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Received 15 March 2013
 Accepted 29 April 2013

Crystallization and preliminary X-ray study of a thermostable alanine racemase from *Thermoanaerobacter tengcongensis* MB4

Alanine racemase (Alr_{MB4}), a dimeric PLP-dependent thermostable enzyme from the anaerobic eubacterium *Thermoanaerobacter tengcongensis* MB4, was expressed and purified with a His₆ tag in a form suitable for X-ray crystallographic analysis. Crystals were grown by the hanging-drop vapour-diffusion method at 289 K using a solution consisting of 0.1 M bis-tris pH 7.0, 22% (w/v) polyethylene glycol 4000. X-ray diffraction data were collected to 2.6 Å resolution. The crystal belonged to the orthorhombic space group *P*2₁2₁2₁, with two protein molecules in an asymmetric unit.

1. Introduction

Alanine racemase (EC 5.1.1.1) belongs to the type III group of pyridoxal 5'-phosphate (PLP)-dependent enzymes and catalyzes the interconversion of L-alanine and D-alanine. Alanine racemase is ubiquitous among prokaryotes, but is typically absent in eukaryotes, and thus offers an attractive target for the development of new antibacterial drugs (Lambert & Neuhaus, 1972; Silverman, 1988; Mustata & Briggs, 2002; Halouska *et al.*, 2007).

Since the first crystal structure of alanine racemase Alr_{BS} from *Bacillus stearothermophilus* was reported (PDB entry 1sft; Shaw *et al.*, 1997), about 36 PDB files for alanine racemase from 16 strains have been deposited in the PDB, such as alanine racemase from *Staphylococcus aureus* (Mu50) (PDB entry 4a3q; Scaletti *et al.*, 2012), *Bacillus anthracis* BA0252 (PDB entries 2vd8 and 2vd9; Au *et al.*, 2008), *Streptomyces lavendulae* (PDB entries 1vfh, 1vfs and 1vft; Noda *et al.*, 2004), *Enterococcus faecalis* (PDB entries 3e5p and 3e6e; Priyadarshi *et al.*, 2009) *etc.* Based on the crystal structure analysis and catalytic mechanisms of alanine racemases, the entrance corridor to the active site consists of three layers divided into the inner, middle and outer layers (LeMagueres *et al.*, 2005). The residues contributing to the middle (Arg316', Ile362, Arg296' and Asp177) and inner (Tyr271', Tyr364, Tyr290' and Ala176) layers of the Alr_{Mtb} from *Mycobacterium tuberculosis* entryway are highly conserved in alanine racemases across different strains such as *S. aureus* (Scaletti *et al.*, 2012), *B. anthracis* (Couñago *et al.*, 2009) and *Escherichia coli* (Wu *et al.*, 2008). The residues of the outer layer are not well conserved between these enzymes (Im *et al.*, 2011).

However, a thermostable alanine racemase Alr_{MB4} (GenBank accession No. AAM24437.1) from *Thermoanaerobacter tengcongensis* MB4, in which the middle and inner layers of the substrate entryway are comprised of six highly conserved residues (middle layer, Arg293', Arg312' and Ile358; inner layer, Tyr287', Tyr268' and Ala172) and two nonconserved residues (Ser173 and Gln360), has gained our attention. Compared with the wild type, mutation at residue Ser173 (Ser173→Asp173) yielded a decrease to 46.4% in enzyme activity, while mutation at residue Gln360 (Gln360→Tyr360) resulted in a 147.9% increase in enzyme activity (J. Ju, unpublished work).

In order to elucidate the biochemical properties of this novel enzyme and the role of both nonconserved residues (Ser173 and Gln360), a crystal of Alr_{MB4} has been obtained. Here, we report the crystallization and preliminary X-ray diffraction data analysis of the

recombinant Alr_{MB4} protein with a His₆ tag. The three-dimensional structure of this protein is being determined.

2. Materials and methods

2.1. Cloning, expression, and purification

Using the *T. tengcongensis* MB4 genome DNA as a template, gene *alr*_{MB4} (GenBank accession No. AAM24437.1) was amplified using the primers 5'-GCTAGCGTGAAATTTGACGGGGTAAGA-3' and 5'-CTCGAGCTTTAAGTAATTTACTTCTCCA-3'; bold sequences represent *NheI* and *XhoI* restriction sites, respectively. The PCR products were digested with *NheI* and *XhoI* and the digests were then inserted into the corresponding cloning sites of plasmid pET-28a(+) (Novagen, USA). The recombinant pET-MBAIr was introduced into *E. coli* BL21 (DE3) (Novagen, USA). The transformants of *E. coli* BL21 (DE3) were grown in 500 ml LB containing 40 µg ml⁻¹ kanamycin at 310 K until the OD₆₀₀ reached 0.5. Expression of the recombinant protein Alr_{MB4} was induced by the addition of 0.1 mM IPTG (final concentration) and growth of the culture continued at 310 K overnight.

The cells were harvested by centrifugation (7585g, 10 min) and the cell pellets were resuspended in 25 ml lysis buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 20 mM imidazole, 10 µM PLP) and disrupted by sonication. Cell debris was removed by centrifugation (26 664g, 10 min). The supernatant was loaded onto a Ni²⁺-chelating affinity chromatography column (GE Healthcare, USA) and the column was rinsed with 25 ml washing buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 50 mM imidazole, 10 µM PLP) and then eluted with 4 ml elution buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 500 mM imidazole, 10 µM PLP). Subsequent purification was performed on FPLC by gel-filtration chromatography (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 10 µM PLP) on a Superdex 200 10/300 GL column (GE Healthcare, USA). The protein fraction was pooled, concentrated and dialyzed against 10 mM Tris-HCl buffer pH 8.0 with 10 µM PLP by ultrafiltration with an Amicon Ultra-15 Centrifugal Filter Device (30 000 Da molecular-weight cutoff, Millipore). The purity of the protein Alr_{MB4} was evaluated by dynamic light scattering and SDS-PAGE [12.5%(w/v)]. The protein concentration was

determined using the BCA Protein Assay Reagent Kit (Pierce, USA) with BSA as a standard.

2.2. Crystallization

Purified protein Alr_{MB4} with a His₆ tag at both the N- and C-termini (N, MGSSHHHHHHSSGLVPRGSHMAS; C, LEHH-HHHH) was used for crystallization screening. Initial crystallization screening was carried out using Crystal Screen, Crystal Screen 2, Index, SaltRx and PEG/Ion from Hampton Research at 289 K by the hanging-drop vapour-diffusion method. The protein concentration was between 5 and 20 mg ml⁻¹ in 10 mM Tris-HCl buffer pH 8.0 with 10 µM PLP. Drops consisting of 2 µl protein solution mixed with an equal volume of reservoir solution were equilibrated against 300 µl reservoir solution. Crystallization conditions were optimized based on the initial screenings.

2.3. X-ray data collection and processing

For X-ray diffraction measurements, Alr_{MB4} protein crystals were first soaked in reservoir solution containing 15%(v/v) glycerol for about 30 s. The soaked crystals were then mounted in nylon loops and flash-cooled in a stream of gaseous nitrogen at 100 K (Parkin & Hope, 1998).

Diffraction data were collected using an in-house X-ray source (Rigaku MicroMax-007 desktop rotating-anode X-ray generator with a Cu target operated at 40 kV and 30 mA) and an R-Axis VI⁺⁺ imaging-plate detector with a 220 mm crystal-to-detector distance at a wavelength of 1.5418 Å. The collected intensities were indexed, integrated, corrected for absorption, scaled and merged using HKL-2000 (Otwinowski & Minor, 1997).

3. Results

A 1164 bp DNA fragment was amplified and cloned into the expression vector pET-28a(+) for the construction of plasmid pET-MBAIr. The recombinant protein Alr_{MB4} with a His₆ tag was successfully expressed and purified to apparent homogeneity. Its molecular mass was estimated to be 47 kDa by SDS-PAGE (Fig. 1), corresponding to the calculated molecular mass of recombinant Alr_{MB4} with a His₆ tag at both the N- and the C-termini. The purified

Figure 1
12% SDS-PAGE analysis of Alr_{MB4}. Lane M, molecular-mass standards (labelled in kDa); lane 1, purified Alr_{MB4} protein (47 kDa).

Figure 2
Typical crystals of Alr_{MB4}. The optimized crystals (about 120 × 120 × 80 µm) were grown using 0.1 M bis-tris pH 7.0, 22%(w/v) polyethylene glycol 4000.

Table 1

Diffraction data statistics.

Values in parentheses are for the outer shell.

Space group	$P2_12_12_1$
Wavelength (Å)	1.5418
Unit-cell parameters (Å, °)	$a = 55.33, b = 89.393, c = 79.305,$ $\alpha = 90, \beta = 104.09, \gamma = 90$
Resolution range (Å)	50.00–2.61 (2.66–2.61)
Total no. of reflections	435550
No. of unique reflections	26108
Average multiplicity	2.1 (1.8)
Completeness (%)	98.7 (98.9)
$R_{\text{merge}}^{\dagger}$ (%)	6.9 (16.7)
Average $I/\sigma(I)$	21.3 (5.0)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, 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 all symmetry-related reflections.

protein Alr_{MB4} was analyzed by dynamic light scattering. When the dynamic light scattering was performed using purified protein at a concentration of 1.6 mg ml^{−1}, a single peak revealed a hydrodynamic radius of 3.7 nm and a monodisperse profile with 99.8% identity. This radius corresponded to a molecular mass of 72 kDa, suggesting that the Alr_{MB4} protein was predominantly in a dimeric form in solution.

Initial crystallization screening was performed at 289 K using 16-well tissue-culture plates. After 6–10 d incubation, crystals of different colour and shape were obtained using Index conditions No. 42 (light yellow irregular sheets), No. 43 (deep yellow cubes), No. 76 (deep yellow cubes), No. 78 (light yellow irregular tetrahedra), No. 91 (deep yellow small prisms) and No. 94 (deep yellow needles). A well diffracting crystal was obtained using condition No. 43 of Index, which consisted of 0.1 M bis-tris pH 6.5, 25% (w/v) polyethylene glycol 3350, with a protein concentration of 10 mg ml^{−1}. Optimization of this condition through adjustment of the precipitant concentration and buffer pH resulted in fewer nucleation sites and yielded crystals of identical size and morphology. The best crystal was about 120 × 120 × 80 µm in size (Fig. 2) and grew with good reproducibility using a solution consisting of 0.1 M bis-tris pH 7.0, 22% (w/v) polyethylene glycol 4000.

Crystallographic data statistics are summarized in Table 1. The data set is 98.7% complete to 2.6 Å resolution (Fig. 3), with an R_{merge} of 6.9%. The crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 55.332, b = 89.393, c = 79.305$ Å, and contains two protein molecules per asymmetric unit (the Matthews coefficient was 2.17 Å³ Da^{−1}, corresponding to a solvent content of 43.29%; Matthews, 1968).

Crystallographic model building and structure refinement are in progress. We hope that the three-dimensional structures will provide insights into deciphering the role of the substrate entryway corridor of the alanine racemase Alr_{MB4} from *T. tengcongensis* MB4, in particular the roles of Ser173 and Gln360.

This work was supported by grants from the NSFC (30970628) and from the Natural Science Foundation of Hebei Province (C2011205045), Hebei Science and Technology Development Foun-

Figure 3

Diffraction pattern of a Alr_{MB4} crystal. The frame edge is at 2.6 Å resolution.

dation (10205521D), Hebei Province Foundation for Returnees (20100705) and the Youth Foundation of Hebei Educational Committee (2011102).

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