Recombinant production, crystallization and crystal structure determination of dihydroorotate dehydrogenase from *Leishmania (Viannia) braziliensis*

Renata Almeida Garcia Reis, Eder Lorenzato Jr, Valeria Cristina Silva and Maria Cristina Nonato*

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Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Avenida Café S/N, 14040-903 Ribeirão Preto-SP, Brazil. *Correspondence e-mail: cristy@fcrp.usp.br

The enzyme dihydroorotate dehydrogenase (DHODH) is a flavoenzyme that catalyses the oxidation of dihydroorotate to orotate in the *de novo* pyrimidine-biosynthesis pathway. In this study, a reproducible protocol for the heterologous expression of active dihydroorotate dehydrogenase from *Leishmania (Viannia) braziliensis* (LbDHODH) was developed and its crystal structure was determined at 2.12 Å resolution. *L. (V.) braziliensis* is the species responsible for the mucosal form of leishmaniasis, a neglected disease for which no cure or effective therapy is available. Analyses of sequence, structural and kinetic features classify LbDHODH as a member of the class 1A DHODHs and reveal a very high degree of structural conservation with the previously reported structures of orthologous trypanosomatid enzymes. The relevance of nucleotide-biosynthetic pathways for cell metabolism together with structural and functional differences from the respective host enzyme suggests that inhibition of LbDHODH could be exploited for antileishmanicidal drug development. The present work provides the framework for further integrated *in vitro*, *in silico* and *in vivo* studies as a new tool to evaluate DHODH as a drug target against trypanosomatid-related diseases.

1. Introduction

The leishmaniasis are a group of protozoan diseases caused by more than 20 different species of parasites from the genus *Leishmania*. Endemic in 98 countries of Asia, Africa, South and Central America and Southern Europe (Barrett & Croft, 2012), the leishmaniasis rank as the leading neglected tropical disease in terms of mortality and morbidity, with an estimated 1.3 million new cases and more than 20 000 deaths occurring annually (World Health Organization, 2015).

There are two major clinical manifestations of leishmaniasis: visceral leishmaniasis (VL; also known as kala-azar) and cutaneous leishmaniasis (CL; Croft & Coombs, 2003). Variations of VL and CL can also occur, including mucocutaneous leishmaniasis (MCL) and diffuse leishmaniasis (DCL), which are forms of CL, and post-kala-azar dermal leishmaniasis (PKDL), a dermatosis that occurs as a sequela of VL.

Cutaneous leishmaniasis is a clinical condition characterized by multiple cutaneous lesions with or without mucosal involvement. The disease is usually caused by parasites of the subgenus *Viannia*, and is often resistant to standard antileishmanial therapy. Mucosal leishmaniasis is considered one of the most important infectious diseases owing to its high infection rates and its potential to cause permanent deformities in infected people (World Health Organization, 2015).

In this work, we report the crystal structure of dihydroorotate dehydrogenase (DHODH) from *L. (V.) braziliensis*. *L. (V.) braziliensis* is recognized as the most important aetiological agent of MCL and is mainly active in Brazil, Colombia, Venezuela and the Andes mountain range (de Oliveira Guerra *et al.*, 2011).

DHODH is a flavoenzyme that participates in the redox reaction of the *de novo* pyrimidine-biosynthesis pathway (Nonato & Costa-Filho, 2013) and has been exploited as a drug target for the treatment of rheumatoid arthritis, cancer and parasitic diseases (Vyas & Ghate, 2011; Nonato & Costa-Filho, 2013). There is now additionally a deep interest in investigating the potential of DHODH from *L. (V.) braziliensis* (*LbDHODH*) as a drug target against MCL.

Besides the relevant role of nucleotides in cellular metabolism and cell growth, the rate-limiting step catalyzed by *LbDHODH* in nucleotide metabolism relies on distinct structural and functional features when compared with the human DHODH homologue enzyme. *LbDHODH* is member

Table 1

Macromolecule production.

Source organism	<i>L. (V.) braziliensis</i>
DNA source	<i>L. (V.) braziliensis</i>
Forward primer†	5'-GACGGATCCATGAGCCTTCAGGTGGGC-3'
Reverse primer†	5'-CTGCTCGAGTTAATCCATTGCCTTAACC-3'
Cloning vector	pET-28a
Expression vector	pET-28a
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	MGSSHHHHHSSGLVPRGSHMASMTGGGQMGRGS-MSLQVGILGNTFANPFMNAAGVMCSTEEELAA-MTESTSGSLITKSCTPALREGNPAPRYTLP-LGSINSMGLPNKGDFYLAYSARHHDYSRKPLF-ISISGFSAEENAEMCKRLAPVAAEKGVILELN-LSCPNVPGKPQVAYDFDAMRRYLAAISEAYPH-PFGVKMPYFDFAHFDDAAEILNQFPKVQFIT-CINSIGNGLVIDVETESVVIKPKQGFGGLGGR-YVFPTALANVNAPYRRCPGKLIFGCGGVYTGE-DAFLHVLGASMVQVGTALHEEGAIFERLTA-ELLDVMAKKGYKALDEFGRKVKAMD

† The underlined sequence indicates the BamHI and XhoI restriction sites in the forward and reverse primers, respectively.

Figure 1

Purification and crystallization of recombinant *LbDHODH*. (a) Coomassie Blue-stained reduced SDS-PAGE. Lanes 1 and 2, samples of the whole cell lysate and the soluble fraction; lane 3, unbound fraction after passage over the affinity column; lane 4, sample of fraction washed with 25 mM imidazole; lane 5, sample of fraction washed with 50 mM imidazole; lanes 6 and 7, samples of fractions of pure *LbDHODH* eluted with 100 mM imidazole. Lane M, molecular-weight marker (labelled in kDa). (b) Crystals of *LbDHODH* grown at pH 7.5 using ammonium sulfate as precipitant.

of the class 1A DHODHs, which are homodimeric proteins located in the cytosol that utilize fumarate to oxidize FMNH₂. In contrast, human DHODH is a class 2 enzyme; these are monomeric proteins that are bound in the inner membrane of the mitochondria of eukaryotes and require quinones as their physiological oxidizing agent (Liu *et al.*, 2000; Löffler *et al.*, 2002; Nagy *et al.*, 1992). Together, these features suggest that species-selective inhibition of DHODH is feasible and could be used to suppress pyrimidine biosynthesis as a strategy for antileishmanicidal drug development.

The development of a reproducible protocol for heterologous expression and purification together with the three-dimensional structure determination of *LbDHODH* corresponds to an important step towards exploiting *LbDHODH* as a drug target against leishmaniasis and other trypanosomatid-related diseases.

2. Materials and methods

2.1. Protein production

Genomic DNA from the promastigote form of *L. (V.) braziliensis* was kindly provided by Professor Angela Kaysel Cruz from the Medical School of Ribeirão Preto, University of São Paulo and was used as a template for polymerase chain reaction. In this study, a predicted open reading frame (ORF) encoding *LbDHODH* was amplified and the product was cloned into the BamHI and XhoI restriction sites of the pET-28a expression vector. Sequencing experiments identified an ORF of 942 bp that encodes a protein with 313 amino acids (Table 1). This pET-28a(+)-*LbDHODH* construct produces *LbDHODH* as an N-terminal six-histidine fusion protein that was functionally expressed as a soluble protein in *Escherichia coli* BL21 (DE3) cells. The cells were grown in Luria Broth (LB) supplemented with 30 µg ml⁻¹ kanamycin and protein expression was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 h at 25°C. The cells were harvested and lysed by sonication in 50 mM sodium

Table 2

Data processing.

Values in parentheses are for the outer shell.

Space group	$P2_12_12_1$
a, b, c (Å)	61.16, 105.69, 106.24
α, β, γ (°)	90, 90, 90
Mosaicity (°)	0.73
Resolution range (Å)	40.10–2.12 (2.23–2.12)
Total No. of observed reflections	434200 (45366)
No. of unique reflections	39526 (5529)
Completeness (%)	99.3 (97.1)
Multiplicity	11.0 (8.2)
$\langle I/\sigma(I) \rangle^\dagger$	7.0 (2.0)
$R_{\text{meas}}^\ddagger$	0.243 (1.351)
Overall B factor from Wilson plot (Å ²)	24.6

$^\dagger \langle I/\sigma(I) \rangle \geq 2$ was used as a criterion to determine the high-resolution limit. $^\ddagger R_{\text{meas}} = \sum_{hkl} [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th measurement of a reflection and $\langle I(hkl) \rangle$ is the average intensity of all reflections with indices hkl . $N(hkl)$ corresponds to the number of observations for each unique reflection $I_i(hkl)$ (Diederichs & Karplus, 1997).

phosphate, 300 mM NaCl pH 8, 1 mM phenylmethylsulfonyl-fluoride (lysis buffer). The insoluble debris was separated by centrifugation at 16 000g for 30 min at 4°C. The supernatant was loaded onto Ni-NTA agarose affinity resin (Qiagen) equilibrated with lysis buffer. The resin was washed with lysis buffer supplemented with a step gradient of 0, 25 and 50 mM imidazole and the pure protein was eluted with lysis buffer supplemented with 100 mM imidazole (Fig. 1a). The yield of pure *Lb*DHODH was typically 4 mg per litre of culture.

2.2. Crystallization

The recombinant *Lb*DHODH was dialyzed against HEPES buffer consisting of 50 mM HEPES pH 7.2, 150 mM NaCl and was concentrated to 1.65 mg ml⁻¹. As previously standardized for other trypanosomatid DHODHs, the concentration of *Lb*DHODH was estimated based on its molar extinction coefficient, which was determined considering equimolar concentrations of free and enzyme-bound FMN (Padua *et al.*, 2014). This method gave an calculated extinction coefficient of 11 352 M⁻¹ cm⁻¹ at 459 nm for the recombinant protein (Supplementary Fig. S1)

Prior to crystallization, the *Lb*DHODH activity was determined by monitoring the formation of orotate at 300 nm. The reaction was initiated by the addition of pure enzyme to reaction buffer containing saturated concentrations of substrates: 50 µM DHO and 500 µM fumarate in 50 mM HEPES pH 7.2, 150 mM NaCl.

The sparse-matrix method (Jancarik & Kim, 1991) was used for initial crystallization experiments as implemented in the commercially available screening kits Crystal Screen, Crystal Screen 2, PEG/Ion and PEG/Ion 2 (Hampton Research). Equal volumes (3 µl) of protein and reservoir solution were mixed, equilibrated against 500 µl reservoir solution and kept at 21°C. The first crystals appeared within 4 d in 100 mM HEPES pH 7.5, 100 mM NaCl, 1.6 M ammonium sulfate (Crystal Screen 2 formulation 32; Hampton Research). Several efforts were made to optimize the crystallization experiment by screening a wide range of different crystallization variables (protein concentration, drop volume, pH,

Table 3

Structure solution and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	33.579–2.12 (2.17–2.12)
Completeness (%)	99.3 (97.1)
No. of reflections, working set	39159
No. of reflections, test set	1975
Final R_{work}	0.176 (0.280)
Final R_{free}	0.224 (0.323)
Cruickshank DPI (Å) (Cruickshank, 1999)	0.2327
No. of non-H atoms	
Protein	4566
Ligand	83
Water	269
Total	4918
R.m.s. deviations	
Bonds (Å)	0.008
Angles (°)	1.101
Average B factors (Å ²)	38.0
Ramachandran plot	
Most favoured (%)	97
Allowed (%)	3

precipitant concentration and additives). Yellow rectangular crystals were obtained in the presence of 100 mM HEPES pH 7.5 and 1.5–1.7 M ammonium sulfate within 4 d and reached maximum dimensions of 0.1 × 0.1 × 0.7 mm in 10 d (Fig. 1b).

2.3. Data collection and processing

A single *Lb*DHODH crystal was transferred into a cryo-protectant solution consisting of 1.8 M ammonium sulfate, 100 mM HEPES pH 7.5, 20%(v/v) glycerol and flash-cooled directly in a nitrogen stream at 100 K. Diffraction data were collected at 100 K on beamline MX2 at the Brazilian Light Source (LNLS), Campinas, Brazil using a Dectris Pilatus 2M detector. 360 images of 1° oscillation per frame were collected with a 150 mm crystal-to-detector distance and an exposure time of 5.2 s per image. The data were processed using *iMosflm* (Battye *et al.*, 2011) and scaled with *SCALA* (Evans, 2006). Data-collection and processing statistics are summarized in Table 2.

2.4. Structure solution and refinement

Initial phases were obtained by molecular replacement (MR) performed using the coordinates of *L. major* dihydro-orotate dehydrogenase as a search model (Cordeiro *et al.*, 2012). A unique solution was found by *Phaser* (McCoy *et al.*, 2007) in the orthorhombic space group $P2_12_12_1$ with two molecules in the crystallographic asymmetric unit. The structure was refined using *phenix.refine* (Afonine *et al.*, 2012), with manual map inspection and model building being performed in *Coot* (Emsley *et al.*, 2010). The quality of the model was regularly checked for steric clashes, incorrect stereochemistry and rotamer outliers using *MolProbity* (Chen *et al.*, 2010). All structural figures were produced using *PyMOL* (Schrödinger; <http://www.pymol.org>). Refined atomic coordinates and experimental structure factors have been deposited in the Protein Data Bank (PDB entry 4wzh). Data-collection and refinement statistics are given in Table 3.

3. Results and discussion

DHODH enzymes have been considered to be promising targets for the development of antitrypanosomatid therapies (Takashima *et al.*, 2002; Arakaki *et al.*, 2008). Thus, considerable attention has been paid to the structural and functional characterization of trypanosomatid DHODHs (TrypDHODHs), including the *Trypanosoma brucei* (Arakaki *et al.*, 2008), *T. cruzi* (Pinheiro *et al.*, 2008; Inaoka *et al.*, 2008; Cheleski, Wiggers *et al.*, 2010) and *L. major* (Feliciano *et al.*, 2006; Cordeiro *et al.*, 2006, 2012) enzymes. Selective inhibitors have already been identified for TrypDHODHs (Cheleski, Rocha *et al.*, 2010; Pinheiro *et al.*, 2013) and meticulous analyses of protein structures have also highlighted the presence and the dynamics of five different pockets, named S1

Figure 2

Ribbon representation of *LbDHODH*. (a) A central barrel composed of eight parallel β -strands ($\beta 1$ – $\beta 8$) illustrated in pink is surrounded by eight α -helices ($\alpha 1$ – $\alpha 8$) illustrated in blue. Two N-terminal antiparallel β -strands (βa – βb) are found at the bottom of the barrel and additional secondary-structural elements and loops form a protuberant subdomain present at the top of the barrel (green, salmon and light blue). A stick representation of the prosthetic FMN group is illustrated in yellow. (b) The functional dimeric structure of *LbDHODH* is formed by a noncrystallographic twofold axis.

to S5, to be targeted in the search for ligands (Pinheiro *et al.*, 2013).

In this work, we moved further by initiating the characterization of *L. (V.) braziliensis* DHODH. Firstly, a protocol was developed for overexpression and purification of *LbDHODH* with excellent yield and high purity (Fig. 1a) on a scale suitable for kinetic and inhibitory studies and crystallization. Our preliminary activity assays showed that recombinant *LbDHODH* is able to catalyse the conversion of dihydro-orotate to orotate using fumarate as the oxidizing substrate (Supplementary Fig. S2). *LbDHODH* crystallized in space group $P2_12_12_1$ using ammonium sulfate as the precipitant agent (Fig. 1b) and its crystal structure has been solved by molecular replacement using the structure of *L. major* DHODH as the search model (PDB entry 3gye; Cordeiro *et al.*, 2012). The structure of *LbDHODH* was refined at 2.12 Å resolution and revealed the presence of the functional dimeric structure described for class 1A DHODHs in the asymmetric unit (Nonato & Costa-Filho, 2013; Fig. 2b). Each monomer of *LbDHODH* consists of an α/β -barrel fold with a central barrel composed of eight parallel β -strands ($\beta 1$ – $\beta 8$) surrounded by eight α -helices ($\alpha 1$ – $\alpha 8$) (Fig. 2a). The bottom and top of the barrel are protected by additional conserved secondary-structural elements and loops.

Chains *A* and *B* comprise residues Met1–Ser130 and Gln139–Met312 and bind noncovalently to the prosthetic group FMN (flavin mononucleotide) found at the top of the barrel. Residues Cys131–Pro138 and the C-terminal residue (Asp313) were excluded from the final structure owing to a lack of interpretable electron density. The solvent structure is made up of 271 molecules, including one glycerol and one 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), assigned on the basis of electron density, *B* factors and local coordination. A small number of water molecules were assigned partial occupancies that were either associated with alternative side-chain conformations or alternative positions. In the *LbDHODH* crystal form the ligand-binding pockets are solvent-exposed and are accessible to the inhibitor by channel diffusion, which is important for the characterization of their mechanism of action (Fig. 3).

The superposition of structurally equivalent C^α atoms from chains *A* and *B* shows an r.m.s.d. of 0.187 Å evenly distributed along the chain. The structural flexibility within the asymmetric unit was then evaluated by comparing the *B*-factors for the individual chains. Chain *A* reveals an average value of approximately 45 Å² and chain *B* of approximately 32 Å². Analysis of the *B*-factor distribution for each residue along the protein sequence shows that the largest differences are found in the $\beta 2$ – βc connecting loop (residues Thr47–Arg58) and in the βd – $\alpha 2$ connecting loop (residues Met70–Asp78) (Fig. 2), where chain *A* is disordered relative to chain *B*. This distortion is caused by crystal contacts involving chain *B* that are absent in chain *A*.

The superposition of C^α atoms between the dimeric structure of *LbDHODH* and other TrypDHODH crystal structures shows r.m.s.d. of 0.544 Å for *L. major* DHODH (PDB entry 3gye; Cordeiro *et al.*, 2012), 0.625 Å for *L. donovani* DHODH

Figure 3

Stereoview of *Lb*DHODH crystal packing. Chain *A* is coloured white and chain *B* is coloured grey. Target sites described for TrypDHODHs are found exposed to solvent channels: S1 (pink), S2 (blue), S3 (orange), S4 (green) and S5 (red).

(PDB entry 3c61; Structural Genomics of Pathogenic Protozoa Consortium, unpublished work), 0.439 Å for *T. brucei* DHODH (PDB entry 2b4g; Arakaki *et al.*, 2008) and 0.922 Å for *T. cruzi* DHODH_Y (PDB entry 3c3n; Pinheiro *et al.*, 2008). Changes within the core of the α/β barrel are minimal, and the positions of the active-site residues are essentially the same in all of the structures. The high similarity identified among TrypDHODHs highlights the importance of both conserved steric and chemical interactions for catalytic activity.

Human DHODH is member of the class 2 DHODHs and shares approximately 25% sequence identity with *Lb*DHODH. A structural comparison between *Lb*DHODH and human DHODH (PDB entry 2fpv; Baumgartner *et al.*, 2006) shows an r.m.s.d. of 1.275 Å for the C α atoms. Although their overall α/β fold is well conserved, there are many regions that differ. Firstly, the extra N-terminal region, which is responsible for membrane association and is recognized as the

binding site for inhibitors and electron acceptors (Couto *et al.*, 2008, 2011; Liu *et al.*, 2000; Baumgartner *et al.*, 2006), is only present in human DHODH (Fig. 4). Another major difference is the presence of additional secondary-structure elements (β_e , β_f , β_g and η_1) located at the top of the barrel and involved in the dimerization of *Lb*DHODH, an intrinsic feature of class 1A DHODHs (Nonato & Costa-Filho, 2013). Relevant differences between TrypDHODHs and human DHODH have been previously assigned as sites S1 to S5, and were found to be fully conserved within TrypDHODHs, including *Lb*DHODH (Pinheiro *et al.*, 2013).

Altogether, our results provide evidence that a single compound might display both selectivity and broad-spectrum inhibitory activity against all TrypDHODHs. The heterologous expression and purification together with determination of the X-ray crystallographic structure of *Lb*DHODH presented here allows comparison between target-based inhibition assays and phenotype-based studies of *L. (V.) braziliensis*. Experiments towards this goal are currently in progress.

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Figure 4

Superposition of human DHODH (PDB entry 2fpv) and *Lb*DHODH (PDB entry 4wzh). Major structural differences are found at the extra N-terminal helical domain (blue) of human DHODH, which is recognized as the site for membrane interaction within class 2 DHODHs (arrow 1), and the dimeric interface (arrow 2), which is composed of additional secondary-structure elements (green) localized at the top of the barrel.

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