Mapping the stereospecificity of peptidyl prolyl cis/trans isomerases

ARTICLE *in* FEBS LETTERS · SEPTEMBER 1998

Impact Factor: 3.17 · DOI: 10.1016/S0014-5793(98)00871-0 · Source: PubMed

4 AUTHORS, INCLUDING:

Ulf Reimer
Jpt Peptide Technologies
55 PUBLICATIONS **1,687** CITATIONS
SEE PROFILE

Mike Schutkowski
Martin Luther University Halle-Wittenberg
114 PUBLICATIONS **3,496** CITATIONS
SEE PROFILE

Gunter Fischer
Max Planck Institute for Biophysical Chemi…
296 PUBLICATIONS **11,977** CITATIONS
SEE PROFILE

FEBS Letters 432 (1998) 202–206 FEBS 20633

Mapping the stereospecificity of peptidyl prolyl *cis/trans* isomerases

Cordelia Schiene, Ulf Reimer, Mike Schutkowski, Gunter Fischer*

Max-Planck Society, Research Unit 'Enzymology of Protein Folding', Kurt-Mothes-Str. 3, D-06120 Halle/Saale, Germany

Received 15 June 1998; revised version received 10 July 1998

Abstract The stereospecificity of peptidyl prolyl *cis/trans* isomerases (PPIases) was studied using tetrapeptide substrate analogs in which one amino acid residue was replaced by the cognate D-amino acid in various positions of the peptide chain. Reversed stereocenters around proline markedly increased the rate of the spontaneous *trans* to *cis* isomerization of the prolyl bond whereas *cis* to *trans* isomerizations were less sensitive. PPIases like human cyclophilin18, human FKBP12, *Escherichia coli* parvulin10 and the PPIase domain of *E. coli* trigger factor exhibited stereoselectivity demanding at the P_1 to P_2' position of the substrate chain. The discriminating factor for stereoselectivity was the lack of formation of the Michaelis complexes of the diastereomeric substrates. However, D-alanine at the P_1 position preserved considerable affinity to the active site, and largely prevented activation of the catalytic machinery for all PPIases investigated.

Key words: Peptidyl prolyl *cis/trans* isomerase; Stereospecificity; Proline; *Cis/trans* isomerization; Enzyme mechanism

1. Introduction

Peptidyl prolyl *cis/trans* isomerases are enzymes specifically evolved to catalyze the *cis/trans* isomerization of prolyl bonds[1]. They comprise three families of proteins, cyclophilins, the FK506-binding proteins (FKBPs) [2] and its related trigger factor [3], and the parvulins [4]. They differ in the amino acid sequence of the catalytic core, the substrate specificity and the sensitivity for inhibitors. For example the immunosuppressants cyclosporin A and FK506 selectively inhibit their partner cyclophilins and FKBPs. PPIases appear to be important for a number of cellular processes including folding of nascent polypeptide chains, transport of proteins [5], viral replication [6–8], and regulation of the cell cycle [9–12]. They also provide a tool to identify proline-limited rate processes during the refolding of denatured proteins [13] like RNase T1 [14], human carbonic anhydrase II [15] and antibody Fab fragment [16].

Powerful catalysis up to a diffusion controlled rate limit for the bimolecular catalytic constant was frequently observed. Nevertheless, it is still unclear how PPIases mediate catalysis. However, it is quite obvious that binding of an aminoacylprolineamide to the active site of PPIases, which completely encompasses the P_1 and P_1' secondary binding sites[2], is insufficient to liberate even a portion of the catalytic power. On the other hand a strict requirement for important features of the P_1-P_1' moiety, namely the prolyl carbonyl group and some essential ring system has been noted [17,18], indicating that extended subsites can only form productive interactions in the context of an authentic prolyl segment. The positions P_2 to P_3' of a substrate were already shown to interact with the active site of PPIases in a productive manner as shown by bimolecular rate constants of truncated substrates [2,19]. In a human Cyp18/oligopeptide complex, in which a 25-mer peptide of the HIV-1 p24 capside protein may represent a segment of a natural substrate, 12 amino acids could be traced as an extended backbone supplemented by a 3_{10} helix using the X-ray structure as a probe for the structure [20]. Nine substrate residues flanking the rotating prolyl bond interacted with the protein and these secondary binding sites may potentially enhance enzyme catalysis. Quantifying catalysis of protein folding [21] did not provide the hoped-for insight into the extension of the catalytically relevant subsites because of the limited accessibility of the targeted prolyl bond in rapidly formed folding intermediates.

Once a PPIase is catalytically activated by interactions of remote subsites various chemical mechanisms are hypothesized to become operative for rate acceleration [2,22–26]. They may act either alone or in combination. The specificity of FKBP12 and *E. coli* parvulin10 regarding the P_1 position seems to be dependent only on the hydrophobicity of the side chain of the amino acid preceding proline [4,27,28]. In this respect Cyp18 has an even more promiscuous substrate characteristics [27]. Contrastingly, the human parvulin-like PPIase Pin1 and the yeast ESS1 catalyze phosphorylation-dependent prolyl isomerizations in a highly specific manner for phosphoSer/Thr-Pro moieties. Even if enzyme activity of Pin1 has been found for other charged groups at the P_1 position of substrates, full activity of catalysis occurs only for the covalently linked dianionic form of the phosphoSer/Thr-Pro motif. It has been proposed that a kinetically significant, single proton dissociation at His^{59} of Pin1 leads to catalytically active enzyme [29]. On the other hand, a cluster of basic amino acid residues in the S_1 site seems to be responsible for the extraordinary phosphopeptide specificity [10,30], which may be closely related to the cellular substrates of the enzymes.

Evaluating the stereoselectivity of the currently known

*Corresponding author. Fax: (49) (345) 55 11 972.

Abbreviations: Abz, 2-aminobenzoyl residue; CsA, cyclosporin A; Cyp18, recombinant human cytosolic cyclophilin with molecular mass of 18 kDa; DMSO, dimethyl sulfoxide; FKBP, FK506 binding protein; HPLC, high performance liquid chromatography; -NH-Np, 4-nitroanilide; PPIase, peptidyl prolyl *cis/trans* isomerase; TF domain, *Escherichia coli* trigger factor PPIase domain (segment Arg^{145} to Glu^{251}); TLC, thin layer chromatography

[1] The term prolyl isomerization is used throughout the paper for the *cis/trans* isomerization of the peptide bond preceding proline in an amino acid sequence. Similarly, the term prolyl bond is synonymous with the peptide bond preceding proline.

[2] Derived nomenclature of subsites according to Schechter and Berger [1].

PII: S0014-5793(98)00871-0

three PPIase families and the FKBP subfamily would identify not only subsites critically important for approaching the transition state but may also indicate minor differences in the activation of the catalytic machinery of the enzyme families. We used a D-amino acid scan from position P_2 to P_2' of the common substrate Ala-Ala-Pro-Phe-NH-Np to examine both enzyme binding and catalysis. We were able to demonstrate that the marked stereoselectivity for human Cyp18, human FKBP12, *Escherichia coli* parvulin10 and *E. coli* trigger factor is coupled to different steps of enzyme catalysis.

2. Materials and methods

2.1. Materials

Subtilisin Carlsberg type VIII was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin was a product from Roth (Karlsruhe, Germany). Buffers were from Serva (Heidelberg, Germany). Human recombinant Cyp18 [31], *E. coli* parvulin10 [32], *E. coli* trigger factor domain [33] and human recombinant FKBP12 [34] were purified according to published procedures. Suc-Ala-Ala-Pro-Arg-NH-Np was purchased from Bachem (Heidelberg, Germany).

2.2. Peptide synthesis

The synthesis of 4-nitroanilide derivatives Phe-NH-Np, D-Phe-NH-Np and Ala-Ala-Pro-Phe-NH-Np were described previously [35,17]. The tetrapeptide-4-nitroanilides were synthesized using standard procedures and the corresponding diastereomers were generated in an analogous manner using the protected amino acid in D-configuration as a building block.

The *tert*.-butyloxycarbonyl (Boc) moiety was used for temporary protection of the amino function. The mixed anhydride method (isobutylchloroformiate/*N*-methylmorpholine/THF) was used for the generation of amide bonds. All compounds were deprotected acidolytically using 1.3 N HCl in acetic acid. After deprotection of the tetrapeptide derivatives the products were purified by preparative HPLC using different acetonitrile water mixtures containing 0.1% trifluoroacetic acid as eluent yielding white powders after lyophilization.

All peptides were characterized by ES-MS, ^{13}C- and ^{1}H-NMR spectroscopy. The purity of all compounds was checked by TLC, HPLC and for the deprotected substances by capillary electrophoresis.

2.3. Kinetic studies

2.3.1. Catalytic efficiency of subtilisin. Sufficiently high k_{cat} and k_{cat}/K_m values for the subtilisin mediated cleavage of the chromogenic substrates are the prerequisites for determining prolyl isomerization rates by isomer-specific proteolysis. Measurements of the initial rates of 4-nitroanilide cleavage were initiated by addition of protease stock solution (76 μM, final concentration 1 μM) to the reaction mixture containing x μl peptide stock solution (40 mM, final concentration 40–70 μM) and y μl 35 mM HEPES buffer pH 7.8 ($x+y=1200$ μl), and enzyme parameters were evaluated according to Schutkowski et al. [17]. Except for the peptide Ala-Ala-Pro-D-Phe-NH-Np that has a very low cleavage rate for the 4-nitroanilide bond, D-Ala-Ala-Pro-Phe-NH-Np ($k_{cat}=0.4\pm0.06$ s^{-1}; $K_m=474\pm37$ μM), Ala-D-Ala-Pro-Phe-NH-Np ($k_{cat}=2.6\pm0.27$ s^{-1}; $K_m=3030\pm410$ μM), Ala-Ala-D-Pro-Phe-NH-Np ($k_{cat}=0.2\pm0.02$ s^{-1}; $K_m=1800\pm380$ μM), Ala-Ala-Pro-Phe-NH-Np ($k_{cat}=6.4\pm0.47$ s^{-1}; $K_m=581\pm75$ μM) and Abz-Ala-Ala-Pro-Phe-NH-Np ($k_{cat}=2.8\pm0.05$ s^{-1}; $K_m=16.5\pm1.2$ μM) met the kinetic criteria of the protease-coupled PPIase assay.

2.3.2. Catalytic constants of PPIases. Using the subtilisin coupled assay the kinetic constants of the *cis*/*trans* isomerization of the peptide derivatives were measured on a Applied Photophysics stopped-flow reaction analyzer at 10°C. Peptide derivatives were dissolved in 100 μl DMSO (2 mM) and diluted with 900 μl degassed HEPES buffer (35 mM) pH 7.8. The enzyme stock solution was prepared by dissolving 22 mg of subtilisin Carlsberg in 200 μl 1 mM HCl. Finally, the mixture was diluted with reaction buffer. The final concentrations in a typical assay were 400 μM subtilisin, 10 μM peptide derivatives and variable concentrations of PPIase in the range of 0.1–5 μM. PPIase concentration was determined by spectral analysis at 280 nm for FKBP12, parvulin and TF domain. Concentration of active Cyp18 was determined by fluorimetric titration with cyclosporin A [52].

Determination of the kinetic constants of the *cis*/*trans* isomerization of the peptide containing D-Phe in P_2' position was performed using fluorescence measurements according to Garcia-Echeverria et al. [36]. These measurements were performed on a Hitachi F-3010 fluorescence spectrophotometer at 10°C with Abz-Ala-Ala-Pro-Phe-NH-Np and Abz-Ala-Ala-Pro-D-Phe-NH-Np as substrate and in the presence of PPIase. Peptide derivatives (10 mM) were dissolved in anhydrous trifluoroethanol/0.5 M LiCl. The measurement of the *cis*/*trans* isomerization was initiated by addition of the peptide derivative solution to a mixture of 35 mM HEPES pH 7.8 (final concentration 20 μM) and the PPIase in concentrations of 0.1–5 μM. Excitation wavelength was 320 nm with a spectral bandwidth of 3 nm. Emission was detected at 416 nm with a spectral bandwidth of 10 nm. Data analysis was performed by single exponential non-linear regression using SigmaPlot Scientific Graphing System Vers. 2.00 (Jandel Corp., USA). Determination of the IC_{50} values was performed according to Schutkowski et al. [17]. Briefly, measurements of the rate constant for the PPIase catalyzed isomerization of Suc-Ala-Ala-Pro-Arg-NH-Np (40 μM) in the absence and presence of the peptides containing the D-amino acid (5 concentrations up to 2 mM competing peptide) were performed using the coupled assay with trypsin as an isomer-specific protease at 10°C.

2.4. NMR measurements

All NMR measurements were carried out on a Bruker ARX 500 NMR spectrometer. Spectra were referenced to internal TSP at 0 ppm. The *cis* content of the peptides was determined by integration of the *cis* and *trans* signals respectively. For measurement of catalysis we recorded phase sensitive NOESY spectra to observe the existence or lack of exchange peaks between the *cis* and *trans* conformer. We used a mixing time of 300 ms for these NOESY experiments. The spectral width was typically 5050 Hz. As many as 512 t1 increments were recorded with 32 scans per increment. For the corresponding peptides a set of three experiments was performed. In the case of Ala-Ala-Pro-D-Phe-NH-Np the first EXSY spectrum was measured for the peptide in solution. After addition of 30 μM cyclophilin a set of exchange peaks was observable. These exchange peaks disappeared after addition of CsA. In the case of the peptide containing D-Pro no exchange peaks were observed after addition of up to 60 μM cyclophilin. After

Table 1
Thermodynamic equilibrium and kinetic constant of the *cis* to *trans* isomerization

D-Amino acid in position	Tetrapeptide derivative	*cis* content (%) in H_2O	$k_{cis\rightarrow trans}$ (10^{-3} s^{-1})[a]	$k_{trans\rightarrow cis}$ (10^{-3} s^{-1})[d]
	Ala-Ala-Pro-Phe-NH-Np	10.7[b]	6.8 ± 0.47	0.81
	Abz-Ala-Ala-Pro-Phe-NH-Np	20.1	4.7 ± 0.41	1.18
P_2	D-Ala-Ala-Pro-Phe-NH-Np	11.1[b]	4.4 ± 0.27	0.55
P_1	Ala-D-Ala-Pro-Phe-NH-Np	22.5[b,c]	5.7 ± 0.11	1.65
P_1'	Ala-Ala-D-Pro-Phe-NH-Np	24.5[c]	4.5 ± 0.31	1.46
P_2'	Ala-Ala-Pro-D-Phe-NH-Np	6.5[c]	n.d.	n.d.
P_2'	Abz-Ala-Ala-Pro-D-Phe-NH-Np	16.0[c]	6.6[d]	1.26

[a]Error limits are standard deviations of the means of 3 or 4 determinations.
[b]The data were obtained using subtilisin as isomer-specific protease according to Fischer et al. [35] in 35 mM HEPES pH 7.8 at 10°C.
[c]The data were obtained using ^{1}H-NMR spectroscopy.
[d]Data are calculated.

Table 2
Catalytic efficiency of Cyp18, parvulin, *E. coli* trigger factor domain and FKBP12

Tetrapeptide derivative	k_{cat}/K_m ($M^{-1}s^{-1}$) Cyp18	k_{cat}/K_m ($M^{-1}s^{-1}$) Parvulin	k_{cat}/K_m ($M^{-1}s^{-1}$) TF domain	k_{cat}/K_m ($M^{-1}s^{-1}$) FKBP12
Ala-Ala-Pro-Phe-NH-Np	$(5.1 \pm 0.5) \times 10^6$	$(2.6 \pm 0.3) \times 10^5$	$(1.3 \pm 0.1) \times 10^5$	$(1.4 \pm 0.09) \times 10^5$
D-Ala-Ala-Pro-Phe-NH-Np	$(3.4 \pm 0.2) \times 10^6$	$(1.1 \pm 0.1) \times 10^5$	$(8.4 \pm 0.9) \times 10^4$	$(3.3 \pm 0.2) \times 10^4$
Ala-D-Ala-Pro-Phe-NH-Np	$(1.3 \pm 0.1) \times 10^2$	$(1.6 \pm 0.09) \times 10^3$	$(2.5 \pm 0.09) \times 10^2$	[b]
Ala-Ala-D-Pro-Phe-NH-Np	[b]	[b]	[b]	[b]
Abz-Ala-Ala-Pro-Phe-NH-Np[a]	$(7.2 \pm 0.2) \times 10^6$	$(3.4 \pm 0.7) \times 10^5$	$(4.3 \pm 0.2) \times 10^5$	$(7.7 \pm 1.5) \times 10^4$
Abz-Ala-Ala-Pro-D-Phe-NH-Np[a]	$(2.6 \pm 0.4) \times 10^3$	[b]	[b]	[b]

All data were obtained using the coupled assay with subtilisin as isomer-specific protease in 35 mM HEPES pH 7.8 at 10°C.
[a]Data were obtained by using fluorescence measurements in 35 mM HEPES pH 7.8 at 10°C.
[b]The catalytic efficiency was below the detection limit of 100 M^{-1} s^{-1}.

heating of the peptide sample to 42°C the exchange cross peaks appeared to indicate a faster uncatalyzed prolyl isomerization.

3. Results

After establishing conditions for isomer-specific proteolysis in the presence of subtilisin comparative studies of diastereomeric pairs of tetrapeptides were performed at pH 7.8 and 10°C. Due to the unfavorable stability of the anilide bond of Ala-Ala-Pro-D-Phe-NH-Np toward subtilisin and chymotrypsin an uncoupled fluorogenic assay was used to measure directly the *cis*/*trans* isomerization rates of Abz-Ala-Ala-Pro-Phe-NH-Np and Abz-Ala-Ala-Pro-D-Phe-NH-Np. Rate constants of the spontaneous *cis* to *trans* isomerization are listed together with isomer ratios in Table 1. Considerable influence of the chirality of the Cα atom on the ratio of prolyl bond isomers was recognized for the diastereomeric peptides except for D-alanine in P_2 position. Because the *cis* to *trans* isomerization rates remained almost constant the reversed *trans* to *cis* direction of rotation proved to be sensitive to Cα stereochemistry.

The kinetic consequences of reversing a stereocenter at various positions of a tetrapeptide substrate was investigated for the PPIase activity of human recombinant Cyp18, *E. coli* parvulin10, *E. coli* trigger factor domain and human FKBP12, each representing the catalytic core of the respective enzyme family (Table 2).

The incorporation of D-alanine into the P_2 position caused an constantly small decrease of k_{cat}/K_m for all examined PPIases, thus representing the most insensitive position within the peptides series. An up to 10^4 fold reduction in k_{cat}/K_m was found for the derivative with D-alanine in P_1 position for the catalysis by Cyp18, parvulin and TF domain. The reduction for the FKBP12 may be very large because the activity was below the detection limit of catalysis. Even more detrimental to enzyme function is D-proline in P_1' position, yielding peptides devoid of substrate properties in the measurement limits (Table 2).

Due to its protease stability described above Ala-Ala-Pro-D-Phe-NH-Np, which is stereochemically altered in P_2' position, was investigated by ^{1}H-NMR spectroscopy for a qualitative description of Cyp18 catalysis. As much as 30 μM Cyp18 was required to produce an additional set of NOESY crosspeaks indicating its poor substrate nature. Crosspeaks disappeared after inhibition of Cyp18 enzyme activity by cyclosporin A [37]. A fluorogenic assay similar to that of Garcia-Echeverria et al. [36] was performed for derivatives containing an N-terminal 2-aminobenzoyl fluorophore and a C-terminal 4-nitroanilide quencher. The fluorescence signal at 416 nm subsequent to a solvent jump was evaluated in the time-course in the presence of PPIases (Fig. 1). In this assay, the D-Phe substitution at P_1' position decreased catalytic efficiency by at least three orders of magnitude for all PPIases (Table 2).

In order to evaluate whether the stereoselectivity happens at the formation or interconversion of the Michaelis complexes both diastereomeric peptides and the reporter all-L substrate Suc-Ala-Ala-Pro-Arg-NH-Np were allowed to compete for entry into the active site. Since the diastereomeric peptides were inert toward 30 μM trypsin, the trypsin-coupled isomer-specific assay was carried out. The affinity of the reporter substrate for the active site of Cyp18 was indicated by the K_m value of 0.5 mM *cis* peptide. The IC_{50} values represent remaining enzyme activity, and were calculated from a series of assays in the presence of 0.2–2 mM concentrations of diastereomeric peptide derivatives (Table 3). In a reference experiment the standard peptide Ala-Ala-Pro-Phe-NH-Np had an affinity to the active site of the PPIases similar to the reporter substrate. However, a D-amino acid in P_1' or P_2' position prevented from or markedly reduced binding to PPIases. Only the *E. coli* trigger factor catalytic domain is less effective in discriminating L and D stereochemistry at these positions.

Table 3
IC_{50} values of tetrapeptide derivatives interacting with peptidyl prolyl *cis*/*trans* isomerases

Tetrapeptide derivative	IC_{50} Cyp18 (μM)	IC_{50} parvulin (μM)	IC_{50} TF domain (μM)	IC_{50} hFKBP12 (μM)
Ala-Ala-Pro-Phe-NH-Np	511 ± 50	274 ± 22	94 ± 7	299 ± 25
D-Ala-Ala-Pro-Phe-NH-Np	664 ± 93	398 ± 47	155 ± 16	137 ± 11
Ala-D-Ala-Pro-Phe-NH-Np	1029 ± 78	362 ± 27	189 ± 12	2620 ± 280
Ala-Ala-D-Pro-Phe-NH-Np	[a]	[a]	681 ± 53	[a]
Ala-Ala-Pro-D-Phe-NH-Np	[a]	4560 ± 490	2350 ± 190	[a]

All measurements were obtained using the coupled assay with trypsin as isomer-specific protease and Suc-Ala-Ala-Pro-Arg-NH-Np (40 μM) as substrate in 35 mM HEPES pH 7.8 at 10°C.
[a]The enzymatic activity was not affected up to final peptide concentrations of 2 mM.

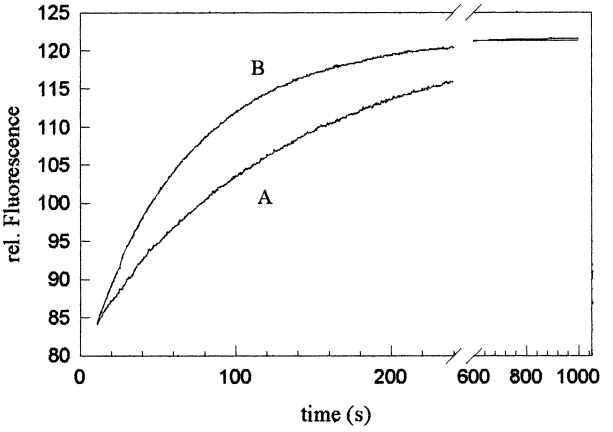

Fig. 1. Time course of fluorescence at 416 nm after jumping from the peptide stock solution in TFE/LiCl into the final buffer solution using 20 μM Abz-Ala-Ala-Pro-Phe-NH-Np at 10°C. A, uncatalyzed ($k = 7.9 \times 10^3$ s^{-1}); B, 1 nM rhCyp18cy ($k = 15.3 \times 10^3$ s^{-1}). Measurements were done in 35 mM HEPES pH 7.8, $\lambda_{ex} = 320$ nm.

Surprisingly, the P_1 stereocenter in D configuration which dramatically inhibits the catalysis retained considerable affinity to the active sites of the PPIases, except for FKBP12 having low affinity to Ala-D-Ala-Pro-Phe-NH-Np.

As was already shown in Tables 2 and 3 the chirality of the P_2 position did not contribute much to active site affinity, nor affects enzymatic activity.

4. Discussion

It is evident from our studies that prolyl *cis*/*trans* isomerization of diastereomeric tetrapeptides is sensitive to reversed stereochemistry at the Cα atom in a positional manner. The altered chirality at P_2 position, did not substantially affect rates of spontaneous rotation or catalysis by PPIases (Tables 1 and 2). We speculate that the remote sites from the prolyl bond in protein substrates have little effect on catalysis of *cis*/*trans* isomerization.

Positions adjacent to proline were found to be critical for spontaneous bond rotation. Compared to L-amino acids, D-amino acids in P_1 and P_1' position favored the amount of *cis* conformer in the equilibrium although both D- and L-amino acids have similar *cis* to *trans* isomerization rates. Therefore Cα chirality affects prolyl isomerizations mainly due to destabilization of the planar *trans* isomer relative to the twisted transition state of rotation. In dimethylsulfoxide a similar preference of the *cis* conformer has been observed for D-Phe at the site preceding proline of morphiceptin analogs [38], whereas the amount of *cis* conformer is decreased for the D-amino acid substitutions in charged dipeptides [39].

Positions of reversed chirality shown to be critical for spontaneous isomerizations have also been found to exert considerable influence on catalysis of prolyl isomerization by PPIases. In fact, the presence of an amino acid in D configuration had markedly detrimental effects on the catalysis by all PPIase families. The failure of PPIases to utilize a D-Pro containing peptide could be experimentally attributed to the lack of formation of the non-covalent enzyme/substrate complex. Only the trigger factor catalytic domain acts as a promiscuous binder for the D-Pro peptide, underlining the special nature of the trigger factor subfamily of FKBPs [40]. This finding is paralleled by the fact that trigger factor has considerable affinity to proteins lacking proline within their binding segments. [41]. This ligand promiscuity, originally assigned to the complementary domains of the full-length protein, can now be attributed to the catalytic domain as well.

A result that we consider obvious is that reversing the P_1 stereocenter did not markedly affect formation of the adsorptive enzyme/peptide complex but greatly reduced or abolished catalysis. The X-ray structure revealed that binding of Ala-D-Ala-Pro-Phe-NH-Np to the active site of Cyp18 is similar to the all-L amino acid congener (Zhao et al., submitted). Thus, non-specific binding cannot be identified as the cause of insensitivity to catalytic interconversions. Since -Gly-Pro- peptides can be effectively interconverted by Cyp18 [42] the Ala side chain methyl preceding proline is not absolutely required to assist the activation of the catalytic machinery of the PPIase. Taken together these results point out that a ground state P_1 diastereomeric complex may be resistant to undergo considerable spatial rearrangements toward of the transition state due to steric hindrance of the wrongly positioned Ala side chain. Thus, the pathway toward the enzymatic transition state has to be considered sterically demanding. Indeed, essential feature of several mechanisms discussed for PPIase catalysis is the large change of the spatial geometry when isomerization proceeds [22,25,43–45].

Formation of a covalent tetrahedral intermediate nucleophilic catalysis belongs to these mechanisms [46]. Recently the X-ray investigation showed that human Pin1 assembled a prolyl peptide in a slightly tetrahedral geometry of the prolyl carbonyl group. Location of nucleophilic side chains adjacent to the distorted carbonyl supports the hypothesis [30].

Similarly, an electrophilic mechanism assuming stabilization of deconjugated prolyl bonds by nitrogen protonation or hydrogen bond formation [25,47] has been supported by intramolecular general acid catalysis of *cis*/*trans* isomerization for the protonated His-Pro moiety in water [48]. In contrast, the mechanisms of ground state distortion and desolvation by binding to a hydrophobic cavity [22,27,49] appear to be sterically less restrictive. Therefore only a minor catalytic contribution should be attributed to desolvation of bound substrates. Small catalytic activities found for the P_1 D-Ala substrate with most of the PPIases might indicate this part of the catalytic machinery, and can be compared quantitatively with micellar rate enhancements of the prolyl isomerization [24].

Reminiscent of the effect of P_1 position, the reversed stereochemistry in the remote P_2' position was found to be rather deleterious to catalysis, but affinity for the PPIases was lowered too. Cyp18 was an exception because catalysis was still visible but binding was already weak.

Here, analyses become difficult due to the lack of detailed knowledge about the influence of altered P_2' position side chains on PPIase catalysis. Again the peculiarity of the trigger factor catalytic domain in the context of other FKBPs became obvious due to the preserved affinity toward the diastereomeric peptide. In the course of a structure-based inhibitor design Hauske et al. reported that a compound with a D chiral center at the P_2' position has a 17-fold higher affinity to FKBP12 than the compound with the L stereochemistry [50].

The pronounced stereoselectivity of PPIases, following from our data, is in large contrast to the stereochemical promiscuity of chaperones which represents the other class of folding help-

er proteins. Chirality variants on the N-terminal helix in rhodanese which are represented by an all-D version and an alternating L and D version bound to GroEL as good as the protein-derived peptide [51]. In contrast, function of PPIases sensitively depends on the proper alignment of enzyme and substrate chains for several interacting sites at the active center in order to release catalytic power in a stepwise manner.

Acknowledgements: We are grateful to G. Stoller, H. Bang, A. Schierhorn and J.-U. Rahfeld for discussions, mass spectrometry and providing peptidyl prolyl *cis/trans* isomerases. We are indebted to Hengming Ke for providing X-ray data and critical reading of the manuscript. We thank I. Höckelmann and I. Kunze for the excellent technical assistance. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (Fi 455/1-3), the Fonds der Chemischen Industrie, and the Boehringer-Ingelheim Stiftung.

References

[1] Schechter, J. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162.
[2] Fischer, G. (1994) Angew. Chem. Int. Ed. 33, 1415–1436.
[3] Stoller, G., Rücknagel, K.P., Nierhaus, K.H., Schmid, F.X., Fischer, G. and Rahfeld, J.U. (1995) EMBO J. 14, 4939–4948.
[4] Rahfeld, J.U., Schierhorn, A., Mann, K. and Fischer, G. (1994) FEBS Lett. 343, 65–69.
[5] Galat, A. and Metcalfe, S.M. (1995) Prog. Biophys. Mol. Biol. 63, 67–118.
[6] Thali, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C.T., Sodroski, J. and Göttlinger, H.G. (1994) Nature 372, 363–365.
[7] Franke, E.K., Yuan, H.E.H. and Luban, J. (1994) Nature 372, 359–362.
[8] Sherry, B., Zybarth, G., Alfano, M., Dubrovsky, L., Mitchell, R., Rich, D., Ulrich, P., Bucala, R., Cerami, A. and Bukrinsky, M. (1998) Proc. Natl. Acad. Sci. USA 95, 1758–1763.
[9] Lu, K.P., Hanes, S.D. and Hunter, T. (1996) Nature 380, 544–547.
[10] Yaffe, M.B., Schutkowski, M., Shen, M., Zhou, X.Z., Stukenberg, T., Rahfeld, J.-U., Xu, J., Kuang, J., Kirschner, M.W., Fischer, G., Cantley, L.C. and Lu, K.P. (1997) Science 278, 1957–1960.
[11] Crenshaw, D.G., Yang, J., Means, A.R. and Kornbluth, S. (1998) EMBO J. 17, 1315–1327.
[12] Shen, M., Stukenberg, P.T., Kirschner, M.W. and Lu, K.P. (1998) Genes Dev. 12, 706–720.
[13] Lang, K., Schmid, F.X. and Fischer, G. (1987) Nature 329, 268–270.
[14] Schönbrunner, E.R., Mayer, S., Tropschug, M., Fischer, G., Takahashi, N. and Schmid, F.X. (1991) J. Biol. Chem. 266, 3630–3635.
[15] Kern, G., Kern, D., Schmid, F.X. and Fischer, G. (1995) J. Biol. Chem. 270, 740–745.
[16] Lilie, H., Lang, K., Rudolph, R. and Buchner, J. (1993) Protein Sci. 2, 1490–1496.
[17] Schutkowski, M., Wöllner, S. and Fischer, G. (1995) Biochemistry 34, 13016–13026.
[18] Kern, D., Schutkowski, M. and Drakenberg, T. (1997) J. Am. Chem. Soc. 119, 8403–8408.
[19] Fischer, G., Wöllner, S., Schönbrunner, R. and Scherer, G. (1994) in: Proceedings of the 5th Akabori Conference, pp. 142–146, MPG, Dresden.
[20] Zhao, Y., Chen, Y., Schutkowski, M., Fischer, G. and Ke, H. (1997) Structure 1, 139–146.
[21] Schmid, F.X., Frech, C., Scholz, C. and Walter, S. (1996) Biol. Chem. 377, 417–424.
[22] Radzicka, A., Acheson, S.A. and Wolfenden, R. (1992) Bioorg. Chem. 20, 382–386.
[23] Stein, R.L. (1993) Adv. Protein Chem. 44, 1–24.
[24] Kramer, M.L. and Fischer, G. (1997) Biopolymers 42, 49–60.
[25] Fischer, S., Michnick, S. and Karplus, M. (1993) Biochemistry 32, 13830–13837.
[26] Orozco, M., Tiradorives, J. and Jorgensen, W.L. (1993) Biochemistry 32, 12864–12874.
[27] Harrison, R.K. and Stein, R.L. (1992) J. Am. Chem. Soc. 114, 3464–3471.
[28] Park, S.T., Aldape, R.A., Futer, O., DeCenzo, M.T. and Livingston, D.J. (1992) J. Biol. Chem. 267, 3316–3324.
[29] Schutkowski, M., Bernhardt, A., Zhou, X.Z., Shen, M., Reimer, U., Rahfeld, J.-U., Lu, K.P. and Fischer, G. (1988) Biochemistry (in press).
[30] Ranganathan, R., Lu, K.P., Hunter, T. and Noel, J.P. (1997) Cell 89, 875–886.
[31] Liu, J., Albers, M.W., Chen, C.M., Schreiber, S.L. and Walsh, C.T. (1990) Proc. Natl. Acad. Sci. USA 87, 2304–2308.
[32] Rahfeld, J.U., Rücknagel, K.P., Schelbert, B., Ludwig, B., Hacker, J., Mann, K. and Fischer, G. (1994) FEBS Lett. 352, 180–184.
[33] Stoller, G., Tradler, T., Rücknagel, K.P., Rahfeld, J.-U. and Fischer, G. (1996) FEBS Lett. 384, 117–122.
[34] Standaert, R.F., Galat, A., Verdine, G.L. and Schreiber, S.L. (1990) Nature 346, 671–674.
[35] Fischer, G., Bang, H. and Mech, C. (1984) Biomed. Biochim. Acta 43, 1101–1111.
[36] Garcia-Echeverria, C., Kofron, J.L., Kuzmic, P., Kishore, V. and Rich, D.H. (1992) J. Am. Chem. Soc. 114, 2758–2759.
[37] Kern, D., Drakenberg, T., Wikstrom, M., Forsen, S., Bang, H. and Fischer, G. (1993) FEBS Lett. 323, 198–202.
[38] Yamazaki, T., Ro, S., Goodman, M., Chung, N.N. and Schiller, P.W. (1993) J. Med. Chem. 36, 708–719.
[39] Grathwohl, C. and Wüthrich, K. (1976) Biopolymers 15, 2025–2041.
[40] Tradler, T., Stoller, G., Rücknagel, K.P., Schierhorn, A., Rahfeld, J.U. and Fischer, G. (1997) FEBS Lett. 407, (2) 184–190.
[41] Scholz, C., Mücke, M., Rape, M., Pecht, A., Pahl, A., Bang, H. and Schmid, F.X. (1998) J. Mol. Biol. (in press).
[42] Reimer, U., Drewello, M., Jakob, M., Fischer, G. and Schutkowski, M. (1997) Biochem. J. 326, 181–185.
[43] Zhao, Y. and Ke, H. (1996) Biochemistry 35, 7356–7361.
[44] Ke, H., Mayrose, D. and Cao, W. (1993) Proc. Natl. Acad. Sci. USA 90, 3324–3328.
[45] Fischer, G., Berger, E. and Bang, H. (1989) FEBS Lett. 250, 267–270.
[46] Fischer, G., Wittmann Liebold, B., Lang, K., Kiefhaber, T. and Schmid, F.X. (1989) Nature 337, 476–478.
[47] Texter, F.L., Spencer, D.B., Rosenstein, R. and Matthews, C.R. (1992) Biochemistry 31, 5687–5691.
[48] Reimer, U., Elmokdad, N., Schutkowski, M. and Fischer, G. (1997) Biochemistry 36, 13802–13808.
[49] Rosen, M.K., Standaert, R.F., Galat, A., Nakatsuka, M. and Schreiber, S.L. (1990) Science 248, 863–866.
[50] Hauske, J.R., Dorff, P., Julin, S., DiBrino, J., Spencer, R. and Williams, R. (1992) J. Med. Chem. 35, 4284–4296.
[51] Gierasch, L.M., Wang, Z., Hunt, J., Landry, S.J., Weaver, A. and Deisenhofer, J. (1995) Protein Eng. 8, (Suppl.) 14.
[52] Handschumacher, R.E., Harding, M.W., Rice, J. and Drugge, R.J. (1984) Science 226, 544–547.