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Crystal structure of murine cyclophilin C complexed with immunosuppressive drug cyclosporin A

(x-ray crystallography/immunophilin/peptidyl-prolyl isomerase)

HENGMING KE*†, YINGDONG ZHAO*, FENG LUO*, IRVING WEISSMAN‡, AND JEFF FRIEDMAN‡§

*Department of Biochemistry and Biophysics, School of Medicine, The University of North Carolina, Chapel Hill, NC 27599; and ‡Departments of Pathology and Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305

Contributed by Irving Weissman, September 9, 1993

ABSTRACT **Cyclophilin is a cellular receptor for the immunosuppressive drug cyclosporin A (CsA). Cyclophilin C (CyPC) is highly expressed in murine kidney, making it a potential mediator of the nephrotoxic effects of CsA. The structure of murine CyPC complexed with CsA has been solved and refined to an *R* factor of 0.197 at a 1.64-Å resolution. Superposition of the CyPC–CsA structure with the unligated cyclophilin A (CyPA) revealed significant migration of three loops: Gln-179 to Thr-189, Asp-47 to Lys-49, and Met-170 to Ile-176. The proximity of the loop Gln-179 to Thr-189 to the CsA binding site may account for the unique binding of a 77-kDa glycoprotein, CyPC binding protein (CyCAP), to CyPC. The binding of CsA to CyPC is similar to that of CsA to human T-cell cyclophilin A (CyPA). However, the conformation of CsA when bound to CyPC is significantly different from that when bound to CyPA. These differences may reflect conformational variation of CsA when bound to different proteins. Alternatively, the previous CyPA–CsA structure at low resolution may not provide sufficient details for a comparison with the CyPC–CsA structure.**

Cyclophilins (CyP) are cellular binding proteins for the immunosuppressive drug cyclosporin A (CsA) (1), which possess peptidyl-prolyl cis ⇌ trans isomerase activity (2, 3). Isomerization of a peptidyl-prolyl amide bond is considered to be a rate determining step in protein folding (4). The immunosuppressive drug CsA is a cyclic undecapeptide that has been widely used to prevent allograft rejection, presumably via inhibition of signal transduction that occurs in T cells after antigen recognition by the T-cell receptor. The discovery that the CyP–CsA complex binds to and inhibits the enzyme activity of calcineurin, a serine/threonine phosphatase and a Ca^{2+}-dependent calmodulin binding protein (5), was a key step toward understanding the mechanism by which CsA suppresses T-cell activation (6, 7). While CsA is an effective immunosuppressive agent, there are side effects associated with its use, most notably acute and chronic nephrotoxicity (8–10). CyPs are abundant and apparently ubiquitous in both prokaryotic and eukaryotic organisms (11–14). CyP C (CyPC) is a tissue-specific CyP with 212 amino acids, whose cDNA was originally isolated from a murine bone marrow stromal cell line (7). CyPC is highly expressed in murine kidney, making it a potential mediator of the nephrotoxic effects of CsA (unpublished data). CyPC is also unique in that it binds with high affinity to a cellular protein termed CyPC binding protein (CyCAP) in the absence of CsA; CsA displaces CyCAP from CyPC (16). Three-dimensional structures of CyP have been determined for the unligated recombinant human CyP A (CyPA) (17, 18), CyPA complexed with a proline-containing tetrapeptide substrate (19, 20), CyPA complexed with a dipeptide substrate Ala-Pro (21), and the CyPA–CsA complex (22, 23). Here, we report the structure of murine CyPC complexed with CsA.¶

METHODS

Crystals of murine CyPC complexed with CsA were grown by two steps: (*i*) mixing purified CyPC (16 mg/ml) with 10 mM CsA in ethanol in a molecular ratio of 1:2 overnight and (*ii*) crystallizing the complex at ≈13 mg/ml by dialysis against a buffer containing 20 mM Tris-base, 2 mM dithiothreitol, 2 mM EDTA, 0.5 mM NaN_3, 14% (wt/vol) PEG (M_r, 3350), 6% (vol/vol) ethanol, 2% (vol/vol) isopropanol, 0.2 M ammonium sulfate, and 0.01 mM CsA (pH 6.0) at 4°C. The CyPC crystal has cell dimensions of $a = 60.3$, $b = 74.0$, $c = 97.9$ Å, $\alpha = 90°$, $\beta = 90°$, and $\gamma = 90°$. The true space group is $P2$, but has pseudo symmetry of a pseudo space group $I2_12_12$. There are eight molecules in the crystallographic unit cell. Four of them in cluster A have a perfect fourfold symmetry of $P2_12_12$ and the other four in cluster B only retain half the $P2_12_12$ symmetry. In addition, the molecules in cluster A are related to those in cluster B by an approximate but not exact translation of (1/2, 1/2, 1/2). Therefore, the crystal structure of the CyPC–CsA complex is a superstructure with two sublattices and with a pseudo body-centered symmetry.

Diffraction data of the CyPC–CsA complex was collected on the Rigaku image plate system at the University of North Carolina and processed as the space group $P2_1$ because the common symmetries of the two sublattices in the atomic space [(x, y, z) and $(1/2 - x, 1/2 + y, -z)$] are equivalent to a twofold symmetry in the diffraction space. A total of 139,812 measurements was reduced to 73,850 symmetry-independent reflections with an R_{merge} of 0.040 at 1.64-Å resolution. The structure of murine CyPC was solved by the molecular replacement method, using the unligated human T-cell CyPA as the initial model. Because of the two sublattices and the pseudo body-centered translation in the CyPC crystal, identification of a true space group and a true solution from the molecular replacement was very difficult and will be discussed elsewhere. The molecular model was built using the program FRODO installed in an ESV10 graphic system (24) and refined by program XPLOR (25). The structure is presently refined in a monoclinic space group $P2_1$ that has symmetries of (x, y, z) and $(1/2 - x, 1/2 + y, -z)$, to an R factor of 0.197 against 72,159 reflections at 6.0- to 1.64-Å resolution. The

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Abbreviations: CyP, cyclophilin; CyPA, CyP A; CsA, cyclosporin A; CyPC, CyP C; CyCAP, CyPC binding protein; MeBmt, (4*R*)-4-[(*E*)-2-butenyl]-4-*N*-dimethylthreonine; Abu, 2-aminobutyric acid; Sar, sarcosine.

†To whom reprint requests should be addressed.
§Present address: Childrens' Hospital, Department of Medicine, 300 Longwood Avenue, Boston, MA 02115.

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

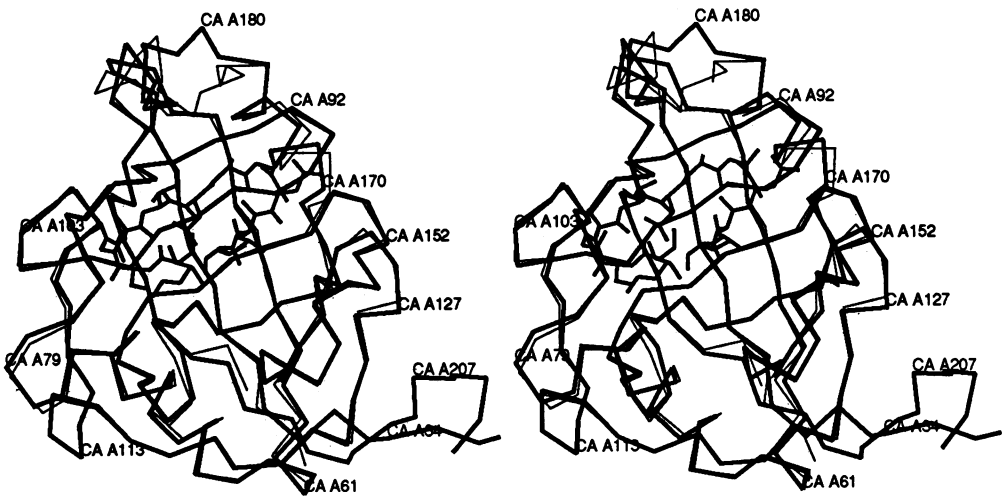

FIG. 1. Superposition of murine CyPC over human T-cell CyPA. N-terminal residues 15–30 in CyPC and the fragment of Gly-Ser-Pro-Gly-Ile-Ser-Leu from the fusion protein were not traceable because of their disorder. Only Cα atoms are shown for CyPC (thicker lines) and CyPA (thinner lines). Some residues of CyPC are labeled. It is obvious that both CyPC and CyPA have the same β-barrel structures except for the significant migration of three loops: Asp-47 to Lys-49 of CyPC (Asp-13 to Glu-15 of CyPA), Met-170 to Ile-176 (Met-136 to Met-142 of CyPA), and Gln-179 to Thr-189 of CyPC (Phe-145 to Lys-154 of CyPA, at the top of the figure). CsA binds on the surface of the barrel and is drawn in thick lines. The extra N and C termini of CyPC extend to outside of the barrel and may serve as a sequence to interact with cellular membrane or other organelles.

rms deviations of bond length and bond angle are 0.012 Å and 2.68° from the ideal geometries.

RESULTS AND DISCUSSION

The structure of CyPC (212 amino acids) is an eight-stranded antiparallel β-barrel with a hydrophobic core closed by two helices on the top and bottom of the barrel (Fig. 1). The present structure includes four molecules of CsA and CyPC (Lys-31 to Trp-212), and 401 water molecules in the crystallographic asymmetric unit. The first 15 N-terminal residues and 7 residues from the fusion protein could not be traced. The N- and C-terminal residues of CyPC extend to the outside of the β-barrel bulk (Fig. 1) and may, therefore, serve as a sequence to interact with the cell membrane or other cellular organelles (7). The N terminus of CyPC is hydrophobic (7) and serves as an endoplasmic reticulum signal sequence (26).

The murine CyPC (212 amino acids) and the human CyPA (165 amino acids) have the same β-barrel structure although they have only ≈56% identity for 165 amino acids and completely different C-terminal sequences after His-174 of CyPC (Glu-140 of CyPA). The alignment of the two structures showed a 1-residue insertion after Thr-182 of CyPC (Fig. 2). The superposition of the CyPC and CyPA structures revealed an averaged positional displacement of 0.53 Å for Cα atoms and 0.79 Å for all atoms (Fig. 3). Significant migration of the Cα atoms has been observed for three loops: >8-Å shift of Gln-179 to Thr-189, >2-Å shift of Asp-47 to Lys-49, and ≈1-Å shift of Met-170 to Ile-176 (Figs. 1 and 3). The loops of Gln-179 to Thr-189 and Asp-47 to Lys-49 have different backbone conformations between CyPC and CyPA (Fig. 1). These positional displacements and conformational changes may correspond to the different functions of CyPC and CyPA. In the absence of CsA, CyPC binds to a 77-kDa glycoprotein CyCAP, but CyPA does not (16). The competitive inhibition between CsA and CyCAP for CyPC binding implies that CyCAP may share fully or partially the binding site with CsA. The structure of CyPC–CsA showed that the observed N and C termini of CyPC aggregated together and pointed away from molecular bulk (Fig. 1). The N and C termini as well as the loops of Asp-47 to Lys-49 and Met-170 to Ile-176 are unlikely to be involved in the binding of CyCAP since they are far away from the CsA binding site. The loop of Gln-179 to Thr-189, which has a distance of ≈7 Å from the nearest atom of CsA, is the only fragment close to the CsA binding site (Fig. 1). Therefore, it is reasonable to propose that its different position and amino acid sequence may account for the unique binding of CyCAP to CyPC. In addition, CyPC residues Lys-92, Asp-93, and Asp-105, which

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                    14          24          34          44          54
Murine CyPC     GSPGIS LGLGALVSSS GSSGVRKRGP SVTDKVFFDV RIDGKDVGRI
Human CyPA                                  M.NPT....I AV..EPL..V
                                                    10          20

         64         74          84      *    * * *      104 *       114
VIGLFGNVVP KTVENFVALA TGEKGYGYKG SIFHRVIKDF MIQGGDFTAR DGTGGMSIYG
SFE..ADK.. ..A...R..S .....F.... .C...I.PG. .C......RH N....K....
        30         40          50          60          70          80

        124        134***      144* *      154**   *  164         174
ETFPDENFKL KHYGIGWVSM ANAGPDTNGS QFFITLTKPT WLDGKHVVFG KVLDGMTVVH
.K.E....I. ..T.P.IL.. .....N.... ....CTA.TE .......... ..KE..NI.E
        90         100         110         120         130         140

          185         195         205
SIELQATDGHD RPLTDCTIVN SGKIDVKTPF VVEVPDW (212 amino acids)
AM.RFGS-RNG KTSKKITIAD CGQLE (165 amino acis)
         150         160
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FIG. 2. Sequence alignment of murine CyPC and human T-cell CyPA, based on the three-dimensional structures. *, Residues for the binding of CsA; dots, identical residues.

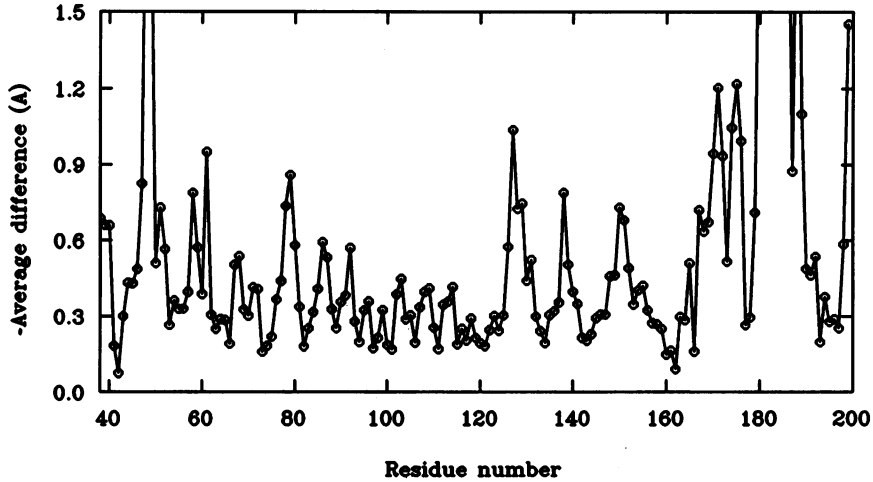

FIG. 3. Average displacement of Cα atoms between CyPC and CyPA. The four molecules of CyPC in the crystallographic asymmetric unit were, respectively, superimposed over CyPA and then the differences were averaged over four molecules.

are 8–10 Å away from the nearest atom of CsA, have the same backbone position (Fig. 1) but are different from the CyPA amino acids (Fig. 2). Although it is conceivable, it is unlikely that they contribute to the binding of CyCAP to CyPC since another human CyP, CyP B, which has the same three residues, does not bind CyCAP.

The immunosuppressive drug CsA binds to the hydrophobic pocket of the active site of CyPC (Fig. 4). Six residues of CsA are involved in interactions with CyPC: (4*R*)-4-[(*E*)-2-butenyl]-4-*N*-dimethylthreonine (MeBmt) 1, 2-amino-butyric acid (Abu) 2, sarcosine (Sar) 3, MeLeu-9, MeLeu-10, and MeVal-11. In addition to numerous hydrophobic contacts between CsA and CyPC, five hydrogen bonds are formed between carbonyl oxygen (CO) of MeBmt-1 of CsA and NE2 Gln-97, the backbone nitrogen of Abu-2 of CsA and CO Asn-136, CO MeLeu-9 of CsA and NE1 Trp-155, CO MeLeu-10 of CsA and both NH1 and NH2 of Arg-89. The residues of CyPC that are located within a 4-Å radius from a nearest CsA atom and may form hydrophilic or hydrophobic interactions with CsA include Arg-89, Phe-94, Met-95, Gln-97, Gly-106, Ala-135, Asn-136, Ala-137, Gln-145, Phe-147, Trp-155, Leu-156, and His-160 (Fig. 2). One water molecule that is hydrogen-bonded to NE2 of His-88 is near the carbonyl oxygen of MeBmt-1 of CsA, in distances of 2.97, 3.38, 3.38, and 3.45 Å, respectively, in the four molecules of CyPC in the crystallographic asymmetric unit.

The binding of CsA to CyPC is essentially the same as that between CsA and human CyPA (22, 23). This is not surprising because the 13 residues involved in CsA binding are actually identical between CyPC and CyPA (7). However, there are some minor differences in the binding of CsA to CyPC and the reported binding of CsA to CyPA. For example, only one hydrogen bond between MeLeu-10 and Arg-55 was identified in the x-ray or NMR structure of CyPA–CsA (22, 23) and the hydrogen bond between MeBmt-1 and Gln-63 of CyPA was not observable in the NMR data (22). These minor differences may simply reflect the higher resolution of the current study, which provides more details than the previous lower-resolution structure by x-ray crystallography or NMR (22, 23).

The cyclic undecapeptide CsA, when it binds CyPC, has trans peptide conformations for all of its 11 amino acids (Fig. 5 and Table 1), consistent with previous observations that CsA has the trans peptide conformation when it binds to

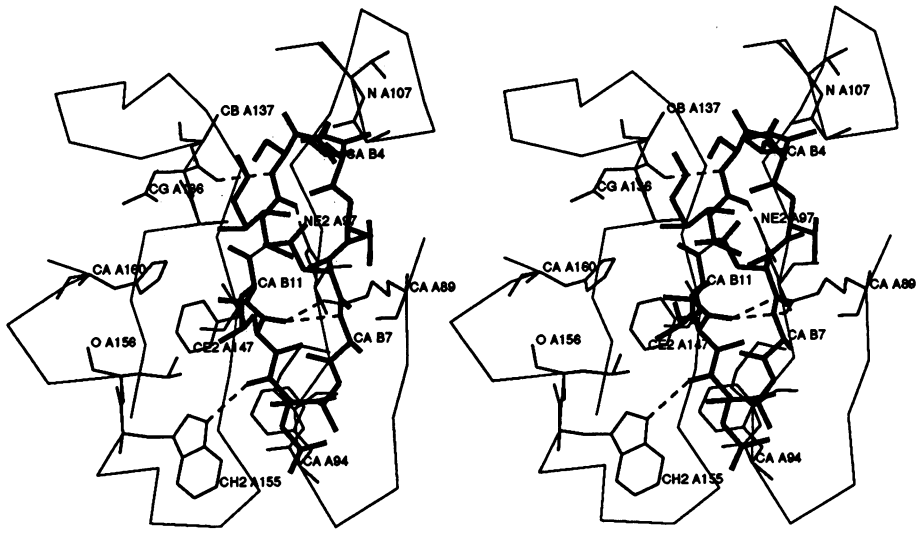

FIG. 4. Stereo drawing of the CsA binding site of murine CyPC. Thin lines represent the backbone traces of residues 88–109 and 131–161, and the nonhydrogen atoms of the active site residues Arg-89, Phe-94, Met-95, Gln-97, Gly-106, Thr-107, Ala-135, Asn-136, Ala-137, Phe-147, Trp-155, Leu-156, and His-160. CsA is shown in thick lines. The dotted lines represent the five hydrogen bonds between the O of Bmt-1 from CsA and the NE2 of Gln-97 from CyPC, the N of Abu-2 from CsA and the O of Asn-136, the O of MeLeu-9 from CsA and the NE2 of Trp-155, and the O of MeLeu-11 and both NH1 and NH2 of Arg-89.

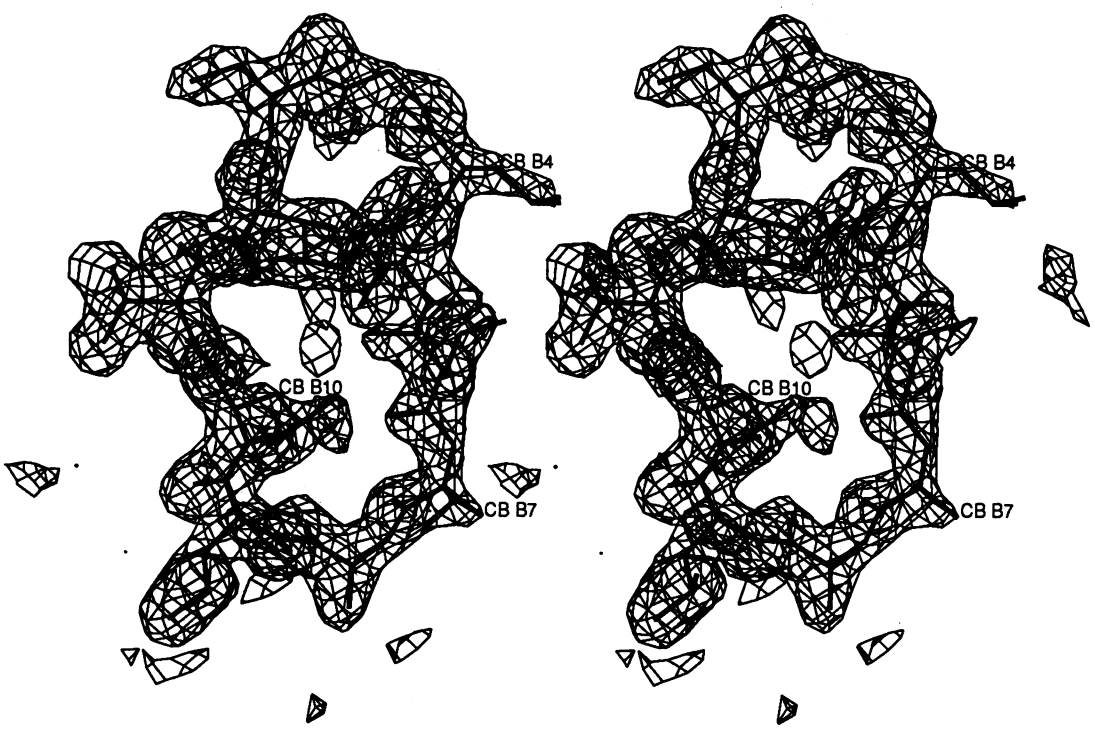

FIG. 5. Stereo plot of the CsA conformation (thick lines) and its electron density (thin lines). The (F_o–F_c) map was calculated from the coordinates without CsA and contoured at 2.5 σ. All the peptide conformational angles of the bound CsA are trans, consistent with that of CsA bound to CyPA (22, 23).

CyPA (27, 28) or Fab fragment of an antibody (29). Our results support the argument that CsA, which has the cis peptide bond between MeLeu-9 and MeLeu-10 when unbound in organic solvent (30, 31), is trapped in the trans conformation when it binds to CyP (27, 28) or Fab (29). In addition, several residues of CsA, such as Val-5, MeLeu-9, MeLeu-10, and MeVal-11, have similar backbone conformational angles in both structures of CyPA–CsA and CyPC–CsA (Table 1). However, significant differences in the backbone conformational angles were observed for MeBmt-1, Sar-3, MeLeu-4, etc. For example, MeBmt-1, which is a key residue for the immunosuppression action of CsA (15, 32), has differences of $\phi = 19°$ and $\psi = 23°$, much larger than the standard deviations of 2.5° and 1.0° in our CyPC–CsA structure. In general, some of the differences may illustrate the multiple conformation states of CsA, corresponding to different functions or responding to different crystallization environments. Alternatively, they may reflect that previous structure of CyPA–CsA determined by NMR (27) was not of sufficient resolution to demonstrate the CsA conformation at the particular region.

Note Added in Proof. Detailed comparison revealed that the bound CsA conformation in the crystal structure of CyPC-CsA is essentially the same as that of CyPA-CsA determined by the x-ray (23) or NMR (22) technique.

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Table 1. Backbone conformational angles of the bound CsA from x-ray and NMR

Amino acid	X-ray, degrees			NMR, degrees		
	ϕ	ψ	ω	ϕ	ψ	ω
MeBmt-1	−105.8 (2.5)	170.0 (1.0)	−174.5 (0.5)	−125	−167	−176
Abu-2	−118.8 (1.3)	87.8 (1.1)	−178.8 (0.4)	−131	99	180
Sar-3	127.0 (2.5)	−74.3 (0.9)	−177.5 (1.5)	138	−41	−179
MeLeu-4	−103.3 (0.4)	93.0 (2.0)	−178.5 (0.8)	−150	92	180
Val-5	−69.8 (2.3)	127.0 (2.0)	178.8 (0.8)	−75	136	177
MeLeu-6	−106.0 (0.5)	−173.8 (7.2)	−176.0 (1.0)	−114	−159	176
Ala-7	−83.0 (8.5)	158.5 (2.3)	176.3 (0.4)	−80	172	178
D-Ala-8	88.8 (2.3)	−132.0 (3.0)	−176.5 (1.5)	83	−156	−177
MeLeu-9	−126.8 (1.9)	74.0 (2.5)	179.3 (1.3)	−126	86	180
MeLeu-10	−107.5 (1.8)	170.8 (0.8)	173.5 (0.5)	−117	153	180
MeVal-11	−125.8 (1.3)	81.3 (1.8)	−179.5 (0.5)	−131	80	177

Average value from the four molecules in the asymmetric unit of the x-ray structure of murine CyPC complexed with CsA is shown. Values in parentheses are the standard deviations. NMR structure of the T-cell CyPA complexed with CsA (27) is shown.

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