stereospecific discrimination is lost for both FKBPs and *E. coli* Par10. Of course, there are two obvious differences between the catalyzed isomerization of peptides and proteins. First, inspection of amino acids flanking the P1′ site reveals differences for both types of substrates, because the tetrapeptide and the barstar variants contain -Ser-4-FPro-Phe- and -Tyr-4-FPro-Leu- segments, respectively. However, we would argue that both substrate types containing the parent proline exhibited a qualitatively similar pattern of k_{cat}/K_m values for all PPIases investigated. Second, slow kinetics observed in barstar refolding correspond to the *trans* to *cis* isomerization in the reverse direction of the reaction monitored by the protease-coupled assay in tetrapeptides. However, direct proportionality of both k_{cat}/K_m values can be assumed according to the Haldane equation (*52*). These points are important to bear in mind in attempts to analyze protein versus peptide substrates. Our observations would suggest that a P1′ subsite-mediated deterioration in k_{cat}/K_m can be compensated by remote subsites of the protein substrates, arguing for cumulative subsite interactions as the driving force determining the substrate specificity of FKBPs and *E. coli* Par10. Assuming that a corresponding relationship exists for position P1, the prediction of protein substrates of FKBP12 simply on the basis of preference for hydrophobic side chains derived from tetrapeptide assays (*17*, *53*) must be considered with caution. The 4-F_2Pro48 barstar variant deserves special attention as, in this case, substituent effects vary over an extreme range: from a value of k_{cat}/K_m ~22-fold higher for *E. coli* TF to values up to 90-fold lower for *E. coli* Par10 when compared with that of the Pro48 barstar variant. Unfortunately, high spontaneous rates of *cis* to *trans* isomerization prevented us from assessing PPIase catalysis in 4-F_2Pro-substituted tetrapeptides. *E. coli* TF belongs to the FKBP family of PPIases with hydrophobicity being a dominant property of its active site (*54*). The higher hydrophobicity that probably accompanies fluorine substitution in a proline CH group (*45*) might favor transfer of the substrate from bulk water to the transition state configuration of the active site and thus increases k_{cat}/K_m values.

A possible cause of the detrimental impact of the second fluorine atom on catalysis by the other FKBPs and *E. coli* Par10 might be rooted in its electron-withdrawing effect that lowers the immonium pK_a of 4-F_2Pro ~2 orders of magnitude when compared to that of the 4-monosubstituted fluoroproline (*46*). Considering the high electron density on the ring nitrogen in the perpendicularly arranged prolyl bond of the putative transition state of isomerization (*9*), an enzyme can exhibit a marked free energy difference between transition state binding and ground state binding at the P1′ subsite. According to the immonium pK_a value, the inductive effect of two fluorine atoms markedly reduces this energy difference, disfavoring transition state binding and thus catalysis. The overall conclusion is that the P1′ specificity of cyclo-

philins and FKBPs as well as prototypic parvulin was strongly dependent on an extended binding region of a substrate backbone or a sequence context of the polypeptide chain and not necessarily on the residues adjacent to the prolyl bond.

From the foregoing account, it can be hypothesized that individual PPIases can utilize different transition states or even distinct catalytic pathways. Evidence implicating family-specific mechanisms of catalysis has been found (*5*, *55*−*57*). With the identification of intrafamilial variability in the response of FKBP toward P1′ subsite variations, we can now expect considerable differences for the activation of the catalytic machinery among members of a particular family of PPIases.

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