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Crystallization and preliminary X-ray diffraction analysis of alanine racemase from *Pseudomonas putida* YZ-26

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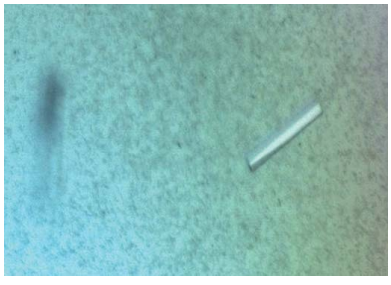

A recombinant form of alanine racemase (Alr) from *Pseudomonas putida* YZ-26 has been crystallized by the sitting-drop vapour diffusion method. X-ray diffraction data were collected to 2.4 Å resolution. The crystals belong to the space group $C222_1$, with unit-cell parameters $a = 118.08$, $b = 141.86$, $c = 113.83$ Å, and contain an Alr dimer in the asymmetric unit. The Matthews coefficient and the solvent content were calculated to be 2.8 Å^3 Da^{-1} and approximately 50%, respectively.

1. Introduction

Alanine racemase (Alr) is an enzyme which catalyses the interconversion of L-alanine to D-alanine with pyridoxal-5′-phosphate (PLP) as a cofactor. Alr is constitutively expressed in bacterial cells and plays a critical role in bacterial growth by providing D-alanine, which is essential for peptidoglycan biosynthesis in the cell wall (Yoshimura & Esak, 2003; Saito *et al.*, 2007; Strych *et al.*, 2000). Since the Alr active pocket is relatively small, the conserved substrate entryway of the protein has been proposed as a potential target for drug design (Wu *et al.*, 2008). Alr has attracted much interest as a candidate for anti-anthrax drugs, as D-alanine is a vital component of the bacterial cell wall. Furthermore, Alr is also accessible in the exosporium and plays a key role in the inhibition of germination in *Bacillus anthracis* (Au *et al.*, 2008). Crystal structures of Alr from different bacteria have been reported, *i.e. Bacillus anthracis* (Au *et al.*, 2008), *Staphylococcus aureus* (MU50) (Scaletti *et al.*, 2012), *Bacillus pseudofirmus* OF4, *Geobacillus stearothermophilus* (Alr GS) (Fenn *et al.*, 2005; Shaw *et al.*, 1997), *Streptomyces lavendulae* (Alr SL) (Noda *et al.*, 2004) and *Mycobacterium tuberculosis* (Alr MT) (LeMagueres *et al.*, 2005).

In order to reveal the structure and function of Alr from *Pseudomonas putida* YZ-26, we cloned the gene of this protein and expressed it in *Escherichia coli*. This is the first report of the crystallization of Alr from *Pseudomonas putida* YZ-26 and the preliminary analysis by X-ray crystallography.

2. Materials and methods

2.1. Expression and purification of Alr

The Alr gene was cloned into the pET32M vector. The recombinant pET-*alr* was transformed into *E. coli* BL21. The cells were grown in LB medium supplemented with 100 µg ml^{-1} ampicillin at 310 K. When the OD_{600} of cells reached 0.6–0.8, the culture flasks were transferred to 289 K and 0.25 m*M* IPTG was added to induce protein expression overnight. Cells were harvested by centrifugation at 4000*g* for 10 min; the cell pellets were resuspended in buffer *A* (50 m*M* Tris–HCl, pH 8.0, 500 m*M* NaCl and 5 m*M* imidazole) and lysed by sonication on ice (SONICS Vibra Cell VC 455, Germany, with 10 s pulses at 40% every 25 s for 15 min). After centrifugation at 13 000 rev min^{-1} (Sorvall SS-34 rotor) for 30 min at 277 K, the supernatant was mixed with 10 ml of Ni^{2+}-NTA agarose (Qiagen, China) in a column (2.6 × 10 cm) and incubated with gentle mixing at 277 K for 2 h. Proteins not bound to Ni-NTA agarose were removed using 100 ml buffer *A*. The target protein was then eluted with 15 ml buffer *B* (50 m*M* Tris–HCl, pH 8.0, 500 m*M* NaCl and 500 m*M*

Table 1
X-ray data-collection statistics for the Alr crystal.

The values in parentheses refer to the highest-resolution shell.

Space group	$C222_1$
Unit cell	a = 118.08, b = 141.86, c = 113.83 Å $\alpha = \beta = \gamma = 90^\circ$
Resolution (Å)	50.00–2.40 (2.40–2.54)
Unique reflections	37702
R_{merge}† (%)	10.2 (54.0)
I/σ	9.4 (1.6)
Average redundancy	4.1 (4.0)
Completeness (%)	99.6 (99)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - I(hkl)| / \sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the mean observations $I_i(hkl)$ of reflection hkl.

imidazole). The enzyme was collected and further polished using a Sephacryl S-200 column (2.6 × 100 cm) pre-equilibrated with 50 m*M* Tris–HCl, pH 8.0 containing 100 m*M* NaCl. The purified target protein was then collected and stored at 277 K for further analysis.

2.2. Analytical ultracentrifugation

Sedimentation velocity was measured in a Beckman ProteomeLab XL-l analytical ultracentrifuge. Sample (400 µl) and buffer (400 µl) solutions were loaded into the double-sector centrepiece, which was built up in a Beckman An-60 Ti rotor. Experiments were performed at 293 K and with a speed of 55 000 rev min^{-1}. Protein sample was monitored by UV absorbance at 275 nm in continuous mode with a time interval of 10 min and a step size of 0.003 cm. Multiple scans at different time points were fitted to a continuous size distribution model by the program *SEDIMENTATION ANALYSIS* (Beckman Coulter Inc.). All size distributions were solved at a confidence level of P = 0.96, a best-fitted average anhydrous frictional ratio (f/f_o), and a resolution N of 200 sedimentation coefficients between 0.1 and 5.2 S. Data were processed using ORIGIN-based *XL-A* analysis software version 6.03 (Beckman Coulter Inc.).

2.3. Crystallization of Alr

A preliminary crystallization screen was carried out by the sitting-drop vapour-diffusion technique (290 K) using Hampton Research Crystal Screen with a protein concentration of approximately 4 mg ml^{-1} (in buffer containing 50 m*M* Tris, pH 8.0, 100 m*M* NaCl and 1 m*M* EDTA). For crystallization screening, 48-well tissue culture plates were used. Typically, 1 µl protein solution was mixed with 1 µl precipitant solution in the drop and equilibrated over 200 µl precipitant solution. After 1 week, crystals of Alr appeared in 0.2 *M* ammonium sulfate and 0.1 *M* sodium acetate trihydrate pH 4.6, 25% PEG 4000. After some improvements of the sample treatment, the qualified crystals of Alr were finally obtained in 0.2 *M* ammonium sulfate and 0.1 *M* sodium acetate trihydrate pH 4.8, 25% PEG 4000.

2.4. Data collection and processing

The crystals were first soaked in cryoprotectant containing paraffin oil (Hampton Research) for 30 s and were then mounted on nylon loops and flash-cooled in a nitrogen-gas stream at 95 K. Diffraction data were collected at the Shanghai Synchrotron Radiation Facility BL17U with a wavelength of 0.979 Å at 100 K, and processed and scaled using *HKL-2000* (Otwinowski & Minor, 1997). Data-collection statistics are summarized in Table 1.

3. Results and discussion

The gene encoding Alr from *Pseudomonas putida* YZ-26 (GenBank ADW54426) was cloned and the protein was overexpressed and purified. The purified Alr showed a single band of around 42 kDa on SDS–PAGE (Fig. 1). During gel filtration on a Superdex-200 column Alr was mainly eluted at the volume expected for a molecular weight of about 80 kDa (Fig. 1). In order to determine the oligomeric state of Alr from *Pseudomonas putida* YZ-26 in solution, we performed the ultracentrifugation assay, which yielded two peaks, a major peak with a calculated molecular weight of 84.2 kDa and a minor peak with a calculated molecular weight of 42.2 kDa (Fig. 2). This result is most consistent with the gel-filtration result, suggesting that the protein mainly exists as a dimer in solution. Most alanine racemases are in dynamic equilibrium between monomeric and dimeric forms, and alanine racemases with high enzymatic activities usually associate with dimeric forms of the enzyme (Ju *et al.*, 2011). The Alr's crystals appeared within 7 d (Fig. 3) and belonged to space group $C222_1$, with unit-cell parameters a = 118.08, b = 141.86, c = 113.83 Å, $\alpha = \beta = \gamma = 90^\circ$. Crystals of Alr can diffract X-rays well and a data set to a resolution of 2.4 Å with good quality has been collected. The asymmetric unit was estimated to contain one dimer with a Matthews coefficient of 2.8 Å^3 Da^{-1} and a solvent content of approximately 50% (Table 1, Fig. 4). Studies to date indicate that Alr is a homo-dimeric enzyme formed by a head-to-tail association of two

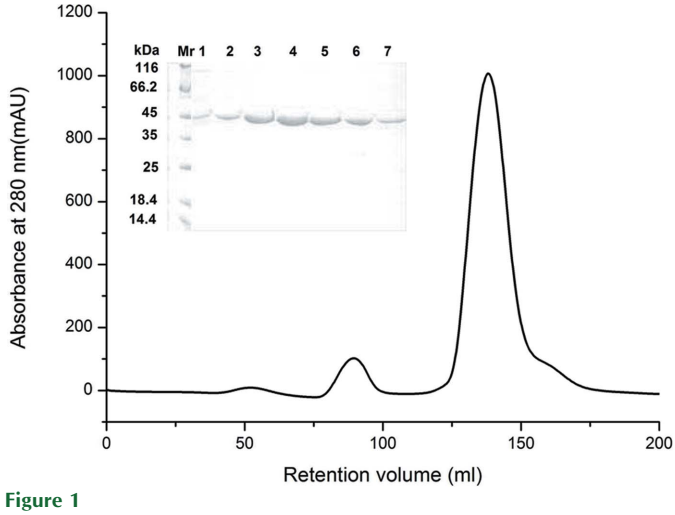

Figure 1
Gel-filtration chromatogram of recombinant Alr using a 26/100 Superdex 200 column and 12% SDS–PAGE of Alr eluted from the gel-filtration column (inset).

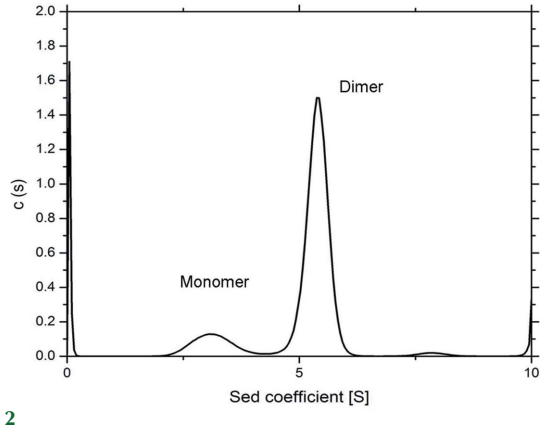

Figure 2
Analytical ultracentrifugation analysis of Alr.

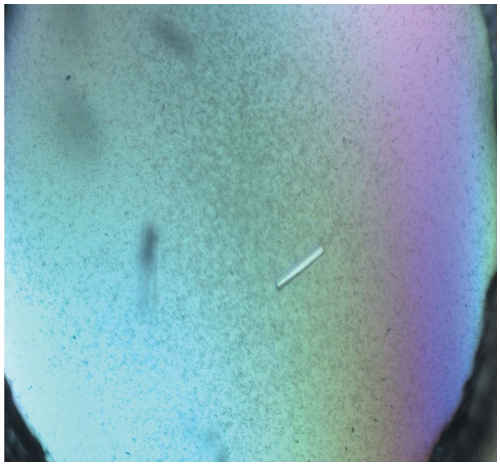

Figure 3
Photograph of the crystals of Alr used for diffraction analysis.

monomers. Each monomer is composed of an N-terminal α/β-barrel and an extended β-strand domain at the C-terminus. Compared with these known structures of Alr, the sequence similarity of Alr from *P. putida* is between 19 and 41% (LeMagueres *et al.*, 2005; Ju *et al.*, 2009; Scaletti *et al.*, 2012). Although the degree of identity among these Alr is low, these structures share the conserved active site and topology.

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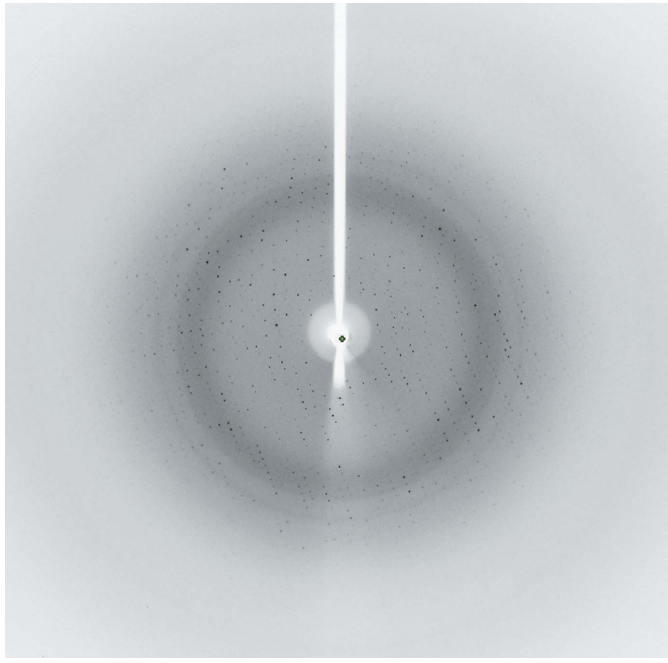

Figure 4
X-ray diffraction image of Alr from *P. putida* YZ-26. The frame edge is at 2.4 Å resolution.

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