Peptidyl−Prolyl *Cis*−*Trans* Isomerase of *Bacillus subtilis*: Identification of Residues Involved in Cyclosporin A Affinity and Catalytic Efficiency[†]

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ABSTRACT: The 17-kDa peptidyl−prolyl *cis*−*trans* isomerase from *Bacillus subtilis* (PPiB) is a member of the cyclophilin family and shows strong homology to PPIases of eukaryotic origin (40%) and less identity to PPIase sequences of Gram-negative bacteria (27−32%). Although the majority of residues that form the PPIase active site are highly conserved, three residues, V52, H90, and H109 in the sequence of the *B. subtilis* PPIase, were found to differ from the sequences found in human (hCyP) and *Escherichia coli* (eCyP). Also, the binding affinity of cyclosporin A (CsA) to the different PPIases varies in IC_{50} values from 6 nM for human PPIase hCyPA and 84 nM for the human hCyPB to over 120 nM for *B. subtilis* and 3000 nM for *E. coli*. In addition, a variety of k_{cat}/K_m values, ranging from 1.1 mM^{-1} s^{-1} for the *B. subtilis* PPIase to over 10 mM^{-1} s^{-1} for human and 13 mM^{-1} s^{-1} for *E. coli*, were detected using the common substrate suc-Ala-Ala-Pro-Phe-pNA. Through site-specific mutagenesis we demonstrate that the differences in the three mentioned residues are mainly responsible for the variations in IC_{50} and k_{cat}/K_m values. Replacement of H90 to N90, or H109 to W109, resembling the amino acid sequence of human hCyPA, resulted in more efficient CsA binding (IC_{50} value for H90N, 60 nM, and for H109W, 95 nM), whereas replacement of H90 to R90, or H109 to F109, resembling the amino acid sequence of *E. coli* eCyP, resulted in less efficient CsA binding (IC_{50} value for H90R, 2000 nM, and for H109F, 5000 nM). In addition to lower CsA affinity, mutant protein H109F shows a k_{cat}/K_m value of 10.5 mM^{-1} s^{-1}, comparably high to that of the wild-type *E. coli* protein. In contrast, other mutants like C57F, H90N, H90R, and H109W do not differ significantly in k_{cat}/K_m values from wild-type PPiB. Replacement of V52 to M52, which is conserved in *E. coli* and all known eukaryotic PPIases, does not show any effect in CsA binding affinity (IC_{50} value for V52M, 120 nM), but it raises the catalytic efficiency by 12-fold to k_{cat}/K_m of 14 mM^{-1} s^{-1}. In conclusion, our studies suggest that the unique histidine residues H90 and H109 in *B. subtilis* PPIase are, at least in part, responsible for its intermediate CsA affinity and that the V52 residue confers the low conversion rate.

Peptidyl−prolyl *cis*−*trans* isomerases (PPIases)[1] catalyze the interconversion of the peptidyl−prolyl imide bonds in peptide and protein substrates, and in some cases it has been shown that isomerization is the rate-limiting step in protein folding *in vitro* (Fischer & Schmid, 1990). PPIases catalyze this isomerization and accelerate folding of some proteins *in vitro* (Fischer & Schmid, 1990) and *in vivo* (Steinmann *et al*., 1991; Lodish & Kong, 1991) and possess greatly varying affinities toward binding of immunosuppressive drugs such as cyclosporin A, FK506, and rapamycin. By use of this binding criterion, two unrelated superfamilies of PPIases can be distinguished: cyclophilins (Cyps), which bind the immunosuppressive drug cyclosporin A, and FK506-binding proteins (FKBPs), which bind the drugs FK506 and rapamycin. The human Cyp/CsA and FKBP/FK506 complexes inhibit the signal transduction pathway for activating human T-cells [for review see Fischer et al. (1994)].

One of the best-characterized cyclophilins is the human hCyPA, which has a characteristically high CsA sensitivity (IC_{50} of 6 nM), in contrast to other eukaryotic cyclophilins like hCyPB (IC_{50} of 84 nM), and a high conversion rate with synthetic tetrapeptides. X-ray structure analysis has revealed the structure of the uncomplexed hCyPA (Ke *et al*., 1991), the hCyPA/CsA complex (Thériault *et al*., 1993), and the hCyPA bound to the tetrapeptide AC-Ala-Ala-Pro-Ala-AMC (Kallen & Walkinshaw, 1992). According to these structure data, R55, N102, and W121 are involved in CsA binding by hydrogen bonds. The important role of W121 in CsA binding has been confirmed by site-directed mutagenesis (Bossard *et al*., 1991; Liu *et al*., 1991). Exchange of W121 to A121 resulted in a 200-fold decrease of CsA affinity, and replacement of W121 to F121 revealed a 400-fold drop.

Whereas eukaryotic PPIases, especially the human hCyPA, are well-characterized, little information is available for prokaryotic PPIases. The best-studied prokaryotic PPIase in terms of structure/function relationship is the periplasmatic eCyP of *Escherichia coli* (Liu & Walsh, 1990). CsA sensitivities differ dramatically between eukaryotic hCyPA and prokaryotic eCyP (IC_{50} 3000 nM). Replacement of F112e to W112e in eCyP increases CsA affinity by 23-fold,

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[1] Abbreviations: PPiB, *Bacillus subtilis* peptidyl−prolyl *cis*−*trans* isomerase, PPIase, peptidyl−prolyl *cis*−*trans* isomerase, hCyPA, human cyclophilin-type peptidyl−prolyl *cis*−*trans* isomerase isoform A; eCyP, *Escherichia coli* cyclophilin-type peptidyl−prolyl isomerase; CsA, cyclosporin A; pNA, *p*-nitroanilide; suc, succinyl; FKBP, FK506-binding protein; AC, *N*-acetyl; AMC, amidomethylcoumarin.

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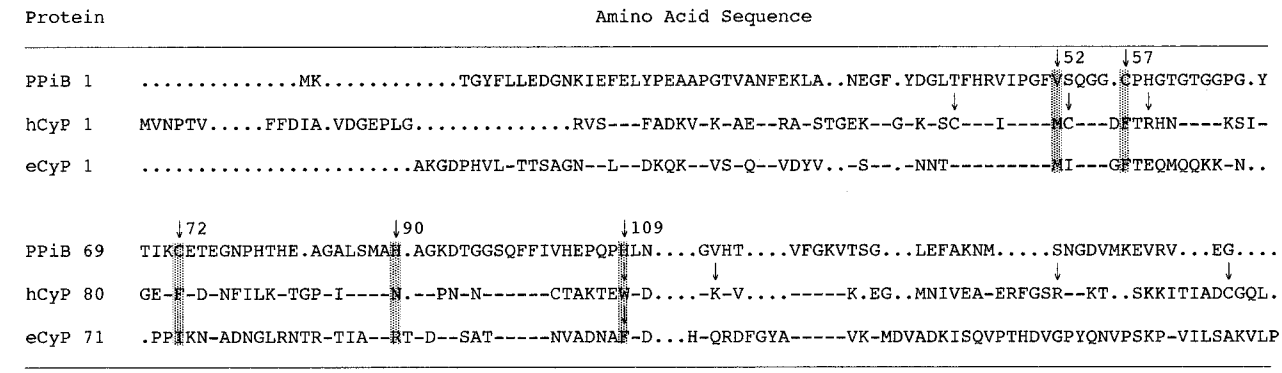


FIGURE 1: Sequence alignment of the peptidyl-prolyl *cis*−*trans* isomerase of *B. subtilis* (PPiB) with the human hCyPA (Haendler *et al*., 1987) and the periplasmatic eCyP of *E. coli* (Hayano *et al*., 1991). Positions of amino acid substitutions are boxed, indicated by arrows and position numbers. Mutants constructed by Liu *et al*. (1990), Bossard *et al*. (1991), and Etzkorn *et al*. (1994) for the hCyPA and eCyP are indicated by arrows.

whereby F112e is the corresponding residue to W121h in hCyPA (Liu *et al*., 1991). The F122W mutant structure was determined by NMR analysis (Fejzo *et al*., 1994) and revealed a high degree of similarity to hCyPA.

The *Bacillus subtilis* PPIase was purified and characterized (Herrler *et al*., 1992) and the coding gene was cloned (Herrler *et al*., 1994). The 17-kDa protein shows strong homology to PPIases of eukaryotic origin (40%) and less identity to PPIase sequences of Gram-negative bacteria (27−32%). Treatment of *B. subtilis* PPIase with CsA revealed an intermediate inhibition constant (IC_{50} 120 nM) compared to hCyPA and eCyP. Sequence alignment showed that three residues, V52, H90, and H109, in the highly conserved active site differ from the corresponding residues in eukaryotic and other prokaryotic PPIases (Figure 1). Therefore we have performed site-directed mutagenesis of these residues to determine their role in CsA affinity and isomerase activity. Furthermore we studied the role of cysteines C57 and C72 with regard to their presumed function in the reaction mechanism (Fischer *et al*., 1989, 1990; Liu *et al*., 1990).

MATERIALS AND METHODS

Cyclosporin A, used for inhibition of peptidyl−prolyl *cis*−*trans* isomerization activity, was a generous gift of Sandoz Pharmaceutical Corp. (Basel, Switzerland). The substrate tetrapeptides suc-Ala-Ala-Pro-Phe-pNA, suc-Ala-Phe-Pro-Phe-pNA, suc-Ala-Gly-Pro-Phe-pNA, and suc-Ala-Lys-Pro-Phe-pNA were obtained from Bachem (Basel, Switzerland). HiLoad 16/60 Superdex 75 preparation-grade column was obtained from Pharmacia LKB-Biotechnology, and Superperformance 150-10 Fractogel EMD TMAE-650(s) column was from Merck.

Protein concentrations were determined by OD_{280} using the equations of Gill and von Hippel (1989).

Mutagenesis. To introduce mutations into the *B. subtilis ppiB*. gene, the plasmid pPPiB was used (Herrler *et al*., 1994). This plasmid contains a 2-kb *Ava*I fragment bearing the structural gene of *ppiB* cloned into the pBluescript KS^+ vector under the control of the T7 promoter. Site-directed mutagenesis of PPiB was accomplished by the method of Taylor *et al*., (1985) with the *in vitro* mutagenesis kit of Amersham/Buchler (Braunschweig, Germany). Primers used for mutagenesis and sequencing were synthesized by M. Krause (Institut für Molekularbiologie und Tumorforschung, Marburg, Germany). The following mutagenic primers were used: V52M, 5′-CCTGGCTTCATGAGCCAAGCC-3′; C57F, 5′-CAAGGCGGATTCCCGCACGG-3′; C72F, 5′-CACAATCAAATTTGAAACAGAAG-3′; H90N, 5′-CTATGGCAAATGCCGGAAAAGAC-3′; H90R, 5′-CTGTGGCACGTGCCGGAAAAGAC-3′; H109W, 5′-CTGTCATGAGCCACAGCCTTGGTTGAACGGTGTTCAC-3′; H109F, 5′-CTGTCATGAGCCACAGCCTTTTTTGAACGGTGTTCAC-3′. Each mutant plasmid was purified using a plasmid miniprep kit from Qiagen. The altered *ppiB* genes were sequenced using the chain termination method of Sanger *et al*. (1977) with the T7 sequencing kit (U.S. Biochemical Corp.) to verify the mutagenesis reaction.

Protein Expression and Purification. For overproduction of PPiB mutants H90N, H90R, H109W, and H109F, the corresponding plasmids were transformed into *E. coli* strain K38 (Russel & Model, 1984), containing the pGP1-2 plasmid (Tabor & Richardson, 1985) encoding for T7 RNA polymerase. Cells were grown in 2× YT medium at 30 °C to $OD_{600} = 0.7$ and subsequently shifted to 42 °C to induce the T7 promoter-dependent expression, and cells were allowed to grow for a further 2 h. Overproduction of V52M, C57F, and C72F was not sufficient with the method by Tabor and Richardson. Therefore the 2-kb *Ava*I fragments, containing the mutant *ppiB* genes, were subcloned into the low-copy-number vector pPD101 (Dersch *et al*., 1994). After transformation in *E. coli* strain BL21, cells were grown at 37 °C in M9 medium to $OD_{600} = 0.7$ and induced with 1 mM IPTG. After induction, cells were allowed to grow for 2 h.

The purification scheme for both expression systems was the same. Cell suspension was centrifuged for 15 min and 6000*g* at 4 °C. The cell pellet was resuspended in buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA, and 1 mM DTT), and the suspension was sonicated at intervals for a total of 3 min. The broken cells were centrifuged for 30 min at 15000*g* and the supernatant was applied to a Superperformance 150-10 Fraktogel EMD TMAE-650(S) anion-exchange column. Bound proteins were eluted with an NaCl gradient ranging from 0 to 0.5 M in buffer B (50 mM Tris-HCl, pH 8.5, 4 mM DTT, and 5 mM EDTA) at a concentration of 120 mM NaCl. PPiB-containing fractions were lyophilized and the concentrated solution was applied to HiLoad 16/60 Superdex 75 prep-grade gel-filtration column. Mutant and wild-type protein eluted with a retention time of 110 min and led to purification of PPiB to apparent homogenity as judged by a single band in Coomassie brilliant blue-stained SDS−PAGE and western blot analysis.

PPIase Activity and CsA Inhibition Assay. Assays for PPIase activity, using the synthetic tetrapeptides suc-Ala-Ala-Pro-Phe-pNA, suc-Ala-Phe-Pro-Phe-pNA, suc-Ala-Gly-Pro-Phe-pNA, and suc-Ala-Lys-Pro-Phe-pNA as substrates in an isomerization reaction coupled to a chymotryptic cleavage of the chromophore *p*-nitroanilide, were performed as described previously (Fischer *et al*., 1984). To measure the inhibition of enzyme activity by CsA (cyclosporin A), the enzyme was preincubated 3 min at 10 °C with the immunosuppressive agent before the addition of chymotrypsin and the suc-Ala-Ala-Pro-Phe-pNA substrate. Different final concentrations of CsA, ranging between 10 nM and 10 μM, were used to determine the inhibition constants (IC_{50} values). The final concentration of EtOH, used to dissolve CsA in a 1 mM stock solution, in the reaction mixture did not exceed 1% of the volume, a concentration shown not to affect PPIase catalysis. Catalytic activity (k_{cat}/K_m values) were determined following the equation $k_{cat}/K_m = (k_{obs}\text{-}k_u)/[E]$, where k_{obs} is the PPiB-catalyzed first-order rate constant, k_u is the first-order rate constant in the presence of substrate but the absence of PPiB, and [E] is the concentration of PPiB. k_{obs} and k_u were determined by an equation for the first-order rate constant (Harrison & Stein, 1990a). PPiB inhibition by CsA (IC_{50} values) was assayed when the ratio of $k_{inhib}/k_{control}$ was 0.5 (Harrison & Stein, 1990b).

Sequence Alignement. On the GENIUSnet in the DKFZ-Heidelberg, the programs FASTA (Lipman & Pearson, 1990) and TREE (Feng & Doolittle, 1990) of the HUSAR package were used for sequence alignment. The HUSAR package is an extended UNIX version of the UWGCG program package (Devereux *et al*., 1984).

RESULTS

Although PPIases of eukaryotic and prokaryotic origin are highly homologous, noticeable differences in residues located in the active sites of the *B. subtilis* PPiB, human hCyPA, and *E. coli* eCyP were observed (Figure 1). In order to understand their role, substitutions in PPiB with the corresponding residues of hCyPA and eCyP were carried out leading to the following mutations: V52M, C57F, C72F, H90N, H90R, H109W, and H109F.

Mutant and wild-type proteins were overexpressed in *E. coli* strain K38 using the method of Tabor and Richardson. With this system only mutants H90N, H90R, H109W, and H109F could be successfully overexpressed. For overexpression of the V52M, C57F, and C72F mutants the 2-kb *Ava*I fragment, containing the mutant *ppiB* genes, were cloned into a low-copy-number vector pPD101 for successful overexpression.

Mutant proteins were purified to apparent homogenity as judged by a single band in Coomassie-stained SDS−PAGE analysis in a two-step protocol, consisting of anion exchange and following gel filtration. The purification profiles of the wild-type PPiB and the mutants were almost identical, suggesting that gross alterations were not induced by the mutations. PPIase activity was determined using synthetic tetrapeptides as substrates in a two-step reaction coupled to chymotrypsin as described by Fischer *et al*. (1984). Wild-type and mutant protein, except the C72F mutant protein, did show significant PPIase activities *in vitro*, indicating that no gross alterations in the structural integrity of the mutant proteins were caused by the introduced mutations.

Table 1: CsA Affinities of Wild-Type and Mutant Proteins[a]

mutation	IC_{50} (nM)
WT	120 (±10)
V52M	120 (±15)
C57F	>10000
C72F	n.d.[b]
H90N	60 (±6)
H90R	2000 (±150)
H109W	95 (±15)
H109F	5000 (±300)

[a] Inhibition of PPIase activity was measured at different concentrations of CsA using succinyl-Ala-Ala-Pro-Phe-pNA as substrate; the data were analyzed with the program ISOMHITA (obtained from H. Bang, University of Erlangen, Germany). PPIase assays were carried out in three independent experiments. [b] No detectable PPIase activity.

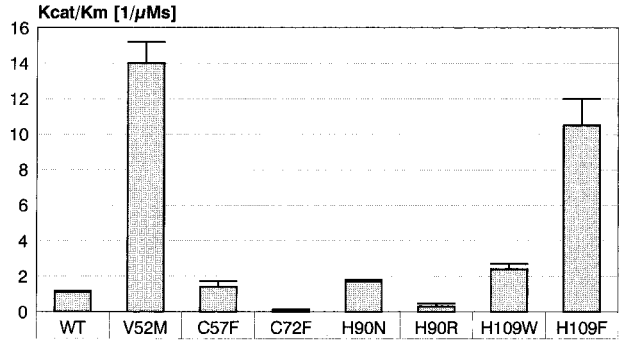
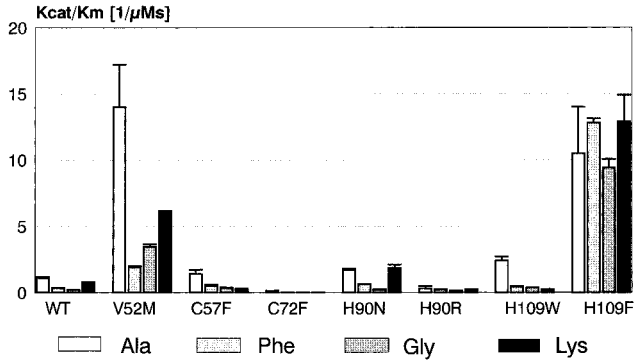


FIGURE 2: Catalytic efficiency, k_{cat}/K_m values, of wild-type and mutant proteins. k_{cat}/K_m values were determined using suc-Ala-Ala-Pro-Phe-pNA as substrate. Final concentration of wild-type and mutant protein was in the range of 100 nM.

As seen in Table 1 the *Bacillus* wild-type enzyme showed an IC_{50} value for CsA binding affinity of 120 nM (±10 nM) while the mutant H109W displayed an IC_{50} of 95 nM (±15 nM). In contrast, the H109F mutant revealed an IC_{50} of 5000 nM (±300 nM) (Table 1). In addition to the CsA affinity, we determined the catalytic efficiencies, k_{cat}/K_m values, of wild-type and mutant proteins (Figure 2). The wild-type *B. subtilis* PPIase has a low conversion rate of 1.1 mM^{-1} s^{-1} (±0.06 mM^{-1} s^{-1}) in contrast to the human hCyPA (k_{cat}/K_m 10 mM^{-1} s^{-1}) and the *E. coli* eCyP (k_{cat}/K_m 13 mM^{-1} s^{-1}). Exchange of H109 to W109 in the *B. subtilis* PPIase revealed a 2-fold higher catalytic efficiency, and replacement of H109 to F109 increased the k_{cat}/K_m values by 10-fold (Figure 2) to a k_{cat}/K_m ratio of 10.5 mM^{-1} s^{-1} (±1.5 mM^{-1} s^{-1}).

Peptidyl−prolyl *cis*−*trans* isomerization is also catalyzed by an structurally unrelated group of enzymes named FK506-binding proteins (FKBPs). In contrast to cyclophilins, these proteins bind specifically to the immunosuppressive agents FK506 and rapamycin. On the basis of the preferred suc-Ala-Xaa-Pro-Phe-pNA substrate, one can distinguish between both types of PPIases. Cyclophilin homologs like PPiB prefer Xaa = Ala as substrate, whereas other isomerases like FKBPs show the highest conversion rate with Xaa = Phe or Leu as substrate. Therefore the ratio of conversion rates measured with Ala and Phe substrates (Ala/Phe ratio) can be used to distinguish between cyclophilin homologs and FKBPs. In our assays wild-type PPiB showed a typical ratio of 3.3 and mutant protein H109W had a ratio of 5.4, whereas the H109F mutant protein showed an unusual ratio of 0.82 (Figure 3, bottom panel).

The H90 position in *B. subtilis* PPiB and comparable positions in other PPIases have not been studied so far,



mutation	Ala/Phe ratio
WT PPiB	3.3
V52M	7.4
C57F	2.8
H90N	2.7
H90R	1.5
H109W	5.4
H109F	0.82

FIGURE 3: (Top) Substrate specificity of wild-type and mutant proteins. Substrate specificity was determined for the substrates suc-Ala-Ala-Pro-Phe-pNA (Ala), suc-Ala-Gly-Pro-Phe-pNA (Gly), suc-Ala-Phe-Pro-Phe-pNA (Phe), and suc-Ala-Lys-Pro-Phe-pNA (Lys). Final protein concentration was in the range of 100 nM. (Bottom) Relative substrate specificity of suc-Ala-*Ala*-Pro-pNA to suc-Ala-*Phe*-Pro-Phe-pNA (Ala/Phe) ratio. For substrate specificity and Ala/Phe ratio of hCyPA and eCyP, see Compton *et al*. (1992) and Harrison and Stein (1990b).

although the corresponding N102 in the human hCyPA PPIase forms hydrogen bonds to CsA according to X-ray structure data. Exchange of H90 to N90 increased the IC_{50} from 120 to 60 nM (Table 1). This 2-fold enhancement of CsA affinity places the H90N mutant in the range of eukaryotic PPIases, as the IC_{50} for the hCyPB was determined to 84 nM (Price *et al*., 1991). The mutant protein H90R, on the other hand, corresponding to the *E. coli* eCyP binding site, showed a 17-fold decrease in CsA affinity leading to an IC_{50} value of 2000 nM (±150 nM). The k_{cat}/K_m value for the H90N mutation increased to a scale of 1.7 mM^{-1} s^{-1} (±0.1 mM^{-1} s^{-1}), similar to the range of the H109W mutant. Both mutations correspond to residues present in the binding site of the human PPIase. In contrast, exchange from H90 to R90 revealed a 3-fold decline in catalytic efficiency.

According to CsA affinity the V52M mutant protein differed from the mutant proteins mentioned above. V52M and wild-type protein share the same CsA affinity (Table 1). For both enzymes we determined an IC_{50} of 120 nM. On the other hand, V52M possesses a 12-fold higher conversion rate (Figure 2) compared to the wild-type protein, indicating the important role of the methionine residue 52 for efficient peptidyl−prolyl *cis*−*trans* isomerization.

In previously published work (Fischer *et al*., 1989) it has been proposed that cysteine residues are implicated in the reaction mechanism. We found that exchange of C57 to F57 in the *B. subtilis* PPIase had little effect on catalytic efficiency but inhibition with cyclosporin A was not detectable up to a final concentration of 10 000 nM. Replacement of C72 to F72 resulted in a 10-fold drop of catalytic function, such that mutant C72F has lost the ability to catalyze peptidyl−prolyl *cis*−*trans* isomerization.

DISCUSSION

Cyclophilins share sequence identities of over 30%, but they vary significantly in CsA affinities, catalytic efficiencies, and substrate specifities. The *B. subtilis* PPIase, PPiB, is characterized by an intermediate inhibition constant for cyclosporin A with an IC_{50} of 120 nM (±10 nM) in relation to the human hCyPA (IC_{50} = 6 nM) and the *E. coli* PPIase eCyP (IC_{50} = 3000 nM). The catalytic efficiency, on the other hand, expressed by k_{cat}/K_m values, is 10−13 fold lower than the human and *E. coli* PPIases.

Sequence alignment disclosed single amino acid residues that differ in sequence from other PPIases in the highly conserved active site, namely, V52, H90, and H109. Therefore, we replaced these residues with the corresponding residues in the best-studied eukaryotic enzyme, the human hCyPA, and the best studied prokaryotic Gram-negative enzyme, the eCyP of *E. coli*, to determine their role in CsA inhibition and peptidyl−prolyl *cis*−*trans* isomerization.

The corresponding residues to H109 in PPiB are W121 in the human enzyme and F112 in *E. coli*. It has been shown by site-directed mutagenesis that high CsA affinity of the human enzyme is mediated by residue W121h, whereas the low CsA affinity of the *E. coli* enzyme is contributed by the F112e residue. Support for these biochemical data was obtained from structural studies. The structure of hCyPA cocrystallized with CsA exposed a hydrogen bond of the W121h side-chain ϵNH group to MeLeu9 (CO) explaining the high affinity to CsA in hCyPA (Thériault *et al*., 1993). The low CsA affinity of eCyP could be explained by the inability of the Phe 112e side chain to form hydrogen bonds (Clubb *et al*., 1994; Fejzo *et al*., 1994).

Furthermore, X-ray structure analysis of hCyPA and eCyP show a high degree of structural homology despite only 34% sequence identity. In contrast, PPiB and hCyPA are found to have 40% sequence identity (Herrler *et al*., 1994), suggesting that PPiB adopts an intermediate structure between that of hCyPA and eCyP. In analogy to the structure of hCyPA, the NH bond of the imidazole side chain of H109 may be in the position to form a hydrogen bond to the backbone CO of MeLeu9 of CsA. This might explain the relatively high affinity for CsA (IC_{50} = 120 nM) for the PPiB and why the substitution of H109 to W109 resulted only in a slightly higher affinity for CsA. However, this difference seems to be significant according to the statistical error. Because the side chain of phenylalanine is not able to form hydrogen bonds to CsA, this is likely to be the cause for the 40-fold decrease in CsA sensitivity we determined after replacing H109 with F109. Bossard *et al*. (1991) demonstrated that exchange of W121h to A121h also caused a noticeable decrease in CsA sensitivity, as alanine is also unable to form hydrogen bonds. These substitution data supported the common hypothesis that a tryptophan in the discussed position causes high CsA affinity. Substitution experiments on the *B. subtilis* PPIase might extend the hypothesis, that the tryptophan can be principally replaced by other hydrogen-bond-forming residues like histidine.

In contrast, the catalytic efficiency of the H109F mutant was about 12-fold higher than that of the wild-type protein (k_{cat}/K_m values: wild-type PPiB, 1.1 mM^{-1} s^{-1}; H109F, 10.5 mM^{-1} s^{-1}) and almost in the range found for the *E. coli* wild-type protein (k_{cat}/K_m 13 mM^{-1} s^{-1}; Liu & Walsh, 1990). According to CsA sensitivity and catalytic efficiency, this

single substitution of H109 to F109 changed the *B. subtilis* PPIase into an *E. coli*-like PPIase.

Interestingly, the H109F mutant revealed a low Ala/Phe ratio of 0.8 (Figure 3, bottom panel) that is comparable with a novel 10.1-kDa PPIase of *E. coli* called parvulin (Ala/Phe ratio 0.6). This novel PPIase cannot be inhibited by CsA or FK506 but shows the typical pattern of substrate specificity of FKBPs (Rahfeld *et al*., 1994).

No site-directed mutagenesis experiments were carried out for the residue H90 or comparable positions in other PPIases. However, the corresponding N102 residue of hCyPA was found to form hydrogen bonds to CsA, but only with the backbone NH and CO (Thériault *et al*., 1993). Therefore, it was expected that an exchange to histidine or arginine should be conservative or should have only indirect effects on the CsA affinity (Fejzo *et al*., 1994). In contrast, we found a 2-fold enhancement in CsA sensitivity in the H90N mutant and a 17-fold drop in CsA sensitivity in the H90R mutant. The H90 residue of the MA*H*AG binding motif seems to have an equivalent function in CsA binding as the H109 residue, although the H90 side chain might not be involved in CsA binding (see above).

Substitution of V52 to M52 does not show any effect in CsA binding affinity but caused a 12-fold increase in isomerization activity. Transferring the structure of hCyPA onto PPiB (see above), V52 might form out the *cis*-proline binding side. X-ray structure analysis (Kallen & Walkinshaw, 1992) of hCyPA cocrystallized with AC-Ala-Ala-Pro-Ala-AMC as substrate suggests that the *cis*-proline of AC-Ala-Ala-Pro-Ala-AMC lies in a pocket made principally by F60, *M61*, F113, and L122, whereas M61 is the corresponding residue to V52 of PPiB. Thus a steric clash of V52 with substrate might cause the low conversion rate of wild-type PPiB.

In addition to the substitutions discussed above, we analyzed the function of the two cysteine residues placed at positions 57 and 72 in connection with a proposed two-step mechanism for peptidyl−prolyl *cis*−*trans* isomerization. An earlier hypothesis proposed that *cis*−*trans* isomerization of peptidyl−prolyl bonds requires cysteine residues to form a tetrahedral hemithioorthoamide intermediate (Fischer *et al*., 1989). The C57F mutant did not show any CsA affinity; however, it generates a slightly higher conversion rate which is not explainable by the a cysteine-requiring mechanism and also indicates that no major changes in structure took place. We found that only one cysteine substitution mutant (C72F) lost the ability for *cis*−*trans* isomerization.

In conclusion, these studies suggest that the three residues V52, H90, and H109 affect CsA binding affinity and substrate conversion rate. Thereby V52 is partially responsible for the low conversion rate of PPiB and the two histidines, H90 and H109, are mainly responsible for the intermediate CsA affinity.

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