Solution Conformation of a Cyclophilin-Bound Proline Isomerase Substrate†

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ABSTRACT: Cyclophilin (CyP) is the 17.8-kDa cytosolic receptor of the immunosuppressant cyclosporin A (CsA) and also a peptidyl prolyl *cis–trans* isomerase (PPIase). In order to gain insights into the PPIase mechanism, transferred nuclear Overhauser effect (TRNOE) measurements by two-dimensional ^{1}H NMR were used to determine the conformation of the isomerase-bound standard model substrate suc-AAPF-pNA. Results indicate a *cis*-like conformation for the CyP-bound substrate with the A–P peptide bond being no more than 40° out of planarity.

Cyclophilin (CyP)[1] is the 17.8-kDa cytosolic receptor of the immunosuppressive drug cyclosporin A (CsA) and a peptidyl-prolyl *cis–trans* isomerase (PPIase) that catalyzes the *cis–trans* isomerization of X-Pro imide bonds, a catalysis strongly inhibited by CsA (Handschumacher et al., 1984; Takahashi et al., 1989; Fischer et al., 1989a). However, the lack of correlation between PPIase inhibition and immunosuppression for certain CsA analogues (Sigal et al., 1991) and the inability of CsA to inhibit the PPIase activity of an *Escherichia coli* CyP (Liu & Walsh, 1990) suggest that PPIase inhibition and immunosuppression are probably unrelated. Further evidence is the ability of PPIase inactive site-directed mutants of human CyP to bind CsA and subsequently inhibit the phosphatase activity of calcineurin (Zydowsky et al., 1992), an inhibition considered to be important for immunosuppression (Schreiber et al., 1992). Nevertheless the X-Pro isomerization and its catalysis by CyP are important per se for their role in protein folding (Freskgård et al., 1992; Gething & Sambrook, 1992; Schmid et al., 1993). Moreover, structural information on PPIase catalysis and the substrate transition state in particular may benefit the design of biologically active peptidomimetic ligands to PPIase receptors.

Proposed mechanisms for the PPIase catalysis include nucleophilic attack on the carbonyl carbon of the X-Pro peptide bond (Fischer et al., 1989b) or protonation of the X-Pro peptide bond nitrogen (Kofron et al., 1991), both leading to the formation of tetrahedral intermediates with decreased double bond characters for the X-Pro peptide bonds and lowered activation energy barriers for the *cis–trans* interconversion. However, steady-state kinetic investigations of the CyP activity and specificity (Harrison & Stein, 1990a,b, 1992) and site-directed mutagenesis studies (Liu et al., 1990) argue against either a nucleophile or an acid/base catalyzed mechanism. A "catalysis by distortion" mechanism (Harrison & Stein, 1990a; Liu et al., 1990; Stein, 1993) whereby noncovalent enzyme–substrate interactions stabilize a substrate transition state with a nonplanar X-Pro peptide bond is awaiting experimental support. The elucidation of the CyP-bound substrate conformation should then provide important insight into the PPIase mechanism.

The X-ray structure of recombinant human CyP (rhCyP) complexed with the model substrate *N*-acetyl-AlaAlaProAla-amidomethylcoumarin (ac-AAPA-amc) at 2.8-Å resolution showed the AlaPro imide bond in the *trans* conformation with the substrate binding site being identical to that of CsA (Kallen et al., 1991). Subsequent work at 2.3-Å resolution identified the CyP residues in the active site and revealed a structure consisting of a dimer of CyP–substrate complexes, each CyP molecule accommodating a *cis* ac-AAPA-amc molecule in its active site while also being associated with a partially disordered *trans* tetrapeptide (Kallen & Walkinshaw, 1992). This dimer arrangement, however, could be the result of the observed stacking of six aromatic groups (four tetrapeptide coumarins and two CyP Trp indoles) and may not be biologically relevant. A third X-ray study at 1.64-Å resolution of rhCyP complexed with the dipeptide AlaPro identified only the *cis* conformer as protein-bound and provided a detailed description of the protein active site (Ke et al., 1993). The likelihood that this dipeptide is a poor CyP substrate as it is for FKBP (Harrison & Stein, 1992) should be kept in mind though when conclusions pertaining to PPIase catalysis are drawn. In view of the hydrophobic nature of the CyP active site and the enchanced *cis–trans* interconversion of Pro-containing compounds in nonpolar solvents (Eberhardt et al., 1992; Radzicka et al., 1992), a solvent-assisted catalysis mechanism for CyP was proposed that involves the desolvation of the substate and the stabilization of the transition state by a protein-bound water molecule (Ke, 1992; Ke et al., 1993).

Information concerning the solution structure of a CyP-bound substrate without any artifacts from crystal packing forces may be obtained by nuclear magnetic resonance (NMR) spectroscopy and transferred nuclear Overhauser effect (TRNOE) measurements in particular (Clore & Gronenborn, 1982, 1983; Rosevear & Mildvan, 1989; Campbell & Sykes, 1991a). The method is an extension of NOE measurements to systems undergoing chemical exchange (e.g., a ligand weakly

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[1] Abbreviations: CyP, cyclophilin; PPIase, peptidyl prolyl isomerase; CsA, cyclosporin A; rhCyP, recombinant human CyP; ac-AAPA-amc, *N*-acetyl-AlaAlaProAla-amidomethylcoumarin; suc-AAPF-pNA, *N*-succinyl-AlaAlaProPhe-*p*-nitroanilide; deuterated TSP, sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$; Bz-FAP, benzoyl-PheAlaPro; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DMSO, dimethyl sulfoxide; FKBP, FK506 binding protein; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; TRNOE, transferred NOE; COSY, correlation spectroscopy; TPPI, time proportional phase incrementation; FT, Fourier transform; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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binding to a macromolecule) and has been extensively used, among other things, to determine the conformation of protein-bound peptides (Clore et al., 1986; Meyer et al., 1988; Ni et al., 1989a,b, 1990, 1992; Anglister & Naider, 1991; Zilber et al., 1990; Scherfet et al., 1992; Landry & Gierasch, 1991; Landry et al., 1992; Bushweller & Bartlett, 1991; Campbell & Sykes, 1991b; Sukumar & Higashijima, 1992; Lippens et al., 1993). It is based on the transfer of information concerning cross-relaxation between two nuclei of the bound ligand to the free ligand resonances via chemical exchange. In the free state the ligand is characterized by short correlation times ($\tau_c \sim 100$ ps) being either at the extreme narrowing limit ($\omega\tau_c \ll 1$) where the NOEs are small and positive or near the limit ($\omega\tau_c \sim 1$) where the NOEs approach zero. When bound, the ligand's correlation time becomes that of the protein ($\tau_c \sim$ 10 ns or larger) and it is thus at the spin diffusion limit ($\omega\tau_c \gg 1$), a condition in which the NOEs are large and negative. In the presence of fast chemical exchange of the ligand between the free and the bound state, negative bound-state NOEs are transferred to the free or exchange-averaged ligand resonances where they can be easily measured. These negative NOEs by virtue of their larger magnitude dominate the observed NOEs which then reflect the bound ligand conformation.

MATERIALS AND METHODS

Protein Preparation. The recombinant human cyclophilin gene, cloned into the pHN1+ expression vector, was kindly provided by S. Schreiber and C. Walsh and was subsequently transformed into the *E. coli* host XA90 FlacI^{Q1} (Maniatis et al., 1982). The protein was overexpressed, harvested, and purified as previously detailed (Liu et al., 1990). The progress of the purification was monitored by SDS–PAGE (Laemmli, 1970) on a PhastSystem (Pharmacia LKB, Uppsala, Sweden). Total protein concentration was measured using the Bio-Rad protein assay (Bradford, 1976) with bovine serum albumin as standard and a conversion factor of 0.65 for CyP (R. E. Handschumacher, personal communication). Concentrations of active CyP at different stages were determined by the LH-20 Sephadex (Pharmacia) column assay (Koletsky et al., 1986). The purified CyP (ca. 20 mg from a 1-L growth) was homogeneous and essentially 100% active. The protein solution was sterile filtered and stored under Ar at 4 °C at or below 1 mg/mL in 20 mM Tris, 0.2 M NaCl, 5 mM BME, 0.02% NaN_3 buffer, pH 7.8.

NMR Sample Preparation. The requisite amount of CyP solution was dialyzed overnight at 4 °C against 2 L of a 50 mM phosphate buffer, pH 6.8, that also contained 150 mM NaCl, 0.02% NaN_3, 0.5 mM DTT, 0.1 mM EDTA, and 0.6 mM PMSF (from a 100 mM stock solution in DMSO). It was then concentrated under N_2 by ultrafiltration to 0.5–1.0 mL using an Amicon Microconcentrator (YM-10 membrane) at 4 °C and buffer-exchanged against 20 mL of Ar-flushed 50 mM potassium phosphate, 150 mM NaCl, 0.02% NaN_3, 0.5 mM DTT, 0.1 mM EDTA buffer, pH 6.8 (uncorrected pH meter reading) made in either metal-free D_2O (>99.9 atom % D, MSD Isotopes, Merck) or a mixture of 90% H_2O/10% D_2O. NMR samples were prepared by dissolving 0.3 mg of *N*-succinyl-AlaAlaProPhe-*p*-nitroanilide (suc-AAPF-pNA, Sigma) in 0.5 mL of CyP stock solution and/or buffer. The final concentration of peptide was 0.86 mM (assuming 90% peptide content) and that of CyP 92 μM. In control experiments, the CyP–CsA complex was formed by adding ~2 mol equiv (to CyP) of finely ground CsA directly to the NMR samples that have been previously used for the TRNOE measurements followed by gentle overnight agitation at 4 °C.

NMR Measurements. One- and two-dimensional proton NMR spectra were acquired on a Bruker AM500 NMR spectrometer operating at 500.13 MHz. Two-dimensional correlation spectroscopy, COSY (Aue et al., 1976; Bax & Freeman, 1981), and NOE spectroscopy, NOESY (Jeener et al., 1979; Macura & Ernst, 1980), measurements were recorded in the pure absorption mode by employing time proportional phase incrementation, TPPI (Redfield & Kunz, 1975; Marion & Wüthrich, 1983). Selection of desirable signals and artifact suppression were accomplished by 8-step (COSY) or 32-step (NOESY) phase cycles. NOESY spectra were acquired with mixing times of 200 and 400 ms that were randomly varied by 10% in order to eliminate zero-quantum coherence transfer (Macura et al., 1981).

Typically, 512 t_1 increments of 2K complex data points over a 6-kHz spectral width were collected with 16 (COSY) or 32 (NOESY) scans per t_1 value that were preceded by 4 dummy scans. All spectra were acquired with the carrier offset placed on the water resonance which was suppressed by coherent decoupler irradiation (Zwiderweg et al., 1986) applied during the relaxation delay (1.5 or 2 s for 90% H_2O and D_2O, respectively) and the NOESY mixing time with field strengths of 37 Hz for 90% H_2O and 14 Hz for D_2O. The peptide NH resonances must not be in fascile exchange with water since they were readily observed for samples in 90% H_2O in the presence of solvent presaturation. The replacement of the NOESY read pulse by a "jump-and-return" pulse sequence (Plateau & Guéron, 1982) did not result in any NH intensity enhancement. Baseline distortion was addressed by properly adjusting the sampling delay and the signal phase (Marion & Bax, 1988). All spectra were acquired at 5 °C and referenced to the HDO resonance of the D_2O samples (5.02 ppm vs deuterated TSP, sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$).

All data sets were processed and analyzed on Silicon Graphics 4D/25TG or Indigo workstations using the FELIX software package (Biosym Technologies, San Diego, CA). Convolution difference of the time domain data (Marion et al., 1989) was used to remove the residual water resonance of 90% H_2O data sets. Unshifted (COSY) or $\pi/6$ (t_2) and $\pi/4$ (t_1) shifted (NOESY) sine-bell window functions were used. Datasets were zero-filled once in the t_1 dimension yielding digital resolutions of 5.9 Hz/point (t_2) and 11.8 Hz/point (t_1). In order to decrease t_1 ridges arising from incorrect treatment of the first data point in the discreet Fourier transform (FT) algorithm, the spectrum corresponding to the first t_1 value was divided by 2 prior to FT along t_1 (Otting et al., 1986). Final matrices were 1K × 1K and have not been symmetrized.

NOE Quantitation and Distance Constraints. NOEs and TRNOEs were quantitated for all data sets by measuring cross-peak volumes and using empty areas adjacent to cross-peaks for baseline correction. Whenever required, corrections for the free peptide contribution to cross-peak volumes were also made. The corrected cross-peak volumes V_o of assigned reference proton pairs such as Ala αCH/βCH$_3$ (r_o = 2.5Å) or geminal CH$_2$ (r_o = 1.8Å) were used to obtain distances from their corresponding corrected cross-peak volumes V according to

$$r = (V_o/V)^{1/6} r_o \quad (1)$$

Prior to comparison, cross-peak NOE intensities were divided by a normalizing factor of $2N_AN_B/(N_A+N_B)$, where N_A and N_B are the proton numbers of the parent diagonal peaks A and B (Williamson & Neuhaus, 1987; Campbell & Sykes,

Table 1: Proton NMR Resonance Assignments for suc-AAPF-pNA in Aqueous Solution, pH 6.7, at 5 °C

	chemical shift (ppm)[a]				
residue	NH	αCH	βCH	others	
A1					
trans	8.34	4.28	1.37		
cis	8.20	4.34	1.33		
A2					
trans	8.52	4.58	1.34		
cis	8.35	4.11	1.27		
P3					
trans		4.44	2.28, 1.86	2.00 (γCH_2)	3.80, 3.63 (δCH_2)
cis		4.57	2.13	1.62	3.48, 3.45
F4					
trans	8.74	4.60	3.27, 3.10	7.29 ($\delta\delta'$CH)	7.32 ($\epsilon\epsilon'$CH)
cis	8.82	4.69	3.20	*b*	*b*

[a] Values (±0.01 ppm) vs the residual water resonance, at 5.02 ppm vs TSP (0.00 ppm). [b] Not identified, most likely overlapping with the *trans* $\delta\delta'$, $\epsilon\epsilon'$ CH resonances. The same must be true for the *trans* and *cis* resonances ζCH.

1991b). In the case of stereospecifically unassigned resonances (e.g., AlaβCH_3), pseudostructures with the appropriate distance constraint corrections (Wüthrich, 1986) were used in molecular models. The software package INSIGHT II (Biosym Technologies, San Diego, CA) running on Silicon Graphics 4D/25TG or Indigo workstations was used for the interactive display and manipulation of molecular structures.

RESULTS

Proton Resonance Assignments and Conformation of the Unbound Substrate. The amino acid spin systems were identified from the tetrapeptide COSY spectrum in D_2O. The NH and the sequential assignments were made from the αCH_i-NH_{i+1} cross-peaks of the corresponding NOESY spectrum (400-ms mixing time) in 90% H_2O/10% D_2O. Linear oligopeptides are present in solution as an ensemble of rapidly interconverting conformers, and their NMR parameters are a population-weighted average over all conformations. Only the *cis–trans* isomerization of the peptide bond, usually X-Pro, is sufficiently slow (20 kcal/mol activation energy) to allow for the observation of two distinct species by NMR. The dominant conformer of suc-AAPF-pNA is *trans*, as evidenced by the strong NOEs between the major A2αCH and P3δCH_2 resonances (Wüthrich, 1986). From the relative peak intensities of the two populations, the minor *cis* conformer is estimated at 5–10%. The low *cis* concentration did not allow the obvervation of the diagnostic A2αCH–P3αCH NOE. The *cis* conformer assignments were based on the corresponding *trans* conformer resonances and the NOESY chemical exchange cross-peaks in the presence of CyP (peptide:CyP ~ 400:1). The assignment results are summarized in Table 1.

In a pure phase absorption NOESY spectrum, a negative NOE gives rise to a cross-peak with the same sign as that of the diagonal peaks (usually phased positive) whereas a positive NOE gives rise to a cross-peak of opposite sign. Surprisingly, the unbound suc-AAPF-pNA (monomer MW of 625) showed positive NOESY cross-peaks that correspond to negative NOEs. This presumably results from the combined effect of slower rotational tumbling at 5 °C particularly in the ca. 20% more viscous D_2O solution (Merck Index, 10th ed.) and self-association at low temperature as evidenced by the slowly exchanging NH protons: suc-AAPF-pNA showed negative NOEs in D_2O at 5 and 25 °C as well as in 90% H_2O at 5 °C, but it showed positive NOEs in 90% H_2O at 25 °C where no NH resonances could be observed in the presence of solvent

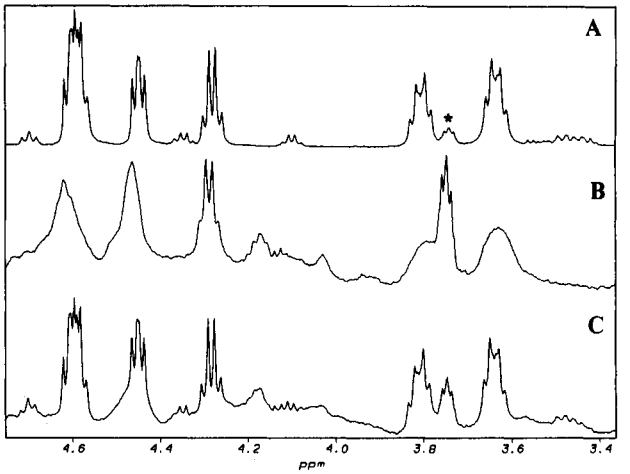

FIGURE 1: CyP-induced resonance broadening of suc-AAPF-pNA and the effect of CsA in buffered D_2O solutions at pH 6.8 and 5 °C: (A) Substrate only, 0.86 mM. (B) Substrate plus CyP, 92 μM (mole ratio 9:1). (C) Substrate plus CyP plus CsA (mole ratio 9:1:2). Acquisition and processing conditions were constant throughout. The peak marked with an asterisk in A is from reduced DTT.

presaturation. The absence of positive NOEs for unbound oligopeptides at low temperature has been noted before (Clore et al., 1986; Campbell & Sykes, 1991b). The situation is not without merit as it fascilitates the correction for the contribution of the free peptide to the TRNOEs.

Substrate Interaction with CyP. In the presence of CyP, there is a marked broadening of all peptide resonances which is completely removed upon the addition of the PPIase inhibitor CsA (Figure 1). This broadening is, therefore, not simply the result of the sample's increased viscosity upon addition of the protein or a nonspecific substrate–protein interaction but must reflect specific substrate binding to the CyP active site. The backbone resonances of A2, P3, and F4 as well as those of the P side chain seem to be affected the most. This differential line broadening is consistent with the reported location of the complexed substrate pyrrolidine ring in a CyP hydrophobic pocket (Kallen & Walkinshaw, 1992; Ke et al., 1993). The broadening could also result from exchange averaging with resonances that are ring current shifted in the bound state as a result of their proximity to protein aromatic group (e.g., F60 or F113 of CyP; Ke et al., 1993). In either event, the conclusion about the identity of the substrate groups that are most intimately involved in binding to CyP is unaffected by the origin of the differential line broadening.

The addition of CyP does not give rise to a new set of resonances for the substrate consistent with the fact that the peptide is in the intermediate or fast exchange limit. Under the experimental conditions used (free to bound ratio of ca. 20) the positions of the averaged substrate resonances are approximately the same as those of the free peptide. The minor *cis* resonances are no longer observable as a result of exchange averaging with the corresponding *trans* resonances (Figure 1B): at this high enzyme to substrate ratio, the interconversion rate well exceeds even the largest *cis–trans* chemical shift difference (235 Hz for A2α). In the presence of CsA, the minor *cis* resonances are again discernible (Figure 1C).

The substrate–CyP interaction may also be followed in NOESY spectra (Figure 2). In the absence of CyP the dominant Ala-Pro conformation is *trans* as evidenced by the diagnostic A2αCH–P3δCH_2 NOE cross-peaks (Wüthrich, 1986) boxed in Figure 2A. When CyP is present in catalytic amounts (substrate:enzyme ~400:1) cross-peaks from the

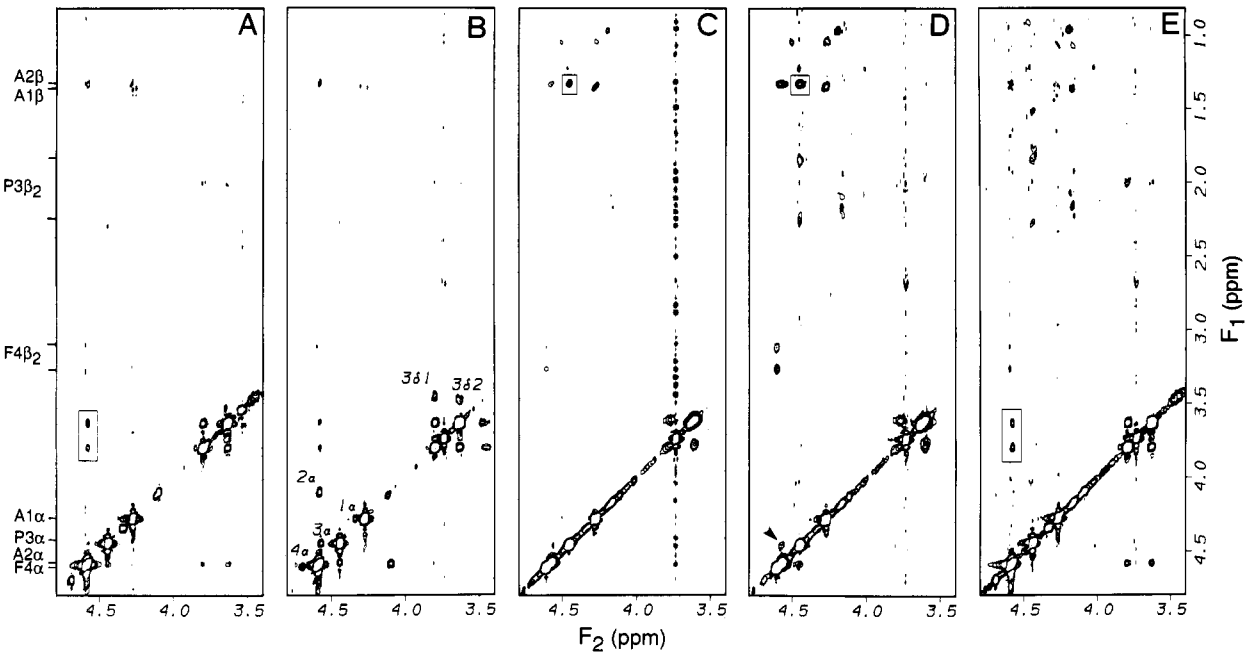

FIGURE 2: αCH/δCH (F_2)–aliphatic (F_1) region of the pure phase absorption 500- MHz NOESY spectra of suc-AAPF-pNA (0.86 mM) in buffered D_2O solutions, pH 6.8 at 5 °C. The identically acquired and processed spectra are directly comparable. (A) Peptide only, 400-ms mixing time; the A2αCH–P3δCH_2 NOE cross-peaks are boxed. (B) Peptide plus CyP (2.2 μM), 400-ms mixing time; the chemical exchange cross-peaks are identified. (C) Peptide plus CyP (92 μM), 200-ms mixing time; the A2βCH_3–P3αCH transferred NOE is boxed. (D) As in panel C, 400-ms mixing time; the A2αCH–P3αCH transferred NOE is indicated with an arrow. (E) Peptide plus CyP (92 μM) plus CsA (0.18 mM), 400-ms mixing time; the A2αCH–P3δCH_2 NOE cross-peaks are boxed.

enhanced *cis–trans* interconversion appear (Figure 2B). These positive cross-peaks are due to chemical exchange and do not carry any structural information. When the CyP concentration is further increased (substrate:enzyme ~9:1), the chemical exchange cross-peaks disappear (Figure 2C and D) as a result of the previously mentioned *cis–trans* exchange averaging. Two new positive TRNOE cross-peaks (A2βCH_3–P3αCH in Figure 2C and A2αCH–P3αCH in Figure 2D) become apparent. The latter (A2α–P3α) is characteristic for a *cis* Ala-Pro conformation (Wüthrich, 1986). In the presence of the PPIase inhibitor CsA (Figure 2E), the TRNOE cross-peaks disappear and the A2αCH–Pro3δCH_2 NOE cross-peaks for the dominant *trans* conformation of unbound suc-AAPF-pNA are again visible.

Transferred NOEs and Distance Constraints. The NOESY spectrum of the 9:1 peptide:protein sample is dominated by the NOEs of the CyP-bound peptide. The observed intraresidue TRNOEs in D_2O with the corresponding distances (Clore et al., 1986; Ni et al., 1989b, 1990, 1992) are provided in Table 2. Most of these NOEs albeit of reduced intensity are also observed in the 400-ms NOESY spectrum of the peptide-only sample and being trivial are of no interest except as distance reference standards. The limited number of TRNOEs, unfortunately, does not permit the accurate determination of the bound substrate structure (Lippens et al., 1993). The focus here is on the interresidue TRNOEs, A2αCH–P3αCH and A2βCH_3–P3αCH, and their ability to define the A–P peptide bond conformation of the CyP-bound suc-AAPF-pNA.

Peptide protons within chemical groups that exhibit local motion such as the flipping F ring or the puckering P side chain can have significantly shorter correlation times than backbone protons (Clore et al., 1986). Their use as internal reference standards leads to underestimates of other interproton distances. As discussed below, the use of protons within rotating methyl and probably methylene groups appears to have little effect on TRNOE-derived distances (Campbell & Sykes, 1991c; Nirmala et al., 1992). This consideration limits the choice of reference distance(s) to A1αCH–βCH_3 or A2αCH–βCH_3 (2.5 Å) and possibly F4αCH–βCH (2.5 Å) or F4βCH_2 (1.8 Å). Both cross-peaks corresponding to the AαCH–βCH_3 reference proton pairs displayed linear buildup of magnetization up to 400 ms and so did the A2αCH–P3αCH and A2βCH_3–P3αCH TRNOE cross-peaks. On the other hand, the buildup of any geminal CH_2 cross-peak was not linear presumably because of the larger susceptibility of shorter distances between strongly coupled protons to spin diffusion (Campbell & Sykes, 1991a). The calculated A2βCH_3–P3αCH distance was 2.3 Å using F4βCH_2 (200 ms) or AαCH–βCH_3 (200 or 400 ms) as a reference distance whereas the A2αCH–P3αCH distance turned out to be 2.4–2.6 Å using the same standards. Use of either the PδCH_2 or PβCH_2 (200 ms) as reference gave a A2αCH–P3αCH distance of 1.8–2.0 Å, the underestimate being attributed to the local motion of the P ring. Inspection of contour plots suggests that a 2.3 Å A2βCH_3–P3αCH distance is consistent with the observed

Table 2: Observed[a] TRNOEs and Corresponding Distances (Å)

intraresidue	AlaαCH–A1βCH_3	2.5[b]
	A2αCH–A2βCH_3	2.5[b]
	P3αCH–P3βCH_2[c]	2.5, 3.0
	P3βCH_2–P3γCH_2[d]	2.5–3.1
	P3βCH_2	1.8
	P3δCH_2	1.8
	F4αCH–F4βCH_2[e]	2.5
	F4βCH_2	1.8
	F4αCH–F4δCH[e]	3.0
	F4βCH_2–F4δCH[e]	2.5
interresidue	A2αCH–P3αCH	2.6 ± 0.5
	A2βCH_3–P3αCH	2.3 ± 0.5

[a] In D_2O solutions. Additional TRNOEs observed in 90% H_2O/10% D_2O solutions are the intraresidue A1NH–A1βCH_3 and A2NH–A2βCH_3 and the sequential A1NH–sucCH_2, A2NH–AlaαCH, and F4NH–P3αCH. [b] Defined as $\langle r^{-6}\rangle^{-1/6}$. [c] The shorter distance corresponds to the PβCH *cis* to the PαCH. [d] Four different distances in this range. [e] Rotation-averaged distances.

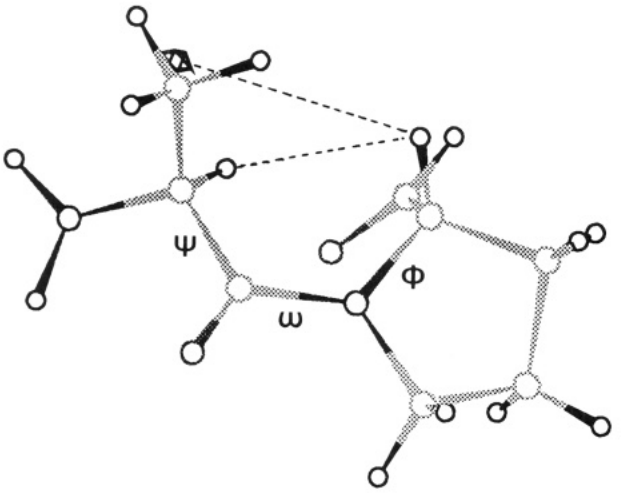

FIGURE 3: Dihedral angles, ψ, ω, and ϕ for the dipeptide AP that determine the distances (dotted lines) A2αCH–P3αCH and A2βCH$_3$ pseudoatom–P3αCH corresponding to observed TRNOEs for the CyP-bound suc-AAPF-pNA. Only the ψ angle of A2 and the ω angle of the A2–P3 peptide bond may be varied, the ϕ angle of P3 being fixed by the pyrrolidine ring at ca. –70°.

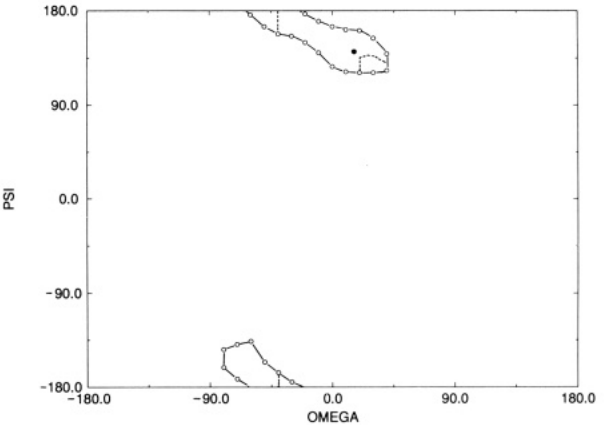

FIGURE 4: The A2 ψ and A2–P3 ω dihedral angle values that satisfy within 0.5 Å both A2αCH–P3αCH and A2βCH$_3$–P3αCH TRNOE-derived distances for CyP-bound suc-AAPF-pNA. The dotted lines separate areas with unlikely conformations (see text). The filled circle corresponds to the ψ = +141°, ω = +16° conformation for which A2βCH$_3$–P3αCH = 2.3 Å and A2αCH–P3αCH = 2.6 Å. The imide bond *cis* and *trans* conformations correspond to ω values of 0° and ±180°, respectively.

cross-peak intensities. The intensity of the A2αCH–P3αCH cross-peak appeared slightly lower than those of other cross-peaks corresponding to 2.5 Å (e.g., F4αCH–βCH). That could result from a cross-peak volume overestimate due to its incomplete resolution from the diagonal. It seems that 2.6 Å may be a better estimate for the A2αCH–P3αCH distance.

The A2βCH$_3$–P3αCH = 3.3 Å (including the 1-Å correction for the A2βCH$_3$ pseudoatom) and the A2αCH–P3αCH = 2.6 Å distances depend on the dihedral angles ψ of A2 and ω of the A–P peptide bond (Figure 3). Monitoring the above distances with an error estimate of 0.5 Å while varying these two angles resulted in a ψ,ω conformational space map satisfying both distance requirements with ω in the –80° to +40° range (Figure 4). Values of ω below –40° are unlikely though since they would result in an A2βCH$_3$–P3δCH distance of 3.0 Å or less and a measurable TRNOE which was not observed. Also, conformations with ω larger than +20° and ψ less than +135° can be excluded because of steric overlap between the A2αCH and the P3 carbonyl carbon.

DISCUSSION

The TRNOE approach requires fast exchange of the ligand between the bound and the free states. In the case of CyP the simplest scheme for catalysis can be depicted as follows

$$cis - S + E \leftrightarrow X \leftrightarrow trans - S + E \qquad (2)$$

with S and E being the substrate and enzyme, respectively, and X being the enzyme–substrate complex intermediate. For the rhCyP catalysis of the suc-AAPF-pNA *cis* → *trans* isomerisms $k_{cat}^{ct} \sim 12\,700$ s^{-1} and $K_M^{ct} \sim 0.87$ mM at 0 °C (Kofron et al., 1991). It is important to realize that k_{cat}^{ct} is a lower limit for all unimolecular steps in the *cis* → *trans* direction, including the off-rate of the *trans*-S from the X complex and that k_{cat}^{ct}/K_M^{ct} is a lower limit for all bimolecular steps in the same direction, i.e., the *cis*-S and E association (Peller & Alberty, 1959). Although no such data are available for the *trans* → *cis* direction to complete the picture, it appears that suc-AAPF-pNa binds weakly to CyP [$K_M \sim 0.9$ mM being a measure of the dissociation constant K_d for Scheme 2 (Fersht, 1985)] and there is fast exchange between the free and bound substrate states. The observation of TRNOEs confirms that. In the case of the PPIase FKBP the measured $K_d \sim 0.3$ mM is comparable to the $K_M \sim 0.5$ mM (Park et al., 1992).

However, not all Pro-containing CyP substrates may be amenable to study by TRNOE measurements. The first-order rate constants k^{tc} for the CyP-catalyzed *trans* → *cis* AP conversion for benzoyl-PheAlaPro (Bz-FAP) and H-AlaAla-ProAla-OH have been measured by ^{1}H NMR saturation transfer at 25 °C (Hsu et al., 1990; L. T. Kakalis and I. M. Armitage, unpublished results) and were found to be 100-fold slower than the k^{tc} rate constants, calculated according to k^{tc} [*trans*-S] = k^{ct} [*cis*-S] and eq 3

$$k^{ct} = \frac{k_{cat}^{ct}[CyP]}{K_M^{ct} + [cis\text{-}S]} \qquad (3)$$

using the k_{cat}^{ct} and K_M^{ct} values of Kofron et al. (1991) for 0 °C, a temperature correction factor of 2 (Harrison & Stein, 1990a), and the fact that [*cis*]:[*trans*] ~ 1:9. This is in agreement with the observed inability of Bz-FAP and other unprotected oligopeptides such as AAPA to compete effectively against the standard substrate suc-AAPF-pNA (CyP) or suc-ALPF-pNA (FKBP) in the PPIase assay (Handschumacher et al., unpublished results; Harrison & Stein, 1992). This may be due to the unfavorable accommodation of the C-terminal charge in the hydrophobic active site thus resulting in suboptimal k_{cat} and/or K_M. It should be noted that in the same assay for the PPIase FKBP, suc-ALPF-NH_2 has been found to compete effectively against the standard substrate suc-ALPF-pNA (Park et al., 1992).

A major concern in NMR investigations of macromolecular structure is the effect of spin diffusion on NOE-derived distances (Wüthrich, 1986), hence the use of short mixing times (ca. 100 ms or less) in NOESY data acquisition. In the present study longer mixing times (up to 400 ms) were required since TRNOEs develop more slowly than regular NOEs, but so does spin diffusion (Neuhaus & Williamson, 1989), whose effect is further limited by the low bound peptide fraction (ca. 5% assuming a $K_d \sim 1$ mM; Campbell & Sykes, 1991a).

Another complicating factor is the possible presence of internal motion(s) for the bound suc-AAPF-pNA as perhaps suggested in the aforementioned differential line broadening. Fortunately, the TRNOEs of interest concern the more rigid A2–P3 backbone segment and the A2βCH$_3$ whose rapid

rotation while dampening the effects of spin diffusion has little effect on the buildup of TRNOEs (Campbell & Sykes, 1991c). In addition, the low bound peptide fraction and the fast peptide dissociation rate (Nirmala et al., 1992), together with the appropriate choice of a spin pair for reference distance can minimize the internal motion effect on TRNOE-derived distances.

The *cis*-like conformation for the CyP-bound substrate is consistent with the increased hydrophobic character of *cis* vs *trans* (Melander et al., 1982). The allowed values of A2 ψ and A2–P3 ω for the rhCyP-bound suc-AAPF-pNA (Figure 4) are in general agreement with reported X-ray results for the rhCyP-bound *cis* ac-AAPA-amc (Kallen & Walkinshaw, 1992), where the respective ψ and ω values are +134° and +20° to +45°, and for the rhCyP-bound *cis* AP (Ke et al., 1993), where the ω value is −3°. Conformations of Figure 3 are by no means unusual: the A2 ψ values are in the high frequency range of a *cis* X–P conformation survey (MacArthur & Thornton, 1991) whereas most of the A2–P3 ω values are within the main distribution (−10° to +15°) of a *cis* imide bond conformation survey (Stewart et al., 1990). Thus, there is no experimental evidence for an unusual conformation of the CyP-bound substrate such as the previously hypothesized 90° out of planarity X–P imide bond (Liu et al., 1990). With regard to the PPIase mechanism, a 20° rotation around the X–P CO–NH peptide bond would facilitate *cis–trans* interconversion but cannot account by itself for the PPIase catalytic efficiency. In addition to geometric distortion, other factors such as substrate desolvation in the hydrophobic PPIase active site (Radzicka et al., 1992; Eberhardt et al., 1992) and the increased sp^2 character of the amide carbonyl therein may also contribute (Stein, 1993).

One of the many differences between the uncomplexed CsA structure in apolar solvents (Kessler et al., 1990) and the CyP-bound CsA structure in aqueous solution (Weber et al., 1991; Fesik et al., 1991; Hsu & Armitage, 1992) is the *trans* 9,10 peptide bond in the latter (Fesik et al., 1990), in contrast to the *cis* 9,10 peptide bond in free CsA. This may appear contradictory to the determined *cis* X-Pro conformation for CyP-bound model PPIase substrates since CsA binds CyP at the PPIase active site. Comparison of the CsA–CyP and substrate–CyP complex structures indicates that it is the CsA MeVal11 Cα–CO bond which corresponds to the substrate isomerizing CO–N bond with the MeVal11 side chain fitting into the same CyP hydrophobic pocket as the substrate P ring and that the bound CsA 9,10 peptide bond is farther away, not corresponding to any segment of the bound model substrate (Thériault et al., 1993; Spitzfaden et al., 1992). Furthermore, the observed CyP-bound CsA conformation may preexist in aqueous solution (Altschuh et al., 1992), and the structural differences with uncomplexed CsA in organic solvents may be due to the solvent change rather than CyP binding. Thus the difference in the conformation of the 9,10 CsA peptide bond may be unrelated to the PPIase catalysis, and it is uncertain whether insights into the PPIase mechanism can be gained from the study of the CsA conformation in CsA–CyP complexes.

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