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Overexpression, purification, crystallization and structure determination of AspB, a putative aspartate aminotransferase from *Mycobacterium tuberculosis*

A recombinant version of a putative aspartate aminotransferase, AspB (encoded by the ORF Rv3565), from *Mycobacterium tuberculosis* (*Mtb*) was overexpressed in *M. smegmatis* and purified to homogeneity using liquid chromatography. Crystals of AspB were grown in a condition consisting of 0.2 M ammonium phosphate monobasic, 0.1 M calcium chloride dihydrate employing the hanging-drop vapour-diffusion method at 298 K. The crystals diffracted to a limit of 2.50 Å resolution and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 93.27$, $b = 98.19$, $c = 198.70$ Å. The structure of AspB was solved by the molecular-replacement method using a putative aminotransferase from *Silicibacter pomeroyi* (PDB entry 3h14) as the search model. The template shares 46% amino-acid sequence identity with *Mtb* AspB. The crystal asymmetric unit contains four AspB molecules (the M_r of each is 42 035 Da).

1. Introduction

Aspartate aminotransferase (EC 2.6.1.1; AspAT) is a pyridoxal-5'-phosphate (PLP)-dependent enzyme which catalyses the reversible transamination between aspartate and 2-oxoglutarate to give oxaloacetate and glutamate (Kirsch *et al.*, 1984; Fig. 1). It is a key metabolic enzyme that links amino-acid metabolism to carbohydrate metabolism; it also plays a role in ureogenesis and is involved in the transfer of reducing equivalents to the mitochondria *via* the aspartate/malate shuttle (Wilkie *et al.*, 1996). These multiple important roles have led to AspAT being conserved throughout evolution (Jeffery *et al.*, 1998). In eukaryotes, two genetically distinct isoenzymes of AspAT (cytosolic and mitochondrial) occur, whereas only a single isoform has been reported in prokaryotes (Sung *et al.*, 1991). Structures of AspAT from various organisms such as *Escherichia coli* (Smith *et al.*, 1989; Jäger *et al.*, 1994; Okamoto *et al.*, 1994), chicken (Borisov *et al.*, 1980; Ford *et al.*, 1980; Malashkevich *et al.*, 1995; McPhalen *et al.*, 1992), pig (Rhee *et al.*, 1997) and yeast (Jeffery *et al.*, 1998) have been elucidated and have shown that most of the amino acids that constitute the active site are conserved. Moreover, several inhibitors against AspAT, such as maleate and 2-methyl-DL-aspartate (Jeffery *et al.*, 2000; Okamoto *et al.*, 1994), hydroxylamine (Wrenger *et al.*, 2011) and oxamate (Thornburg *et al.*, 2008), have been identified.

Aspartate biosynthesis is an essential pathway required for the growth and survival of *Mycobacterium tuberculosis* (*Mtb*; Sassetti *et al.*, 2003). In this respect, structure analysis of aspartate aminotransferase (AspB) from *Mtb* will not only aid in elucidating the detailed mechanism of its catalysis but will also provide a platform for the structure-based design of small-molecule inhibitors. The *Mtb* genome codes for two aspartate aminotransferases, AspB and AspC (Cole *et al.*, 1998). Reported here are the details of enzyme preparation, crystallization and preliminary X-ray studies of AspB from *Mtb*.

Figure 1

A schematic representation of the transaminase reaction catalysed by AspAT.

Table 1

Sequences of the primers used in PCR for amplification of the Rv3565 gene (aspB), and the amino-acid sequence of recombinant protein AspB.

In the primers, flanking nucleotides are shown in bold and restriction sites are underlined. The forward primer contains four directional cloning specific nucleotides (bold), an *NdeI* restriction site (underlined) and the first 39 nucleotides of the open reading frame (ORF) Rv3565 and the reverse primer contains a *HindIII* restriction site (underlined), flanking nucleotides (bold) and the reverse complement of last 20 nucleotides of the ORF.

Forward primer	5'- CACCCATATG CACCATCATCATCATCACATG- ACGGATCGTGTCCGCCCTG-3'
Reverse primer	5'- TATAAGCTT CTATTGGCTCGGCAGCCAGG-3'
Recombinant protein sequence	MHHHHHMTDRVALRAGVPPFYVMDVWLAARQRTGDLVNLNLS- MTDRVALRAGVPPFYVMDVWLAARQRTGDLVNLNLSAGQP- SAGAPEPVRAAAAAALHNLQGYSLGPELRLDAIAADYQ- RRHGITVEPDVAVITGSSGGFLAFLACFDAGDRVAMASP- GYPCYRNILSALGCEVVEIPCGPQTRFQTAQMLAEIDPPL- RGVVVASPANTGTVIPPEELAAIASWCADSVRLISDEVY- HGLVYQGAQPTSCAWQTSRNVVNSFSKYYAMTGWRLGWL- LVPTVLRRAVDCLTGNFTICPPVLSQIAAVSAFTPEATAEA- DGNLASAIINRSLLDGLRRIGIDRLAPTDFGAFVYVAVDS- FTSDSLAFCSKLLADTGVAIAPGIDFDFTARGGSFVRISFAG- PSGDIEEALRRIGSWLPSQ

2. Materials and methods

2.1. Cloning

The gene (Rv3565) encoding AspB was amplified by the polymerase chain reaction (PCR) method. The PCR consisted of *Mtb* H37Rv genomic DNA as a template, gene-specific primers (Table 1), Phusion polymerase (Finnzymes, Finland), dNTPs and MgCl₂. The PCR product was inserted into an entry vector pENTR using the pENTR/TOPO directional cloning kit (Invitrogen, USA) as per the manufacturer's protocol. AspB was then cloned into an *E. coli*/*M. smegmatis* shuttle vector pYUB1062 (Wang *et al.*, 2010), for which both the recombinant entry construct and the final vector pYUB1602 were digested with the *NdeI* and *HindIII* restriction enzymes. Ligation was carried out using T4 DNA ligase and the reaction mixture was transformed into chemically competent *E. coli* DH5α cells, which were then selected on hygromycin B (150 µg ml⁻¹) Luria–Bertani (LB) agar plates. The final recombinant construct was confirmed by colony PCR, restriction digestion and DNA sequencing (Macrogen Inc, India).

2.2. Overexpression

The recombinant expression plasmid pYUB1062 containing the gene of interest was electroporated into *M. smegmatis* mc²4517 expression host at 2500 V, 1000 Ω and 5 µF using a 2 mm diameter electroporation cuvette (Bio-Rad, USA). The cells were then plated on a 7H10 agar plate with oleic acid–albumin–dextrose–catalase (OADC) nutrient supplements and the antibiotics hygromycin B (100 µg ml⁻¹) and kanamycin (25 µg ml⁻¹) for selection of the transformed cells. Colonies appeared after approximately 72 h, one of which was inoculated into a 50 ml flask containing 10 ml LB broth with 0.05% Tween 80 and 0.2% glycerol (LBTG) to revive the colony. The culture was grown for about 24 h at 310 K at 180 rev min⁻¹. A primary culture was inoculated from the revival culture in 100 ml

LBTG. The culture was incubated for 12 h at 310 K with shaking until the OD₆₀₀ reached around 1. A 4 l secondary culture was set up from the primary culture and was induced with 4.0 mM acetamide at an OD₆₀₀ of about 0.7. The cells were harvested 20 h post-induction by centrifugation at 10 000g for 40 min at 277 K and were frozen at 253 K until further processing.

2.3. Purification

The enzyme AspB was fused with a 6×His tag at the N-terminus to facilitate protein purification using Ni–NTA metal-affinity chromatography. All purification steps were carried out at 277 K. The protein was purified using an ÄKTAexplorer chromatographic system (GE Healthcare Life Sciences, USA). The harvested cells were resus-

Figure 2

Purification profile of AspB. (a). Gel-filtration chromatography profile showing that AspB exists as a dimer in solution. (b). SDS–PAGE analysis of the purified recombinant AspB. Lane M, molecular-mass marker (in kDa).

Table 2

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 93.27$, $b = 98.19$, $c = 198$
Molecules in asymmetric unit	4
Matthews coefficient ($\text{\AA}^3 \text{Da}^{-1}$)	2.71
Solvent content (%)	54.54
Temperature (K)	100
Detector	R-AXIS IV ⁺⁺
Wavelength ($\text{\AA}$)	1.5418
Resolution ($\text{\AA}$)	50.00–2.50 (2.59–2.50)
Unique reflections	63937 (6241)
Multiplicity	4.6 (3.7)
$\langle I/\sigma(I) \rangle$	14.4 (2.0)
Completeness (%)	99.5 (98.1)
$R_{\text{merge}}^\dagger$ (%)	9.3 (62.7)

$^\dagger R_{\text{merge}}(I) = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. $\langle I(hkl) \rangle$ is the average intensity of the i observations.

pended in buffer *A* (50 mM Tris, 300 mM NaCl, 20 μM PLP, 5% glycerol pH 8.0) containing a Complete EDTA-free protease-inhibitor tablet (Roche). The resuspended cells were then homogenized at 186 MPa using a cell-disruptor system (Constant Systems Ltd, UK). The supernatant containing soluble proteins was collected by centrifugation at 10 000g at 277 K for 40 min. After loading the lysate onto the pre-equilibrated Ni-NTA Sepharose High Performance affinity matrix (GE Healthcare Biosciences) column with buffer *A*, the unbound and nonspecifically bound proteins were washed out with buffer *A*, followed by an additional wash with buffer *A* containing 2 M NaCl. The column was further stringently washed with increasing concentration of imidazole in buffer *A* (10, 20, 30 and 40 mM imidazole). The protein was eluted using buffer *A* with 300 mM imidazole. The pooled eluate was then further purified and resolved by gel-filtration chromatography on a Superdex 200 prep-grade 16/60 column pre-equilibrated in buffer *A*. The purity of the protein was analyzed on SDS-PAGE (12%) and the identity of the protein was confirmed by MALDI-TOF analysis (TechnoConcept, India).

2.4. Crystallization and data collection

To determine the appropriate protein concentration for setting up medium-throughput crystallization trials, a pre-crystallization test (PCT), using Hampton Research PCT screens, was performed at

Figure 3

Crystals of AspB. The approximate dimensions of the crystals were $800 \times 200 \times 50 \mu\text{m}$.

293 K with the protein concentration varying from 5 to 20 mg ml^{-1} in buffer *A*. Crystallization experiments encompassing approximately 400 different conditions were set up in 96-well plates employing the hanging-drop method at 296 K using a Mosquito robot (TTP LabTech, UK). A drop size of 1 μl with a 1:1 protein solution (15 mg ml^{-1}) to precipitant ratio and 100 μl of the precipitant solution in the corresponding reservoir was used. Micro-crystals appeared in a few conditions after a week. Among these conditions, crystals grew further in size in 10 d in one of the screens containing 0.4 M ammonium phosphate monobasic. This condition was further optimized by varying the precipitant concentration from 0.1 to 0.6 M. The drop size was kept at 5 μl while maintaining the protein concentrations in a 24-well plate with 750 μl of the precipitant solution in the reservoir. Relatively larger sized crystals began to appear after 2 d in 0.2 M ammonium phosphate monobasic precipitant. However, these crystals did not produce workable quality diffraction data. This prompted further optimization with Additive Screen (Hampton Research). 5 μl drops maintaining a 5:4:1 ratio of protein solution, precipitant and additive, respectively, and 750 μl of the precipitant solution in the reservoir were used. Diffraction-quality rod-shaped crystals were observed in 3 d in an optimized condition consisting of 0.2 M ammonium phosphate monobasic, 0.1 M calcium chloride dihydrate. A single crystal from this drop was mounted on a cryo-loop and a few test diffraction images were collected. The unit-cell parameters of the crystal were computed using these images and a complete diffraction data set was collected on an R-AXIS IV⁺⁺ detector at 100 K. The data set was processed using HKL-2000 (Otwinowski & Minor, 1997). The statistics pertaining to data collection and processing are given in Table 2.

2.5. Structure solution

The structure of AspB was solved in space group $P2_12_12_1$, which was determined on the basis of systematic absences, by the molecular-replacement method using the crystal structure of a monomeric

Figure 4

A representative diffraction image collected at 1.0° oscillation range from a single AspB crystal. The resolution shells are shown by concentric circles.

molecule of a homologous aminotransferase from *Silicibacter pomeroyi* (PDB entry 3h14; New York SGX Research Center for Structural Genomics, unpublished work) as the search model. The search model shares 46% sequence identity with *Mtb* AspB. *Phaser* (McCoy *et al.*, 2007) was used to solve the structure and yielded a model which comprised four molecules in the crystal asymmetric unit. The corresponding Matthews coefficient and solvent content are $2.72 \text{ \AA}^3 \text{ Da}^{-1}$ and 54.76%, respectively (Matthews, 1968). The

molecular-replacement solution was corroborated by self-rotation function analysis of the experimental data. The self-rotation function computed using *MOLREP* from *CCP4* (Winn *et al.*, 2011) in the resolution range 40–3.5 Å revealed the presence of twofold noncrystallographic axes. To start with, the model was subjected to 50 cycles of rigid-body refinement followed by 50 cycles of maximum-likelihood restrained positional refinement using *REFMAC5* (Murshudov *et al.*, 2011) from *CCP4*. At this stage, the values of R_{work}

Figure 5
Stereographic projections of various χ sections of the self-rotation function computed using data for the $P2_12_12_1$ crystal.

and R_{free} were 0.435 and 0.494, respectively. Subsequently, rebuilding of the model by replacing the *S. pomeroi* aminotransferase sequence with the corresponding *Mtb* AspB amino acids, wherever necessary, was carried out by the macromolecular model-building program *Coot* (Emsley & Cowtan, 2004) using its model-manipulation tools. Cycles of positional refinement continued after every round of model rebuilding. The current model has R_{work} and R_{free} values of 0.223 and 0.290, respectively.

3. Results

AspB was successfully cloned in pYUB1062 expression vector and milligram quantities of soluble AspB from *Mtb* were obtained by overexpression in an *M. smegmatis*-based expression system, which is reported to be a better host for overexpressing *Mtb* proteins (Bashiri *et al.*, 2007; Goldstone *et al.*, 2008; Nasir *et al.*, 2012). Protein of crystallographic grade purity was achieved by Ni-NTA metal-affinity and gel-filtration chromatography (Fig. 2). The yield of the His-tagged AspB is approximately 10 mg per litre of culture. Crystals suitable for crystallographic analysis were grown by optimizing the initial condition employing the hanging-drop vapour-diffusion method using 0.2 M ammonium phosphate monobasic, 0.1 M calcium chloride dihydrate (Fig. 3). Crystals of AspB diffracted to a resolution limit of 2.50 Å (Fig. 4) and its three-dimensional structure was solved by the molecular-replacement method using the structure of a putative aminotransferase from *S. pomeroi* as the search model. Analysis of the self-rotation function revealed that the four molecules in the crystal asymmetric unit seem to have 222 molecular symmetry ($\chi = 180^\circ$ section; Fig. 5).

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