Zhen Chen,^{a,b} Zengqiang Gao,^b
Haidai Hu,^c Jianhua Xu,^b Heng
Zhang^{b*} and Yuhui Dong^{b*}

^aSchool of Life Sciences, Anhui University, Hefei 230601, People's Republic of China, ^bBeijing Synchrotron Radiation Facility, Institute of High Energy Physics, Chinese Academy of Sciences, Beijing 100049, People's Republic of China, and ^cKey Laboratory of Molecular Virology and Immunology, Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai 200025, People's Republic of China

Correspondence e-mail: zhangheng@ihep.ac.cn, dongyh@ihep.ac.cn

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Cloning, purification, crystallization and preliminary X-ray studies of the putative type VI secretion immunity protein Tli5 (PA5088) from *Pseudomonas aeruginosa*

The putative protein PA5089 from *Pseudomonas aeruginosa* has recently been identified as a Tle5 phospholipase effector from a type VI secretion system (T6SS), and its toxicity can be neutralized by the cognate immunity protein Tli5 (PA5088). Here, the expression, purification, crystallization and preliminary crystallographic analysis of PA5088 are reported. X-ray diffraction data were collected from selenomethionine-derivatized PA5088 crystals to a resolution of 2.55 Å. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 64.002$, $b = 104.744$, $c = 90.168$ Å.

1. Introduction

The type VI secretion system (T6SS) of Gram-negative bacteria plays an important role in interspecies interactions by injecting effector proteins into the periplasmic compartment or the cytoplasm of the recipient cells to mediate interbacterial competition and many other virulence-related processes (Mougous *et al.*, 2006; Hood *et al.*, 2010; Russell *et al.*, 2011). Meanwhile, the cognate immunity proteins in the donor cells bind and neutralize the toxicities of the effectors to protect themselves (Russell *et al.*, 2011). A diverse superfamily of bacterial T6SS effectors Tle1–Tle5 (type VI lipase effectors 1–5) with the role of mediating antagonistic bacterial interactions has recently been identified (Russell *et al.*, 2013). In this superfamily, Tle1, Tle2 and Tle5 possess phospholipase A2, A1 and D (PLA2, PLA1 and PLD) activities, respectively. These proteins can hydrolyze the membrane phospholipids of neighbouring cells with an increase in cellular permeability after being injected into their periplasmic space by T6SS, whereas these antibacterial activities can be inhibited by their cognate immunity proteins Tli1, Tli2 and Tli5 (type VI secretion lipase immunity), respectively (Russell *et al.*, 2013). These families lack significant homology to known lipase enzymes, suggesting that they may represent new members with a distinct substrate recognition and catalytic mechanism in the lipase superfamily.

The putative protein PA5089 from the pathogen *Pseudomonas aeruginosa* PAO1 has been predicted to be a phospholipase effector with PLD activity from a T6SS (Russell *et al.*, 2013). Its toxicity can be neutralized by the cognate immunity protein PA5088 (~33 kDa), which has not been characterized until now. In order to further investigate the inhibition mechanism of PA5088, we report its expression, purification, crystallization and preliminary X-ray crystallographic studies. Structure determination will be pursued using experimental phasing methods. Determination of its three-dimensional structure will help in understanding how this protein recognizes and binds to its cognate effector to inhibit the activity.

2. Results

2.1. Cloning and protein expression

The gene encoding PA5088 (without the N-terminal 21-residue signal peptide) was amplified from the genomic DNA of *P. aeruginosa* PAO1. The PCR product was digested and then cloned into the pET28at-plus vector with an N-terminal His tag followed by a

Figure 1

Solution characteristics of PA5088. (a) Purified PA5088 eluted from gel-filtration chromatography (Superdex 200 10/300 GL) at 15.7 ml corresponding to a molecular mass of ~70 kDa. (b) SDS-PAGE analysis of purified PA5088 visualized using Coomassie Blue. Lane *M* contains molecular-mass markers (labelled in kDa).

Tobacco etch virus (TEV) cleavage site. The expression plasmid was transformed into *Escherichia coli* strain BL21 (DE3) for expression.

2.2. Protein purification

Bacterial cells were grown to mid-log phase in LB medium at 37°C in the presence of 50 mg ml⁻¹ kanamycin. Induction of the culture was then carried out with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16°C. Cells were pelleted after 20 h by centrifugation at 8000 rev min⁻¹ for 10 min at 4°C. The cell pellet was resuspended in buffer consisting of 20 mM Tris pH 8.0, 100 mM NaCl, 2 mM β-mercaptoethanol, 1 mM PMSF and lysed by ultrasonication

on ice. The cell debris and membranes were pelleted by centrifugation at 16 000 rev min⁻¹ for 60 min at 4°C. The soluble N-terminally hexahistidine-tagged PA5088 was purified by affinity chromatography with nickel-nitrilotriacetic acid resin (Bio-Rad) and the tag was removed by overnight hydrolysis with TEV protease and reloading with 20 mM imidazole. The protein was further purified by ion-exchange chromatography and subsequent gel-filtration chromatography (Superdex 200, GE Healthcare) equilibrated in buffer consisting of 20 mM Tris pH 8.0, 150 mM NaCl, 2 mM DTT using an ÄKTApurifier System (Amersham). The recombinant PA5088 has an apparent molecular mass of ~70 kDa according to analytical size-exclusion chromatography (Fig. 1a). Considering its molecular weight of ~33 kDa (Fig. 1b), it is likely to be a dimer in solution. Highly purified protein fractions were pooled and concentrated to 8 mg ml⁻¹ as quantified by the Bio-Rad protein assay kit for crystallization trials by ultrafiltration in an Amicon cell (Millipore, California, USA).

Selenomethionine-derivatized (SeMet) PA5088 was produced in minimal medium supplemented with 100 mg l⁻¹ lysine, phenylalanine and threonine and 50 mg l⁻¹ isoleucine, leucine, valine and selenomethionine. The production and purification of the SeMet protein were the same as described above.

2.3. Crystallization

Crystallization screens were performed with Hampton Research and Qiagen kits using the sitting-drop vapour-diffusion method at 20 and 4°C. The purified protein was crystallized under PEG-containing conditions. The SeMet PA5088 crystal was obtained using a reservoir solution consisting of 0.1 M HEPES sodium pH 7.5, 10% (v/v) 2-propanol, 20% (w/v) PEG 4000 after 5 d at 4°C. The SeMet PA5088 crystal quality was optimized by adjusting the concentration of the precipitant and buffer. A hexagon-shaped crystal was obtained in a solution of 0.1 M HEPES sodium pH 7.3, 7.5% (v/v) 2-propanol, 25% (w/v) PEG 4000 after 4 d at 4°C (Fig. 2).

Figure 2

A crystal of SeMet PA5088 from *P. aeruginosa* as grown by the sitting-drop method.

Figure 3

X-ray diffraction image from a SeMet PA5088 crystal recorded using a MAR165 CCD detector (a) and a magnification (b) of the region in the rectangle in (a). The edge of the detector corresponds to a resolution of 2.55 Å.

Table 1

Data collection for SeMet PA5088.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.9792
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 64.002$, $b = 104.744$, $c = 90.168$, $\beta = 102.818$
Resolution (Å)	2.55 (2.59–2.55)
No. of unique reflections	36409 (1650)
Completeness (%)	96.6 (89.6)
Multiplicity	7.3 (7.1)
No. of molecules in asymmetric unit	3 or 4
Solvent content (%)	57.8 or 43.7
Matthews coefficient (Å ³ Da ⁻¹)	2.91 or 2.18
Mean $I/\sigma(I)$	19.2 (3.9)
R_{merge} (%)	12.2 (53.5)

2.4. Data collection and processing

The SeMet PA5088 data were collected on beamline 3W1A at the BSRF (Beijing Synchrotron Radiation Facility) using a MAR165 CCD detector at a wavelength of 0.9792 Å (Fig. 3). Before data collection, the crystals were soaked in reservoir solution supplemented with 20%(v/v) glycerol for a few seconds and then flash-cooled in liquid nitrogen. All data were processed using *HKL-2000* (Otwinowski & Minor, 1997). The final data-collection and processing statistics are given in Table 1.

3. Analysis and discussion of preliminary X-ray diffraction results

A total of 36 409 measured reflections were merged into 1650 unique reflections with an R_{merge} of 12.2%. The merged data set was 96.6% complete to 2.55 Å resolution. The relevant data-collection statistics are given in Table 1.

The PA5088 crystals belonged to space group $P2_1$, with unit-cell parameters $a = 64.002$, $b = 104.744$, $c = 90.168$ Å (Table 1). Based on the molecular weight of PA5088 and space group $P2_1$, solvent-content analysis indicated that three or four molecules could be accommodated per asymmetric unit. The crystal structure of SeMetPA5088 is under determination by the single-wavelength anomalous dispersion (SAD) method.

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