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Crystallization and preliminary X-ray analysis of cyclophilin from *Leishmania donovani*

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Cyclophilin from the parasite *Leishmania donovani* is a protein with peptidylprolyl *cis–trans* isomerase activity, in addition to being a receptor for the drug cyclosporin. Crystals of the enzyme have been obtained in space group $P4_32_12$, with unit-cell parameters $a = b = 48.73$, $c = 140.93$ Å, and diffract to 3.5 Å resolution. One molecule per asymmetric unit gives a solvent content and Matthews coefficient of 46% and 2.3 Å^3 Da^{-1}, respectively. Molecular-replacement calculations with human cyclophilin A as the search model give an unambiguous solution in rotation and translation functions.

1. Introduction

Cyclophilins are a ubiquitous class of proteins with peptidylprolyl *cis–trans* isomerase activity (Koletsky *et al.*, 1986). In addition, they act as a receptor for the antifungal immunosuppressive drug cyclosporin (Handschumacher *et al.*, 1984). The cyclophilin–cyclosporin complex formed in the cytoplasm binds to calcineurin, inhibiting its serine–threonine protein phosphatase activity and thereby leading to immunosuppression (Liu *et al.*, 1991). The inhibition of peptidylprolyl *cis–trans* isomerase activity is however unrelated to immunosuppression. Perhaps owing to the multiple cellular localization of cyclophilins, they have been implicated in a wide variety of cellular processes ranging from signal transduction to playing the role of a chaperone in protein folding (Schreiber, 1991).

Several isoforms of cyclophilins have been identified in mammalian tissues (Friedman *et al.*, 1993), of which human cyclophilin A has been structurally well characterized. Several crystal structures are now available of the protein in its unligated form (Ke, 1992) or complexed with cyclosporin and its derivatives (Mikol *et al.*, 1993; Kallen *et al.*, 1998). The overall shape of the molecule is that of a barrel constituted of eight antiparallel β-strands, with two helices on either end. The cyclosporin-binding site is a hydrophobic crevice defined by 13 residues on the face of the barrel (Mikol *et al.*, 1993).

Leishmania donovani is a dimorphic protozoan responsible for kala-azar in humans. Although cyclosporin is highly toxic to a wide variety of parasites (*e.g. Plasmodium*, *Schistosoma*; Page *et al.*, 1995), there is considerable ambiguity in its effects on various *Leishmania* species. *L. major* and *L. donovani* exhibit a relative insensitivity to the drug. Initially, the drug resistance in *L. major* was attributed to the mutation of Arg69 in human cyclophilin A to Asn in the corresponding position of the parasitic enzyme, the correctly located arginine being thought to be essential for the subsequent binding of the cyclophilin–cyclosporin complex to calcineurin. Identical mutations in *Toxoplasma gondii* and *Plasmodium falciparum* cyclophilins have however cast doubt on the hypothesis, as both are sensitive to the drug (Berriman & Fairlamb, 1998). There is evidence to suggest that drug resistance in *L. donovani* is a consequence of the low expression of cytoplasmic cyclophilin in the parasite leading to reduced cyclosporin binding activity (Dutta *et al.*, 2001).

Given the variability in the toxicity of the drug to *Leishmania* species, crystallographic studies have been initiated on cyclophilin from *L. donovani* to elucidate its mode of binding to cyclosporin and to indicate the possible interactions of the enzyme–drug complex with calcineurin. Despite the importance of cyclosporin as an immunosuppressive drug, no crystal structure of the cyclophilin–cyclosporin–calcineurin ternary complex is yet available. Comparison of the parasite and human enzyme structures is also of interest.

2. Methods and results

2.1. Preparation of cyclophilin

Recombinant cyclophilin from *L. donovani* was purified from *Escherichia coli* M15 cells harbouring the chimeric plasmid containing amino acids 22–187 of the encoded cyclophilin gene sequence into the expression vector pQE32 (Qiagen). Residues 1–21 are a signal sequence which is post-translationally cleaved

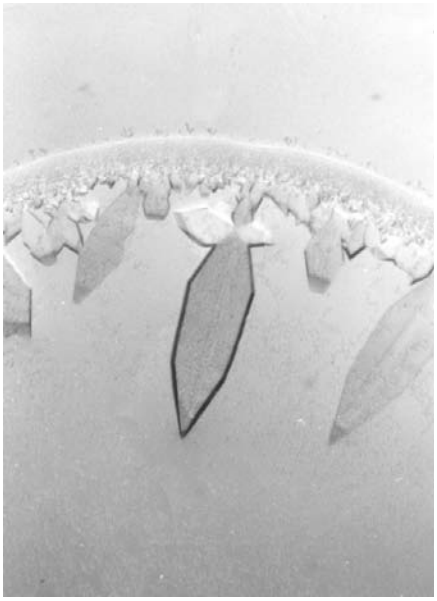

Figure 1
Crystal of cyclophilin from *L. donovani*, approximately 0.8 mm in the longest dimension.

in the native protein. Induction of the above clone resulted in production of cyclophilin containing His_6-tagged fusion protein, which was purified to apparent homogeneity using a nickel–nitrilotriacetic acid–agarose column following the manufacturer's suggested procedure. For all the experiments, imidazole-eluted His_6-tagged cyclophilin was extensively dialyzed against buffer containing 10 m*M* Tris pH 7.5 and 1 m*M* dithiothreitol.

2.2. Crystallization

The purified protein was concentrated to about 20 mg ml^{-1} and then dialysed against 20 m*M* Tris pH 8.5 and 0.02% NaN_3. All crystallization screening experiments were performed by the hanging-drop vapour-diffusion method on Linbro tissue-culture plates at 293 K. Crystals were obtained in 10 µl drops of concentrated protein sample containing 0.5–3% PEG 6000 as a precipitant inverted over a reservoir of 40% PEG 3350. Crystals were also obtained using PEG 3350 and 8000 as precipitants under similar crystallization conditions. Cyclophilin crystals appeared in about two weeks and grew to average dimensions of 0.7 × 0.3 × 0.1 mm (Fig. 1). Crystals from all batches were highly sensitive to externally added mother liquor, but were reasonably stable only in 40% PEG 3350 in 20 m*M* Tris pH 8.50.

2.3. Data collection and analysis

Diffraction data sets were collected using a MAR image-plate scanner at 293 K on an in-house Cu $K\alpha$ rotating-anode X-ray source (40 kV, 50 mA). The crystals were mounted in locally produced glass capillary tubes sealed with wax at both ends. The crystal-to-detector distance was set to 130 mm with a 1° oscillation range. The data was processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) in the Laue symmetry 4/*mmm*. Although the crystal displayed diffraction spots to 3.0 Å resolution, two independent data sets were processed at 3.0 and 3.5 Å resolution. The latter (3.5 Å; Table 1) was retained for subsequent calculations owing to an increase in $R_{\rm merge}$ beyond 3.5 Å ($R_{\rm merge}$ is 0.14 for the entire 3.0 Å data set and 0.40 in the 3.07–3.00 Å resolution shell). The 3.5 Å data set had a mosaicity of 0.27 as output by *SCALEPACK*.

Given the molecular weight of the protein to be 18.5 kDa (including the His_6 tag), a single cyclophilin molecule per asymmetric unit gave a solvent content of 46% ($V_{\rm M}$ = 2.3 Å^3 Da^{-1}; Matthews, 1968). All other possibilities for the number of molecules in the asymmetric unit were not physically viable. Molecular-replacement calculations were performed using the program *AMoRe* (Navaza, 1994) with human cyclophilin A as the search model, with which the parasitic enzyme exhibited a 56% sequence identity. Based on these coordinates, three different models were used: model 1, the complete cyclophilin A molecule; model 2, all-Ala model of the same structure; model 3, residues identical to *Leishmania* cyclophilin included, the remainder being truncated to alanine (only main-chain atoms were retained where Gly was present in the parasitic molecule). The molecular-replacement solutions of all the three models were in good agreement. The calculations were carried out in the resolution range 15.0–4.0 Å, with a radius of integration of 24.7 Å. For model 3, the best solution in the rotation-function map gave a correlation coefficient of 13.4 (next best 9.6). The translation function was then calculated for all possible space groups in the class 422. Space group $P4_32_12$ yielded the best solution, with a final correlation coefficient (CC) of 50.9 (next best 29.9, in the same space group) and an *R* factor (RF) of 42.7 (next best RF = 48.6), subsequent to a few cycles of fast rigid-body refinement in *AMoRe*. $P4_12_12$ (CC = 27.2, RF = 49.1) did not give a prominent peak, along with other space groups. Examination of packing in space group $P4_32_12$ was also found to be sterically reasonable. It thus appears that the molecular-replacement solution obtained in $P4_32_12$ is unambiguous. Currently, efforts are under way to improve crystal diffraction to a higher resolution.

Table 1
Data-collection statistics.

Values in the parentheses refer to the highest resolution shell (3.58–3.50 Å).

Space group	$P4_32_12$
Unit-cell parameters (Å)	$a = b = 48.73$, $c = 140.93$
No. of measured reflections	6556
No. of unique reflections	2278
Redundancy	2.9 (3)
Resolution (Å)	15.0–3.5
$R_{\rm merge}$† (%)	10.7 (15.8)
Completeness (%)	94.2 (95.1)
$I/\sigma(I)$	10.1 (6.5)

† $R_{\rm merge} = \sum|I(k) - \langle I\rangle|/\sum I(k)$, where $I(k)$ is the kth intensity measurement of a reflection, $\langle I\rangle$ is the average intensity value of that reflection and the summation is over all measurements.

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