Purification, crystallization and preliminary X-ray crystallographic analysis of the phosphatase domain (PA3346PD) of the response regulator PA3346 from *Pseudomonas aeruginosa* PAO1

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The phosphatase domain (PA3346PD) of the response regulator PA3346 modulates the downstream anti-anti- σ factor PA3347 to regulate swarming motility in *Pseudomonas aeruginosa* PAO1. PA3346PD, which comprises the protein phosphatase 2C domain (PP2C), is classified as a Ser/Thr phosphatase of the Mg²⁺- or Mn²⁺-dependent protein phosphatase (PPM) family. The recombinant PA3346PD, with molecular mass 26 kDa, was overexpressed in *Escherichia coli*, purified on an Ni²⁺-NTA agarose column and crystallized by the sitting-drop vapour-diffusion method. X-ray diffraction data were collected from PA3346PD crystals to a resolution of 2.58 Å and the crystals belonged to space group *I*₄32 or *I*₄32, with unit-cell parameter *a* = 157.61 Å. Preliminary analysis indicates the presence of a monomer of PA3346PD in the asymmetric unit with a solvent content of 58.4%.

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that possesses a remarkable capacity to adapt to various environments and survive with limited nutrients (Wenner *et al.*, 2014). *P. aeruginosa* commonly causes severe infections in patients with compromised immunity such as those suffering from cystic fibrosis and burns (Goldberg & Pier, 2000). The great capability of the bacterium to adapt to various environments might partly result from the presence of more than 60 sets of two-component systems (TCSs; Rodrigue *et al.*, 2000; Stover *et al.*, 2000). Most TCSs can be classified into the 'classical' type, where a sensor histidine kinase (HK) responds to a signal by modifying the phosphorylation state of a cognate response regulator (RR) directly, and 'phosphorelay-type' systems, where three sequential phosphoryl-transfer events result in the phosphorylation of a regulatory protein mediating the response. Certain bacterial pathogens, including *P. aeruginosa*, possess 'hybrid'-type TCSs, where the phosphoryl group is transferred from an HK to a histidine-containing phosphotransfer (Hpt) protein and subsequently to the downstream cytoplasmic RR, as shown in Fig. 1 (Wu *et al.*, 2011).

A hybrid-type TCS in *P. aeruginosa* PAO1, consisting of four orphan sensor HKs (PA1611, PA1976, PA2824 and PA4856), one phosphotransfer protein HptB (PA3345) and one specific RR (PA3346), has been identified and shown to be related to the bacterial swarming motility (Hsu *et al.*, 2008).

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Swarming motility has been strongly implicated in the virulence of *P. aeruginosa* (O'Toole & Kolter, 1998). PA3346 is a protein of 571 amino acids comprising a N-terminal receiver (REC) domain, a stalk linker, a PP2C-like Ser/Thr protein phosphatase domain (PA3346PD) and a C-terminal ATP-binding domain. After the N-terminal REC domain of PA3346 receives a stimulus, the phosphatase activity of PA3346 is increased to dephosphorylate the anti-anti- σ factor PA3347 to regulate the downstream genes (Fig. 1; Wu *et al.*, 2011; Bhuwan *et al.*, 2012). This signal pathway has been demonstrated to be involved in flagellar biosynthesis and the chemotaxis response that plays an essential role in its virulence (Feldman *et al.*, 1998; Bhuwan *et al.*, 2012). The phosphatase activity of PA3346 requires magnesium (Mg^{2+}) or manganese (Mn^{2+}) ions as a cofactor to dephosphorylate the downstream PA3347 (Gooderham *et al.*, 2009).

Multiple sequence alignment shows that PA3346PD shares low overall sequence identities with the C-terminal phosphatase domains of *P. aeruginosa* RssB (15% sequence identity; PDB entry 3es2; I. Levchenko, R. A. Grant, R. T. Sauer & T. A. Baker, unpublished work) and a Ser/Thr protein phosphatase from *Moorella thermoacetica* (15% sequence identity; PDB entry 3zt9; Quin *et al.*, 2012). To further understand the mechanism of dephosphorylation catalyzed by PA3346PD, it is thus necessary to elucidate the detailed three-dimensional structure of the protein and how the structure relates to its function. Here, we report the overexpression, purification, crystallization and preliminary X-ray diffraction characterization of PA3346PD to determine its structure.

2. Materials and methods

2.1. Molecular cloning and protein expression

The DNA region comprising the phosphatase domain (PA3346PD; Cys170–Ser390) of PA3346 was amplified from *P. aeruginosa* PAO1 chromosomal DNA by PCR with the primer pair forward, 5'-GGAATTCCATATGTCCGGCTG-CCTGAAC-3', and reverse, 5'-CGGAATTCTTAGCTATC-CGGCTGCAG-3'. After DNA-sequencing analysis, the PCR-amplified DNA was subcloned into the NdeI- and EcoRI-

digested pET-28a vector and transformed into *Escherichia coli* BL21-CodonPlus-RIL cells for recombinant protein expression and purification. One colony of the transformant was inoculated into 10 ml Luria–Bertani (LB) broth containing 50 $\mu\text{g ml}^{-1}$ kanamycin and propagated at 310 K overnight. The overnight culture was then transferred into 2 l fresh LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin. After the OD_{600} of the culture had reached 0.4–0.6, isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.05 mM to induce protein synthesis. Incubation was continued for 20 h with vigorous shaking at 291 K. Information on recombinant PA3346PD production is summarized in Table 1.

2.2. Protein purification

The cells were harvested by centrifugation (6000g) at 277 K for 30 min. The supernatant medium was discarded and the cells were resuspended in lysis buffer (30 ml) consisting of 150 mM NaCl, 20 mM Tris–HCl pH 7.5 and then subjected to cell disruption by ultrasonication (SONICS VCX 750) using a pulse cycle of 2 s on and 4 s off with a total duration of 15 min sonication at 40% energy on ice. The soluble protein extract was then collected by centrifugation (10 000g) at 277 K for 30 min and passed through an Ni^{2+} –NTA agarose column (length 5 cm; GE Healthcare) that had been pre-equilibrated with 50 ml binding buffer (150 mM NaCl, 20 mM Tris–HCl pH 7.5). The column was washed with 100 ml binding buffer containing 40–100 mM imidazole; the purified target protein was eluted with 50 ml binding buffer containing 200 mM imidazole. The eluted fractions were collected and analyzed by 15% SDS–PAGE. The collected purified PA3346PD protein was dialyzed in 20 mM Tris–HCl buffer pH 8.5 containing 300 mM NaCl, 5 mM EDTA and 20 mM β -mercaptoethanol at 277 K overnight. The protein sample was concentrated (Centricon, MWCO 10 000; Sartorius Vivaspinn 20) before crystallization. The yield of the protein was approximately 12 mg ml^{-1} ; the purity exceeded 95% as analysed by 15% SDS–PAGE with Coomassie Brilliant Blue R-250 staining (Fig. 2).

Figure 1
The hybrid two-component regulatory system.

Table 1
Molecular cloning and protein expression of PA3346PD.

Source organism	<i>P. aeruginosa</i> PAO1
DNA source	<i>P. aeruginosa</i> PAO1 chromosomal DNA
Forward primer (NdeI site)	GGAATTCCATATGTCCGGCTGCCTGAAC
Reverse primer (stop codon and EcoRI site)	CGGAATTCTTAGCTATCCGGCTGCAG
Cloning vector	pET-28a
Expression vector	pET-28a
Expression host	<i>E. coli</i> BL21-CodonPlus-RIL
Complete amino-acid sequence of the construct produced	MGSSHHHHHSSGLVPRGSHMSGCLNASNVRYLQ-SPLALFNGDLLLAAYKPSGGMHVLGDFTHG-LPAAIGAMPLAEVFGMTAKGYPMADILREMN-AKLKRILPVGVFCCATLLNLSFQRELVEVWNG-GLPDGYLLRAASGERVALVSRHLPLGILEPAA-FSDRCETYPLDIEDRIFLFSFGVLEASNKAGE-MFGEARLLQLFERNRQPSALFDEIQRSLAQFR-GAVQDDVSMLEVRLLQPD

2.3. Protein crystallization

Prior to crystallization trials, the purified PA3346PD protein was prepared and concentrated to 10 mg ml⁻¹ in 20 mM Tris-HCl buffer pH 8.5 containing 300 mM NaCl, 5 mM EDTA, 20 mM β-mercaptoethanol and protease-inhibitor cocktail (1%; Roche). Crystallization trials were performed using crystal-screening kits with 96-well plates (Corning) by the sitting-drop vapour-diffusion method using 1 μl sitting drops consisting of equal volumes of protein solution (0.5 μl) and reservoir solution (0.5 μl) equilibrated against 100 μl reservoir solution at 291 K (Table 2).

2.4. X-ray data collection and processing

The crystals were transferred from a crystallization drop into 5 μl cryoprotectant solution with precipitant solution containing 20%(v/v) glycerol for a few seconds, mounted on a synthetic nylon loop (0.1–0.2 mm; Hampton Research) and then flash-cooled in liquid nitrogen. The protein crystals were initially tested and characterized with synchrotron X-rays on

Figure 2
Purification and analysis of PA3346PD. Coomassie Blue-stained 15% SDS-PAGE of pools from PA3346PD expression and purification. All samples were boiled for 10 min in 5× sample buffer. Lane M, molecular-weight markers (labelled in kDa); lane 1, pool from supernatant after sonication; lane 2, pool from flowthrough; lane 3, pool from 40 mM imidazole wash; lane 4, pool from 70 mM imidazole wash; lane 5, pool from 100 mM imidazole wash; lane 6, pool from 200 mM imidazole elution.

Table 2
Crystallization.

Method	Vapour diffusion (sitting drop)
Plate type	CrystalEx plates (Corning)
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM Tris-HCl pH 8.5, 300 mM NaCl, 5 mM EDTA, 20 mM β-mercaptoethanol, 1%(v/v) protease-inhibitor cocktail
Composition of reservoir solution	1.3 M NaCl, 15%(v/v) ethanol
Volume and ratio of drop	1 μl protein:1 μl precipitant
Volume of reservoir (μl)	150

beamlines BL13B1, BL13C1 and BL15A1 equipped with CCD detectors (Q315r, ADSC) at the National Synchrotron Radiation Research Center (NSRRC), Taiwan and on beamline BL12B2 equipped with a CCD detector (MX225-HE, Rayonix) at SPring-8, Japan. For complete data collection to the best resolution, a total rotation of 50° was measured with 1.0° oscillations on beamline BL44XU at SPring-8 equipped with a CCD detector (MX300-HE, Rayonix) at an X-ray wavelength of 0.9 Å with 1 s exposures and a crystal-to-detector distance of 350 mm at 110 K under a nitrogen stream provided by a cryosystem (X-Stream, Rigaku/MSC). All data were indexed, integrated and scaled with the *HKL-2000* program suite (Otwinowski & Minor, 1997).

3. Results and discussion

The recombinant PA3346PD comprising the phosphatase domain (221 residues; Cys170–Ser390) was purified on an Ni²⁺-NTA column and was eluted with 200 mM imidazole. Under SDS denaturing and reducing conditions, the protein showed a single band corresponding to a molecular mass of about 26 kDa on SDS-PAGE (Fig. 2), which was confirmed by a MALDI-TOF analysis (data not shown) and was in agreement with prediction for the recombinant PA3346PD. In order to determine the structural differences between the apo form and the divalent-cation bound form of PA3346PD, we first prepared the apo form with a relatively high concentration of

Figure 3
Single crystals of PA3346PD grown by the sitting-drop vapour-diffusion method.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL44XU, SPring-8
Wavelength (Å)	0.90
Temperature (K)	110
Detector	MX300-HE, Rayonix
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	1
Total rotation range (°)	50
Exposure time per image (s)	1
Space group	$I4_132$ or $I4_332$
Unit-cell parameters (Å, °)	$a = b = c = 157.61$, $\alpha = \beta = \gamma = 90$
Mosaicity (°)	0.30
Resolution range (Å)	30–2.58 (2.67–2.58)
Total No. of reflections	301354
No. of unique reflections	10860
Completeness (%)	99.5 (100)
Multiplicity	11.9 (12.3)
$\langle I/\sigma(I) \rangle$	26.3 (4.9)
$R_{\text{r.i.m.}}$ † (%)	2.9 (17.0)
Overall B factor from Wilson plot (Å ²)	37.95

† The redundancy-independent merging R factor $R_{\text{r.i.m.}}$ was estimated by multiplying the conventional R_{merge} value by the factor $[N/(N-1)]^{1/2}$, where N is the data multiplicity.

EDTA to chelate possible bound divalent cations in the expression and purification environment.

Small crystals were first observed from a screening condition consisting of 1.5 M NaCl, 10%(v/v) ethanol (Structure Screen condition No. H11, Molecular Dimensions) within a few days after the initial crystallization setup at 291 K. Protein crystals were further optimized using 2 µl sitting drops consisting of equal volumes of protein solution (1 µl) and reservoir solution (1 µl) equilibrated against 150 µl reservoir solution consisting of 1.3 M NaCl, 15%(v/v) ethanol and grew to a final size of $0.2 \times 0.2 \times 0.3$ mm within two weeks in an incubator at 291 K. Crystals with satisfactory quality were identified by careful screening and were selected for data collection (Fig. 3), as the crystals typically exhibited a fairly high mosaicity ($>1.0^\circ$). Radiation damage was observed after protracted exposure during data collection, which caused a decrease in $\langle I/\sigma(I) \rangle$ and an increase in R_{merge} . X-ray diffraction data were collected over a total rotation of 50° and processed. Analysis of the diffraction pattern indicated that these crystals exhibited a cubic symmetry; systematic absences indicated the space group to be $I4_132$ or $I4_332$, with unit-cell parameter $a = 157.61$ Å. Assuming the presence of one molecule per asymmetric unit, the Matthews coefficient was estimated to be $2.95 \text{ Å}^3 \text{ Da}^{-1}$, corresponding to a solvent content of 58.4% (Matthews, 1968), which is within the normal range for protein crystals. Details of the data statistics are given in Table 3.

Initial attempts to solve the crystal structure of PA3346PD by the molecular-replacement method were performed with the monomer structure of a C-terminal phosphatase domain

(15% sequence identity; PDB entry 3es2; I. Levchenko, R. A. Grant, R. T. Sauer & T. A. Baker, unpublished work) or a Ser/Thr phosphatase (15% sequence identity; PDB entry 3zt9; Quin *et al.*, 2012) as the search model, but neither of these attempts provided a clear or correct solution. Owing to low sequence identities based on sequence alignment, the structure of PA3346PD is expected to differ markedly from the available structures. A search for useful heavy-atom derivatives or the preparation of a selenomethionine derivative for phasing by single-wavelength or multiple-wavelength anomalous dispersion and *ab initio* structure determination is therefore in progress. Structural comparison of the apo form and the Mg^{2+} - or Mn^{2+} -bound form of PA3346PD obtained by crystal soaking will be described in the structure paper.

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