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Preliminary crystallographic analysis of the antibiotic discharge outer membrane lipoprotein OprM of *Pseudomonas aeruginosa* with an exceptionally long unit cell and complex lattice structure

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Crystals of the drug-discharge outer membrane protein OprM (MW = 50.9 kDa) of the MexAB-OprM multidrug transporter of *Pseudomonas aeruginosa* have been grown at 293 K in the presence of 2-methyl-2,4-propanediol and a combination of surfactants. The crystal belonged to space group *R*32, with unit-cell parameters $a = b = 85.43$, $c = 1044.3$ Å. Diffraction data for OprM were obtained using the undulator synchrotron-radiation beamline at SPring-8 (BL44XU, Osaka University), which allowed an extra-long specimen-to-detector distance with a wide detector area. The crystal diffracted to 2.56 Å resolution using 0.9 Å X-rays from the synchrotron-radiation source. A heavy-atom derivative for isomorphous replacement phasing was obtained using iridium chloride.

1. Introduction

Xenobiotic/antibiotic efflux transporters are ubiquitous to all living organisms and protect cells from small noxious compounds. When such transporters are expressed in neoplastic cells, they render the cancer cells resistant to chemotherapeutic agents; in bacteria, they render cells resistant to many structurally and functionally dissimilar antibiotics (termed multidrug-resistance). Development of agents that inhibit the function of the multi-antibiotic efflux pump and can shift back antibiotic resistant cells to being susceptible, lower the antibiotic dosage and thus lower the adverse effect of antibiotics is awaited. A short-cut to this goal may be protein structure-based drug design and development.

The OprM (MW = 50.9 kDa; 468 amino acids) subunit of the MexAB-OprM efflux pump of *Pseudomonas aeruginosa* is a lipoprotein-type outer membrane protein that is assumed to form a transmembrane drug-discharge duct across the outer membrane and to facilitate antibiotic disposal (Nakajima *et al.*, 2000). Thus, it is of interest to reveal the structure of the drug-disposal duct and to determine whether OprM possesses multiple modes of membrane anchoring. The structure of OprM may differ from that of the porin-like open duct, as the protein facilitates the exit of antibiotics but not the entry. The cognate partners of the OprM subunit in the pump assembly are the intrinsic inner membrane protein MexB and the periplasmic MexA subunit, which serve as a drug-recognition/energy-conversion subunit and a molecular clamp connecting the MexB and OprM subunits, respectively.

MexAB-OprM belongs to the resistance-nodulation cell-division (RND) superfamily assembly. The structures of three proteins from this superfamily have been reported to date. The structure of AcrB, a MexB homologue, has been resolved at 2.7–3.5 Å resolution and was revealed to mainly consist of three domains: a transmembrane domain, an extra-membranous channel/vestibule domain and a funnel domain (Murakami *et al.*, 2002; Pos *et al.*, 2004). Of the membrane-fusion family proteins (MFP), the structure of MexA has been solved at 2.40–3.00 Å resolution, which showed a long sickle-shaped protein mainly consisting of four domains: an α-helical hairpin domain, a β-domain consisting of eight β-sheets, a third domain containing seven β-sheets and a short α-helix and an addi-

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Native-1	Native-2	$IrCl_3$ derivative
Data collection	BL44XU	BL44XU	BL44XU
Wavelength (Å)	0.9000	0.9000	1.1056
Resolution (Å)	40.0–2.56 (2.69–2.56)	40.0–2.95 (3.06–2.95)	40.0–3.30 (3.42–3.30)
Space group	*R*32	*R*32	*R*32
Unit-cell parameters (Å)			
$a = b$	85.43	84.92	85.31
c	1044.3	1042.6	1043.0
Observed reflections	548330	471037	322693
Independent reflections	48709	30962	41952
Percentage of rejected reflections (%)	0.05	1.34	0.56
$R_{\rm merge}$†	0.047 (0.362)	0.069 (0.432)	0.056 (0.227)
Completeness (%)	99.1 (94.5)	97.5 (99.9)	99.0 (100.0)
Multiplicity	11.4	15.2	7.7
$I/\sigma(I)$	6.4 (2.0)	35.5 (8.7)	31.7 (8.8)

† $R_{\rm merge} = \sum\sum_j |\langle I(h)\rangle - I(h)_j| / \sum\sum_j \langle I(h)\rangle$, where $\langle I(h)\rangle$ is the mean intensity of symmetry-equivalent reflections. Friedel pairs were merged as individual data.

tional disordered domain (Akama *et al.*, 2004; Higgins *et al.*, 2004). Of the outer membrane family proteins, the structure of TolC has been solved at 2.1 Å resolution and consisted of two major domains: an outer membrane-spanning β-barrel and a long periplasmic α-barrel (Koronakis *et al.*, 2000). Both ends of the barrel had pore openings. OprM differs from TolC in that the protein participates solely in xenobiotic discharge, whereas TolC is a multifunctional outer membrane protein involved in protein secretion and xenobiotic extrusion; OprM also has N-terminal fatty-acid modification but TolC does not. Therefore, it is important to clarify the structure of this medically relevant pro-xenobiotic discharge protein of the RND-efflux pump.

2. Preparation of OprM

P. aeruginosa TNP072 cells lacking the chromosomal *oprM* harboured pMMB67EH, which carries the *oprM-(his)*$_6$ fusion gene (Nakajima *et al.*, 2000). Cells were grown overnight in 800 ml Luria broth supplemented with 50 µg ml^{-1} sulbenicillin at 310 K under constant rotation at 200 rev min^{-1}. The culture was diluted with 9200 ml Luria broth and rotation continued as above; isopropyl-β-D-thiogalactoside (IPTG; Wako Pure Chemical Industries Ltd) was then added to 1 m*M* when $A_{600}^{1\,\rm cm}$ reached 0.7 and the flask was rotated for an additional 3 h. Cells were harvested by centrifugation at 10 000*g* for 10 min at 283 K, washed once with 100 ml of a solution containing 20 m*M* sodium phosphate buffer with 150 m*M* NaCl pH 7.2 and suspended in 45 ml of the same buffer containing one tablet of protease-inhibitor mixture (Complete Mix, Roche Applied Science) and about 5 µg ml^{-1} of DNaseI (Worthington Biochemical Corp.). Cells were disintegrated by passing them three times through a French pressure cell at 82.7 MPa cm^{-2} and unbroken cells were removed by centrifugation at 10 000*g* for 15 min at 277 K. The supernatant fraction was subjected to high-speed centrifugation at 150 000*g* for 60 min at 277 K, the pellets were washed once by centrifugation under the same conditions as above and were suspended in 80 ml 20 m*M* sodium phosphate buffer pH 7.2. Freshly prepared *N*-lauryl sarcosine solution (*N*-dodecanoyl-*N*-methylglycine sodium salt; MW = 293.4; nakalai tesque Corp.) was added to 0.8%(*w*/*v*) and the mixture was incubated at 277 K for 20 min under constant stirring and then centrifuged at 150 000*g* for 60 min at 277 K. The pellets were washed with 80 ml 20 m*M* sodium phosphate buffer pH 7.2 supplemented with 5%(*v*/*v*) glycerol and were suspended in 50 ml of 20 m*M* sodium phosphate, 20 m*M* imidazole, 2.5%(*w*/*v*) *n*-octyl-β-D-glucopyranoside (Octyl-Glc; CMC = 19 m*M*, MW = 292.4; Alexis Corp.) and 0.3 *M* NaCl. The mixture was gently stirred at 277 K for 30 min and centrifuged at 150 000*g* for 60 min. The supernatant fraction was mixed with a 4 ml volume of Ni–NTA Sepharose resin (Invitrogen, ProBond) equilibrated with a mixture of 50 m*M* sodium phosphate, 20 m*M* imidazole, 1%(*w*/*v*) Octyl-Glc and 0.3 *M* NaCl and gently stirred at 277 K for 120 min and packed into two open columns. The columns were washed with 60 ml of buffer containing 50 m*M* sodium phosphate, 40 m*M* imidazole, 2%(*w*/*v*) Octyl-Glc, 1 *M* NaCl pH 7.2 and eluted with 14 ml of buffer containing 50 m*M* sodium phosphate, 250 m*M* imidazole and 2%(*w*/*v*) Octyl-Glc. Fractions containing homogeneous OprM-His$_6$ (MW = 51.7 kDa, 474 amino acids) were used for crystallization.

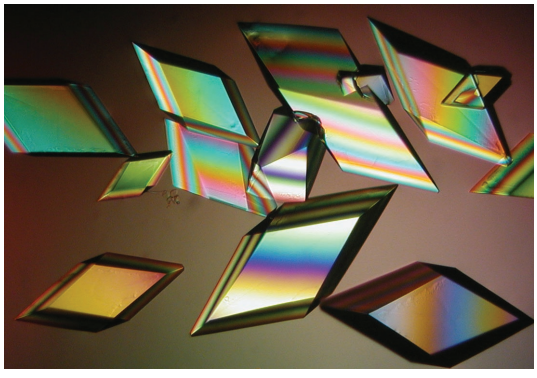

Figure 1
Crystals of native OprM from *P. aeruginosa*.

3. Crystallization and crystallographic studies

Crystal Screen kits (Hampton Research Corp.) were used for initial screening. The setup was kept at 293 K in an incubator for two to there weeks. The following refinements were subsequently made. The OprM protein was dialyzed against a solution of 50 m*M* Tris–HCl pH 8.0, 100 m*M* imidazole pH 8.0, 450–600 m*M* sodium acetate, 30 m*M* cyclohexyl-propyl-β-D-maltoside (CYMAL-3; CMC = 34.5 m*M*, MW = 466.5; Anatrace Inc.), 0.1%(*v*/*v*) *n*-octyl-oligo-oxyethylene (Octyl-POE; CMC = 6.6 m*M*, MW = 400; Alexis Corp.) and 5%(*v*/*v*) 2-methyl-2,4-pentanediol (MPD; Wako Pure Chemical Industries Ltd) for 6 h by changing the dialysis buffer every 2 h and was concentrated to about 12 mg ml^{-1} protein using a Vivaspin concentrator (VivaScience). The hanging-drop vapour-diffusion technique

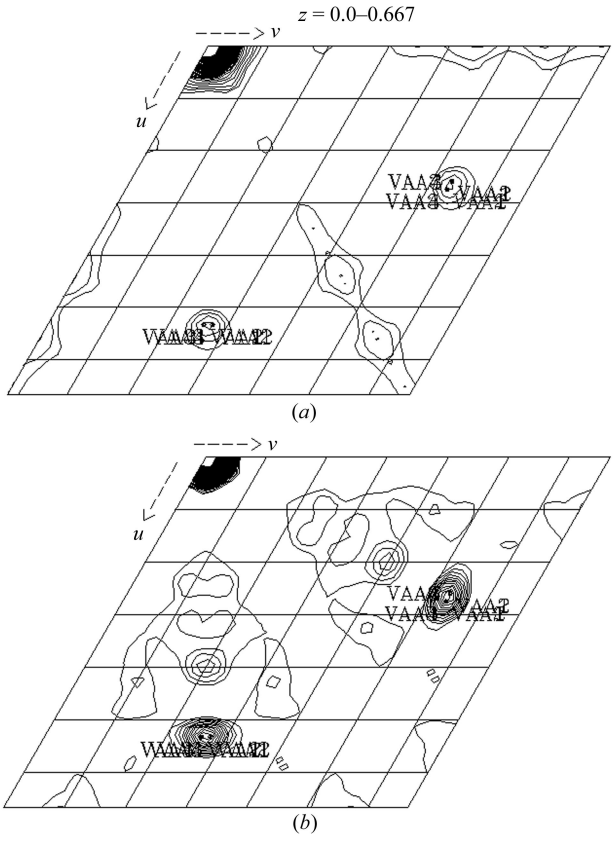

Figure 2
Difference Patterson map in the Harker plane. (*a*) shows the isomorphous difference map and (*b*) the Bijvoet anomalous difference map. Both peaks are from atomic vector site *A* (x, y, z) = (0.0645, 0.4028, 0.3220). The maps were contoured at intervals of 1.0 times the r.m.s. density of the map.

was used throughout screening and optimization. The reservoir contained 0.5 ml of the same buffer used for dialysis except that the MPD concentration was varied from 22 to 27%(*v*/*v*). Rhombohedral crystals grew to dimensions of 0.8 × 0.4 × 0.2 mm in a few weeks (Fig. 1). The crystals were flash-cooled in an N_2-gas stream at 95 K and stored in liquid nitrogen for further data collection.

Diffraction data of native OprM and of the iridium derivative were collected at the synchrotron-radiation source at SPring-8 (Hyogo, Japan) using a DIP6040 imaging-plate detector (Bruker AXS/MAC Science) at 95 K. For data collection, the crystal-to-detector distance, oscillation range and exposure time were set to 550 mm, 1° and 30 s per frame, respectively. The space group of the native crystal was *R*32, with unit-cell parameters $a = b = 85.43$, $c = 1044.3$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. The diffraction images were reduced, scaled and merged with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) or *MOSFLM* (Leslie, 1992) and *SCALA* (Kabsch, 1988). The intensities were then converted to structure-factor amplitudes with *TRUNCATE* from *CCP*4 (Collaborative Computational Project, Number 4, 1994). A set of X-ray diffraction data was collected to 2.56 Å resolution from crystal Native-1. A summary of the crystallographic data is given in Table 1. The presence of two OprM-His_6 monomer molecules per asymmetric unit gave a crystal volume per protein weight (V_M) of 3.5 $Å^3$ Da^{-1} and a solvent content of 65.1% by volume.

The single isomorphous replacement with anomalous scattering (SIRAS) method using the iridium-derivative crystal was applied to solve the crystallographic phase problem. Native-2 data were used as the native data set for phase determination. The heavy-atom derivative was obtained by soaking the crystal in 2 m*M* $IrCl_3$ for 16 h. A difference Patterson map of Native-2 and iridium-derivative data showed clear peaks corresponding to the heavy atom–heavy atom vectors (Fig. 2). The self-rotation function map only shows a clear peak at (φ, ψ, κ) = (0.0, 0.0, 120.0), which corresponds to crystallographic threefold symmetry. Since OprM is expected to be a trimeric structure, as in the AcrB and TolC structures, it is assumed that the trimeric structure of OprM is located at the crystallographic threefold axes in the crystal. The unusually long *c* axis is caused by the complex lattice in the hexagonal setting *R*32 and the trimeric structure of OprM on the crystallographic threefold axis. Further refinement of the heavy-atom parameters and phasing are in progress.

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