Mechanistic Implication of Crystal Structures of the Cyclophilin−Dipeptide Complexes[†,‡]

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ABSTRACT: The structures of cyclophilin A complexed with dipeptides of Ser-Pro, His-Pro, and Gly-Pro have been determined and refined at high resolution. Comparison of these structures revealed that the dipeptide complexes have the same molecular conformation and the same binding of the dipeptides. The side chains of the N-terminal amino acid of the above dipeptides do not strongly interact with cyclophilin, implying their minor contribution to the *cis*−*trans* isomerization and thus accounting for the broad catalytic specificity of the enzyme. The binding of the dipeptides is similar to that of the common substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide in terms of the N-terminal hydrogen bonding and the hydrophobic interaction of the proline side chain. However, substantial differences between these structures are observed in (1) hydrogen bonding between the carboxyl terminus of the peptides and Arg55 and between Arg55 and Gln63, (2) the side chain conformation of Arg55, and (3) water binding at the active site. These differences imply either that dipeptides are not substrates but competitive inhibitors of peptidyl-prolyl *cis*−*trans* isomerases or that dipeptides are subject to different catalytic mechanisms from tetrapeptides.

Cyclophilin (CyP)[1] is a binding protein for the immunosuppressive drug cyclosporin A (CsA; Handschumacher et al., 1984) and also an enzyme with peptidyl-prolyl *cis*−*trans* isomerase activity (Fischer et al., 1989a; Takahashi et al., 1989). As the immunosuppressant receptor, the CyP−CsA complex binds to calcineurin, a serine/threonine phosphatase, and a Ca^{2+}-dependent calmodulin binding protein (Klee et al., 1988; Liu et al., 1991; Friedman & Weissman, 1991). Recently, the structures of calcineurin and calcineurin−FK506−FKBP12 have been reported (Kissinger et al., 1995; Griffith et al., 1995).

The isomerization of peptidyl-prolyl amide bonds is a slow step in protein folding. CyP has been reported to accelerate protein folding (Fischer & Schmid, 1990; Schmid et al., 1993). Several mechanisms have been proposed for the *cis*−*trans* interconversion of the peptidyl-prolyl bond by CyP: (1) catalysis by formation of a covalent tetrahedral intermediate (Fischer et al., 1989a,b), (2) catalysis by distortion (Harrison & Stein, 1990a), (3) protonation of the amide nitrogen (Kofron et al., 1991), (4) catalysis by desolvation (Wolfenden & Radzicka, 1991; Radzicka et al., 1992), and (5) a solvent-assisted mechanism (Ke et al., 1993a). However, many questions about the *cis*−*trans* isomerization remain to be answered. For example, what is the catalytic specificity for substrates of proteins and peptides of various lengths? What is the impact of the N- and C-terminal residues on *cis*−*trans* isomerization? Do peptide and protein substrates have the same catalytic mechanisms?

Three-dimensional structures have been determined for the unligated recombinant human T cell CyPA (Ke et al., 1991; Ke, 1992), CyPA complexed with *N*-acetyl-Ala-Ala-Pro-Ala-amidomethylcoumarin (Kallen et al., 1991; Kallen & Walkinshaw, 1992), CyPA−Ala-Pro (Ke et al., 1993a), CyPA complexed with CsA or its derivative (Pflügl et al., 1993, Mikol et al., 1993; Thériault et al., 1993; Ke et al., 1994), CyPB−CsA (Mikol et al., 1994), and CyPC−CsA (Ke et al., 1993b).

We report here the structures of CyPA−Ser-Pro, CyPA−His-Pro, and CyPA−Gly-Pro, as well as further refinement of CyPA−Ala-Pro, to illustrate the specificity of the amino acid Xaa in Xaa-Pro. The structural comparison between the dipeptide and tetrapeptide complexes revealed significant differences in interactions at the active site. The question, whether the proline-containing dipeptides are competitive inhibitors of cyclophilin or whether the dipeptides and tetrapeptides have different catalytic mechanisms of *cis*−*trans* isomerization, is discussed on the basis of the structural information.

METHODS

Recombinant human CyPA was purified as previously described (Liu et al., 1990). Dipeptides of Ser-Pro, Gly-Pro, and His-Pro were purchased from BACHEM (catalog no.: G3230, G2190, and G2335). The unligated crystals of the recombinant human T cell CyPA were grown by dialyzing 10−15 mg/mL CyPA against 20 mM Tris−base (pH 8.5), 2 mM EDTA, 2 mM 2-mercaptoethanol, 0.5 mM NaN_3, 6.7% ethanol, and 11−13% polyethylene glycol (PEG 3350) at 4 °C, as described previously (Ke et al., 1991). The complexes of CyPA with the dipeptides were prepared by soaking the unligated CyPA crystals in the buffer of 20 mM Tris−base (pH 8.0), 30−38% PEG 3350, and the

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[‡] The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973. The entry codes are 2CYH for CyPA−Ala-Pro, 3CYH for CyPA−Ser-Pro, 4CYH for CyPA−His-Pro, and 5CYH for CyPA−Gly-Pro.

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[1] Abbreviations: CyP, cyclophilin; CyPA, cyclophilin A; CyPC, cyclophilin C; AAPF, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; CsA, cyclosporin A; FKBP, FK506 binding protein.

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Table 1: Data Collection of CyPA Complexed with Dipeptides[a]

	CyPA−Ser-Pro	CyPA−His-Pro	CyPA−Gly-Pro	CyPA−Ala-Pro
cell (*a*, *b*, *c*) (Å)	40.9, 52.4, 90.4	40.7, 52.4, 89.5	40.8, 52.4, 90.1	40.7, 52.3, 90.4
total measurements	56836	34148	35845	55553
unique reflections	14311	10112	8924	21601
R_{merge}	0.056	0.038	0.062	0.051
resolution (Å)	1.9	2.1	2.1	1.64
completeness (%)				
overall	90.0	86.8	75.5	88.8
last shell	74.4 (1.9−2.0 Å)	80.9 (2.1−2.2 Å)	66.0 (2.1−2.2 Å)	71.1 (1.64−1.71 Å)

[a] $R_{merge} = \sum_{hkl}\sum_i|I_i - \langle I\rangle|/\sum_{hkl}\langle I\rangle$, where $\langle\ \rangle$ means average.

Table 2: Structure Refinement of the CyPA−Dipeptide Complexes

	CyPA−Ser-Pro	CyPA−His-Pro	CyPA−Gly-Pro	CyPA−Ala-Pro
resolution (Å)	8.0−1.9	8.0−2.1	8.0−2.1	6.0−1.64
reflections	14075	9944	8732	21119
R-factor	0.186	0.177	0.175	0.185
R_{free}	0.231	0.230	0.250	0.228
no. of protein atoms	1258	1258	1258	1258
no. of ligand atoms	14	18	12	13
no. of solvent atoms	54	51	48	88
RMS bond deviation (Å)	0.014	0.015	0.015	0.013
RMS angle deviation (deg)	2.9	3.1	3.0	2.6
average *B*-value (Å^2)				
CyPA	19.5	19.8	27.2	25.5
dipeptide	27.4	40.3	31.4	23.2

dipeptides of 40 mM His-Pro, 50 mM Gly-Pro, and 75 mM Ser-Pro at 4 °C for 14 days. The unopened dialysis buttons which contain the unligated crystals were soaked in the buffers to prevent dissolving of the crystals. The unligated CyPA crystals have the space group $P2_12_12_1$ and cell dimensions of $a = 43.0$ Å, $b = 52.6$ Å, and $c = 89.2$ Å with one molecule in the crystallographic asymmetric unit. The CyPA−dipeptide complexes have the same space group as the unligated CyPA but slightly different cell dimensions (Table 1). Diffraction data were collected at room temperature on the Rigaku phosphate image plate system and are summarized in Table 1.

The structures of the CyPA−dipeptide complexes were determined either by the molecular replacement method using the unligated CyPA as the initial model or by difference Fourier synthesis. The structures were refined using the program X-PLOR (Brünger et al., 1987) on a Dec Alpha computer and were rebuilt using the program FRODO (Jones, 1982) linked to an ESV10 graphic system. There is no restraint applied to the *B* refinement. The occupancy was not refined but fixed as 1 for all atoms of CyPA, the dipeptides, and solvent molecules. The *cis* proline peptide bonds of the dipeptides were built using the program FRODO and refined under a restraint of a *trans* peptide bond with a low force constant of 5 in comparison to 100 for other peptide bonds. All models were first refined as rigid bodies and then subjected to several rounds of positional and *B*-factor refinement for individual atoms. Solvent molecules were automatically picked up by our program WATFIND in $(F_o - F_c)$ maps and incorporated into the refinement. After each round of refinement, water molecules with high *B*-factors were deleted, and new solvent molecules were picked from the $(F_o - F_c)$ map and added into the refinement. This water-picking−refining procedure was repeated until no peaks stronger than 3.5σ were found in the $(F_o - F_c)$ map. The results of the refinements are given in Table 2.

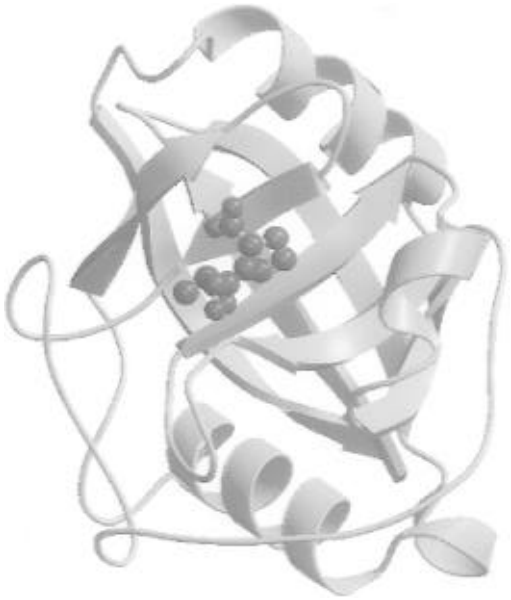

FIGURE 1: Ribbon presentation of the CyPA−Ser-Pro structure. Ser-Pro is in a ball-and-stick model. The structures of CyPA−His-Pro, CyPA−Gly-Pro, and CyPA−Ala-Pro are the same as that of the CyPA−Ser-Pro.

The structure comparison was performed with the program SUPERIMP or X-PLOR.

RESULTS AND DISCUSSION

The Cis Form of Dipeptides Bound to CyPA. The structures of CyPA complexed with the dipeptides of Ser-Pro, Gly-Pro, and His-Pro are an eight-stranded β-barrel (Figure 1), similar to those of the unligated CyPA (Ke et al., 1991; Ke, 1992) and the CyPA−cyclosporin A complex (Ke et al., 1994). The Ramachandran plot (Ramachandran, 1968), in which all amino acids of the finally refined structures have backbone conformations located at the

Table 3: Interactions between Dipeptides and CyPA

hydrogen bond dipeptide−CyPA[a]	distance (Å)			
	CyPA−Ser-Pro	CyPA−His-Pro	CyPA−Gly-Pro	CyPA−Ala-Pro
Xaa:N-Asn102:O	2.79	2.79	2.74	2.88
Xaa:N-water	2.62 (Wat52)	2.64 (Wat39)	2.69 (Wat48)	2.78 (Wat33)
Xaa:O-Asn102:N	3.05	2.95	3.07	3.02
Pro:O-Arg55:NE	2.84	2.91	2.84	2.85
Pro:O-Gln63:OE1	3.21	3.35	3.11	3.04
Pro:O-water	2.91 (Wat52)	2.95 (Wat39)		3.23 (Wat33)
Pro:OT-Arg55:NH1	2.66	2.73	2.73	2.78
Pro:OT-water	2.84 (Wat21)	2.82 (Wat40)	2.77 (Wat18)	2.75 (Wat16)
Pro:OT-water	3.10 (Wat48)			
water-Gln63:NE2	2.99 (Wat52)	2.93 (Wat39)	2.68 (Wat48)	2.81 (Wat33)
Ser:OG-Asn102:O	3.02			
Hydrophobic or Polar Interactions[b]				
Xaa: Ala101, Asn102, His126				
Pro: Phe60, Met61, Gln63, Phe113, Leu122				

[a] Xaa represents the amino acid before proline such as Ser in Ser-Pro etc. [b] A distance of 3.2−4.0 Å is defined as hydrophobic or polar interactions.

Table 4: Torsional Angles of the Dipeptides[a]

dipeptide	ψ_1	ω	ϕ_2	ψ_2	χ_1	χ_2	χ_3
Ser-Pro	155	5	−67	165	−5	27	−39
His-Pro	145	1	−78	14	37	−40	28
Gly-Pro	166	5	−76	9	−7	28	−38
Ala-Pro	148	−4	−77	6	37	−43	32

[a] The torsional angle of the proline side chain is listed.

energy-favored regions (data not shown), together with reasonable statistics (Table 2) ensures good stereochemistry of the structures. The first residue of the N-terminus was not included in the model for its poor electron density. For each molecule of CyPA, one molecule of the dipeptides Ser-Pro, Gly-Pro, His-Pro, or Ala-Pro binds to the hydrophobic pocket on the surface of the β-barrel. The four dipeptides bind to CyPA with similar interactions and molecular conformations. Residues of CyPA, which are involved in hydrogen bonds or van der Waal interactions with the dipeptides, include Arg55, Phe60, Met61, Gln63, Ala101, Asn102, Phe113, Leu122 and His126 (Table 3). The binding of dipeptides can be divided into three portions: hydrophobic interactions of the proline side chain and C- and N-terminal hydrogen bonding (Figure 2). The proline side chains of the dipeptides sit in the hydrophobic pocket made up of the side chains of Phe60, Met61, Phe113, Leu122, and His126 of CyPA. The C-terminus of the dipeptides forms two hydrogen bonds with the side chain of Arg55 while the N-terminus of the dipeptides hydrogen-bonds to the backbone carbonyl oxygen of Asn102 and a water molecule. The carbonyl oxygen of the amide bond is at a hydrogen bond distance to the backbone nitrogen of Asn102.

All four dipeptides are bound to CyPA in a *cis* conformation with peptide amide bonds of −4° to 5° (Table 4). The electron density for the dipeptides is excellent (Figure 3). Average *B*-factors were 27.4, 40.3, 31.4, and 23.2 Å^2 for dipeptides of Ser-Pro, His-Pro, Gly-Pro, and Ala-Pro, respectively, in comparison to the average *B*-values of 19.5, 19.8, 27.2, and 25.5 Å^2 for the corresponding proteins. The side chain of histidine in His-Pro has slightly higher *B*-factors with an average value of 44.6 Å^2. This is probably due to lack of polar interactions or hydrogen bonds between the histidine side chain and CyPA. Overall, our crystallographic evidence, well-shaped electron density, and reasonable *B*-values indicate the ordered binding, good occupancy, and a single *cis* conformation of the dipeptides under our experimental conditions.

Similar Structures of the Dipeptide Complexes Imply a Broad Specificity. The superposition between the structures of CyPA complexed with the dipeptides of Ser-Pro, His-Pro, Gly-Pro, and Ala-Pro revealed average positional displacements of 0.09−0.14 Å for C_α atoms and 0.21−0.26 Å for all atoms in the structures. The maximum positional displacement for residues, except for the N- and C-termini, is less than 0.4 Å for C_α atoms. However, a few residues such as Arg148 have side chain displacement as large as 4 Å. Since these residues have charged and long side chains and are located at the surface of the molecule, their large displacements most likely reflect conformational disorder without biological relevance.

There are no widely accepted standards to evaluate the coordinate errors in protein structures. A frequently used method called the Luzzatti plot (Luzatti, 1952) revealed that the coordinate errors for the CyPA structures complexed with dipeptides of Ser-Pro, His-Pro, Gly-Pro, and Ala-Pro ranged from 0.2 to 0.25 Å. If Luzzatti's analysis is taken as an estimation of structural errors although it has been argued that Luzzatti's analysis underestimates errors of structures determined by X-ray crystallography (Kuriyan et al., 1986; Brünger, 1988), the small positional displacements indicate that the structures of the CyPA−dipeptide complexes have the same overall structures and a similar conformation of individual residues.

In addition to the similar interactions and bound conformations of dipeptides, as discussed in the last section, the side chain of the Xaa residue in the dipeptides of Xaa-Pro makes weak interactions with CyPA. In the structure of CyPA−His-Pro, the histidine side chain of His-Pro forms a hydrogen bond with a water molecule and is within van der Waals distance of the carbonyl oxygen of Asn102 (about 3.6 Å^2). In the structure of CyPA−Ser-Pro, the side chain oxygen (OG) of serine hydrogen-bonds to the carbonyl oxygen of Asn102. In short, the structures of CyPA complexed with the dipeptides revealed that the side chain of Xaa in Xaa-Pro does not significantly contribute to the binding of the dipeptides, implying that cyclophilin has a broad catalytic specificity with respect to the side chain of Xaa in Xaa-Pro. This conclusion is consistent with the

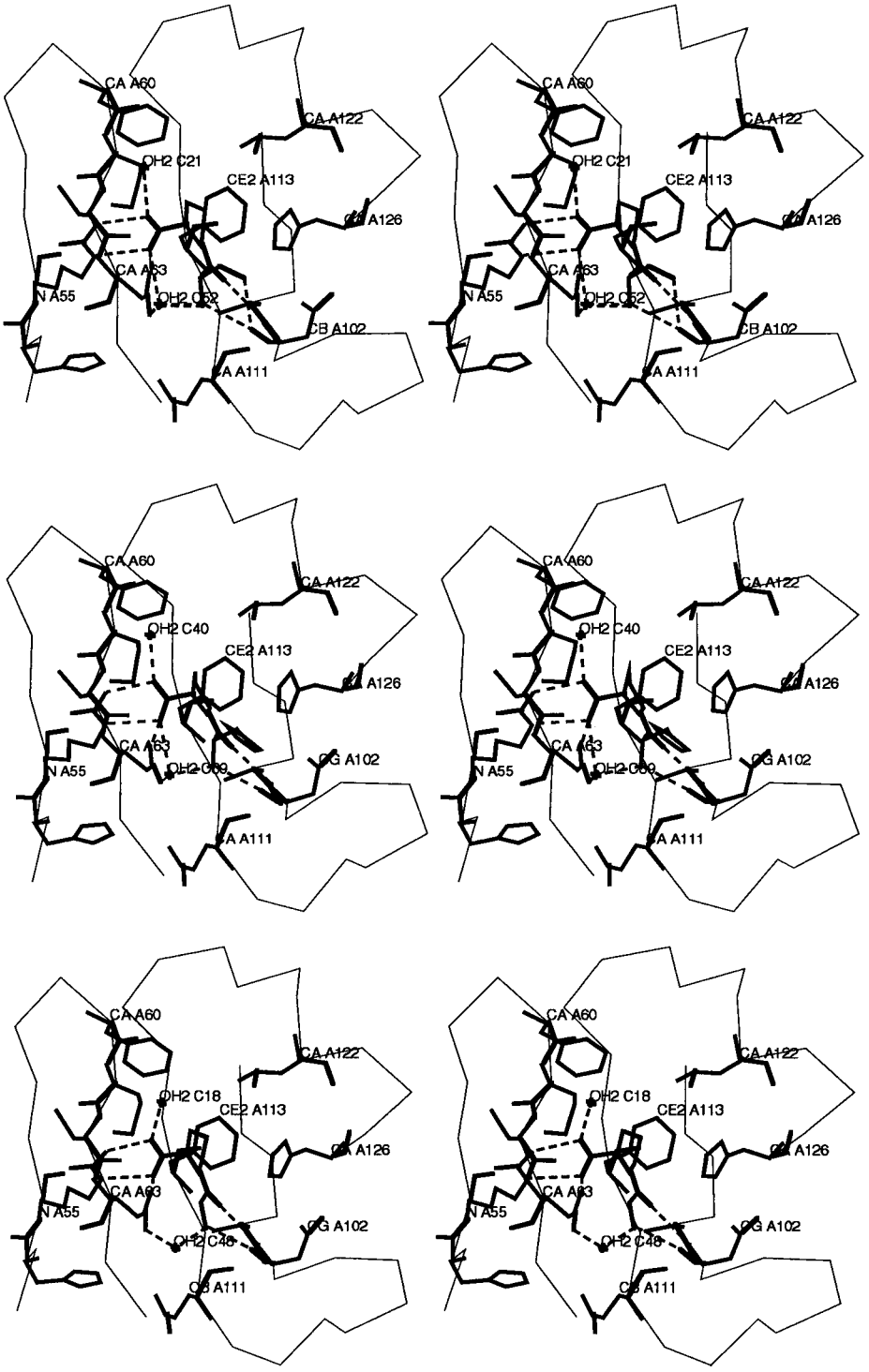

FIGURE 2: Stereo plot of the active site of CyPA complexed with (a, top) Ser-Pro, (b, middle) His-Pro, and (c, bottom) Gly-Pro. Thin lines represent the backbone traces of Ser52 to Phe65 and Gly97 to His126 while thick lines show non-hydrogen atoms of the active site residues. The water molecules are marked as "x", and dotted lines represent hydrogen bonds among the dipeptides, waters, and protein atoms. The detailed interactions are listed in Table 3.

kinetic data that the effect of the side chain of Xaa on the k_{cat} is less than 10-fold (Harrison & Stein, 1990b).

Binding of Dipeptides Is Overall Similar to That of AAPF but Different in Detail. The superposition between the structures of CyPA−AAPF and CyPA−dipeptides revealed average displacements of 0.23−0.26 Å for C_α atoms and 0.47−0.53 Å for all atoms, indicating similarity of these structures. In addition, the overall binding of the dipeptides to CyPA also closely resembles that of the tetrapeptide AAPF (Figure 4). First, the N-terminal portion of the peptides has the same hydrogen-bonding pattern. The nitrogen atom of Xaa in Xaa-Pro interacts with the carbonyl oxygen of A102 of CyPA [Table 3 of this paper and Table 2 in Zhao and Ke (1996)]. Second, the proline side chains of both dipeptides and AAPF sit in the hydrophobic pocket made up of the side chains of Phe60, Met61, Phe113, Ile122, and His126. Third, both dipeptides and the tetrapeptide have the *cis* peptidyl-prolyl amide bond (−4° to 5° for the dipeptides and 17.5° for AAPF).

On the other hand, significant differences in binding are observed between the dipeptides and AAPF. First, the two carboxyl-terminal oxygens of the dipeptides form three

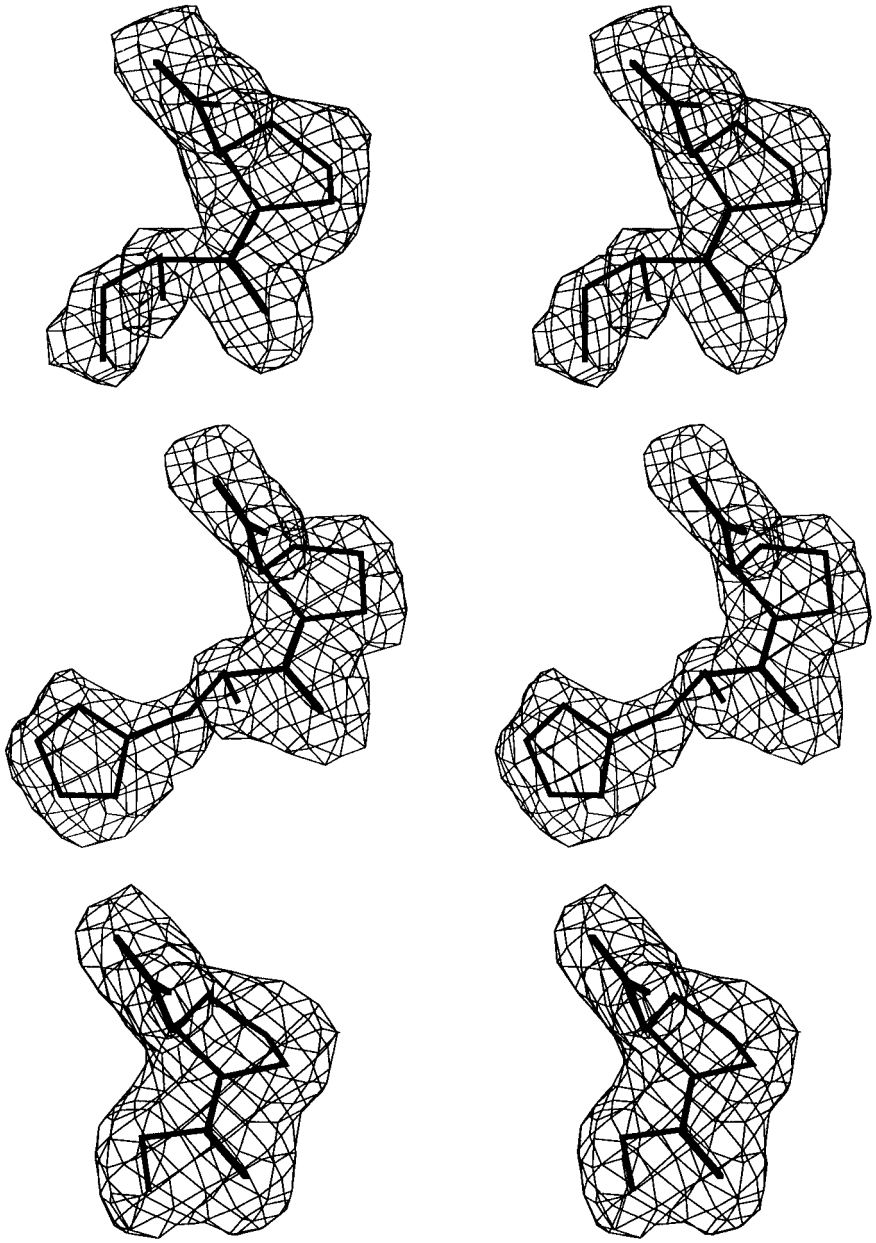

FIGURE 3: Stereo plot of electron density for the dipeptides (a, top) Ser-Pro, (b, middle) His-Pro, and (c, bottom) Gly-Pro. The electron density is calculated from the refined structure in which the dipeptides were omitted. The ($F_o - F_c$) map is contoured at 2.5σ. The electron density revealed that all dipeptides are orderly bound and have a single *cis* conformation.

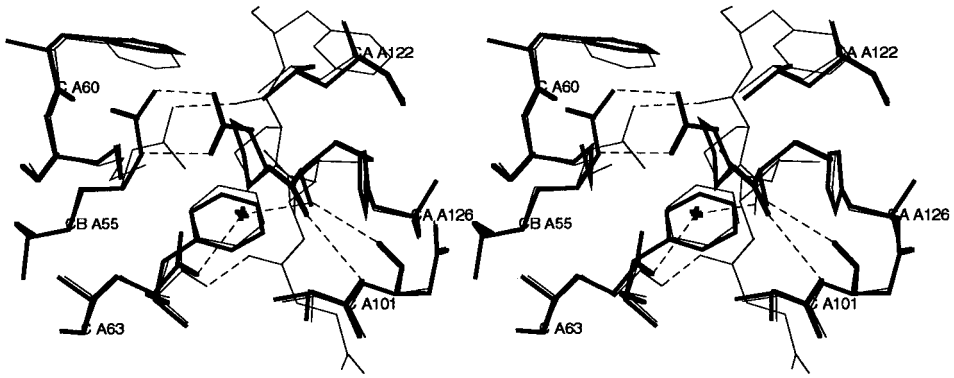

FIGURE 4: Superposition of the active sites of CyPA−Ser-Pro over CyPA−AAPF. Residues of CyPA−Ser-Pro are drawn in thicker lines while those of CyPA−AAPF are in thin lines. The dotted lines represent the hydrogen bonds. The water molecule marked as "x" is from CyPA−Ser-Pro. Dramatic changes are observed for the side chain conformations of Arg55 in the dipeptide and tetrapeptide complexes and for the interactions of Arg55 with the carboxyl terminus of the dipeptide or with the proline carbonyl of the tetrapeptide.

hydrogen bonds respectively with the side chain oxygen OE1 of Gln63 and NE and NH1 of Arg55, whereas the carbonyl oxygen of proline in the tetrapeptide forms hydrogen bonds with NH1 and NH2 of Arg55 in the CyPA−AAPF structure

(Figure 4). Second, the hydrogen bond between OE1 of Gln63 and NH2 of Arg55 in the CyPA−AAPF structure (2.6 and 2.7 Å in the dimer) becomes weak van der Waals interactions in the CyPA−dipeptide complexes in which the shortest distance is 3.8 Å between OE1 of Gln63 and the side chain atoms of Arg55. Third, the conformation of the side chain of Arg55 is changed dramatically. The torsional angle around the CD−NE bond of the side chain of Arg55 changed from about 70° (59° to 73°) in the CyPA−dipeptide structures to about −140° (−134° and −145°) for the two molecules in asymmetric unit of the CyPA−AAPF structure, while other torsional angles of the side chain of Arg55 have similar conformational angles about 180° (between −170° and 152°). Fourth, the interaction between the carbonyl oxygen of the proline amide bond and the backbone nitrogen of Asn102 is a normal hydrogen bond with a distance of about 3 Å in the dipeptide complexes (Table 3), whereas the same interaction is much weaker with the distances of 3.30 and 3.55 Å in the dimer of the tetrapeptide complex. Last, the water molecule which is hydrogen-bonded to the side chain nitrogen of Gln63 in the CyPA−dipeptide complexes is displaced by the oxygen of the succinate group of the tetrapeptide AAPF. This water molecule was proposed to assist the *cis*−*trans* isomerization of the peptidyl-prolyl amide bond on the basis of the structure of CyPA complexed with Ala-Pro (Ke et al., 1993a). In short, the first three differences in the peptide binding may result from the special nature of the carboxyl terminus of the dipeptides in corresponding to the amide bond of the tetrapeptide, whereas the last two differences may reflect the presence of the N-terminal moiety of the tetrapeptide.

Are Dipeptides Inhibitors of CyP or Do They Have a Different Catalytic Mechanism from the Tetrapeptide? The binding differences of the dipeptides and tetrapeptide imply either that the dipeptides and tetrapeptide belong to two categories of substrates having different catalytic pathways or that the dipeptides are not substrates but competitive inhibitors of CyP. Indeed, it has been reported that Ala-Pro is a competitive inhibitor of the *cis*−*trans* isomerase (Fischer et al., 1994).

On the basis of the crystal structure of the CyPA−AAPF complex, we proposed in the accompanying paper (Zhao & Ke, 1996) that Arg55 hydrogen-bonds to the lone pair electrons of the amide nitrogen during catalysis and deconjugates the resonance of the amide bond so as to lessen the barrier of the *cis*−*trans* isomerization, as schematically presented in Figure 5a. If this mechanism, which assumes Arg55 plays a key role in the *cis*−*trans* isomerization, is correct and unique, the binding of the carboxyl terminus of dipeptides to Arg55 should inhibit the function of Arg55. In general, it may be further reasoned that all peptides with a C-terminal proline should have C-terminal binding as exhibited by the dipeptides and should thus be inhibitors of cyclophilin.

However, the above argument is inconsistent with identification of benzoyl-Phe-Ala-Pro as a substrate of cyclophilin by NMR (Hsu et al., 1990). The carboxyl terminus of the tripeptide has the same chemical structure as Ala-Pro and thus should have the same binding as the dipeptides. The fact that benzoyl-Phe-Ala-Pro is a substrate of CyP implies that blocking Arg55 is not sufficient to completely abolish the *cis*−*trans* isomerization. From this point of view, dipeptides might be considered as substrates of CyP.

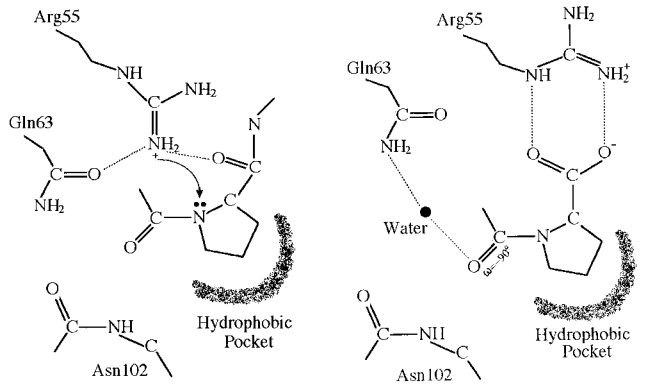

FIGURE 5: Schematic presentation of the mechanism of the peptidyl-prolyl *cis*−*trans* isomerization: (a, left) a general acid mechanism in which Arg55 hydrogen-bonds to the lone pair electrons of the amide nitrogen so as to lower the barrier of the *cis*−*trans* rotation and (b, right) the solvent-assisted mechanism in which a bound water hydrogen-bonds to the carbonyl oxygen of the distorted amide bond and stabilizes binding of the intermediate.

In the nonenzymatic prolyl *cis*−*trans* isomerization, the dipeptides have consistently higher ΔG and lower isomerization rates than the tetrapeptides and thus have been considered to have a different mechanism from the tetrapeptide (Stein, 1993). For the enzymatic prolyl *cis*−*trans* isomerization, the crystal structures revealed that the *cis* ground state of dipeptides is further stabilized by a hydrogen bond between the carbonyl oxygen of the prolyl amide bond and the backbone nitrogen of Asn102 (about 3 Å; Table 3). However, the same interaction becomes much weaker, 3.30 and 3.55 Å in the dimer of the tetrapeptide complex. This hydrogen bond will increase the barrier of the *cis*−*trans* isomerization for the dipeptides and make dipeptides be poor substrates. On the basis of differences of detailed binding between dipeptides and the tetrapeptide and under an assumption that dipeptides are substrates of CyP, it might be argued that dipeptides have a different mechanism from the tetrapeptide in the CyP-catalyzed *cis*−*trans* isomerization.

We previously proposed a solvent-assisted mechanism for the *cis*−*trans* isomerization of dipeptides on the basis of the observation that a water molecule consistently binds to the side chain of Gln63 in both structures of the unligated CyPA and the CyPA−Ala-Pro complex (Ke et al., 1993a). In the crystal structures, the *cis* ground state of the dipeptides is stabilized by a hydrogen bond between the backbone nitrogen of Asn102 and the carbonyl oxygen of the amide bond. The model docking showed that the water molecule will form a hydrogen bond with the carbonyl oxygen and thus stabilizes the intermediate of substrate (Figure 5b), if the amide bond turns to 90° while the orientation of the C-terminus of the substrate is fixed by numerous hydrogen bonds and hydrophobic interactions (Ke et al., 1993a). This solvent-assisted mechanism is similar to the proposal of "catalysis by distortion" (Harrison & Stein, 1990a) but pinpoints water instead of protein residues as the chemical group to cause the distortion of the amide bond. However, this water molecule is not observed in the CyPA−AAPF complex so that the solvent-assisted mechanism is not verified on the basis of the tetrapeptide structure.

As an enzyme, cyclophilin only generates a rate enhancement of 5 orders of magnitude and has the lowest catalytic proficiency in a list of compared enzymes (Radzicka & Wolfenden, 1995). On the other hands, CyP can take

peptides and proteins with various sizes as its substrates. Thus, the catalytic proficiency of CyP may vary from inhibitor-like substrates to "real" substrates and may be affected by many factors such as the binding of the N- and C-termini of a substrate. The comparison between the crystal structures of the dipeptides and tetrapeptide complexes might suggest a possibility that the enzymatic prolyl *cis*−*trans* isomerization has multiple pathways for various substrates. A firm conclusion needs further experiments using NMR or kinetic approaches.

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