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Purification, crystallization and preliminary X-ray studies of MbtN (Rv1346) from *Mycobacterium tuberculosis*

In *Mycobacterium tuberculosis*, the protein MbtN (Rv1346) catalyzes the formation of a double bond in the fatty-acyl moiety of the siderophore mycobactin, which is used by this organism to acquire essential iron. MbtN is homologous to acyl-CoA dehydrogenases, whose general role is to catalyze the α,β -dehydrogenation of fatty-acyl-CoA conjugates. Mycobactins, however, contain a long unsaturated fatty-acid chain with an unusual *cis* double bond conjugated to the carbonyl group of the mycobactin core. To characterize the role of MbtN in the dehydrogenation of this fatty-acyl moiety, the enzyme has been expressed, purified and crystallized. The crystals diffracted to 2.3 Å resolution at a synchrotron source and were found to belong to the hexagonal space group $H3_2$, with unit-cell parameters $a = b = 139.10$, $c = 253.09$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$.

1. Introduction

Iron is an indispensable nutrient for all organisms. However, in most microbial habitats iron is not freely available (Ratledge, 2004). *Mycobacterium tuberculosis* primarily resides in host macrophages, which are low in soluble iron (Rodriguez, 2006; Barry & Boshoff, 2005). *M. tuberculosis* counters this problem by producing specific chelators (siderophores) called mycobactins. Mycobactins can mobilize iron from the intracellular iron pool and also from endocytosed transferrin (De Voss *et al.*, 2000; Miethke & Marahiel, 2007; Ratledge, 2004).

Most of the observed structural variation in different mycobactins is found in the acyl moiety. Generally, this is a long unsaturated fatty-acid chain (C_{14} – C_{21}) with an unusual *cis* double bond which is conjugated to a carbonyl group of the mycobactin core (Snow, 1970; Ratledge & Dover, 2000). MbtN, encoded by Rv1346, is a putative acyl-ACP dehydrogenase that introduces the double bond into the fatty-acyl moiety (Cole & Barrell, 1998; Krithika *et al.*, 2006; Quadri *et al.*, 1998). MbtN belongs to a widespread family of acyl-CoA dehydrogenases (ACADs) which catalyze the α,β -dehydrogenation of fatty-acyl-CoA conjugates (Ghisla & Thorpe, 2004; Ikeda *et al.*, 1985).

MbtN differs from most ACADs in two ways. Firstly, the *in vivo* fatty-acyl carrier for MbtN is believed to be an acyl carrier protein (ACP) rather than a CoA (Krithika *et al.*, 2006). Secondly, ACADs generally catalyze the formation of *trans* isomers, whereas mycobactin contains a *cis* double bond (Krithika *et al.*, 2006). Determination of the crystal structure of MbtN would allow elucidation of the details of its active site and provide insights into its function. Here, we describe the key steps towards the structural characterization of MbtN, including its expression, purification, crystallization and preliminary X-ray diffraction analysis.

2. Materials and methods

2.1. Cloning, expression and purification

The gene coding for MbtN was amplified from *M. tuberculosis* H37Rv genomic DNA using the primers 5'-GTTTTCCCATGGG-AACGGCCGGCTCCGACCTC-3' (*Nco*I site in bold) and 5'-GTTTTCGAATTCCTACGCTTGGAAAGCTCCGACCACG-3' (*Eco*RI

site in bold). The polymerase chain reaction product was digested with the *Nco*I and *Eco*RI enzymes and was ligated into previously digested pProExHtb vector (Invitrogen). The N-terminal methionine of MbtN was not included in the construct. The vector contained a *Tobacco etch virus* (TEV) protease cleavage site in front of the *Nco*I cloning site, and TEV protease cleavage would leave four residues, Gly-Ala-Met-Gly, in front of residue 2 of MbtN, giving a construct consisting of 389 residues with a molecular weight of 41 365.3 Da. MbtN was overexpressed in *Escherichia coli* strain BL21 (DE3) as an N-terminally His₆-tagged fusion protein. An overnight culture of pProExHtb-MbtN-containing *E. coli* cells was used to inoculate 2× YT medium containing ampicillin (100 µg ml⁻¹). Cultures were grown with shaking at 310 K to an OD₆₀₀ of 0.6–0.8, at which point the temperature was reduced to 291 K. Expression was then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

After overnight growth, the cells were harvested by centrifugation and were resuspended in pre-cooled lysis buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 2 mM β-mercaptoethanol) supplemented with DNase, RNase, lysozyme and two cOmplete Protease Inhibitor Cocktail Mini Tablets (EDTA-free). The cells were lysed by cell disruption (Constant Cell Disruption Systems) and the lysate was clarified by centrifugation. The supernatant was purified on a pre-equilibrated HiTrap immobilized metal-affinity column (IMAC; GE Healthcare) charged with Ni²⁺. The bound protein was washed with ten column volumes of wash buffer (lysis buffer containing 10 mM imidazole) and was eluted with the same buffer containing 200 mM imidazole. The eluted protein was dialysed into 2 l lysis buffer and was cleaved with 4 µM recombinant TEV (rTEV) protease at 277 K overnight. MbtN was separated from His-tagged rTEV by passage over the same IMAC column and washing with wash buffer. Soluble cleaved MbtN was further purified by size-exclusion chromatography (SEC) using a Superdex 200 16/60 column (Amersham Biosciences) pre-equilibrated with running buffer consisting of 20 mM Tris–HCl pH 8.0, 300 mM NaCl, 2 mM β-mercaptoethanol. The protein was concentrated to ~20 mg ml⁻¹, flash-frozen in liquid nitrogen and stored at 193 K.

2.2. Crystallization

Initial crystallization screening was carried out at 291 K as a 480-condition sitting-drop vapour-diffusion experiment in 96-well Intelli-Plates (Hampton Research) using a Cartesian Honeybee nanolitre

Figure 1
Crystals of MbtN shown under polarized light.

dispensing robot (Genomic Solutions; Moreland *et al.*, 2005). The screen included PEG/Ion and the sparse-matrix Crystal Screen and Crystal Screen 2 (Hampton Research), PEG/pH and ammonium sulfate screens, the Stura Footprint screen, Grid Screen MPD (Hampton Research) and the Precipitant Synergy and Clear Strategy screens (Moreland *et al.*, 2005). Only one hit was obtained. Subsequent optimizations were carried out by hanging-drop vapour diffusion in 24-well Linbro plates by mixing 1 µl protein solution (10 mg ml⁻¹ in 20 mM Tris–HCl pH 8.0, 300 mM NaCl, 2 mM β-mercaptoethanol) with 1 µl crystallization buffer consisting of 23%(w/v) polyethylene glycol (PEG) 3350, 0.2 M diammonium hydrogen citrate pH 5.5 and equilibrating against 1 ml reservoir solution. Colourless rod-shaped crystals grew to dimensions of 10–20 µm within 2 d.

2.3. Data collection

For data collection, the crystals were transferred to cryoprotectant [crystallization solution supplemented with 25%(v/v) glycerol] and flash-cooled to 110 K. The best crystal diffracted to 2.3 Å resolution and the data were collected on beamline MX2 at the Australian Synchrotron ($\lambda = 0.95369$) using an ADSC Quantum 315r CCD detector. The crystal-to-detector distance was set to 300 mm, the oscillation range was 1.0° and 180 images were collected. Owing to radiation damage, only 100 images were processed. Autoprocessing with XDS (Kabsch, 2010) proposed C2 as the space group. However, analysis of space-group possibilities using POINTLESS (Evans, 2006) identified a different space group, *H*₃₂. The re-indexed data were scaled with SCALA (Evans, 2006) from the CCP4 suite (Winn *et al.*, 2011).

3. Results and discussion

MbtN was expressed in *E. coli* and was purified by IMAC and SEC, eluting as a single peak that was 99% pure based on SDS–PAGE analysis. The expressed protein was found to have FAD bound when

Figure 2
A typical diffraction image obtained from the MbtN crystals.

Table 1

Data-collection statistics for MbtN.

Values in parentheses are for the outermost resolution shell.

Space group	$H3_2$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = b = 139.10$, $c = 253.09$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution range ($\text{\AA}$)	87.25–2.30 (2.42–2.30)
Total reflections	259702 (36262)
Unique reflections	42053 (5886)
Completeness (%)	99.4 (96.5)
Multiplicity	6.2 (6.2)
R_{merge} (%) [†]	11.8 (82.1)
$R_{\text{p.i.m.}}$ (%)	5.1 (35.5)
R_{meas} (%)	12.9 (89.5)
$\langle I/\sigma(I) \rangle$	14.2 (2.8)
$CC_{1/2}$	0.997 (0.779)

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity for multiple measurements.

purified, as indicated by the yellow colour of the solution and the presence of maxima in the UV–visible absorption spectrum at 360 and 450 nm characteristic of oxidized FAD. Crystals of MbtN could be grown by vapour diffusion in hanging-drop format within 2 d. Interestingly, these crystals were colourless, in contrast to the yellow solution, suggesting that either the FAD was reduced during crystallization or it had dissociated from the protein. Since the FAD had remained bound throughout the expression and purification of the protein, we assume it is most likely to be reduced in the crystals. The MbtN crystals (Fig. 1) diffracted to 2.3 $\text{\AA}$ resolution on the MX2 beamline at the Australian Synchrotron (Fig. 2). Data-collection statistics are shown in Table 1. The crystals belonged to the hexagonal crystal system, with unit-cell parameters $a = b = 139.10$, $c = 253.09$ $\text{\AA}$, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The space group was re-indexed from $C2$ to $H3_2$ using *POINTLESS* (Evans, 2006). The Matthews coefficient V_M (Matthews, 1968) of 2.89 $\text{\AA}^3 \text{Da}^{-1}$ suggests the presence of two molecules per asymmetric unit, with a solvent content of 57%.

These crystals had excellent reproducibility and are being used for structure determination by molecular replacement using the *Thermus thermophilus* acyl-CoA dehydrogenase monomer (PDB entry 1ws9; RIKEN Structural Genomics/Proteomics Initiative, unpublished work) as a model. Elucidation of the crystal structure of MbtN will provide structural insights into the function of MbtN.

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