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Liu Chen,[a,b] Demeng Sun,[b] Minhao Wu,[b] Jianye Zang[b]* and Changlin Tian[a,b]*

[a]National Laboratory for Physical Science at Microscale, University of Science and Technology of China, Hefei, Anhui 230026, People's Republic of China, and [b]School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230026, People's Republic of China

Correspondence e-mail: zangjy@ustc.edu.cn, cltian@ustc.edu.cn

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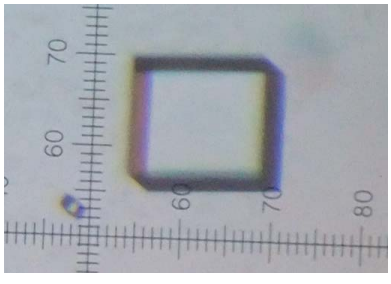

Cloning, expression, purification, crystallization and preliminary crystallographic analysis of Rv1698, an outer membrane channel protein from *Mycobacterium tuberculosis*

Rv1698 has been reported to be an important outer membrane channel protein of *Mycobacterium tuberculosis* with unknown function. Recombinant Rv1698 overexpressed in *Escherichia coli* was purified in detergent solution and crystallized at 295 K using the sitting-drop vapour-diffusion method with ammonium sulfate as a precipitant. The crystals of Rv1698 diffracted to 2.5 Å resolution and belonged to the orthorhombic space group *P*422, with unit-cell parameters $a = b = 122.0$, $c = 88.9$ Å.

1. Introduction

Mycobacterium tuberculosis is a fatal infectious pathogen which causes about two million deaths per year worldwide (Floyd & Glaziou, 2009). The outer membrane of *M. tuberculosis*, the thickest biological membrane presently known, is composed of mycolic acids and a large variety of other lipids and functions as a barrier with an exceptionally low permeability. The outer membrane is an important virulence factor of *M. tuberculosis* that plays a crucial role in its intrinsic drug resistance and survival under harsh conditions (Brennan & Nikaido, 1995; Niederweis, 2003). Protein machines located in the outer membrane are essential for the processes of nutrient uptake, signal transduction and secretion across the outer membrane barrier (Niederweis *et al.*, 2010). More than 60 proteins are known to locate to the outer membrane of *Escherichia coli*; however, only two *M. tuberculosis* outer membrane proteins have been reported to date (Song *et al.*, 2008). Outer membrane protein A from *M. tuberculosis* (OmpATb), the C-terminal region of which is similar to the C-termini of many members of the OmpA family in *E. coli*, is known to form channels and to play an important role in nutrition acquisition (Raynaud *et al.*, 2002). Rv1698 was first predicted to be an outer membrane protein using a bioinformatics approach (Song *et al.*, 2008). Expression of Rv1698 in *M. smegmatis* increased uptake rates of nutrients and the susceptibility of the organism to small antibiotics. Protease K digestion of the modified *M. smegmatis* resulted in full digestion of Rv1698, indicating that Rv1698 is surface-accessible. Single-channel conductance was measured after the reconstitution of purified Rv1698 protein (devoid of signal peptide) into a lipid bilayer. These experiments demonstrated that Rv1698 is a channel protein that is likely to be involved in transport processes across the outer membrane of *M. tuberculosis* (Siroy *et al.*, 2008). To elucidate the molecular mechanism of transport, Rv1698 has been cloned, overexpressed and crystallized.

2. Materials and methods

2.1. Cloning, protein expression and purification

The Rv1698 gene encoding residues 27–314 (devoid of signal peptide) was amplified by polymerase chain reaction (PCR) from a DNA library of *M. tuberculosis* with the primer pair 5′-CGAATCATATGGGCTTTTTCTCCGATACTTTGCTGT (forward) and 5′-CAGCTCGAGTTACTGGGAAACCGTGACTGACATCG (reverse). The amplified target DNA fragment was digested with *Nde*I and

*Xho*I and inserted into the expression vector pET-28a (Novagen) with an N-terminally fused six-His tag. The construct was confirmed by sequencing and transformed into *Escherichia coli* strain BL21 (DE3) (Novagen). Cells were grown in M9 minimal medium supplemented with 30 µg ml^{-1} kanamycin. The culture was grown in shake flasks at 310 K and 225 rev min^{-1} until the optical density $OD_{600\,nm}$ reached 0.8. 0.8 m*M* IPTG was added to induce protein overexpression and the culture was shaken at 285 K for a further 40 h.

E. coli cells expressing recombinant Rv1698 were harvested by centrifugation at 5000*g* and 277 K. The cells were resuspended in buffer *A* [50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 5%(*v*/*v*) glycerol, 10 m*M* imidazole] and lysed by sonication in an ice–water bath. After cell lysis, the detergent C12E8 was added to a final concentration of 2%(*w*/*v*) and the lysate was rotated at 277 K for 2 h. The lysate was then centrifuged at 16 000 rev min^{-1} for 20 min at 277 K. The supernatant was mixed with Ni–NTA resin (Qiagen). The mixture was rotated at 277 K for 20 min before packing onto a gravity column. Contaminants were washed out using buffer *A* with 0.1%(*w*/*v*) C12E8. The target protein was then eluted with elution buffer [50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 5%(*v*/*v*) glycerol, 250 m*M* imidazole and 0.2%(*w*/*v*) C12E8]. The eluate was concentrated in a centrifugal concentrator (Millipore) and further purified on a Superdex 200 size-exclusion column (10/300 GL, GE Healthcare) with running buffer consisting of 50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 5%(*v*/*v*) glycerol and 0.1%(*w*/*v*) C12E8. The peaks were collected in 0.25 ml fractions and analyzed by 12% SDS–PAGE. Only the homogenous fractions were used for crystallization.

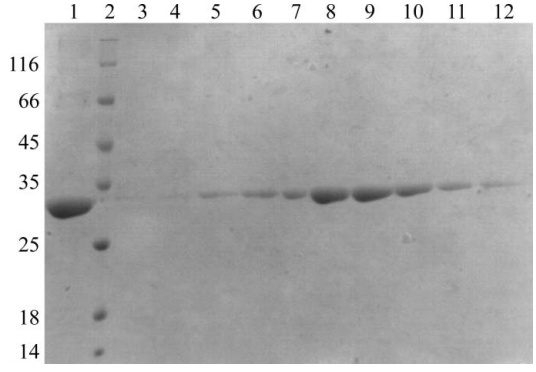

Figure 1
12% SDS–PAGE analysis of the purity of Rv1698. Lane 1, Rv1698 purified on an Ni–NTA column; lane 2, molecular-weight markers (kDa); lanes 3–12, eluted fractions from the Superdex 200 size-exclusion column.

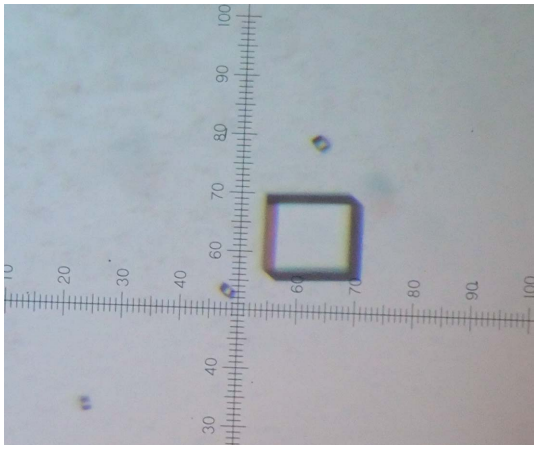

Figure 2
Crystal of Rv1698. The smallest interval on the ruler is 0.015 mm.

2.2. Crystallization

Rv1698 was initially crystallized using the sitting-drop vapour-diffusion method in 48-well plates (XtalQuest Co.) at 295 K. The crystal screening kits Crystal Screen, Crystal Screen 2 (Hampton Research), MemSys and MemStart (Molecular Dimensions) were used for initial crystallization screening. Purified Rv1698 was concentrated to 6 mg ml^{-1} in 50 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 5%(*v*/*v*) glycerol and 0.1%(*w*/*v*) C12E8. 1 µl protein solution was mixed with an equal volume of reservoir solution and the droplet was equilibrated against 100 µl reservoir solution. Microcrystals appeared in a condition consisting of 0.1 *M* sodium acetate pH 4.6, 2 *M* ammonium sulfate after one week. Optimization of the crystallization condition was performed by altering the concentration of ammonium sulfate, the pH, the concentration of Rv1698 and the temperature; a total of approximately 800 conditions were tried. A series of conditions (0.1 *M* sodium acetate pH 4.6, 1.6–2.0 *M* ammonium sulfate) gave crystals, but the best Rv1698 crystals used for data collection were grown in 0.1 *M* sodium acetate pH 4.6, 1.8 *M* ammonium sulfate with a protein concentration of 5 mg ml^{-1} at 295 K.

2.3. Data collection

X-ray diffraction data were collected on beamline BL17U at SSRF (Shanghai). A crystal of Rv1698 was harvested and soaked in a cryoprotectant solution consisting of 20%(*w*/*v*) glycerol, 0.1 *M* sodium acetate pH 4.6, 1.8 *M* ammonium sulfate for several seconds. The crystal was flash-cooled in liquid nitrogen and subjected to X-ray diffraction data collection at 100 K. 180 images were recorded from one single crystal with an oscillation angle of 1° and an exposure time of 2 s per image. The diffraction data were processed using *HKL*-2000 (Otwinowski & Minor, 1997).

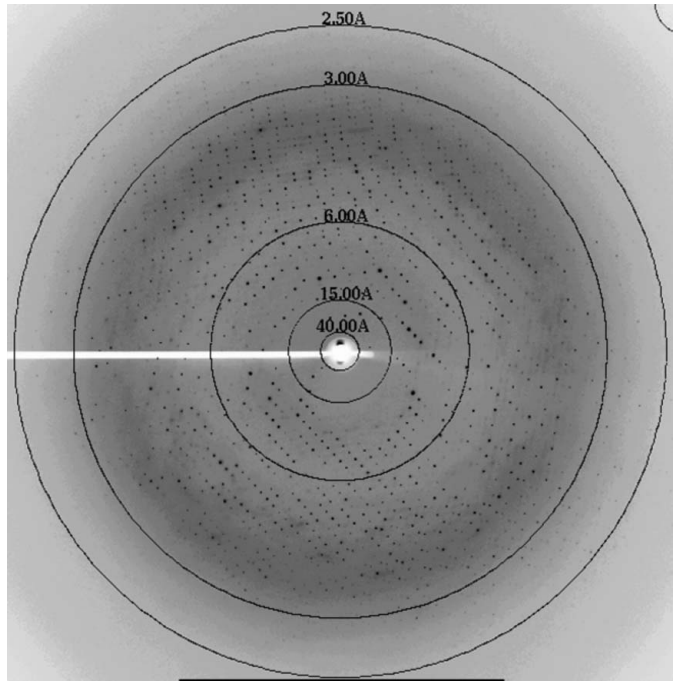

Figure 3
X-ray diffraction frame collected from a crystal of Rv1698.

Table 1
Crystal parameters and data-collection statistics for the crystal of Rv1698.

Values in parentheses are for the outer resolution shell.

Wavelength (Å)	0.9793
Resolution (Å)	50–2.50 (2.54–2.50)
Space group	*P*422
Unit-cell parameters (Å, °)	$a = b = 122.0$, $c = 88.9$, $\alpha = \beta = \gamma = 90$
Unique reflections	28419
Redundancy	15.3 (12.9)
Average $I/\sigma(I)$	66.0 (8.0)
$R_{\rm merge}$† (%)	6.7 (29.6)
Completeness (%)	98.6 (91.2)
$V_{\rm M}$ (Å^3 Da^{-1})	2.47
Solvent content (%)	50.33
No. of molecules in the asymmetric unit	2

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity of all observations i of reflection hkl.

3. Results and discussion

Rv1698 without signal peptide was overexpressed in *E. coli* strain BL21 (DE3). The recombinant protein was solubilized in C12E8 detergent solution and was purified using Ni–NTA agarose affinity chromatography and a Superdex 200 size-exclusion column. About 25 mg of homogenous target protein could be obtained from 1 l cell culture. The purified Rv1698, consisting of 297 residues (30.9 kDa), was >98% pure as estimated by SDS–PAGE (Fig. 1).

After initial screening and optimization of the crystallization conditions, large crystals with dimensions of about 0.2 × 0.2 × 0.1 mm (Fig. 2) were obtained in 0.1 *M* sodium acetate pH 4.6, 1.8 *M* ammonium acetate using a protein concentration of 5 mg ml^{-1} after one month at 295 K. Diffraction data were collected and processed to a resolution limit of 2.5 Å (Fig. 3). Data-collection and processing statistics are shown in Table 1. The crystals belonged to space group *P*422, with unit-cell parameters $a = b = 122.0$, $c = 88.9$ Å, $\alpha = \beta = \gamma = 90^\circ$. A crystal of selenomethionine-labelled Rv1698 was obtained using the same method as described above and a MAD data set was collected. The structure of Rv1698 will be solved by the MAD method and will be reported elsewhere.

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