Crystal structure of cyclophilin from *Leishmania donovani* at 3.5 Å resolution

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The crystal structure of cyclophilin from *Leishmania donovani* has been solved at 3.5 Å resolution. The protein with peptidylprolyl cis–trans isomerase activity is also a receptor for the drug, cyclosporin. The crystal structure of cyclophilin obtained in space group $P4_32_12$ with cell parameters $a = b = 48.73$ Å, $c = 140.93$ Å and one molecule in the asymmetric unit, was solved by molecular replacement using human cyclophilin A as the search model. The refined low resolution structure ($R = 0.218$ and $R_{free} = 0.324$) clearly indicates the conservation of the cyclosporin binding-site geometry with respect to human cyclophilin A.

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CYCLOPHILINS are an ubiquitous class of proteins with peptidylprolyl cis–trans isomerase activity[1]. They are implicated in a wide range of cellular processes, from cell division, signal transduction, acceleration of protein folding and even the dispersion of aggregated proteins[2,3]. In addition, most cyclophilins (with the exception of the same molecule from *E. coli*[4]) are receptors for the immunosuppressive drug cyclosporin (CsA)[5]. The cyclophilin–cyclosporin complex subsequently binds to calcineurin (CN), inhibiting its serine–threonine protein phosphatase activity leading to immunosuppression. Several isoforms of cyclophilin have been identified in mammalian tissues, among which human cyclophilin A (CypA) has been biochemically and structurally well-characterized.

Several X-ray crystal structures of CypA (165 amino acids), both in its unligated form and complexed with CsA (or its derivatives), have been solved[6,7]. The protein molecule is a compact barrel composed of eight antiparallel β-strands with two helices on either side enclosing a prominent hydrophobic core. An unusual feature of the structure are six β-bulges within the sheet system. The CsA binding site is a hydrophobic crevice defined by 11 amino acid residues on the face of the barrel. Single-domain compact CypA shows negligible structural differences between its ligated and unligated forms, though the hydrophobic undecapeptide drug CsA, undergoes major conformational changes on binding to CypA. Residues 3–7 of CsA (effector region) protrude out of the complex and were predicted to interact specifically with CN.

Crystal structures of the CypA–CsA–CN ternary complex[8,9] confirmed the primary interaction of residues 3–7 of CsA with CN. CN is a fairly large molecule with two distinct domains, CNA and CNB. The catalytic domain (CNA) consists of a central β-sheet flanked on either side by α-helices. CNB can be further subdivided into two domains, each of which has a fold similar to calmodulin. Both CNA and CNB form a composite surface which interacts with CypA–CsA to form the ternary complex. Thus, in addition to interactions with CsA, around 25 residues from both CNA and CNB make contact with amino acids Gly 80, Glu 81, Ala 103, Arg 148, Ser 147, Trp 121, Asn 149 and Thr 73 of CypA. Of particular interest is the hydrogen bond between Arg (148) CypA and Arg 122 (CN) which reorients the latter side chain, thereby affecting its catalytic activity.

Leishmania donovani is a dimorphic protozoan responsible for kala-azar in humans. Although CsA is highly toxic for a wide variety of parasites[10] (e.g. *Plasmodium falci-*

parum, *Schistoma*), *L. major* and *L. donovani* are relatively insensitive to the drug. The binding affinity of *Leishmania* cyclophilin (LdCyp) to CsA has been confirmed experimentally[11]. Sequence alignment (Figure 1) of cyclophilin from different sources shows a high sequence conservation of binding-site residues amongst those species sensitive to the drug. Initially, drug resistance was attributed to mutations in LdCyp corresponding to those residues in CypA which make contact with CN. However, variability in the same residue positions for other drug-sensitive parasites, has weakened the above hypothesis[12]. On the contrary, there is evidence to suggest that drug resistance in *L. donovani* is a consequence of the low expression of cyctoplasmic cyclophilin leading to reduced cyclosporin-binding activity[11]. Therefore, given the variability in the toxicity of CsA to *Leishmania* species, crystallographic studies have been initiated to elucidate its mode of binding to CsA and indicate possible interactions of the drug enzyme complex to CN. Here we report the unligated structure of cyclophilin (LdCyp) from *L. donovani* at 3.5 Å resolution.

The purification and crystallization of LdCyp, along with low-resolution data collection from the same crystals have been reported previously[13]. Given the cell parameters, only a single protein molecule (mol. wt 18.5 kDa, including the His_6 tag) is physically possible with a solvent content of 46% ($Vm = 2.3$ Å^3/Da). As reported earlier[13], molecular replacement using CypA (pdb code: 2rma) as the search model with 56% sequence identity gave an unambiguous solution in the space group $P4_32_12$, with a correlation coefficient of 50.9 and an *R*-factor of 42.7, after a few cycles of fast rigid body refinement in AmoRe[14]. CypA was chosen as a search model due to its high resolution (2.10 Å), adequate sequence identity with LdCyp and a binding affinity to cyclosporin similar to LdCyp, perhaps due to the binding site in both molecules being largely conserved sequentially. The search model (CypA with residues identical to LdCyp retained, the rest appropriately truncated to Ala or Gly) was placed in the cell and refined by Henrickson–Konnert restrained least squares positional refinement, initially using XPLOR[15] and then CNS[16]. Manual model building was performed with FRODO[17]. Despite the low resolution, the trace of the main chain was fairly unambiguous. Several cycles of manual model-building with $2Fo\text{-}Fc$ maps and refinement enabled the gradual inclusion of the majority of the side-chain atoms. For most bulky residues (Phe, Tyr, etc.) the orientation of the side chain was clearly visible in the electron density (Figure 2). Attempts to improve the structure by simulated annealing proved abortive. A few cycles of isotropic B-factor refinement with lower and upper limits set to 3 and 200 Å^2 respectively, were performed (average *B*-factor of the final model 16.3 Å^2) and an OMIT map calculated in the closing stages of the refinement process. The final model was assessed using PROCHECK[18]. Mean coordinate error for the working set estimated by Luzzati[19,20] and SigmaA[21,22] plots (5–3.5 Å, with no σ-cut) turned out to be 0.47 and 0.56 Å (figure of merit: 0.84) respectively. A summary of the refined model along with the relevant geometrical parameters is given in Table 1.

The *R*-factor and R_{free} for the final structure are 0.218 and 0.324 respectively (for reflections $F > 2\sigma(F)$). Among the 187 amino acids of LdCyp, residues 26–186 could be traced. No electron density could be found for the side chains of 14 residues (51 Asp, 72 Lys, 81 Gln, 110 Asn, 112 Asn, 116 Phe, 156 Asp, 166 Lys, 169 Thr, 170 Asn, 174 Arg, 177 Lys, 182 Val, 184 Ser). Two breaks were found in the continuity of the main chain electron density between residues 112 and 113 and 168 and 170. Discontinuities in side or main chain electron densities are due to both the limited resolution and intrinsic disorder in the polypeptide chain. The structure had one short contact of 2.6 Å between 139 Ala O and 140 Pro C. About 95.5% of

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Human          ------------------------------------MVNPTVFFDIAVDGEPLGRVSFE 23
Plasmodium     MKNNDNKKNEKISGLEENEEHNNNNIVPYYLSNLLTNPSNPVVFMDINLGNHFLGKFKFE 60
Leishmania     --------------MRFVAVLAVVLCALSFLNVAAEPEVTAKVYFDVMIDSEPLGRITIG 46
E. coli        ----------------MFKSTLAAMAAVFALSALSPAAMAAKGDPHVLLTTS-AGNIELE 43

Human          LFADKVPKTAENFRALSTGEK-----GFGYKGSCFHRIIPGFMCQGGDFTRHNGTGGKSI 78
Plasmodium     LFQNIVPRTSENFRKFCTGEHKINNLPVGYKNTTFHRVIKDFMIQGGDFVNYNGSGCISI 120
Leishmania     LFGKDAPLTTENFRQLCTGEH-----GFGYKDSIFHRVIQNFMIQGGDFTNFDGTGGKSI 101
E. coli        LDKQKAPVSVQNFVDYVNSGF--------YNNTTFHRVIPGFMIQGGGFTEQMQQKKPNP 95
                                                *    *  *        *
Human          YGEKFEDENFILKHTGPGILSMANAG-PNTNGSQFFICTAKTEWLDGKH-----VVFGKV 132
Plasmodium     YGEHFDDENFDIKHDKEGLLSMANTG-PNTNGCQFFIITKKCEWLDGKN-----VVFGRI 174
Leishmania     YGEKFADENLNVKH-FVGALSMANAG-PNTNGSQFFITTAPTPWLDGRH-----VVFGKV 154
E. coli        PIKNEADNGLRNTR---GTIAMARTADKDSATSQFFINVADNAFLDHGQRDFGYAVFGKV 152
                                      ***       *         *    *
Human          KEG--MNIVEAMERFGSR-----NGKTSKKITIADCGQLE 165
Plasmodium     IDNDSLILLKKIENVSVTPY---IYKPKLAINIVECGEL- 210
Leishmania     LDG--MDVVLRIEKTKTN-S---HDRPVKPVKIVASGEL- 187
E. coli        VKG--MDVADKISQVPTHDVGPYQNVPSKPVVILSAKVLP 190
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Figure 1. Sequence alignment[24] of cyclophilin from human[25], *Leishmania donovani*[26], *Plasmodium falciparum*[27] and *Escherichia coli*[28]. The cyclosporin binding site is marked with a star. Of the four sources, only cyclophilin from *E. coli* does not bind to the drug.

the fitted residues were in the favoured or additionally allowed regions of the Ramachandran plot[23] and the rest (six amino acids) in the generously allowed region.

LdCyp is a compact molecule with dimensions $38 \times 35 \times 42$ Å^3. The structure of the molecule consists of a β-barrel constituted by eight anti-parallel strands (Figure 3), with four strands (78–80, 84–87, 119–122, 134–137) approximately orthogonal to the other three (28–35, 45–47, 178–181). The seventh strand of the sheet system extends from 150 to 153. Relaxing the criteria for secondary structure determination in terms of hydrogen-bonding pattern for the second strand (45–47) and including those residues contiguous to 45 Ile lying in the β-sheet region of the Ramachandran plot, extends the strand from 42 to 47. Val 29 and Tyr 30 (strand 1: 28–35) also show distortions from the geometry characteristic of anti-parallel β-sheets. Shortening of the β-strands relative to CypA is most probably due to the low resolution of the present structure which makes the determination of side chain orientations ambiguous (especially for smaller amino acids), thereby perturbing the hydrogen bonding geometry. Two α-helices (53–64 and 158–166) are located on either side of the barrel. Both the barrel and the helices enclose a core, assembled by an extensive network of about 17 hydrophobic amino acids (151 Phe, 59 Phe, 120 Leu, 31 Phe, 136 Ile, 76 Phe, 134 Phe, 79 Val, 71 Tyr, 164 Ile, 85 Ile, 43 Ile, 33 Val, 122 Met, 161 Val, 47 Leu, and Ile 45). The rest of the structure consists of loops linking the individual β-strands and helices.

Superposition of the Cα coordinates of LdCyp onto CypA gives an rms deviation of 2.67 Å. Both sequentially as well as structurally, the C-terminal end of the molecule exhibits marked deviation from human CypA compared to the rest

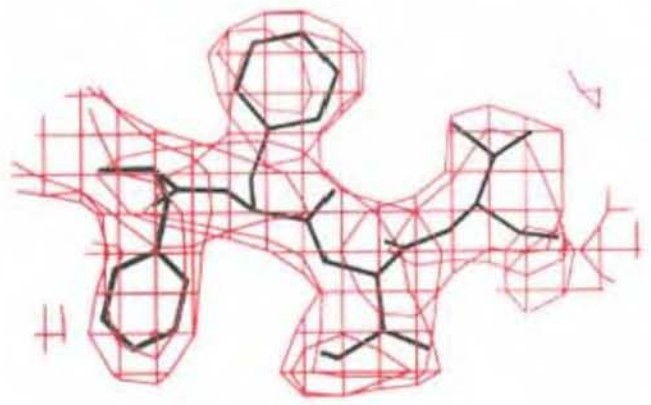

Figure 2. Electron density contoured at 1σ level[29] for residues 134 to 137. Phe 134 is on the extreme left.

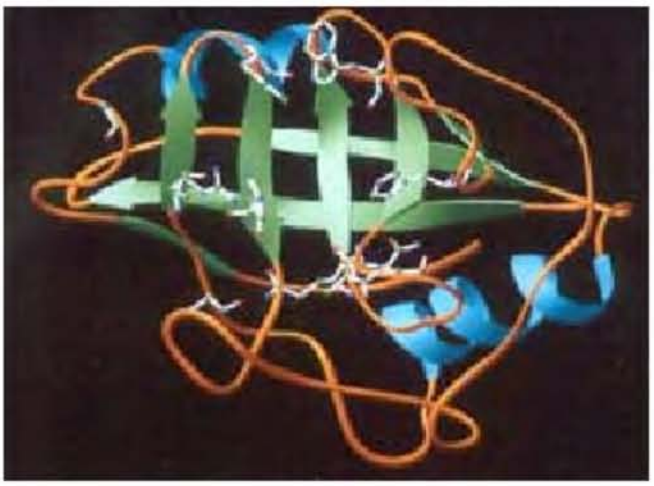

Figure 3. Ribbon diagram[30] of LdCyp. The putative binding site for CsA is indicated in ball and stick. The criterion for secondary-structural assignment has been relaxed for β-strands 1, 2 extending from 28 to 35 and 42 to 47 respectively.

Table 1. Summary of data and geometrical statistics at the end of refinement

Space group	:	$P4_32_12$
Unit cell parameters (Å)	:	$a = b = 48.73$, $c = 140.93$
Resolution (Å)	:	15.0–3.5
R merge (%)	:	10.7
Data cut-off (σF)	:	2.0
Total number of reflections	:	2389
Total number of reflections used	:	2047
Number of reflections in working set	:	1860
Number of reflections in test set	:	187
R-factor	:	0.218 (0.267)*
R_{free}	:	0.324
Number of protein atoms	:	1189
RMS deviation from ideal values		
Bond lengths (Å)	:	0.01
Bond angles (°)	:	2.1
Dihedral angles (°)	:	26.4
Improper angles (°)	:	1.34

*The R-factor of the last resolution shell (3.54–3.50 Å) is given within parenthesis.

Table 2. RMS deviation in Cα coordinates between human (CypA) and superposed *Leishmania* cyclophilin (LdCyp), of those residues responsible for binding to cyclosporin (CsA), as determined from the crystal structure of the CypA–CsA complex[6]. The minimum contact distances of CypA Cαs to CsA are tabulated with the corresponding distances of superposed LdCyp given in parentheses. Nomenclature of atoms for CsA are those found in the pdb file (2rma)

CypA	LdCyp	RMS (Å) deviation	CsA	Minimum contact distance (Å)
Arg 55	Arg 78	0.493	11 Mva CG1	8.152 (8.184)
Phe 60	Phe 83	0.627	9 Mle CD2	6.037 (6.492)
Gln 63	Gln 86	0.234	11 Mva CG1	5.428 (5.584)
Gly 72	Gly 95	0.984	3 Sar CN	4.305 (4.537)
Asn 102	Asn 124	0.558	2 Aba CG	3.851 (3.737)
Ala 103	Ala 125	0.944	1 Bmt CH	4.423 (4.015)
Gly 104	Gly 126	0.422	1 Bmt CE	5.285 (5.514)
Gln 111	Gln 133	0.107	2 Aba CG	5.252 (5.182)
Trp 121	Trp 143	1.115	9 Mle O	7.395 (6.460)
His 126	His 148	0.606	11 Mva CN	7.347 (7.911)
Arg 148	Asn 170	1.621	9 Mle CD2	7.202 (7.176)

of the molecule. The cyclosporin binding site in CypA is constituted by 11 residues, which are less than 3.5 Å from the drug molecule, as seen in the crystal structure of the CypA–CsA complex[6]. The entire set of active-site residues are sequentially conserved in LdCyp with the exception of Arg 148 (human), which is mutated to Asn. The rms deviation in the Cα atoms between the two binding site residue sets is 0.81 Å. Distance calculation between CsA and the putative active site of *Leishmania* LdCyp (Cα) superposed onto CypA gives values comparable to its human homologue (Table 2). Contact calculations involving all atoms also show LdCyp to be quite capable of maintaining the necessary interactions involved in binding to CsA. It is thus fairly certain that the binding-site geometry in case of both CypA and LdCyp is effectively conserved.

Packing of LdCyp within the cell is primarily mediated by two β-strands and three loops, specifically 30 Tyr, 34 Met, 36 Asp, 42 Arg, 91 Thr, 93 Phe, 94 Asp, 117 Val, 127 Pro, 128 Asn, 145 Asp, 159 Asp and 186 Glu which come within 3.5 Å of symmetry-related molecules in the cell.

In conclusion, the low-resolution structure of *Leishmania* cyclophilin LdCyp clearly indicates the structural conservation of the cyclosporin binding site with respect to CypA, rationalizing its binding affinity for the drug, similar to its human homologue. Thus, the present study reinforces a previous report[11] attributing drug resistance in *L. donovani* to low expression of cytoplasmic cyclophilin coupled to efflux of the drug by the parasite, rather than the inability of the drug to bind to its receptor. Currently, efforts are underway to grow crystals which diffract to a higher resolution and to commence the co-crystallization of the molecule with cyclosporin.

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