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Crystallization and preliminary X-ray diffraction analysis of *Leishmania major* dihydroorotate dehydrogenase

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Dihydroorotate dehydrogenases (DHODHs) are flavin-containing enzymes that catalyze the oxidation of L-dihydroorotate to orotate, the fourth step in the *de novo* pyrimidine nucleotide synthesis pathway. In this study, DHODH from *Leishmania major* has been crystallized by the vapour-diffusion technique using lithium sulfate as the precipitating agent. The crystals belong to space group $P6_1$, with unit-cell parameters a = 143.7, c = 69.8 Å. X-ray diffraction data were collected to 2.0 Å resolution using an in-house rotating-anode generator. Analysis of the solvent content and the self-rotation function indicate the presence of two molecules in the asymmetric unit. The structure has been solved by the molecular-replacement technique.

1. Introduction

The *de novo* pyrimidine nucleotide biosynthetic pathway, found in all organisms, results in the formation of UMP and consists of a cascade of six enzymatic reactions (Jones, 1980). The oxidation of (*S*)-dihydroorotate to orotate constitutes the fourth and the only redox step in *de novo* pyrimidine biosynthesis and is catalyzed by the flavoenzyme dihydroorotate dehydrogenase (DHODH). This reaction represents a rate-limiting step in *de novo* pyrimidine biosynthesis and the selective inhibition of DHODH has been used as a strategy in the design and development of antitumour, antimicrobial and antiprotozoal agents (Chen *et al.*, 1992; Ittarat *et al.*, 1995; Ruckemann & Simmonds, 1998; Copeland *et al.*, 2000; Knecht *et al.*, 2000; Christopherson *et al.*, 2002; Baldwin *et al.*, 2002, 2005).

On the basis of sequence comparison, DHODHs can be divided into two classes: class 1, which is further divided into subclasses 1A and 1B, and class 2. This division corresponds to differences in cellular location and in the nature of the electron acceptor. Class 1 enzymes, which use soluble electron acceptors, are widespread in Gram-positive bacteria and are also found in some unicellular eukaryotic organisms. Subclass 1A members are dimeric enzymes composed of two copies of a polypeptide chain encoded by the PyrD gene (Rowland *et al.*, 1998). Subclass 1B members are tetrameric enzymes composed of two polypeptide chains encoded by the PyrD gene and two polypeptide chains encoded by the PyrF gene (Nielse *et al.*, 1996). Class 2 enzymes are monomeric, membrane-bound and use respiratory quinones as electron acceptors.

Here, we report the successful crystallization and preliminary X-ray diffraction analysis of dihydroorotate dehydrogenase from *Leishmania major* (LmDHODH). LmDHODH is a representative of the class 1A enzymes that utilizes fumarate as a redox cofactor. Kinetic studies indicate a sequential ping-pong mechanism for the conversion of dihydroorotate to orotate (Feliciano *et al.*, 2006), where in the first half-reaction, which comprises the reduction of dihydroorotate to orotate, electrons are transferred to the flavine mononucleotide moiety (FMN). In the second half-reaction, orotate dissociates from the enzyme and oxidized dihydroflavin mononucleotide ($FMNH_2$) is regenerated by a fumarate molecule.

The *Leishmania* parasite is a sandfly-transmitted protozoan parasite that causes leishmaniasis in vertebrate hosts. This pathogen targets macrophages and dendritic cells and induces a spectrum of diseases ranging from mild cutaneous to lethal visceral forms (Shaw

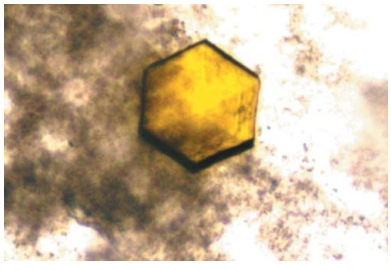

Table 1
Data-collection and processing statistics.

Values in parentheses are for the outer resolution shell (2.1–2.0 Å).

Temperature (K)	100
Wavelength (Å)	1.5418
Space group	$P6_1$
Unit-cell parameters (Å)	a = 143.7, c = 69.8
Resolution range (Å)	28.5–2.0
Unique reflections	58045
Redundancy	5.6 (5.5)
Data completeness (%)	100 (100)
$I/\sigma(I)$	10.5 (2.5)
R_{sym}† (%)	6.4 (30.2)
Molecules per ASU	2
V_M (Å^3 Da^{-1})	2.9
Solvent content (%)	58

† $R_{sym} = \sum |I_{obs} - \langle I \rangle| / \sum I_{obs}$, where $\langle I \rangle$ is the mean intensity obtained of multiple observations of symmetry-related reflections.

& Lainson, 1987). It is estimated that 12 million people in 88 countries around the world are infected with leishmaniasis and 350 million more are threatened (http://www.who.int). There are no approved vaccines for preventing leishmaniasis and current chemotherapeutic agents are either too expensive for widespread use or have toxic side effects. In addition, there has been an increase in drug-resistant infections and there is a relative lack of diagnostics, which have led the World Health Organization to label leishmaniasis as an emerging disease.

It is our aim to evaluate LmDHODH as a target for the development of highly specific anti-leishmaniasis drugs. The therapeutic potential of *Plasmodium falciparum* DHODH inhibitors against malaria (Baldwin *et al.*, 2002, 2005) and the dependence on DHODH enzyme activity for cell viability in *Trypanosoma cruzi* (Annoura *et al.*, 2005) suggest that selective DHODH inhibitors may also be developed for leishmaniasis chemotherapy. The work presented here constitutes the first step towards the structure determination of LmDHODH in complex with substrates and inhibitors. Those models, in combination with the structural information on *Trypanosoma brucei* DHODH (PDB code 2b4g) deposited by the Genomics of Pathogenic Protozoa Consortium (SGPP), will provide a structural basis for the design of potential antiparasitic drugs through the technology of structure-based drug design.

2. Materials and methods

2.1. Crystallization and data collection

L. major dihydroorotate dehydrogenase (LmDHODH) was cloned, expressed, purified and characterized as previously reported (Feliciano *et al.*, 2006). The enzyme is expressed as a His_6-tag fusion protein; the N-terminal sequence of the recombinant protein is MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGS**M**, where the methionine indicated in bold represents residue 1 of the DHODH sequence. The enzyme can be purified to homogeneity by a single affinity chromatographic step using a nickel-chelating resin. Prior to crystallization, the recombinant LmDHODH was dialyzed against 50 m*M* HEPES pH 7.2, 150 m*M* NaCl and concentrated to 8.8 mg ml^{-1} (Millipore Ultrafree membrane; 10 kDa molecular-weight cutoff). The crystallization trials on LmDHODH were carried out using His_6-tagged enzyme.

Initial crystallization conditions were screened by the sparse-matrix method (Jancarik & Kim, 1991) using commercially available screening kits. Vapour-diffusion techniques using sitting and hanging drops were used; equal volumes (3 µl) of protein and reservoir solution were mixed, equilibrated against 500 µl reservoir solution and kept at 293 K. A single crystal appeared within 3 d in 0.1 *M* sodium citrate tribasic dihydrate pH 5.6, 1 *M* lithium sulfate, 0.5 *M* ammonium sulfate (Crystal Screen II, formulation 15; Hampton Research). Additional efforts were made to optimize the crystal quality by screening various crystallization variables (pH, precipitant concentration, temperature and additives). Crystals suitable for X-ray diffraction measurements were obtained at 293 K using both sitting and hanging drops. The drops contained 3 µl protein solution and 3 µl of a reservoir solution consisting of 1–1.3 *M* lithium sulfate, 0.3–0.6 *M* ammonium sulfate in 0.1 *M* sodium citrate tribasic dihydrate pH 5.6. The hexagonal-shaped crystals usually appeared within 5 d and reached maximum dimensions of 0.2 × 0.2 × 0.1 mm after a week (Fig. 1).

Single LmDHODH crystals were transferred to a cryoprotectant solution consisting of 1 *M* lithium sulfate, 0.6 *M* ammonium sulfate in 0.1 *M* sodium citrate pH 5.6, 20%(*v*/*v*) glycerol and were flash-frozen directly in a nitrogen stream at 100 K. Diffraction data were collected on a MAR345dtb detector mounted on a Rigaku X-ray source and equipped with a focusing mirror. Cu $K\alpha$ X-ray diffraction data were collected using 1.0° oscillations with a crystal-to-detector distance of 150 mm. The data were processed using *MOSFLM* (Leslie, 2006) and scaled with *SCALA* (Evans, 2006). The data-collection and processing statistics are summarized in Table 1.

2.2. Phase calculation and refinement

Initial phases were obtained by molecular replacement with the program *MOLREP* (Vagin & Teplyakov, 2000). An initial rotation and translation function were calculated using the coordinates of a homologous DHODH structure from *Lactococcus lactis* (LlDHODH; PDB code 1dor; Rowland *et al.*, 1997) as the search model. LmDHODH and LlDHDOH share approximately 54% sequence identity. The model was initially refined by rigid-body refinement, followed by several rounds of adjusting side-chain rotamers for residues using *Coot* (Emsley & Cowtan, 2004) interspersed with torsion-angle simulated annealing using *CNS* (Brünger *et al.*, 1998) and positional and individual *B*-factor refinement using *REFMAC*5 (Murshudov *et al.*, 1997).

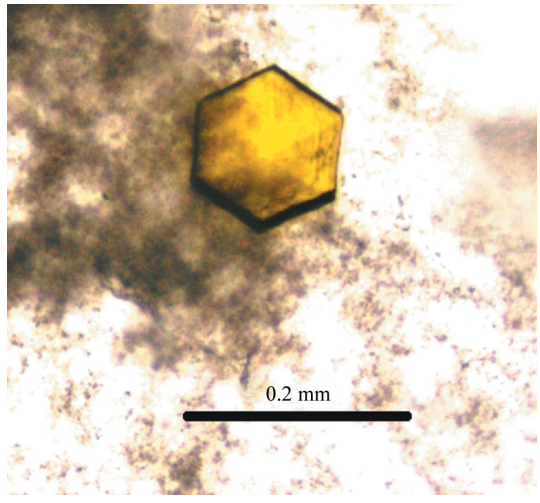

Figure 1
Crystals of *L. major* dihydroorotate dehydrogenase.

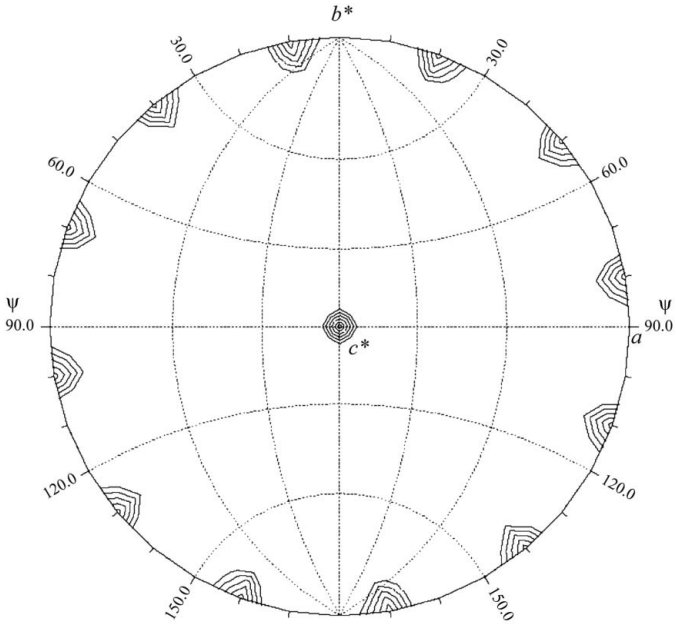

Figure 2
Stereographic projection down the *c* axis of the $\kappa = 180^\circ$ polar section. The plot was generated with the program *GRLF* (Tong & Rossmann, 1997) using data in the resolution range 20.0–5.0 Å; the integration radius is 25 Å.

3. Results and discussion

The yellow crystals of apo LmDHODH diffracted to better than 2.0 Å resolution. The unit-cell parameters (Table 1) and the systematic absences for the 00*l*, $l = 6n$ reflections indicate that the enzyme crystals belong to the hexagonal space group $P6_1$ or $P6_5$. Assuming the presence of two monomers of LmDHODH with a calculated molecular weight of approximately 34 kDa in the asymmetric unit, the calculated Matthews coefficient is 2.9 $\text{Å}^3\ \text{Da}^{-1}$ and, based on a specific volume of 0.74 $\text{cm}^3\ \text{g}^{-1}$, the calculated solvent content is approximately 58%, values which lie within the range commonly observed for protein crystals (Matthews, 1968). Self-rotation function calculations using the program *GLRF* (Tong & Rossmann, 1997) support the assumption of two molecules in the asymmetric unit (Fig. 2). Only the $\kappa = 180^\circ$ section shows significant peaks other than those belonging to the crystallographic space group.

The application of molecular-replacement procedures for both enantiomer possibilities indicated $P6_1$ to be the correct space group, yielding a solution with a correlation coefficient of 31.9% and an *R* factor of 50.6%. Initial rigid-body refinement followed by 30 cycles of restrained refinement, implemented with *REFMAC*5, resulted in $R = 30.4\%$ and $R_{\rm free} = 34.8\%$. Further structural refinement is in progress.

The crystallization and structure determination of LmDHODH in complex with a variety of substrates and inhibitors identified from Brazilian natural products are currently under way. The structural analysis of the ligand-binding regions will help to increase our understanding of the enzyme molecular mechanism and of its substrate-binding and inhibitor-binding specificities. The preparation of diffraction-quality crystals of apo LmDHODH reported here represents an important first step towards this goal.

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