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Cloning, expression, purification, crystallization and X-ray crystallographic analysis of Rv3168 from *Mycobacterium tuberculosis* H37Rv

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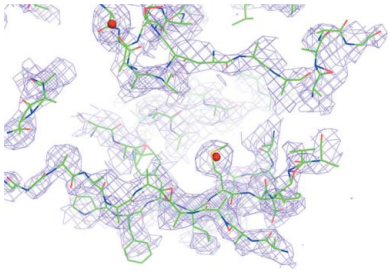
Tuberculosis is a widespread and deadly infectious disease, with one third of the human population already being infected. Aminoglycoside antibiotics have become less effective in recent years owing to antibiotic resistance, which arises primarily through enzymatic modification of the antibiotics. The gene product Rv3168, a putative aminoglycoside phosphotransferase (APH), from *Mycobacterium tuberculosis* was crystallized using the sitting-drop vapour-diffusion method in the presence of 0.2 *M* calcium acetate, 0.1 *M* Tris–HCl pH 7.0 and 20% PEG 3000 at 295 K. X-ray diffraction data were collected to a maximum resolution of 1.67 Å on a synchrotron beamline. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 56.74$, $b = 62.37$, $c = 103.61$ Å. With one molecule per asymmetric unit, the crystal volume per unit protein weight (V_M) is 2.91 Å^3 Da^{-1}. The structure was solved by the single-wavelength anomalous dispersion method and refinement of the selenomethionine structure is in progress.

1. Introduction

Aminoglycosides are a family of broad-spectrum antibiotics that are expressed by soil-derived bacteria (Greenwood, 1995). Streptomycin, kanamycin and amikacin are among the aminoglycoside antibiotics that have been used to combat tuberculosis. The target of these compounds is the bacterial 30S ribosomal subunit's 16S rRNA, where they promote miscoding (Kotra *et al.*, 2000; Wirmer & Westhof, 2006; Karimi & Ehrenberg, 1994). Tuberculosis is a widespread and deadly infectious disease, with one third of the human population already being infected (Dye *et al.*, 1999). A new infection occurs every 4 s and one person dies of tuberculosis every 15 s. With about two billion carriers of *Mycobacterium tuberculosis* worldwide and the emergence of multi-drug-resistant strains (Coker, 2004), there is an urgent need to develop more effective drugs.

Many studies have been performed in order to better understand the antibiotic-resistance mechanisms in *M. tuberculosis* (Johnson *et al.*, 2006). Nowadays, resistance to aminoglycosides is widespread and operates primarily through deactivation of the antibiotics through enzymatic modification. Three families of enzymes have been found to be responsible: ATP-dependent phosphotransferases (APHs), ATP-dependent adenylyltransferases (ANTs) and acetyl CoA-dependent acetyltransferases (AACs).

The complete genome sequence of *M. tuberculosis* strain H37Rv has revealed several mycobacterial protein sequences that have homology to aminoglycoside phosphotransferase enzymes (Philipp *et al.*, 1996; Wright, 1999). The structures of putative aminoglycoside phosphotransferases from *M. tuberculosis* have not been investigated to date. As a step towards elucidating the function and activity of these enzymes, we cloned the *Rv3168* gene and purified the gene product Rv3168, which is one of the potential aminoglycoside phosphotransferase enzymes in *M. tuberculosis* H37Rv. Crystals of the Rv3168 protein were obtained by the sitting-drop method. The crystal diffracted well and data were collected to a resolution of 1.67 Å; the structure was determined by SAD using an SeMet crystal. Here, we describe the cloning, expression, purification, crystallization and X-ray crystallographic analysis of the Rv3168 protein.



2. Expression and purification of the recombinant Rv3168 protein

The *Rv3168* gene was amplified from the chromosomal DNA of *M. tuberculosis* strain H37Rv by polymerase chain reaction (PCR). The PCR product was then subcloned into $pP_{RO}EX$ HTa (Invitrogen) with His_6 at the N-terminus and a recombinant TEV protease (rTEV) cleavage site. The resulting expression vector $pP_{RO}EX$ HTa:*Rv3168* was transformed into *Escherichia coli* strain B834 and the cells were grown in LB medium containing ampicillin at 310 K. After induction with 1.0 m*M* IPTG for a further 20 h at 295 K, the culture was harvested by centrifugation at 5000*g* at 277 K. The cell pellet was resuspended in ice-cold buffer *A* (50 m*M* Tris–HCl pH 8.0, 5 m*M* β-mercaptoethanol) and disrupted by ultrasonication. The cell debris was removed by centrifugation at 11 000*g* for 1 h and the lysate was bound to Ni–NTA agarose (Qiagen). After washing with buffer *A* containing 10 m*M* imidazole, the bound proteins were eluted with 300 m*M* imidazole in buffer *A*. The 6×His tag was released from the Rv3168 by incubation with rTEV protease (Gibco). A trace amount of contamination was removed using HiTrap Q ion-exchange and Superdex 75 size-exclusion chromatography. The purified protein with a three-residue cloning artifact (Gly-His-Met) at the N-terminus showed ∼95% purity on SDS–PAGE (Fig. 1). It was concentrated to 38 mg ml^{-1} in 50 m*M* Tris–HCl pH 8.0 and stored at 193 K for crystallization trials. The SeMet-substituted protein was expressed in a minimal medium supplemented with SeMet and purified under the same conditions as the native.

3. Crystallization

Crystallization of the purified protein was initially performed with commercially available sparse-matrix screens from Hampton Research and Emerald BioSystems using the hanging-drop vapour-diffusion method at 295 K. Each experiment consisted of mixing 2 µl protein solution (38 mg ml^{-1} in 20 m*M* Tris–HCl pH 7.0 and 5 m*M* β-mercaptoethanol) with 2 µl reservoir solution and then equilibrating the drop against 0.5 ml reservoir solution. Rv3168 crystals were observed in several crystallization screening conditions. After several steps that improved the crystallization process using the sitting-drop vapour-diffusion method, crystals with the best quality appeared in 5 d and reached maximum dimensions of approximately 0.2 × 0.2 × 0.8 mm using a reservoir solution consisting of 0.2 *M* calcium acetate, 0.1 *M* Tris–HCl pH 7.0 and 20% PEG 3000. The volumes of the sitting drop and reservoir solution were same as those used in the hanging-drop vapour-diffusion method for the initial crystallization screening. SeMet crystals of Rv3168 were obtained using the same crystallization conditions as used for the native protein crystals.

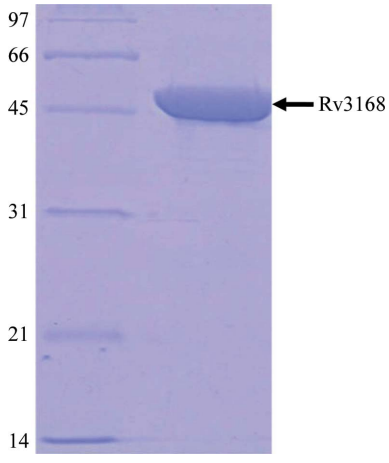


Figure 1
SDS–PAGE of purified Rv3168 protein from *M. tuberculosis*. Molecular-weight markers are labelled on the left of the gel (in kDa) and purified Rv3168 protein with a molecular weight of 42.6 kDa is indicated on the right of the gel.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	Native	Se peak
Beamline	6C1 (MXII), PAL	6C1 (MXII), PAL
Wavelength (Å)	1.23985	0.97950
Temperature (K)	100	100
Oscillation (°)	1.0	1.0
Total rotation range (°)	360	162
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 56.74, b = 62.37, c = 103.61	a = 56.45, b = 62.25, c = 103.43
Resolution limits (Å)	50.00–1.67 (1.73–1.67)	50.00–2.06 (2.13–2.06)
Total reflections	456640	116766
Unique reflections	46378	23072
Completeness (%)	95.5 (90.6)	98.8 (97.4)
R_{merge}† (%)	5.8 (30.5)	7.2 (16.6)
$\langle I/\sigma(I)\rangle$	35.78 (3.09)	32.55 (7.74)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the observed individual and mean intensities of a reflection, respectively. $\sum_i$ is the sum over the individual measurements of a reflection and $\sum_{hkl}$ is the sum over all reflections.

4. X-ray analysis

The crystals were transferred to a cryoprotectant solution consisting of 0.2 *M* calcium acetate, 0.1 *M* Tris–HCl pH 7.0, 20% PEG 3000 and 30% glycerol, fished out with a loop larger than the crystals and flash-frozen by immersion in liquid nitrogen at 100 K. Data were collected to a resolution of 1.67 Å on the 6C1 beamline (MXII) at Pohang Accelerator Laboratory (PAL; Pohang, Republic of Korea) using a Quantum 210 CCD detector (ADSC, USA). The data were then indexed, integrated and scaled using the *HKL*-2000 suite (Otwinowski & Minor, 1997). The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 56.74, b = 62.37, c = 103.61 Å. Assuming one molecule of Rv3168 per asymmetric unit, the crystal volume per

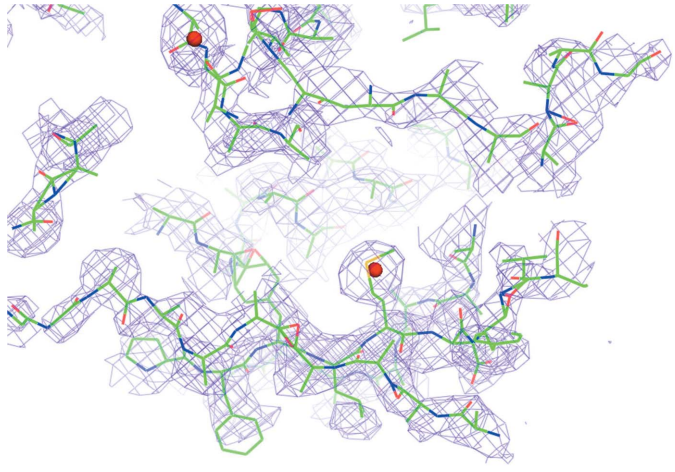


Figure 2
The electron-density map ($2F_o - F_c$) and model of Rv3168 after *RESOLVE*. The electron-density map and model were prepared using *PyMOL*. Se atoms found by *SOLVE* are presented as red spheres.

unit of protein mass was 2.91 Å^3 Da^{-1} (Matthews, 1968), corresponding to a solvent content of approximately 57.8%.

SAD data were collected from an SeMet crystal on the 6C1 beamline (MXII) at PAL using a wavelength of 0.97953 Å. Nine of the ten Se atoms in the asymmetric unit were identified using the program *SOLVE* (Terwilliger & Berendzen, 1999) at 2.2 Å resolution. The electron density was improved by density modification using *RESOLVE* (Terwilliger, 2000), resulting in 80% of the residues being built automatically (Fig. 2). The data statistics are summarized in Table 1. After crystallographic model building and refinement of the seleomethionine structure, we performed molecular replacement using the selenomethionine-substituted structure as a search model for refinement of the selenomethionine-free structure. Refinement of the selenomethionine-free structure to 1.67 Å resolution is in progress. After completing the structure refinement, we will deposit the refined structure in the PDB.

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