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Expression, crystallization and preliminary X-ray crystallographic analysis of alanine racemase from *Acinetobacter baumannii* OXA-23

Acinetobacter baumannii has received much attention owing to its exceptional ability to develop resistance to currently available antibiotics. Alanine racemase (ALR) catalyzes the racemization of L-alanine to D-alanine with pyridoxal 5'-phosphate (PLP) as a cofactor. The D-alanine product is an essential component of the bacterial cell wall and ALR is a potential target for the development of novel antibacterial drugs. The *alr* gene from *A. baumannii* was cloned and the protein (AbALR) was expressed, purified and crystallized. The AbALR crystal diffracted to 2.3 Å resolution and belonged to the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 55.1$, $b = 85.0$, $c = 167.7$ Å. Two protomers were present in the asymmetric unit, with a corresponding V_M value of $2.3 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 47.5%.

1. Introduction

Multidrug-resistant *Acinetobacter baumannii* causes severe infections in immunocompromised and injured patients worldwide, and is considered to be one of the most difficult human pathogens to control and treat (Perez *et al.*, 2008). *A. baumannii* is a Gram-negative bacillus and causes many healthcare-associated infections, such as bacteraemia, pneumonia, meningitis, urinary-tract infections and wound infections (Eliopoulos *et al.*, 2008). In addition, *A. baumannii* has the unique ability to survive for prolonged periods under a wide range of environmental conditions, including the hospital environment, and has an exceptional ability to develop antimicrobial resistance (García-Garmendia *et al.*, 1999; Falagas & Karageorgopoulos, 2008; Lee *et al.*, 2007). We selected alanine racemase as a drug target against *A. baumannii*.

Peptidoglycan is a polymer consisting of sugars and amino acids and is an essential component of bacterial cell walls. Inhibition of its biosynthesis or its specific degradation during bacterial cell growth results in cell lysis. Alanine racemase (ALR; EC 5.1.1.1) catalyzes the racemization of L-alanine to D-alanine, which is a key component of the peptidoglycan layer, especially in cross-linking the bacterial cell walls. Accordingly, ALR is an attractive target for antimicrobial drug development, along with penicillin-binding proteins and D-alanine D-alanine ligase. In this study, the cloning, expression, purification, crystallization and preliminary X-ray crystallographic studies of ALR from *A. baumannii* (AbALR) were carried out. The atomic resolution structure of AbALR would be helpful for the design of a novel antibacterial drug against multidrug-resistant *A. baumannii*.

2. Materials and methods

2.1. Cloning

The *alr* coding sequence of ALR was produced by PCR using *A. baumannii* OXA-23 genomic DNA, which was isolated from a urine specimen of a patient hospitalized in Busan, Republic of Korea (Lee *et al.*, 2007), as a template. The forward and reverse oligonucleotide primers were designed based on the alignment of ten different *alr* coding sequences from *A. baumannii* strains and their sequences were as follows: 5'-CCCCC CAT ATG CGT CAA GCA ACA GTT TAT ATT G-3' and 5'-CCCCC GGA TCC TTA AGT

ACC CTG ACG GAC TGG-3', respectively. The *Nde*I and *Bam*HI restriction sites are shown in bold. The PCR reaction was performed using amfiECO PCR Premix (GenDEPOT, USA). Amplified DNA fragments were purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany); they were then double-digested with *Nde*I and *Bam*HI, cloned into a modified pET11a vector (His-TEV-pET11a, Novagen) containing a 7×His tag upstream of a TEV cleavage site, yielding the recombinant clone His-TEV-pET11a-AbALR, and transformed into *Escherichia coli* BL21 (DE3) cells.

2.2. Overexpression and purification

E. coli BL21 (DE3) cells containing His-TEV-pET11a-AbALR, coding for residues 1–356, were grown at 310 K to an OD₆₀₀ of 0.6 in Luria–Bertani (LB) medium containing 50 µg ml⁻¹ ampicillin. Protein expression was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were cultured at 310 K for an additional 8 h. The cells were harvested by centrifugation at 3000g for 30 min at 277 K (Hanil Supra 30K A1000S-4 rotor,

Seoul, Republic of Korea). The cell pellet was then resuspended in ice-cold lysis buffer [25 mM Tris–HCl pH 7.5, 300 mM NaCl, 15 mM imidazole, 10% (v/v) glycerol, 3 mM β-mercaptoethanol] and homogenized by ultrasonication on ice (Sonomasher). The crude cell extract was centrifuged for 45 min at 19 960g (Hanil) at 277 K to remove cell debris.

The supernatant containing soluble AbALR protein was applied onto Ni–NTA His-Bind resin (Novagen) and affinity purification was performed according to the manufacturer's protocol at 277 K. The 7×His-tagged AbALR protein was then eluted using lysis buffer containing 250 mM imidazole. The eluted AbALR was further purified using a HiTrap Q ion-exchange column (GE Healthcare). Approximately 30 mg of AbALR was purified from 6 l of cell culture. The homogeneity of the purified protein was analyzed *via* SDS–PAGE (Fig. 1). The molecular weight of purified AbALR is 42 kDa instead of the calculated 40 kDa because 19 residues from the His-TEV-pET11a vector remained at the N-terminus of AbALR (Fig. 2). For crystallization, the protein solution was concentrated to a final concentration of 9 mg ml⁻¹ in a buffer consisting of 25 mM Tris–HCl pH 7.5, 15 mM NaCl, 10% (v/v) glycerol, 3 mM β-mercaptoethanol using a Centriprep device (Millipore).

2.3. Crystallization and X-ray data collection

Before setting up the crystallization trials, AbALR stock solution (9 mg ml⁻¹) was incubated with 100 µM PLP (pyridoxal 5'-phosphate) for 30 min. Initial crystallization was carried out at 287 K by the sitting-drop vapour-diffusion method in 96-well Intelli-Plates (Art Robbins) using a Hydra II eDrop automated pipetting system (Matrix) and screening kits from Hampton Research (Index, Crystal Screen, Crystal Screen Cryo, Crystal Screen Lite, PEG/Ion 1 and PEG/Ion 2) and Emerald BioSystems (Wizard Classic 1 and 2 and Wizard Precipitant Synergy). 0.5 µl protein solution was mixed with 0.5 µl reservoir solution and equilibrated against 70 µl reservoir solution. After 2 d, crystals with nine different shapes were observed from 39 conditions (Fig. 3). Crystals were reproduced and optimized using condition 32 from PEG/Ion 2 [2% (v/v) Tacsimate pH 5.0, 0.1 M sodium citrate tribasic dihydrate pH 5.6, 16% (w/v) PEG 3350] by the hanging-drop method, in which drops consisted of 1.0 µl protein solution mixed with 1.0 µl reservoir solution (Fig. 3). Each hanging drop was positioned over 1 ml reservoir solution. Optimization was

Figure 1
Purified AbALR is shown on a 10% SDS–PAGE gel (lane P). Lane M contains molecular-mass markers (labelled in kDa).

Additional residues from the pET11a-HIS-TEV vector remaining at the N-terminus of AbALR:

MGHHHHHHSSENLYFQGH (19 amino acids)

Full-length protein sequence of AbALR (356 amino acids):

(N-terminus)

1-MRQATVYIDRQALQYNLRVKQLAANSKIVSMVKANAYGHGVKDCLAALNASDAFGVACLQEGLEIREL
GFEQPVTLIEGVFSEDEMPVAIEQKFECVHHQQQFEWLIKHKQAYIAQGLKVWVKLNSGMNRLGFKDPEI
IEVIKTLKSEGFTCVLAMHFANADVDHPLNEQQKQFLHIKETCDPILASCCNSAAIFKWPELNFDYVRPG
IMLYGASPFADKTVHDLKPVMTFTAETIALNHIEAGEHVGSTFCAQQAMDIAIVSIGYGDGYPRAYV
KQNFVAIDGKLCPPVGRVSMMDIAIDVTNTQVKLGTQVELWGNHRLVDDVAEANGTIGYELLCLRSSRPVR
QGT-356

(C-terminus)

Figure 2
Amino-acid sequence of the crystallized AbALR (394 amino acids).

achieved by varying the concentration of PEG 3350 and the pH of the 0.1 M sodium citrate tribasic dihydrate. After 2 d, needle-shaped crystals with adequate dimensions were obtained using a reservoir solution consisting of 2% (v/v) Tacsimate pH 5.0, 0.1 M sodium citrate tribasic dihydrate pH 5.5, 16% (w/v) PEG 3350 (Fig. 4). The fully grown crystals ($0.3 \times 0.03 \times 0.01$ mm) were flash-cooled at 100 K in liquid nitrogen using 2% (v/v) Tacsimate pH 5.0, 0.1 M sodium citrate tribasic dihydrate pH 5.5, 16% (w/v) PEG 3350, 20% (v/v) glycerol as a cryoprotectant. X-ray diffraction data were collected from the cryoprotected crystal (at 100 K) with 1° rotation at a crystal-to-detector distance of 250 mm using an ADSC Q270 detector on beamline 7A-SBI of the Pohang Light Source (PLS), Republic of Korea. The crystals diffracted to 2.3 Å resolution. Diffraction data were inte-

grated and scaled using the *HKL-2000* program package (Otwinowski & Minor, 1997).

3. Results and discussion

In the initial crystallization trials, nine different crystal shapes were observed in 39 conditions (Fig. 3). Four different crystals (belonging to four different conditions) that had adequate dimensions were directly picked up from the Intelli-Plates and checked for X-ray diffraction (Figs. 3*b*, 3*c*, 3*d* and 3*e*). Although these crystals diffracted, we could not process the data owing to high mosaicity or multiple crystals. Only the crystals obtained using condition 32 from

Figure 3

Crystals of AbALR with nine different shapes observed from 39 conditions in initial crystallization trials. (a) 2% Tascimate pH 5.0, 0.1 M sodium citrate tribasic dihydrate pH 5.6, 16% PEG 3350 (PEG/Ion 2 condition 32). (b) 0.02 M magnesium chloride hexahydrate, 0.1 M HEPES pH 7.5, 22% poly(acrylic acid sodium salt) 5100 (Index condition 59). (c) 25% PEG 3350, 10% (v/v) 2-methyl-2,4-pentanediol, 200 mM lithium sulfate, 100 mM imidazole-HCl pH 6.5 (Wizard Precipitant Synergy condition 142). (d) 0.17 M ammonium sulfate, 25.5% PEG 4000, 15% glycerol (Crystal Screen Cryo condition 30). (e) 0.1 M magnesium formate dihydrate, 15% PEG 3350 (Index condition 92). (f) 8% Tascimate pH 5.0, 20% polyethylene glycol 3350 (PEG/Ion 2 condition 12). (g) 0.2 M potassium sodium tartrate tetrahydrate, 20% PEG 3350 (Index condition 86). (h) 0.17 M ammonium acetate, 0.085 M sodium acetate trihydrate pH 4.6, 25.5% PEG 4000, 15% glycerol (Crystal Screen Cryo condition 10). (i) 3.5 M sodium formate, 0.1 M sodium acetate trihydrate pH 4.6 (SaltRX condition 30). The scale bars represent 0.01 mm.

Figure 4

The optimized AbALR crystals from Fig. 3(a). AbALR crystals with adequate dimensions ($0.3 \times 0.03 \times 0.01$ mm) were obtained using a reservoir solution consisting of 2% (v/v) Tacsimate pH 5.0, 0.1 M sodium citrate tribasic dihydrate pH 5.5, 16% (w/v) PEG 3350. The scale bar represents 0.02 mm.

Table 1

Data-collection statistics.

Values in parentheses are for the outer shell.

X-ray source	Beamline 7A-SBI, PLS
Wavelength (Å)	0.97951
Unit-cell parameters (Å)	$a = 55.1, b = 85.0, c = 167.7$
Total rotation (°)	360
Mosaicity (°)	1.2
Multiplicity	12.5 (12.9)
Space group	$P2_12_12_1$
Resolution (Å)	50.0–2.3 (2.34–2.30)
No. of observations	386924 (19565)
No. of unique observations	30480 (1494)
Completeness (%)	86.9 (86.3)
$R_{\text{merge}}^{\dagger}$ (%)	8.7 (35.1)
$\langle I/\sigma(I) \rangle$	35.9 (10.5)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl .

PEG/Ion 2 [2% (v/v) Tacsimate pH 5.0, 0.1 M sodium citrate tribasic dihydrate pH 5.6, 16% (w/v) PEG 3350] provided data that could be processed (Figs. 3a and 4). The crystals belonged to the crystallographic space group $P2_12_12_1$. The unit-cell parameters were $a = 55.1$, $b = 85.0$, $c = 167.7$ Å. The space group was assigned by auto-indexing (Otwinowski & Minor, 1997) and the data-collection statistics are provided in Table 1. According to calculation of the Matthews coefficient (Matthews, 1968), there are probably two protomers in

the asymmetric unit, with a corresponding V_M of $2.3 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 47.5%. The structure of AbALR was determined by molecular replacement (MR) using the *PHENIX* package of crystallographic programs (Adams *et al.*, 2010). Alanine racemase from *Pseudomonas aeruginosa* (PDB entry 1rcq; 41% sequence identity; LeMagueres *et al.*, 2003) was used as a search model. MR was successful and indicated the presence of two protomers in the asymmetric unit. The MR solution model was used to run the *AutoBuild* module of the *PHENIX* package (Adams *et al.*, 2010). The best result, with an R factor of 24.0% and an R_{free} of 30.0%, showed good crystal packing. The resulting electron-density maps were clear and fitted the model well. No clashes were found between molecules. Currently, the structure is being refined. The final refined structure will be used to screen for anti-ALR inhibitors and the details will be described in a separate paper.

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