Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9483–9487, November 1991
Biochemistry

Crystal structure of recombinant human T-cell cyclophilin A at 2.5 Å resolution

(x-ray crystallography/immunosuppressive drug binding protein/peptidyl-prolyl isomerase/cyclosporin A)

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Contributed by Christopher T. Walsh, July 22, 1991

ABSTRACT **The structure of the unligated human T-cell recombinant cyclophilin has been determined at 3 Å resolution by multiple isomorphous replacement methods and refined at 2.5 Å resolution to an *R* factor of 0.209. The root-mean-square errors of the bond lengths and bond angles are 0.013 Å and 2.8° from ideal geometry, respectively. The overall structure is a β-barrel, consisting of eight antiparallel β-strands wrapping around the barrel surface and two α-helices sitting on the top and the bottom closing the barrel. Inside the barrel, seven aromatic and other hydrophobic residues form a compact hydrophobic core. A loop of Lys-118 to His-126 and four β-strands (B3–B6) constitute a pocket we speculate to be the binding site of cyclosporin A.**

Cyclophilin (CyP) A is a 17-kDa cytosolic protein originally purified from bovine spleen (1, 2) with a high affinity for the immunosuppressive drug cyclosporin A (CsA). CyPs are widely distributed and abundant proteins found in eukaryotes and prokaryotes. They are known to possess enzymatic activity in the form of peptidyl-prolyl *cis–trans* isomerase (PPIase) catalysis (3, 4), a reaction thought to be involved in the late stages of protein folding (5). This PPIase activity can be completely inhibited by CsA.

An understanding of the mechanism of the PPIase activity of the CyPs and the specificity of the interaction with CsA will require three-dimensional structural analysis. In this paper, we report the structure of recombinant human T-cell CyP A at 2.5 Å resolution by x-ray crystallographic analysis.¶

METHODS AND RESULTS

The cDNA for the 165-residue recombinant human T-cell protein has been cloned and expressed, and recombinant protein was purified from *Escherichia coli* as described (6). Crystals of the unligated CyP were grown by dialyzing 10–15 mg of protein per ml against buffer containing 20 mM Tris base (pH 8.5), 2 mM dithiothreitol, 2 mM EDTA, 0.5 mM NaN_3, and 12.5% (wt/vol) polyethylene glycol (PEG) at 4°C. The crystallization yield varied with different batches of purified protein. On average, ~20% of the dialysis wells generated crystals. The yield was improved by use of 10.5% PEG and 6.7% ethanol as the crystallization precipitant. A dialysis of 2 days to 1 week produced crystals having a shape of a rectangular bar or irregular hexahedron with a typical size of 0.3 × 0.4 × 1.0 mm. The space group is $P2_12_12_1$ with unit cell dimensions of a = 43.0, b = 52.6, and c = 89.2 Å. One monomer exists in the crystallographic asymmetric unit.

The iridium derivatives were prepared by soaking the native crystals in a solution of 20 mM Tris base (pH 7.5), 0.5 mM NaN_3, 20% PEG, 10 mM Na_3IrCl_6 or 5 mM K_3IrCl_6 for 2–4 days. The platinum derivatives were prepared by soaking the native crystals in either a solution of 5 mM maleic acid, 0.5 mM NaN_3, 35% PEG, and 0.5 mM $K_2Pt(NO_2)_4$ at pH 6.5 for 4 days, or a solution of 5 mM Bicine [*N,N*-bis(2-hydroxyethyl)glycine], 30% PEG, and 0.5 mM K_2PtCl_4 at pH 8.5 for 4 days. These two platinum derivatives have slightly different binding sites of the heavy atoms.

Table 1. Data of native CyP and its derivatives

Sample	Total reflections	Unique reflections	Resolution, Å	*R*-merge
Native (T)	53,638	22,219	1.6	0.042
Na_3IrCl_6, pH 7.5 (T)	38,393	12,060	2.1	0.035
$K_2Pt(NO_2)_4$, pH 8.5 (B)	36,953	8,175	2.4	0.054
$K_2Pt(NO_2)_4$, pH 6.5 (G)	32,348	7,661	2.5	0.088

Data were collected either on the Rigaku phosphate image plate in the Molecular Structure Group at Texas (T), the Hamlin multiwire diffractometer at Boston College (B), or on the Siemens multiwire area detector in Gibbs Laboratory at Harvard University (G).

The diffraction data of the native CyP and its derivatives were collected at room temperature on the Siemens multiwire area detector in Gibbs Laboratory (Harvard University), the Rigaku phosphate image plate at the Molecular Structure Corporation (Texas), or on the Hamlin multiwire area diffractometer at Boston College. Four sets of the native and 18 sets of the derivative data were collected, and 3 sets of the derivative data were selected for the final MIR (7) phase calculation. The native data from the image plate, a total of 53,638 diffraction maxima, were reduced to 22,219 symmetry-independent reflections to a resolution of 1.65 Å with an *R*-merge of 0.042 (Table 1).

The difference Patterson map between the iridium derivative and the native data from the Texas image plate showed consistent Harker peaks to 2.2 Å resolution. However, only part of Harker peaks were observable in the platinum maps. Two difference Patterson maps between the iridium derivative and the native data sets from the Hamlin system have substantially variable quality. These observations probably imply that multiple forms of the native crystals might exist and the derivatives are not perfectly isomorphous to the native crystal. The heavy atom positions were refined at 2.5 Å resolution using the program HEAVY (Table 2) (7), but only the MIR phases at 3.0 Å resolution were used for the MIR map calculation, solvent flattening (8), and the phase com-

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Abbreviations: CyP, cyclophilin; CsA, cyclosporin A; PPIase, peptidyl-prolyl isomerase.

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¶The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1CPL).

Table 2. Statistics of heavy atom derivative refinement

Derivative	Centric R factor	Phasing power	Sites	Riso
Na_3IrCl_6, pH 7.5	0.69	1.37	3	0.198
$K_2Pt(NO_2)_4$, pH 8.5	0.78	1.50	3	0.154
$K_2Pt(NO_2)_4$, pH 6.5	0.73	1.59	5	0.157

Overall figure of merit, 0.65 at 2.5 Å resolution. Riso is defined as $\Sigma|F_n - F_d|/\Sigma|F_n|$ and is calculated by using the reflections between 15 and 2.5 Å. F_n and F_d, native and derivative, respectively.

bination (9). Although other heavy metal compounds, such as $OsCl_3$, K_2OsCl_6, $Hg(NO_3)_2$, $K_2Pd(NO_2)_4$, and $KAuCl_4$, bind to the protein, incorporation of those derivatives into the MIR phases did not improve the quality of the map. The structure model was built using the program FRODO (10) and refined using the program XPLOR (11) on a DECstation 3100 in Gibbs Laboratory (Harvard University).

The MIR map and the map from the solvent flattening (8) were not interpretable and only major secondary structural components were marginally recognizable. An initial model with 140 of a total 165 amino acids, which was built as either an alanine or a residue with a side chain arbitrarily assigned according to its shape of electron density, was connected into four fragments. In this model, there were several places having the wrong connectivity and about half of the chains had a wrong N to C trace of the polypeptide chain. This partial structure was refined to an R factor of 0.412 at 2.5 Å resolution and was used to combine with the MIR phases at 3 Å resolution. Seven rounds of the phase combination and model building brought the R factor to 0.316 at 2.5 Å resolution before the structure could be traced.

The final refinement of the complete structure against 6456 reflections, which are 98.5% complete between 8 and 2.5 Å resolution, yielded an R factor of 0.209 with rms deviations of 0.013 Å and 2.8° from ideal geometries of the bond lengths and bond angles, respectively. Solvent molecules have not been included in the structure. Analysis of conformational angles of the polypeptide backbone shows that no pair of ϕ and ψ fell into the energy-disallowed regions of the Ramachandran plot (12). The electron density is excellent for most residues except for the last of the C-terminal residues and the first two of the N-terminal residues. Some long side chains that are located on the surface were partially disordered.

Table 3. Correspondence between secondary structure and sequence of CyP

Secondary element	Sequence range
B1	Pro-4–Ala-11
T1	Val-12–Glu-15
B2	Pro-16–Leu-24
H1	Phe-25–Val-29
H2	Pro-30–Thr-41
T2	Gly-42–Gly-45
B3	Lys-49–Ile-56
T3	Ile-57–Phe-60
B4	Met-61–Gly-64
T4	Ser-77–Gly-80
B5	Gly-96–Ala-101
T5	Gly-104–Thr-107
B6	Gln-111–Thr-116
B7	Val-127–Glu-134
H3	Gly-135–Gly-146
T6	Ser-147–Gly-150
B8	Lys-155–Leu-164

B, β-strand; H, α-helix; T, β-turn.

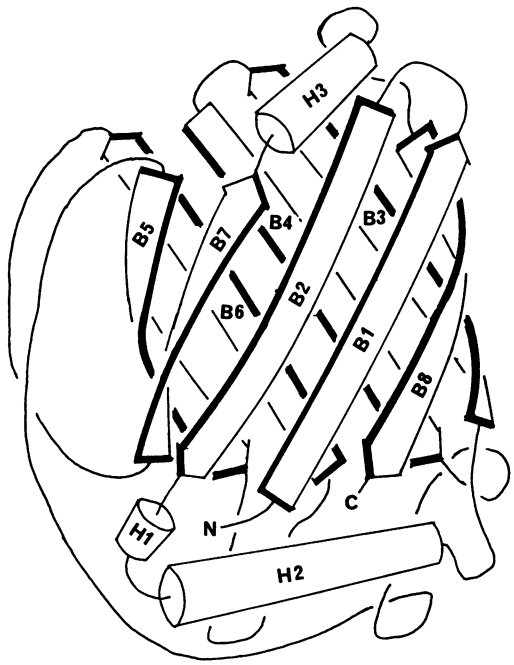

FIG. 1. Schematic drawing of the structure of CyP. The α-helices are represented by a cylinder designated as H while the β-strands are represented by an arrow marked as B. N and C denote the N and C termini of the molecule. Eight antiparallel strands (B1–B8) form a β-barrel and the two α-helices (H2 and H3) are on the top and bottom, respectively, closing the barrel. Inside is the hydrophobic core of the molecule, containing seven aromatic and several other hydrophobic residues.

DISCUSSION

Molecular Architecture of Recombinant Human CyP. The overall shape of the structure is globular with a dimension of approximately 34 × 33 × 30 Å. The structure consists of three α-helices, eight β-strands, and six β-turns corresponding to contents of 17.6%, 35.8%, and 14.5% among the total amino acids, respectively (Table 3). The major component of the molecule is a flattened cylinder with diameter of 16 × 18 Å and a height of 26 Å. All of the eight β-strands wrap in an antiparallel fashion around the cylinder surface and two α-helices (H3 and H2) sit on the top and on the bottom of the cylinder (Fig. 1). Inside the cylinder, six phenylalanines and one tyrosine orient their side chains together in a pattern of edge-to-face to form a very compact hydrophobic core of the molecule. Five β-bulges (13) exist in the strands and are probably responsible for making the strands flexible to wrap around the cylinder.

There is no structural similarity between CyP and other proteins in terms of topology and overall morphology of the structures. However, CyP does share a common feature, "orthogonal packing of the β-sheets," with a family of retinol binding proteins (14–17) and others (18).

The structure of CyP is unique and different from that of FK506 binding protein (FKBP) (19–21) although both CyP and FKBP are binding proteins for immunosuppressant drugs and are active PPIases. A domain from Tyr-48 to Gly-80 of CyP was reported to be homologous with FKBP (22). However, the residues in that "domain" belong to several unrelated regions in the CyP structure: Tyr-48 and Phe-53 are located in the hydrophobic core, Phe-60 belongs to the assumed CsA binding pocket, and Tyr-79 is on the surface of the molecule.

Hydrophobic Core of the Molecule. Two α-helices and eight β-strands form the compact hydrophobic core of the structure. Residues contributing to the hydrophobic core of the molecule include Val-6, Phe-8, Val-20, Phe-22, Leu-24, Phe-

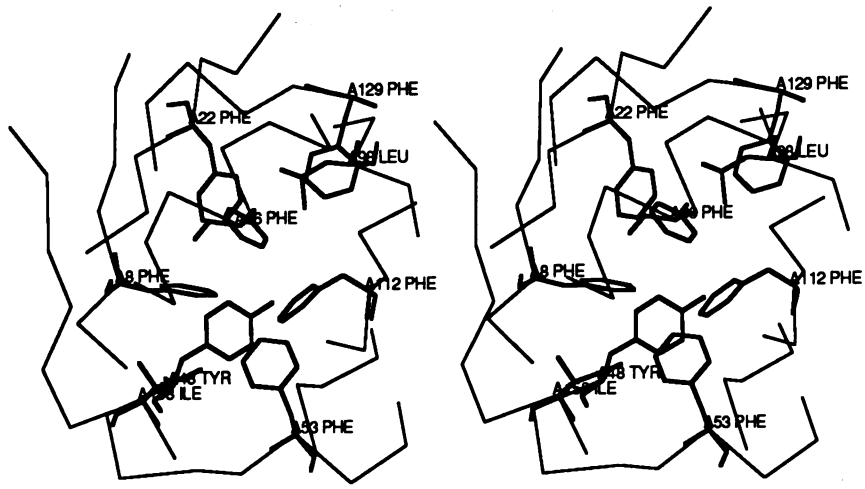

FIG. 2. Stereoview of the hydrophobic core of CyP, viewing down the barrel axis (the verticle axis in Fig. 1). Single lines represent backbone traces of the eight β-strands, while the residues in heavy lines are clockwise: Phe-129, Leu-98, Phe-122, Phe-53, Ile-158, Tyr-48, Phe-8, Phe-36, and Phe-22.

36, Leu-39, Tyr-48, Phe-53, Ile-56, Leu-98, Met-100, Phe-112, Ile-114, and Phe-129 (Fig. 2). These results are consistent with the initial NMR data showing that numerous aromatic residues form a hydrophobic cluster in CyP (23). Inspection of the sequence alignment of various CyPs shows that the residues in the hydrophobic core are highly conserved (Fig. 3). Among them Phe-36, Phe-53, Phe-112, and Phe-129 are fully conserved, while other residues are substituted with analogous residues in some species, except Val-6 and Phe-8, which are mutated to Asn and Lys in *Neurospora crassa* or Lys and Asp in *E. coli*. This conservation of the hydrophobic core residues implies that the CyP family probably has a common β-barrel motif as its molecular core even though the overall sequence homology may be as low as 43%.

Putative Binding Pocket for CsA. On one side of the hydrophobic core, a pocket, with a dimension of approximately 15 × 20 Å and ~10 Å deep, contains several conserved hydrophobic and polar residues. The loop of Lys-118 to His-126 forms one wing of the pocket while four β-strands (Fig. 1)—B5 (Gly-96–Ala-101), B6 (Gln-111–Thr-116), B4

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                                                    * *           * * *
  humana : M.............................VNPTVFFDIAVDGEPLGRVSFELFADK
  bovine : .............................-------------------------
  yeasta : -.............................SQ-Y--VEA--Q-I---V-K-YN-I
  humanb : -kvllaaaliagsvffllpgpsaadekkkgpk-TVK-Y--LRIGD-DV---I-G--GKT
   ninaA : -ksllnriilcsaflavasglsft.........-TSRIYM-VKHNKK-V--IT-G--GKL
  yeastb : -kfsglwcwlllflsvnviasdvgelidqddevITQK-----EHGE-KV--IVIG-YGKV
  E.coli : -...................AAVFALSALSPAAMAAKGDPHVLLTTSA-NIEL--DKQ-
N.crassa :.....................SKVFFDLEWEGPV.LGPNNKPTSEIKAQS-RINFT-YDDV

                 * *          *    *+++*+  ++ +
  humana : VPKTAENFRALS.TGEKGFGYKGSCFHRIIPGFMCQGGDFTRHNGTGGKSIYGEKFEDEN
  bovine : ------------.----------------------------------------D---
  yeasta : -----------C.--------A--P---V--D--L------AG----------G--P---
  humanb : ----VD--V--A.---------N-K---V-KD--I-------GD----------R-P---
   ninaA : A---VA---HIClR-IN-TS-V--R---VVDR-LV----IVNGD---SI----DY-P--D
  yeastb : C----K--YK--t-TNSKK-FI--T---V--N--V------DGT-V-------DT-P---
  E.coli : A-VSVQ--VDY....VNSGF-NNTT---V-----I---G--EQMQQKKP---NPPIKN-A
N.crassa : VPKTARNFKELCT.GQNGFGYKGSS------EE-L-------GN----------A---

                      * * +        +*+*       ++  ++  *
  humana : ..FILKHTGPGILSMANAGPNTNGSQFFI.CTAKTEWLDGKHVVFGKVKEGMNIVEAME.
  bovine : ..----------------------------.----------------------------.
  yeasta : ..-KKH-DR--L----------------.T-VPCP----------E-VD-YD--KKV-.
  humanb : ..-K---Y---WV------KD--------.T-V--A----------L---EV-RKV-s
   ninaA : kaLAVE-NR--Y-G---R--D---C--YV.T-VGAK------T-----L---DTIY-I-.
  yeastb : ..-T---DRK-R-----R-KD--------tT-EEAS----------Q-VD--DV-NYIQ.
  E.coli : .DNG-RN-RGT-AMART-DKDSAT-----.NV-DNAF--HGQRD--YAVF-KVVKGMDV.
N.crassa : ..-AK--VR--L----------------V.T-VPTS-------.....---EVADDESMK

  humana : RF.G.SRNGKTSKKITIADCGQLE
  bovine : --.-.----------------..
  yeasta : SL.-.-PS-A-KAR-VV-KS-E-
  humanb : TK.T.DSRD-PL-DVI-----KI-VEKPFAIAKE
   ninaa : DVkT.DTDDFPVEPVV-SN--EIPTEQFEFYPDDFNILGWIKAAGLPVTSSFCVLLIFHYFF
  yeastb : HV.SrDA-D-PLEAVK--K--EWTPELSS
  E.coli : ADKISQVPTHDVGPYQNVPSKPVVILSAKVLP
N.crassa : VVKALEATGSSSGAIRYSKKPTIVDCGAL
```

FIG. 3. Sequence alignment of CyPs: human A (24), bovine (2), yeast A (27), human B (28), *ninaA* (29, 30), yeast B (31), *E. coli* (26), *N. crassa* (25). *, Residues in the hydrophobic core; +, residues in the putative binding site for CsA.

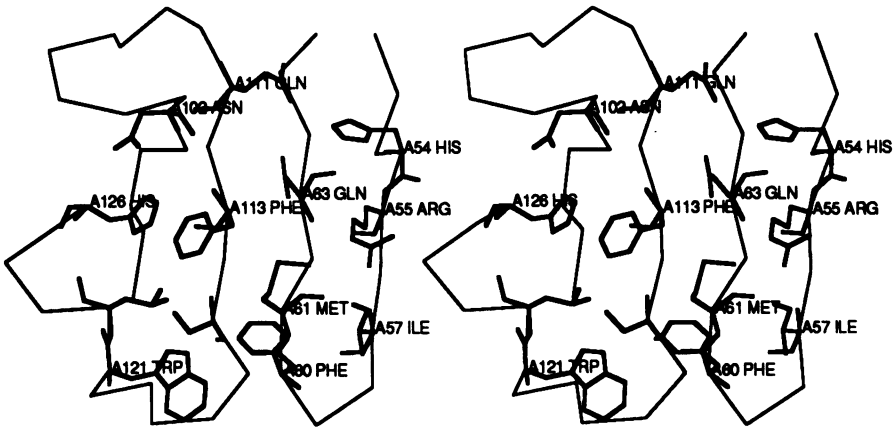

FIG. 4. Stereoview of the putative binding pocket for CsA. Single line represents a backbone trace of the loop around Trp-121 and β-strands B5, B6, B4, and B3 (left to right).

(Met-61–Gly-64), and B3 (Lys-49–Ile-56)—are arranged in order forming the bottom and another wall (Figs. 4 and 5). Loops consisting of residues His-70–Lys-76 and Asn-102–Asn-108 are located nearby. We tentatively assign this pocket as the binding site for CsA since it has a geometry that can most likely adopt the binding of ligands. Residues in this pocket include His-54, Arg-55, Ile-57, Phe-60, Met-61, Gln-63, Asn-102, Gln-111, Phe-113, Trp-121, Leu-122, Lys-125, and His-126. Except for the mutations of Asn-102 to Thr, Trp-121 to Phe, and Lys-125 to Gly in *E. coli*, all other residues are fully conserved or substituted with equivalent residues (Fig. 3). Therefore, this homology supports the hypothesis that this is the binding site for cyclosporin A. The residue Trp-121, which has been identified as the binding residue for CsA by enhancement of the Trp fluorescence upon binding to CsA (1, 5), by NMR studies (23, 32), and by site-directed mutagenesis (33), is located on one edge of the pocket. Moreover, the proton interactions between the aromatic residues of CyP and CsA, which were revealed in the NMR studies (32, 34, 35), may correspond to Phe-60 and/or Phe-113 in our structure.

Characterization of the CsA–CyP complex will be crucial for understanding the molecular mechanism of immunosuppression. Various synthetic CsA analogs have been reported recently to be competitive inhibitors of CyP PPIase activity (36). This suggests that these analogs are binding to the active site of CyP and that this site is also the same as the CsA binding pocket. Site-directed mutagenesis studies on the residues in this active site and three-dimensional structural analysis of CyP complexed with CsA and a proline-containing peptide should help to further elucidate the mechanism of PPIase and the binding domain of CsA.

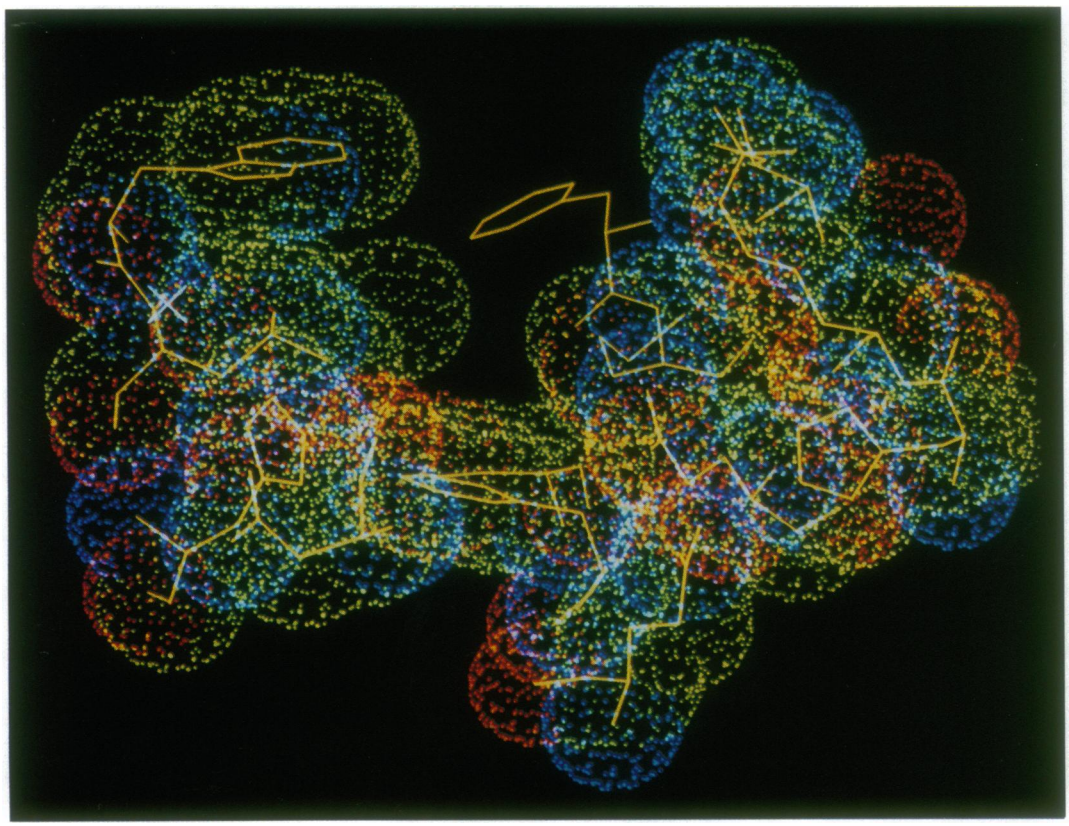

FIG. 5. Presentation of the van der Waals surfaces of the putative binding pocket for CsA. This view is ~90 Å away from the horizontal axis of Fig. 4.

Note Added in Proof. The structure of the recombinant human T-cell cyclophilin has been further refined to an *R* factor of 0.18 at 1.63 Å resolution. We thank Sandoz for a preprint of their paper (37) that shows an independent determination of the CyP structure complexed with a proline-containing peptide at 2.6 Å resolution.

Since most of the structure determination was done in Gibbs Laboratory (Harvard University), H.K. is deeply indebted to Professor William Lipscomb for his generosity and financial support (National Institutes of Health Grant GM06920). H.K. would also like to thank his Gibbsian colleagues (R. Stevens, J. Y. Liang, Y. Zhang, K. Reinisch, H. Kim, Y. M. Chook, S. Huang, and X. Wei) for the hospitality in use of their computer facilities and for helpful discussions. We thank Dr. M. Teeter of Boston College and Dr. J. Troop of Molecular Structure Corporation (Texas) for data collection. We would like to thank C. Hunter Baker, Chih-Ming Chen, and Mark Albers for their involvement in the CyP purification. This work was supported in part by National Institutes of Health Grant GM20011 to C.T.W. and National Institutes of Health National Research Service Award Grant ES05459 to L.D.Z.

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