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## Cloning, expression, purification, crystallization and preliminary X-ray studies of epoxide hydrolases A and B from *Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* epoxide hydrolases A and B, corresponding to open reading frames Rv3617 and Rv1938, are detoxification enzymes against epoxides. The recombinant forms of these enzymes have been expressed in *Escherichia coli* and purified to homogeneity. Diffraction-quality crystals of Rv3617 and Rv1938 were obtained by the hanging-drop vapour-diffusion technique. Crystals of Rv3617 and Rv1938 diffracted to 3.0 and 2.1 Å resolution, respectively, at the ALS synchrotron at Berkeley, CA, USA.

### 1. Introduction

*Mycobacterium tuberculosis* (*Mtb*) is the causative agent of the chronic infectious disease tuberculosis (TB). Currently, 2.2 billion people are infected with *Mtb* worldwide, leading to around two million deaths annually (World Health Organization, 2003). The lack of effective treatment and the appearance of multidrug resistance has led to further studies designed to explore the various factors responsible for the survival of this organism. The determination of the complete sequence of the *Mtb* genome in 1998 has facilitated the understanding of the pathogen in greater detail (Cole *et al.*, 1998). Subsequently, the *Mtb* structural genomics consortium was formed in 2000 (<http://www.doe-mpi.ucla.edu/TB>); the consortium aims to provide a structural basis for the development of therapeutics for tuberculosis (Goulding *et al.*, 2002, 2003; Rupp *et al.*, 2002; Rupp, 2003; Smith & Sacchettini, 2003; Terwilliger *et al.*, 2003).

Recent studies demonstrate that reactive epoxides are responsible for an electrophilic reaction targeting DNA and proteins (Zheng *et al.*, 1997; Szeliga & Dipple, 1998). Although the precise role of epoxide hydrolases (EHs) differs in different organisms, overall these molecules are involved in detoxification, catabolism and regulation of signalling molecules. Sequencing of the complete genome of *Mtb* has revealed the genes for six probable EH proteins (Rv0134, Rv1124, Rv1938, Rv2214c, Rv3617 and Rv3670) that are likely to be involved in detoxification of extraneous host-cell epoxides (Cole *et al.*, 1998).

As a member of the *Mtb* structural genomics consortium, we have targeted ~300 proteins for determination of their X-ray crystal structures in an effort to discover new inhibitors against potential targets responsible for the survival of *Mtb*. In this paper, we report the cloning, expression, purification, crystallization and preliminary X-ray studies of two detoxification enzymes, EH A and EH B. In addition to the two reported here, the other four enzymes are in various stages of cloning, expression, purification or crystallization trials, but thus far no crystals have been obtained for them.

### 2. Experimental methods

#### 2.1. Cloning, expression and purification

H37Rv genomic DNA in a BAC ordered library from L'Institut Pasteur (Brosch *et al.*, 1998; Gordon *et al.*, 1999) was used as the template to amplify Rv1938 and Rv3617 by PCR with primers sense, **GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGAAAACC-TGTATTTTCAGGGCGTGTTCGACAGGTCCATCG**, and antisense, **GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACGGCCG-CAGCCCCGCTAG**, and primers sense, **GGGGACAAGTTTGTAC-**



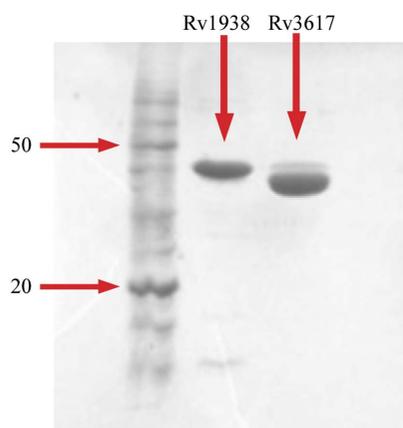
**CAAAAAGCAGGCTCAGAAAACCTGTATTTTCAGGGCAT-**  
GGGCGCACCTACCGAACGG and antisense, **GGGGACCACT-**  
**TTGTACAAGAAAGCTGGGTCTCATCGCAACTCCAACCCCG-**  
TCAGG, respectively.

The amplified open reading frames (ORF) were gel purified (Qiagen) and then inserted directionally into a cloning vector by the homologous recombination sites (bold) using a Gateway Cloning System (Invitrogen). An rTEV protease site (Invitrogen) is encoded by the sense primers (italicised) to cleave the N-terminal affinity tag. Rv1938 and Rv3617 were then transferred by recombination into an expression vector encoding an amino-terminal hexa-His-tag fusion protein. The resulting expression plasmids were confirmed by DNA-sequence analysis (DNA Sequence facility, Department of Biochemistry, University of Alberta, Canada).

Rv1938 and Rv3617 were expressed in *Escherichia coli* BL21 (DE3) pLysS cells (Novagen) in a very similar manner. A 16 h culture of the bacteria transformed with the expression vector in LB supplemented with 100  $\mu\text{g ml}^{-1}$  ampicillin and 34  $\mu\text{g ml}^{-1}$  chloramphenicol (LBAC) was diluted 1:40 into 2 l LBAC. Incubation at 310 K proceeded for 2 h, at which point the  $\text{OD}_{600\text{nm}}$  was  $\sim 0.9$ . The temperature was shifted to 298 K and expression was induced by adding isopropyl thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 16 h incubation, the cells were harvested by centrifugation at 3500g for 20 min. Bacterial pellets were resuspended in PBS, EDTA-free Complete protease inhibitor (Roche) and 1 mM DTT (Fischer Scientific). Resuspended cells were frozen at 193 K. Thawed cells were subjected to sonication on ice. The lysate was cleared by centrifugation (30 min, 20 000g) and the supernatant was loaded onto an Ni-charged immobilized metal-affinity chromatography column (GE Healthcare). 16% SDS-PAGE analysis of the concentrated protein revealed single bands of  $\sim 40$  and  $\sim 35$  kDa consistent with the predicted molecular weights of Rv1938 and Rv3617, respectively (Fig. 1).

## 2.2. Crystallization

The initial crystallization conditions for Rv1938 and Rv3617 were found with the help of the Hydra I robot (Robbins Scientific Corporation), which utilizes the sitting-drop vapour-diffusion method in 96-well plates (Intelli-plates, Hampton Research). Protein samples in 25 mM Tris-HCl buffer pH 8.0 were concentrated to 5–10  $\text{mg ml}^{-1}$ . A medium-throughput crystal screen (Hampton Research) was performed with each protein sample using equal volumes of 0.5  $\mu\text{l}$



**Figure 1**  
16% SDS-PAGE showing the purity of Rv1938 and Rv3617. Lanes 1, 2 and 3 correspond to molecular-weight markers (kDa), Rv1938 and Rv3617, respectively.

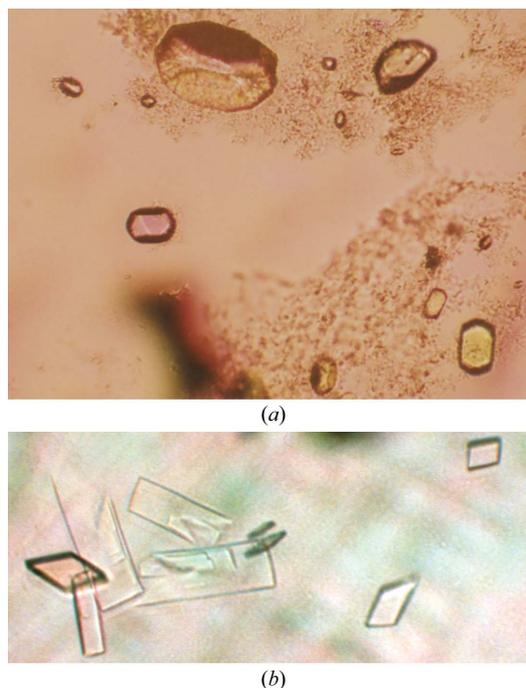
His<sub>6</sub>-tagged protein and precipitating solutions. From this screen, initial hits were found and then used for optimization of the crystallization conditions.

**2.2.1. Rv1938.** Large disordered crystals were obtained in condition B12 (20% 2-propanol, 0.2 M CaCl<sub>2</sub>, 0.1 M sodium acetate buffer pH 4.6). After optimization, X-ray diffraction-quality crystals (Fig. 2a) were grown in hanging drops (in VDX plates from Hampton Research) containing equal volumes (1  $\mu\text{l}$ ) of the protein and reservoir solutions and equilibrated against 1 ml reservoir solution (20% 2-propanol, 0.2 M MgCl<sub>2</sub>, 0.1 M sodium acetate buffer pH 4.5). For data collection, smaller well shaped crystals (50–70  $\mu\text{m}$ ) were gradually transferred to a cryoprotectant solution containing 70% of the reservoir solution and 30% glycerol and flash-cooled to 100 K.

**2.2.2. Rv3617.** Small crystals grew in several conditions containing PEG and salts. Larger crystals were grown in hanging drops from 25% PEG 4000, 0.1 M MES pH 5.5, 0.2 M Li<sub>2</sub>SO<sub>4</sub>. Equal volumes (1–2  $\mu\text{l}$ ) of protein and reservoir solutions were mixed on a plastic cover slip and equilibrated against 1 ml reservoir solution. Crystals grew to maximum dimensions of 0.2  $\times$  0.05  $\times$  0.02 mm within a few days (Fig. 2b). For cryosolvent, mother liquor was diluted with glycerol to a final concentration of 30%. Crystals were then flash-cooled to 100 K.

## 3. Results

Crystals of Rv1938 and Rv3617 diffracted to 2.8 and 4.0 Å, respectively, at the home source, an R-AXIS IV<sup>++</sup> image-plate detector with Cu K $\alpha$  radiation generated by a Rigaku RU-300 rotating-anode X-ray generator. Crystals of Rv1938 and Rv3617 belong to the tetragonal and monoclinic space groups, respectively (Table 1). Rv1938 and Rv3617 crystals have solvent contents of 42.81 and 42.84%, respectively (Matthews, 1968). Higher resolution X-ray data from the native crystals of Rv1938 and Rv3617 were collected at beamline 8.3.1 at the Advanced Light Source, Berkeley, CA, USA. Data were



**Figure 2**  
Crystals of (a) Rv1938 and (b) Rv3617.

**Table 1**

Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

Crystal	Native Rv1938	Native Rv3617
Space group	$P4_12_12$ ( $P4_32_12$ )	C2
Unit-cell parameters		
$a$ (Å)	66.26	142.88
$b$ (Å)	66.26	97.84
$c$ (Å)	157.11	45.14
$\alpha$ (°)	90	90
$\beta$ (°)	90	101.52
$\gamma$ (°)	90	90
$Z$	8	8
Matthews coefficient (Å <sup>3</sup> Da <sup>-1</sup> )	2.15	2.15
Solvent content (%)	42.81	42.84
Data collection		
Temperature (K)	100	100
Detector	ADSCQ210	ADSCQ210
Wavelength (Å)	1.115869	1.115879
Resolution (Å)	40–2.1 (2.18–2.10)	40–3.0 (3.11–3.0)
Unique reflections	20745	11402
Multiplicity	6.4	2.0
$\langle I/\sigma(I) \rangle$	23.4 (2.1)	8.8 (2.0)
Completeness (%)	97.6 (80.4)	92.7 (85.4)
$R_{\text{sym}}^\dagger$ (%)	7.0 (49.2)	8.3 (30.6)

$^\dagger R_{\text{sym}}(I) = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$  for  $n$  independent reflections and  $i$  observations of a given reflection.  $\langle I(hkl) \rangle$  is the average intensity of the  $i$  observations.

integrated and scaled using the programs *DENZO* and *SCALE-PAK* (Otwinowski & Minor, 1997). Details of the data-collection statistics are given in Table 1. Efforts to solve the structures are being made and the detailed structures of Rv1938 and Rv3617 will be reported elsewhere.

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