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A preliminary crystallographic analysis of the putative mevalonate diphosphate decarboxylase from *Trypanosoma brucei*

Mevalonate diphosphate decarboxylase catalyses the last and least well characterized step in the mevalonate pathway for the biosynthesis of isopentenyl pyrophosphate, an isoprenoid precursor. A gene predicted to encode the enzyme from *Trypanosoma brucei* has been cloned, a highly efficient expression system established and a purification protocol determined. The enzyme gives monoclinic crystals in space group $P2_1$, with unit-cell parameters $a = 51.5$, $b = 168.7$, $c = 54.9$ Å, $\beta = 118.8^\circ$. A Matthews coefficient V_M of 2.5 Å³ Da⁻¹ corresponds to two monomers, each approximately 42 kDa (385 residues), in the asymmetric unit with 50% solvent content. These crystals are well ordered and data to high resolution have been recorded using synchrotron radiation.

1. Introduction

Trypanosoma brucei is a unicellular parasitic protozoan with a complex life cycle involving both insects and mammals. The parasites are transmitted into the bloodstream of mammals by the bite of the tsetse fly, causing African sleeping sickness in humans. No vaccines are available, the drugs in current use have severe side-effects and drug resistance is also rapidly increasing. The enzymes of the mevalonate (MVA) pathway of isoprenoid precursor biosynthesis represent potential drug targets in trypanosomes since active sterol biosynthesis has been shown to be essential for growth and survival in *T. cruzi* (Urbina, 1997, 2002). For example, sterol-synthesis inhibitors such as terbinafine (an inhibitor of squalene epoxidase, downstream of the MVA pathway) have been shown to retard growth and lead to parasite death (Buckner *et al.*, 2001) and inhibitors of *T. cruzi* HMG-CoA reductase (a central enzyme of the MVA pathway) have been shown to potentiate the anti-proliferative effects of terbinafine (Urbina *et al.*, 1993). This would suggest that inhibition of the MVA pathway offers hope for future drug development against trypanosomatid parasites. Furthermore, several MVA-pathway enzymes, including MDD, have been shown to be essential in yeast (Oulmouden & Karst, 1990; Tsay & Robinson, 1991; Bergès *et al.*, 1997) which, like trypanosomatids, is dependent on the MVA pathway for isoprenoid precursor biosynthesis.

Isoprenoids, which represent one of the largest families of compounds in nature, contribute to many important and diverse biochemical roles: for example, quinones participate in electron-transport processes, sterols such as cholesterol or ergosterol are components of membrane structures, dolichol is required for glyco-protein synthesis and isoprenyl moieties are involved in membrane anchorage and also signalling processes (Sacchetti & Poulter, 1997). The essential building block for all isoprenoids is the five-carbon isopentenyl diphosphate (IPP). Two pathways lead to IPP biosynthesis, those being either the mevalonate (MVA) pathway or the non-mevalonate (also called the 1-deoxy-D-xylulose-5-phosphate or DOXP) pathway (Eisenreich *et al.*, 1998; Rohmer, 1999). Trypanosomes, as is the case for all eukaryotes, archaeobacteria and a few eubacteria, use the MVA pathway for IPP biosynthesis. This biosynthetic pathway provides new potential drug targets for exploitation in the search for improved therapies against parasitic

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diseases caused by trypanosomatid infection and it is important to study the enzyme components further.

Mevalonate diphosphate decarboxylase (MDD) catalyses the last step in the pathway, using mevalonate diphosphate in an ATP-dependent decarboxylation step to make IPP. MDD is a GHMP kinase superfamily member, so-called because the family originally included galacto-, homoserine, mevalonate and phosphomevalonate kinases. There is interest in this family from a structural perspective since members all share the characteristic α/β -fold of the GHMP kinase family and similar sequence homology (in the range 10–20% identity), yet they catalyze different reactions and utilize very different substrates. Only three ternary complex structures are available from this superfamily, including homoserine kinase (Krishna *et al.*, 2001), galactokinase (Hartley *et al.*, 2004) and 4-diphosphocytidyl-2C-methyl-D-erythritol kinase (Miallau *et al.*, 2003), and thus there is limited detailed structural information as to how individual members achieve substrate specificity. Only a single structure of MDD, that of the *Saccharomyces cerevisiae* enzyme, is available to date and this is in the apo form (Bonanno *et al.*, 2001). Detailed kinetic and mutagenesis studies of this enzyme have suggested a possible mechanism (Dhe-Paganon *et al.*, 1994) and which residues might contribute to substrate binding (Krepkiy & Miziorko, 2004, 2005), but ligand–complex structures remain essential to achieve a detailed understanding of the catalytic mechanism and ligand specificity of MDD necessary to support a programme of structure-based inhibitor design.

Here, we describe the cloning of the putative mevalonate diphosphate decarboxylase gene from *T. brucei*, the construction of a highly efficient protein-expression system and purification of the recombinant enzyme (of approximate molecular weight 42.4 kDa, 385 residues), along with crystallization and preliminary diffraction experiments.

2. Methods and results

2.1. Sample preparation and crystallization

This project was initiated prior to the annotation of the MDD sequence in the online sequencing database (GeneDB) of the Wellcome Trust Sanger Institute Pathogen Sequencing Unit (<http://www.genedb.org>). Similarity matches to all genomic fragments from a preliminary assembly of the *T. brucei* genome were determined by BLAST search (Altschul *et al.*, 1990) against the SWISS-PROT database. Text mining of the annotation for these sequence matches



Figure 1
Monoclinic crystals of the putative *T. brucei* MDD. These samples attain a maximum size of $0.2 \times 0.05 \times 0.05$ mm.

identified a candidate gene sequence for *T. brucei* MDD in contig tryp10.0.000024_21. The predicted gene was amplified from genomic DNA in a PCR reaction using the primers 5'-CATATGTCGGAT-TCCGATCAGTGTTCAGCG-3' and 5'-GGATCCTCATACCCGAG-GTGGAAATG-3', containing restriction-enzyme sites for *NdeI* and *BamHI*, respectively (bold). The resultant PCR product was cloned into the pCR-BluntII-TOPO shuttle vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen). The vector was cut with *NdeI* and *BamHI* to excise the open reading frame and ligated into the pET15b expression vector (Novagen) for expression with an N-terminal hexahistidine tag for ease of purification by metal-ion affinity chromatography. The resulting construct was sequenced to confirm the integrity of the product and then heat-shock transformed into *Escherichia coli* JM109 cells (Stratagene) for plasmid amplification before transformation into BL21 (DE3) cells (Stratagene) for protein expression. Clones containing the desired plasmid were selected on Luria–Bertani agar plates containing $50 \mu\text{g ml}^{-1}$ ampicillin (LB/amp). Bacterial cultures were grown in 1 l LB/amp media at 310 K until a cell density of $\text{OD}_{600} = 0.5\text{--}0.7$ was achieved; protein expression was then induced overnight at 293 K with 0.4 mM isopropyl- β -D-thiogalactopyranoside. Cells were harvested by centrifugation (3000g, 20 min, 277 K), resuspended in 20 mM Tris–HCl pH 7.5, 500 mM NaCl, 5 mM imidazole and 5 mM benzamidine and then incubated on ice for 30 min with lysozyme and DNaseI. Cells were broken by one pass through a French press and the insoluble cell debris removed by centrifugation (31 000g, 30 min, 277 K). The supernatant was filtered and passed over a 5 ml metal-chelating column (Hi-Trap; Amersham Biosciences), which had previously been charged with Ni^{2+} (100 mM nickel chloride) and washed with 25 mM HEPES pH 6.8 containing 50 mM NaCl and 5 mM imidazole. The protein was eluted by running a gradient from 5 mM to 1 M imidazole in 25 mM HEPES pH 6.8 containing 50 mM NaCl (buffer A); fractions containing MDD were then pooled and dialyzed against buffer A. The hexahistidine tag was removed from the protein following incubation at room temperature for 20 h with 10 units of thrombin (Amersham Biosciences) per milligram of protein. The protein solution was filtered and passed through a second metal-chelating column, equilibrated as before and washed with buffer A to remove any uncut protein and tag fragments. After further dialysis against buffer A, the protein was loaded onto a 5 ml cation-exchange column (SP Sepharose HP; Amersham Biosciences) previously equilibrated with buffer A for ion exchange. A salt gradient from 50 mM to 1 M NaCl in 25 mM HEPES pH 6.8 was applied and MDD passed straight through while contaminants remained on the column. The purity of the MDD sample was assessed by SDS–PAGE and matrix-assisted laser desorption time-of-flight (MALDI–TOF) mass spectrometry. The theoretical weight of the protein is approximately 42.4 kDa and that determined by mass spectrometry was within 50 Da. The protein was extensively dialyzed in buffer A, concentrated to 25 mg ml^{-1} and then stored at 277 K. The typical yield of purified protein using this protocol was over 60 mg per litre of bacterial cell culture.

2.2. Crystallization

Crystallization trials were initiated using commercially available screens. MDD at 15 mg ml^{-1} was incubated for at least 2 h on ice with 2 mM adenosine 5'-(β - γ -imido)triphosphate (AMPPNP) and 2 mM MgCl_2 . Sitting drops made up of 1 μl protein and 1 μl reservoir were set up for vapour diffusion against 70 μl reservoir solution at 293 K using 96-well Greiner CrystalQuick plates. An initial hit was obtained in 30% PEG 8000, 200 mM ammonium sulfate and 100 mM sodium

acetate pH 6.5 (Crystal Screen 1; Hampton Research), which produced small needles overnight. These conditions were optimized in hanging-drop vapour-diffusion experiments by changing the protein solution:reservoir ratio and protein concentration to 3 μl protein at 6 mg ml^{-1} plus 2 μl reservoir, with the reservoir ultimately consisting of 22% PEG 8000, 180 mM ammonium sulfate and 100 mM sodium cacodylate pH 6.0 (Fig. 1). These crystals diffracted to medium resolution (~ 2.3 Å) when used on an in-house X-ray source (Fig. 2).

2.3. X-ray diffraction

A suitable cryoprotectant was found by screening glycerol, ethylene glycol and 2-methyl-2,4-pentanediol. Finally, MDD crystals were soaked in 15% glycerol/mother liquor for about 10 s, without any signs of mechanical damage, before being flash-cooled to 103 K in a stream of nitrogen gas. Crystals were tested and stored in liquid nitrogen and then transported to the European Synchrotron Radiation Facility (ESRF) in Grenoble, France for high-resolution data collection.

Data were measured on beamline ID14-EH4 and autoindexed and processed with *MOSFLM* (Leslie, 1992), *SCALA* (Evans, 1997) and the *CCP4* suite of programs (Collaborative Computational Project, Number 4, 1994). The crystals were monoclinic, with unit-cell parameters $a = 51.5$, $b = 168.7$, $c = 54.9$ Å, $\beta = 118.8^\circ$. Collection of 300° of data gave a highly redundant data set of good completeness with an effective resolution near 2.0 Å (Table 1). Examination of systematic absences indicates space group $P2_1$. On the basis of gel-filtration and analytical centrifugation experiments, the *T. brucei* MDD appears predominantly as a monomer, which has a molecular weight of approximately 42.4 kDa and consists of 385 amino acids, with traces of a dimer present in solution (unpublished work). A Matthews coefficient V_M (Matthews, 1968) of $2.5 \text{ Å}^3 \text{ Da}^{-1}$ correlates to two

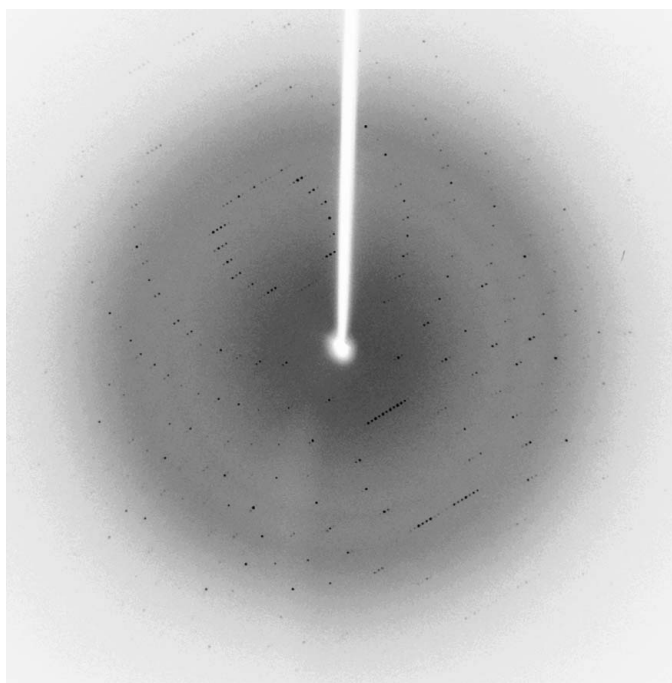


Figure 2
An example of the diffraction obtained in-house using a Rigaku Micromax 007 rotating anode (Cu $K\alpha$, $\lambda = 1.5418$ Å) and R-AXIS IV⁺⁺ image-plate detector. The crystal-to-detector distance is 200 mm, the oscillation angle is 0.5° and the exposure time is 15 min, with X-rays produced at settings of 40 kV and 18 mA.

Table 1

Summary of crystallographic data.

Values in parentheses refer to the highest resolution bin 1.97–1.8 Å.

Resolution range (Å)	25–1.8
Wavelength (Å)	0.939
Unit-cell parameters	
a (Å)	51.5
b (Å)	168.7
c (Å)	54.9
β ($^\circ$)	118.8
Space group	$P2_1$
No. of measurements	454564
No. of unique reflections	73954
Redundancy	6.1
Completeness (%)	99.0
$\langle I/\sigma(I) \rangle$	4.3 (1.2)
R_{sym} (%)	10.6 (65.8)
Wilson B (Å ²)	24

monomers in the asymmetric unit with 50% solvent content. A self-rotation function was calculated using *MOLREP* (Vagin & Teplyakov, 1997; not shown) and confirmed the presence of a non-crystallographic twofold axis of symmetry ($\psi = 66.3$, $\phi = 28.4$, $\kappa = 180.0^\circ$) with a peak height of approximately 25% of that observed for the crystallographic peak.

These crystals are suitable for high-resolution crystallographic analyses and, since there is an appropriate model available (yeast MDD; Bonanno *et al.*, 2001), work is under way to solve the structure by the application of molecular-replacement methods. Structure determination will then facilitate the detailed study of the structure and reactivity of MDD in trypanosomatid protozoans.

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