Crystallization and preliminary X-ray study of biosynthetic alanine racemase from *Pseudomonas aeruginosa* PAO1

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Biosynthetic alanine racemase (Alr_{PA}) from *Pseudomonas aeruginosa* PAO1 carrying a His₆ tag was expressed in *Escherichia coli* BL21 (DE3) cells and purified by Ni²⁺-chelating affinity and anion-exchange chromatography for X-ray crystallographic analysis. Crystals were grown by the hanging-drop vapour-diffusion method at 289 K in a solution consisting of 4% (v/v) Tacsimate pH 5.0, 14% (w/v) polyethylene glycol 3350 with a protein concentration of 8 mg ml⁻¹. The crystal diffracted to 2.76 Å resolution and belonged to the orthorhombic space group *P*₂₁₂₁₂₁, with unit-cell parameters *a* = 74.12, *b* = 76.97, *c* = 154.80 Å, $\alpha = \beta = \gamma = 90^\circ$.

1. Introduction

Pseudomonas aeruginosa is an important Gram-negative pathogen that is widely distributed in nature and in the respiratory tract and intestinal tract of humans. It is one of the most common causes of nosocomial infections, including infections in intensive care units (Jarvis & Martone, 1992). Patients with cystic fibrosis are prone to a higher risk of life-threatening *Pseudomonas* infections (FitzSimmons, 1993). The high antimicrobial resistance and fatality rate of *P. aeruginosa* have constituted a serious threat to the control of clinical infections in recent years. Therefore, it is vital to identify new antibiotics directed against this organism.

Alanine racemases (EC 5.1.1.1) have been isolated, purified and characterized from several different bacteria (Wood & Gunsalus, 1951; Rosso *et al.*, 1969; Diven *et al.*, 1964; Lambert & Neuhaus, 1972). Alanine racemase belongs to the type III group of pyridoxal 5'-phosphate (PLP)-dependent enzymes and catalyzes the racemization of L-alanine to D-alanine, which is an indispensable building block in the synthesis of the peptidoglycan layer of cell walls, utilizing PLP as a cofactor. Alanine racemase is ubiquitous among prokaryotes but is absent in eukaryotes. Therefore, it is an attractive target for the development of new antimicrobial drugs (Lambert & Neuhaus, 1972; Silverman, 1988).

In *P. aeruginosa* PAO1 there are two independent alanine racemase genes that encode biosynthetic alanine racemase (*alr*_{PA}) and catabolic alanine racemase (*dadX*_{PA}) (Strych *et al.*, 2000). The amino-acid sequence of Alr_{PA} is 50% identical to the sequence of DadX_{PA} (PDB entry 1rcq; LeMagueres *et al.*, 2003; Fig. 1). Based on the apparent molecular weight as analyzed by gel filtration, native PAGE and analytical ultracentrifugation, Alr_{PA} and DadX_{PA} are intermediate between monomeric and dimeric forms (Ju *et al.*, 2011). However, crystal structure analysis demonstrated that DadX_{PA} is a dimer (LeMagueres *et al.*, 2003). Moreover, intermolecular complementation analysis between two defective mutants of Alr_{PA} indicated that the enzyme functions exclusively as a homodimer (Strych & Benedik, 2002).

The structure of biosynthetic alanine racemase (Alr_{PA}) from *P. aeruginosa* PAO1 has not previously been reported. In order to elucidate the biochemical properties of Alr_{PA} and its mode of substrate recognition, a structural investigation was initiated. Here, we report our progress on the crystallization and preliminary X-ray diffraction data collection of the recombinant Alr_{PA} protein carrying a His₆ tag. The three-dimensional structure of this protein is being determined.

Figure 1

Multiple sequence alignment of alanine racemases from *P. aeruginosa* PAO1 (PAAI, Alr_{PA}; 1RCQ, DadX_{PA}) and *P. fluorescens* (2ODO). The stars indicate the catalytic residues.

2. Materials and methods

2.1. Cloning, expression and purification

The gene encoding Alr_{PA} (GenBank accession No. NP_253617) was amplified from *P. aeruginosa* PAO1 genomic DNA by PCR using the primers 5'-CGGGATCCTCAGTGGCTGTAGCGTCGCG-3' and 5'-GGCTCGAGATGCGTCCCCTCGTTGCCAC-3', where the bold sequences represent *Bam*HI and *Xho*I restriction sites, respectively. The PCR product was digested with *Bam*HI and *Xho*I and inserted into the expression vector pET-22b(+) (Novagen, USA). The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) cells (Novagen, USA). The transformed cells were grown in 800 ml LB medium containing 100 µg ml⁻¹ ampicillin at 310 K until the OD₆₀₀ reached 0.6–0.8. The culture was cooled and induced overnight at 289 K with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration).

The cells were harvested by centrifugation at 8600g and 277 K for 15 min and resuspended in 25 ml lysis buffer [50 mM Tris–HCl pH 8.0, 5% (v/v) glycerol, 200 mM NaCl]. After the cells had been homogenized using a high-pressure homogenizer (JNBIO, People's Republic of China), the insoluble cell debris was removed by centrifugation at 48 400g for 30 min at 277 K. The supernatant containing crude protein was loaded onto an Ni²⁺-chelating affinity chromatography column (HiTrap Chelating HP, GE Healthcare, USA) and rinsed with 100 ml wash buffer [50 mM Tris–HCl pH 8.0, 5% (v/v) glycerol, 200 mM NaCl, 50 mM imidazole] to remove non-specifically bound proteins. The bound Alr_{PA} protein was eluted with 8 ml elution buffer [50 mM Tris–HCl pH 8.0, 5% (v/v) glycerol, 200 mM NaCl, 200 mM imidazole]. The eluate was dialyzed against 50 mM Tris–HCl buffer pH 8.0 with 5% (v/v) glycerol, and further loaded onto an anion-exchange chromatography column (1 ml Q HP HiTrap column, GE Healthcare, USA) and eluted using a NaCl gradient. The purified Alr_{PA} protein was dialyzed against 50 mM Tris–HCl buffer pH 8.0 with 5% (v/v) glycerol and concentrated to 20 mg ml⁻¹ at 277 K using an Amicon Ultra centrifugal filter device (30 kDa molecular-weight cutoff; Millipore). The purity of Alr_{PA} was analyzed by 12% SDS–PAGE and the protein concentration was

determined using a NanoDrop device (Thermo Scientific, USA) by recording the absorption at 280 nm.

2.2. Crystallization

Initial crystallization screening was performed in 96-well plates at 289 K using the hanging-drop vapour-diffusion method with commercial crystallization kits from Hampton Research (Crystal Screen, Crystal Screen 2, Index and PEG/Ion kits). The protein concentrations for crystallization screening were 5 and 10 mg ml⁻¹ in 50 mM Tris–HCl buffer pH 8.0, 5% (v/v) glycerol with 10 µM PLP. For optimization, drops composed of 1 µl protein solution mixed with an equal volume of reservoir solution were equilibrated against 80 µl reservoir solution. Optimization of crystallization conditions was

Figure 2

12% SDS–PAGE analysis of Alr_{PA}. Lane M, molecular-mass standards (labelled in kDa); lane 1, purified Alr_{PA} (38.3 kDa).

based on the initial screening by adjustment of the precipitant concentration, the protein concentration and the buffer pH.

2.3. X-ray data collection and processing

For X-ray diffraction experiments, the crystals were quick-soaked in reservoir solution containing 15%(v/v) glycol as a cryoprotectant for 15–20 s. The soaked crystals were mounted in nylon loops and flash-cooled in liquid nitrogen at 100 K for data collection (Teng, 1990; Parkin & Hope, 1998). A X-ray diffraction data set was collected at 100 K using an in-house X-ray source (Rigaku MicroMax-007 HF desktop rotating-anode X-ray generator with a Cu target operated at 40 kV and 30 mA) and a Saturn 944+ detector with a 70 mm crystal-to-detector distance at a wavelength of 1.5418 Å. 360 diffraction frames were collected with 0.5° oscillation per image at 100 K. The crystal diffracted to 2.76 Å resolution. The diffraction data set was indexed, integrated and subsequently scaled using HKL-2000 (Otwinowski & Minor, 1997). The data quality was assessed

Table 1
Diffraction data statistics.

Values in parentheses are for the highest resolution shell.

Diffraction source	Rigaku MicroMax-007 HF
Wavelength (Å)	1.5418
Temperature (K)	100
Detector	Saturn 944+ detector
Crystal-to-detector distance (mm)	70
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	60
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	$a = 74.12, b = 76.97, c = 154.80,$ $\alpha = 90, \beta = 90, \gamma = 90$
Mosaicity (°)	0.738
Resolution range (Å)	50.00–2.76 (2.81–2.76)
No. of observed reflections	159438 (5400)
No. of unique reflections	23107 (931)
Completeness (%)	98.1 (81.5)
Multiplicity	6.9 (5.8)
Average $I/\sigma(I)$	23.9 (7.7)
$R_{\text{merge}}^{\dagger}$ (%)	10.6 (19.1)
Overall B factor from Wilson plot (Å ²)	47.03

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th measurement of reflection hkl and $\langle I(hkl) \rangle$ is the mean intensity of symmetry-related reflections.

using *SFCHECK* (Vaguine *et al.*, 1999) and the solvent content was calculated using *MATTHEWS_COEF* from *CCP4* (Winn *et al.*, 2011). Table 1 gives a summary of data collection and processing.

3. Results

The gene encoding the Alr_{PA} protein was amplified from *P. aeruginosa* PAO1 and cloned into the expression vector pET-22b(+). The recombinant Alr_{PA} with a His₆ tag was expressed in *E. coli* strain BL21 (DE3) and purified by Ni²⁺-chelating affinity and anion-exchange chromatography to electrophoretic homogeneity. The molecular weight of Alr_{PA} was estimated to be about 39 kDa by SDS-PAGE analysis (Fig. 2), coinciding with the calculated molecular weight of 38.3 kDa for recombinant Alr_{PA} with a His₆ tag at the C-terminus.

Figure 3
Typical crystals of Alr_{PA} (about 200 × 50 × 40 μm in size).

Figure 4
Typical X-ray diffraction pattern of an Alr_{PA} crystal. The frame edge is at 2.76 Å resolution.

Initial crystallization screening was performed in 96-well plates at 289 K using the hanging-drop vapour-diffusion method. After 5–7 d of incubation, crystals were obtained in PEG/Ion condition Nos. 24 [small needles; 0.2 M lithium acetate dehydrate, 20%(w/v) polyethylene glycol 3350], 29 [small needles; 0.2 M potassium acetate, 20%(w/v) polyethylene glycol 3350] and 57 [irregular polyhedra; 4%(v/v) Tacsimate pH 4.0, 12%(w/v) polyethylene glycol 3350], and Crystal Screen condition No. 57 (irregular tetrahedra; 0.1 M sodium acetate trihydrate pH 4.6, 2.0 M sodium chloride). Condition No. 57 of PEG/Ion was optimized to obtain crystals suitable for structure determination. By adjustment of the precipitant concentration, the protein concentration and the reservoir pH, well diffracting crystals of suitable size were obtained. Crystals of about $200 \times 50 \times 40 \mu\text{m}$ in size (Fig. 3) were obtained using a solution consisting of 4%(v/v) Tacsimate pH 5.0, 14%(w/v) polyethylene glycol 3350 and a protein concentration of 8 mg ml^{-1} .

The crystals diffracted to beyond $2.5 \text{ \AA}$ resolution. Considering that the completeness in the highest resolution shell is below 70% at higher resolution, the highest resolution of the data was cut off at $2.76 \text{ \AA}$. The crystal belonged to the orthorhombic space group $P2_12_12_1$ (Table 1), with unit-cell parameters $a = 74.12$, $b = 76.97$, $c = 154.80 \text{ \AA}$, $\alpha = \beta = \gamma = 90^\circ$ (Fig. 4). This data set has 98.1% overall completeness and 81.5% completeness in the highest resolution shell. The overall R_{merge} is 10.6%. *MOLREP* (Vagin & Teplyakov, 2010) was employed to calculate the self-rotation peaks. A clear twofold axis in the self-rotation function indicated the presence of two molecules in the asymmetric unit. The solvent content and Matthews coefficient are 57.34% and $2.88 \text{ \AA}^3 \text{ Da}^{-1}$, respectively (Matthews, 1968).

Alr_{PA} shares 49% sequence identity with the alanine racemase from *P. fluorescens* (PDB entry 2odo; H. Tsuge, K. Ohnishi & K. Yokoigawa, unpublished work) and 50% sequence identity with DadX_{PA} (PDB entry 1rcq; LeMagueres *et al.*, 2003) (Fig. 1). The BLAST scores of 1rcq and 2odo are 304 and 311, respectively. 2odo

was finally used as a model for molecular replacement (MR). *MOLREP* (Vagin & Teplyakov, 2010) was employed to determine the initial phases using a monomer model of 2odo. We can see sharp rotation peaks and definite translation peaks in the Alr_{PA} MR. A clear solution was obtained with an R_{free} of 26.23% and an R_{work} of 19.32% using data in the resolution range $50.00\text{--}2.76 \text{ \AA}$. The three-dimensional structure of Alr_{PA} will provide insight into the biochemical properties and the oligomerization state of biosynthetic alanine racemase from *P. aeruginosa* PAO1.

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