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Crystallization of the acyl-CoA thioesterase TesB from *Yersinia pestis*

Yersinia pestis is a highly virulent human pathogen and is the causative agent of bubonic plague. Spread through the bite of infected fleas, plague epidemics have marked important events in history, including the Justinian plague (6th century), the Black Death (14th century) which decimated nearly one quarter of the European population, and more recently the Orientalis plague (1894). To date, deaths are still being reported and, without treatment, the disease kills most people within 4 days. One of the thioesterases from *Y. pestis*, TesB, is a broad-range acyl-CoA thioesterase and is highly conserved within prokaryotes and throughout evolution, sharing sequence similarity with the HIV Nef binding protein ACOT8. Here the expression, purification, crystallization and diffraction of TesB are reported. TesB has been recombinantly expressed and crystallized using the vapour-diffusion hanging-drop technique at pH 7.0 and 290 K. After optimization, crystals diffracted to 2.0 Å resolution at the Australian Synchrotron and belong to the space group $P12_11$ ($a = 73.55$, $b = 170.82$, $c = 101.98$ Å), with eight molecules likely to be present in the asymmetric unit.

1. Introduction

Yersinia is a genus of bacteria responsible for causing significant disease in both humans and animals. *Y. pestis*, the causative agent of bubonic plague, is highly virulent and, if not treated, quickly develops into a high-density septicaemia that is usually fatal (Pollitzer, 1954; Perry & Fetherston, 1997; Bertherat *et al.*, 2011). This bacteria has been responsible for infamous epidemic outbreaks, particularly in the Middle Ages, with one epidemic killing one quarter of the European population (Guiyoule *et al.*, 1994; Pollitzer, 1954). Significantly, its use in biological warfare has been reported as recently as World War II and deaths from bubonic plague are still being reported (Westin *et al.*, 2008; Tourdjman *et al.*, 2012).

Acyl-CoA thioesterases (Acots) perform a range of cellular functions by hydrolysing thioester bonds of CoA-activated molecules (commonly fatty acids), to release coenzyme A and the corresponding fatty acid. Substrates of Acots include acyl-CoAs of fatty acids of various chain lengths, as well as a range of methyl-branched and dicarboxylyl-CoAs (Shimazu *et al.*, 2010; Kirkby *et al.*, 2010). The *Escherichia coli* TesB contains an unusual active site, a hydrogen-bonded triad which orientates the thioester for a nucleophilic attack (Li *et al.*, 2000). Despite its high conservation in bacteria and higher organisms, the broad range of Acot activity on acyl-CoA substrates has prevented a specific role of TesB being elucidated (Naggert *et al.*, 1991); however one suggested role for TesB is the prevention of intracellular acyl-CoA accumulating to toxic levels (Zheng *et al.*, 2004; Spencer *et al.*, 1978).

TesB shares a high degree of sequence conservation with other prokaryotic and eukaryotic Acots. It is a homologue of the human Acot family ACOT8, which has received attention for its role in binding the HIV Nef protein (Watanabe *et al.*, 1997). Sharing 45% sequence homology with ACOT8 the *E. coli* thioesterase II (TesB) protein structure provided a model for further analysis of the mechanism by which ACOT8 binds with the HIV Nef protein (Li *et al.*, 2000). The protein crystal presented herein, that of *Y. pestis* TesB, will provide information relating to the structural and functional role

of TesB both in *Y. pestis* and, because of its highly conserved nature within prokaryotes, a range of other related pathogenic organisms such as *Mycobacterium tuberculosis*, *Shigella dysenteriae* and *Brucella ovis*.

2. Methods

2.1. Expression and purification

Y. pestis TesB was recombinantly expressed as a His-tagged fusion protein as previously described (Pollitzer, 1954). Briefly the pMCSG21:tesB expression vector was transformed into *E. coli* BL21 (DE3) pLysS competent cells and used to inoculate a 5 ml Luria-Bertani (LB) starter culture in the presence of spectinomycin ($100 \mu\text{g ml}^{-1}$). This starter culture was used to inoculate 500 ml of LB media which was grown at 310 K until mid-log phase, when expression of TesB was induced by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 21 h at 297 K until an OD_{600} of 6–10 was reached. The cells were then harvested by centrifugation and resuspended in His buffer (50 mM phosphate buffer pH 8.0, 300 mM NaCl, 20 mM imidazole). His-tagged protein was purified using affinity chromatography (HisTrap HP, GE Healthcare) and, following elution of TesB by slowly increasing the concentration of imidazole to a final concentration of 500 mM, 100 μl of TEV protease (3.3 mg ml^{-1}) was added to 10 ml of protein sample to cleave the His tag before incubation for 12 h at 276 K. The protein mixture was then further purified by size-exclusion chromatography (S-200 column, GE Healthcare) and collected in 50 mM Tris pH 7.4, 125 mM NaCl. The protein was concentrated to 21 mg ml^{-1} , assessed by SDS-PAGE to be >95% pure and stored at 253 K.

2.2. Crystallization

Crystallization conditions were screened using the sparse-matrix hanging-drop vapour-diffusion method over a number of commercially available screens (PEG/Ion, PEG/Ion 2, Crystal Screen and Crystal Screen 2 from Hampton Research) using VDX 48-well plates from Hampton Research. Briefly, 1.5 μl of the protein sample was combined with 1.5 μl of reservoir solution, suspended above 300 μl of reservoir solution and incubated at 290 K. Small, cuboidal-shaped crystals were obtained from PEG/Ion conditions (Nos. 13, 14, 16, 36 and 37), PEG/Ion 2 (Nos. 8, 16, 24, 28, 40 and 46), Crystal Screen

(Nos. 19, 21, 22 and 27) and Crystal Screen 2 (No. 37). To obtain large, single crystals suitable for X-ray diffraction, conditions were optimized around different PEG and salt concentrations. Large, single crystals were obtained from the conditions 20% PEG 3350, 235 mM sodium malonate, pH 7.0. The addition of the ligand CoA to the protein, at a concentration of 1 mM, resulted in diffraction-quality crystals from the conditions 20% PEG 3350, 235 mM sodium malonate, pH 7.0.

2.3. Diffraction data collection

Translucent, cube-shaped crystals that grew after 1 d at 290 K were soaked in reservoir solution containing 15% glycerol and flash-cooled in liquid nitrogen at 100 K. Diffraction data were collected at the Australian Synchrotron on the MX2 macromolecular crystallography beamline using crystals grown in conditions containing the CoA ligand. Raw data were auto-indexed, merged and scaled using *iMOSFLM* (Battye *et al.*, 2011). The structure of TesB will be solved by molecular replacement.

3. Results and discussion

Recombinant expression of TesB in *E. coli* BL21 DE3 yielded high levels of soluble protein. Overexpression using IPTG to induce the protein produced approximately 40 mg l^{-1} , which was shown to be predominantly soluble as shown in Fig. 1(a) (lanes 2 and 3).

A two-step FPLC purification protocol was used to produce homogeneous TesB, incorporating affinity and size-exclusion chromatography. The soluble bacterial lysate was applied onto a Ni^{2+} affinity resin (GE Healthcare), with complete binding of the 6-His-tagged TesB observed, as assessed by SDS-PAGE (Fig. 1a lanes 3 and 4). Removal of unbound proteins from the affinity matrix was achieved through extensive washing (ten column volumes) and monitored by UV absorbance (280 nm; Fig. 1b). His-tagged TesB was subsequently eluted from the column by applying an increasing concentration of imidazole up to 500 mM. An elution peak of 2300 mAU was observed that corresponded to approximately 40 mg of protein, indicating almost complete saturation of the column (10 mg ml^{-1} binding capacity).

Removal of the 6-His tag for crystallography resulted in a 3 kDa reduction in the molecular weight of the protein, corresponding to

Figure 1

The purification of TesB. (a) SDS-PAGE was used to confirm the solubility and purity of the protein. Protein present in both the whole cell and soluble extract (lanes 2 and 3, respectively) following centrifugation to remove cell debris and insoluble proteins indicate high protein solubility. (b) HisTrap column elution profile showing unbound proteins (lane 4) and TesB eluting (lane 5) as the concentration of imidazole increases to approximately 250 mM and, following incubation of the protein with TEV protease (lane 6), the presence of a small vector-derived 3 kDa 6-His tag. (c) Size-exclusion chromatography profile revealing a singular symmetrical peak indicative of a highly pure and homogeneous protein (lane 7) and concentrated protein (lane 8).

Figure 2

Diffraction patterns of TesB collected at the Australian Synchrotron in conditions (a) without CoA, (b) containing CoA at 1° and (c) after 360° of data collection (the intensity was maintained).

Table 1

Data-collection statistics.

Values in parentheses are for the highest-resolution shell.

Crystal system	Monoclinic
Space group	$P12_11$
Resolution range (Å)	63.86–2.00 (2.11–2.00)
No. of measured reflections	834860 (116147)
No. of unique reflections	143067 (18486)
Completeness (%)	89.8 (79.9)
Multiplicity	0.43
Mosaicity	5.8 (6.3)
Mean $I/\sigma(I)$	9.9 (7.5)
$R_{\text{merge}}^{\dagger}$ (%)	0.10 (0.17)
Wilson B factor	20.9

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection with Miller indices hkl and $\langle I(hkl) \rangle$ is the mean intensity of that reflection. Calculated for $I \geq 3\sigma(I)$.

the theoretical molecular weight of the N-terminal 6-His tag. The cleaved protein was further purified and buffer exchanged using size-exclusion chromatography. A single elution peak was obtained, indicative of a highly pure, homogeneous protein suitable for crystallization trials (Fig. 1c).

Sparse-matrix screening was undertaken using the commercial kits PEG/Ion, PEG/Ion 2, Crystal Screen and Crystal Screen 2 from Hampton Research to identify positive conditions that support crystallization. Of 190 conditions screened, 15 conditions were observed to produce crystals that were either not of diffraction quality (PEG-based conditions at various pHs), or that were salt crystals (e.g. condition 20% PEG 3350 with 200 mM potassium sodium tartrate). Optimization of crystals from PEG/malonate mixtures at various pHs produced large, single crystals; however these did not diffract past 3.8 Å on the MX2 beamline at the Australian Synchrotron (Fig. 2a). Further optimization, including crystals grown in the presence of CoA, yielded well ordered crystals that diffracted past 2.0 Å (Fig. 2b). These crystals were resistant to radiation damage enabling 360° of data to be collected with no loss in diffraction quality (Figs. 2b and 2c). The crystals belonged to the space group $P12_11$ and data frames were integrated and scaled to 2 Å using *iMOSFLM* 1.0.5 with an R_{merge} of 10 and 17% across all resolution shells and the outer resolution shell, respectively. The data-collection statistics are summarized in Table 1. Unit-cell parameters for the crystal were $a = 73.55$, $b = 170.82$, $c = 101.98$ Å, $\alpha = 90.00$, $\beta = 109.44$, $\gamma = 90.00^\circ$, and the program *Pointless* from the

CCP4 suite (Winn *et al.*, 2011) was used to calculate the minimal probability of the data being twinned using an *L*-test for twinning ($\langle |L| \rangle$ 0.491, where 0.5 signifies untwinned, 0.375 a perfect twin).

Interestingly, preliminary structural analysis of both $2F_o - F_c$ and $F_o - F_c$ electron-density maps revealed a lack of density for the CoA, indicating that the molecule may be acting as an additive rather than binding in the active site. The structure can be solved using molecular replacement against the solved *E. coli* thioesterase II; full determination, refinement and analysis of this protein structure will provide important insight into the substrate specificity, and the conserved nature of the active site and quaternary structure that is maintained throughout evolution.

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