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Overexpression, purification and crystallization of lysine ε-aminotransferase (Rv3290c) from *Mycobacterium tuberculosis* H37Rv

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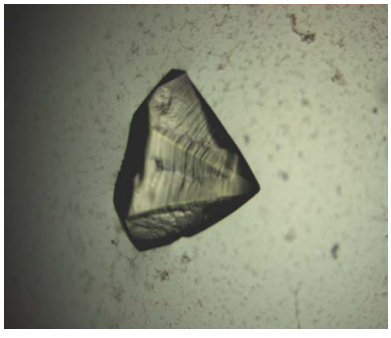
Lysine ε-aminotransferase (LAT) is a protein involved in lysine catabolism; it belongs to the aminotransferase family of enzymes, which use pyridoxal 5′-phosphate (PLP) as a cofactor. LAT probably plays a significant role during the persistent/latent phase of *Mycobacterium tuberculosis*, as observed by its up-regulation by ∼40-fold during this stage. Crystals of recombinant LAT have been grown in 0.1 *M* trisodium citrate dihydrate solution containing 0.2 *M* ammonium acetate and 25% PEG 4000 in the pH range 5.4–6.0. Diffraction data extending to 1.98 Å were collected at room temperature from a single crystal. Crystals are trigonal in shape and belong to space group $P3_121$, with unit-cell parameters a = 103.26, b = 103.26, c = 98.22 Å. The crystals contain a monomer in the asymmetric unit, which corresponds to a Matthews coefficient (V_M) of 3.1 Å^3 Da^{-1}.

1. Introduction

Mycobacterium tuberculosis exhibits significant changes in gene expression during the latent/persistent stage of infection. Proteomic analysis of the nutrient-starved/latent-phase bacteria suggested a decreased expression of proteins involved in energy metabolism, lipid biosynthesis and cell division in addition to induction of stringent-response and several other genes that may play a role in long-term survival within the host (Betts *et al.*, 2002; Stewart *et al.*, 2003). Lysine ε-aminotransferase (LAT) is a member of the superfamily of vitamin B_6-dependent enzymes (Hayashi, 1995) and converts L-lysine to α-aminoadipate-δ-semialdehyde, which is subsequently cyclized to form Δ^1-piperideine-6-carboxylic acid (Soda & Misono, 1968; Soda *et al.*, 1968). It lies within the genomic region upstream of *sigF* involved in mycobacterial stress response and is up-regulated ∼40-fold in nutrient-starved models mimicking the persistent state (Betts *et al.*, 2002).

The degradation of most amino acids starts with transfer of their amino group to α-keto acids. This reaction, called transamination, is catalyzed by aminotransferases, which have pyridoxal 5′-phosphate (PLP) or its converted form pyridoxamine 5′-phosphate (PMP) as the prosthetic group (Miyahara *et al.*, 1994). These enzymes have been divided into four groups based on evolutionary relatedness (Mehta *et al.*, 1993). Lysine ε-aminotransferases (LAT: EC 2.6.1.36), which belong to the group II aminotransferase family (Mehta *et al.*, 1993), catalyse a reversible transamination reaction between L-lysine and an α-ketoacid, which is in this case α-ketoglutaric acid. The LAT subunit has a molecular weight of ∼50 kDa and requires PLP as the cofactor for its catalysis.

PLP-dependent enzymes catalyze a wide variety of reactions in the metabolism of amino acids, including transamination, racemization, deamination, decarboxylation and elimination of β- and α-C atoms (Jansonius, 1998). Alexander *et al.* (1994) reported that they share limited sequence homology and have diversified catalytic specificities. Extensive biochemical and structural studies reveals that PLP-dependent enzymes share a common mechanism of catalysis (Jansonius, 1998). However, determinants of substrate specificities of PLP enzymes vary with each unique enzyme. Proteins of this superfamily catalyze a reaction in which one of the products is usually glutamate, while the other is dictated by the enzyme's specificity for a particular substrate (John, 1995). Each enzyme can therefore recog-



nize at least two substrates. While in the case of glutamate the chemical changes occur at the C^{α} atom, the changes in the case of the unique substrate may occur at the same atom or in many cases at the C atom attached to the distal amino group (Jansonius, 1998). It is generally understood that the overall reaction in this enzyme family proceeds *via* a ping-pong Bi-Bi mechanism (Velick & Vavra, 1962). In case of LAT (Soda & Misono, 1968; Soda *et al.*, 1968) the two half-reactions are the following.

E-PLP + L-lysine ↔ E-PMP + α-aminoadipate-δ-semialdehyde

↓ (spontaneous dehydration)

Δ^1-piperidine-6-carboxylic acid (1)

E-PMP + α-ketoglutarate ↔ E-PLP + glutamate (2)

In addition to its physiological reaction, LAT has been shown to catalyze the first steps in the β-lactam antibiotic biosynthesis pathway in β-lactam-producing actinomycetes (Fujii *et al.*, 2000). The gene encoding *lat* was shown to be located in the β-lactam antibiotic gene cluster in *Streptomyces clavuligerus* (Khetan *et al.*, 2000) and *Nocardia lactamdurans* (Coque *et al.*, 1991), whereas LAT is absent from the genome of most other actinomycetes, confirming that this enzyme is specific for secondary metabolism. LAT is found in a wide range of organisms, such as *Candida guilliermondii* (Der Garabedian & Vermeersch, 1989), *Flavobacterium lutescen* IFO3084 (Soda & Misono, 1968; Soda *et al.*, 1968), *Pseudomonas aeuroginosa* (Fathergill & Guest, 1977) and β-lactam-producing actinomycetes (Madduri *et al.*, 1991). Like most aminotransferases, LAT from various sources is active as a monomer or a homodimer (Fujii *et al.*, 2000).

No crystal structure of the protein is available from any source. Owing to its implied importance in the persistent stage of *M. tuberculosis* infection, we have cloned, expressed, purified and crystallized the *M. tuberculosis* protein (MtLAT), aiming to solve the structure and gain insights into the molecular mechanisms underlying its catalytic activity.

2. Experimental methods

2.1. Protein expression and purification

The *Rv3290c* gene was amplified from *M. tuberculosis* H37Rv genomic DNA using the following primers: sense primer 5′-CAA-**GGATCC**ATGGCCGCCGTCGTGAAG-3′ and antisense primer 5′-ACTA**AAGCTT**CGTCACCACCGGTAACGC-3′, containing *Bam*HI and *Hin*dIII restriction sites (nucleotides in bold). The amplified PCR product was digested with *Bam*HI and *Hin*dIII and ligated in pET23a (Novagen) digested with the same enzyme. Clones were screened by restriction digestion and the resulting construct was transformed into *Escherichia coli* C41 (DE3). The integrity of the insert was verified by sequencing.

A single colony was inoculated into 50 ml YT medium containing 50 µg ml^{-1} ampicillin and grown overnight at 310 K. These cells were used to inoculate 1 l of YT medium containing 50 µg ml^{-1} ampicillin. After 3 h of growth at 310 K, 1 m*M* isopropyl 1-thio-β-D-galactopyranoside was added and the cells were grown for a further 8 h. The IPTG-induced cells were harvested, resuspended in 50 m*M* Tris–HCl pH 7.2, 300 m*M* NaCl and 10 m*M* imidazole (buffer *A*) and sonicated. The crude lysate was centrifuged at 27 000*g* for 30 min. The supernatant was applied onto an Ni^{2+}-IDA column pre-equilibrated with buffer *A*. Protein was eluted using the same buffer supplemented with 500 m*M* imidazole. Fractions containing protein were pooled and precipitated using ammonium sulfate (60%) saturation. The pellet was resuspended in 50 m*M* Tris–HCl pH 7.2, 50 m*M* NaCl, 5 m*M* EDTA, 2 m*M* β-mercaptoethanol (buffer *B*) and further purified on a Superdex S-200 (Amersham Biosciences) gel-filtration column equilibrated with buffer *B*. Protein fractions were pooled and concentrated to 30 mg ml^{-1} using a 30 kDa cutoff Centricon (Amicon). Protein concentrations were determined with the Bradford reagent (Bradford, 1976) using bovine serum albumin as a standard. The protein remained stable at 277 K without degradation for weeks. The purity of the protein was confirmed using 12% SDS–PAGE (Fig. 1).

2.2. Assay

MtLAT activity was determined by the detection of either glutamate or piperidine 6-carboxylate (Soda & Misono, 1968; Soda *et al.*, 1968). Briefly, 15 µ*M* enzyme solution was added to 1.0 ml 200 m*M* phosphate buffer pH 7.2 containing 1 m*M* L-lysine–HCl, 1 m*M* α-ketoglutarate and 15 µ*M* PLP. The mixture was incubated at 310 K for 1 h. The reaction was terminated by the addition of 500 µl 10% trichloroacetic acid in ethanol. Piperidene 6-carboxylate was detected by measuring the colour intensity of its adduct with *o*-amino--aminobenzaldehyde spectroscopically at 465 nm. For detection of the glutamate product, 1 µl of deproteinized mixture was loaded on a TLC plate (coated with silica) and run using *n*-butyl alcohol, methanol, acetic acid and water (4:3:1:1) as the mobile phase. The TLC plate was sprayed with 0.5% ninhydrin solution in ethanol. The position of glutamate was detected visually by the appearance of coloured spots. The production of glutamate was also confirmed by LC–MS mass-spectrophotometric analysis of the deproteinized solution (data not shown).

2.3. Crystallization

Crystallization experiments were set up at 295 K using the hanging-drop vapour-diffusion method (McPherson, 1999), exploiting two different sparse-matrix screening strategies (Mazeed *et al.*, 2003; Jancarik & Kim, 1991; Cudney *et al.*, 1994). Large single crystals (1.4 × 0.7 × 0.4 mm) of Rv3290c were grown over 2–3 d from hanging drops consisting of 2 µl 30 mg ml^{-1} protein and 1 µl reservoir solution containing 0.1 *M* trisodium citrate dihydrate solution in the pH range

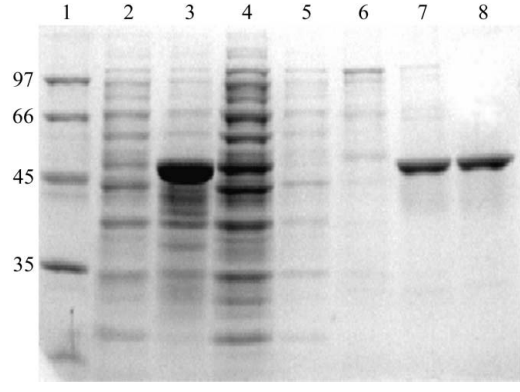


Figure 1
SDS–PAGE analysis of MtLAT during purification. Protein samples were analyzed on 12% SDS–PAGE and stained with Coomassie Blue. Lane 1, molecular-weight markers (kDa); lane 2, uninduced cell lysate; lane 3, induced cell lysate with 1 m*M* IPTG; lane 4, supernatant; lane 5, sample washed with 10 m*M* imidazole; lane 6, sample washed with 80 m*M* imidazole; lane 7, purified protein after Ni^{2+}-IDA column; lane 8, purified protein after size-exclusion chromatography.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell (2.09–1.98 Å).

Wavelength	1.5418
Space group	$P3_121$
Unit-cell parameters (Å)	a = 103.3, b = 103.3, c = 98.2
No. of measured reflections	275297 (38483)
No. of unique reflections	42553 (6123)
Resolution (Å)	24.56–1.98 (2.09–1.98)
R_{merge}	0.120 (0.67)
R_{meas}	0.131 (0.73)
$I/\sigma(I)$	15.9 (3.3)
Completeness (%)	99.9 (100)
Redundancy	6.5 (6.3)

5.4–6.0 containing 0.2 *M* ammonium acetate and 25% PEG 4000. Crystals started appearing within 1–2 d and grew to maximum size in 3–4 d (Fig. 2). Increasing the protein concentration led to a clustering of crystals as well as a reduction in size.

2.4. Data collection and analysis

A single crystal obtained from the trisodium citrate dihydrate and ammonium phosphate condition was mounted in a capillary. Diffraction data were collected on a Rigaku RU300 X-ray generator using a MARdtb image-plate detector. The crystal remained stable at room temperature throughout data collection. All data were indexed and integrated with *MOSFLM* (Leslie, 1992) and reduced with *SCALA* (Evans, 1993) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The crystals are trigonal, with unit-cell parameters a = 103.27, b = 103.27, c = 98.22 Å, and belong to space group $P3_121$. The crystal diffracted to 1.98 Å resolution. The data-collection statistics are summarized in Table 1. The crystal mosaicity refined to around 0.35 and an overall data completeness of 99.9% was obtained.

Size-exclusion chromatography experiments had earlier indicated that the protein exists as a dimer. Calculation of the Matthews coefficient (Matthews, 1968) suggests that the asymmetric unit contains one molecule. Assuming a single molecule in the asymmetric unit corresponds to a Matthews coefficient of 3.1 $Å^3\,Da^{-1}$ or a solvent content of about 59.8%.

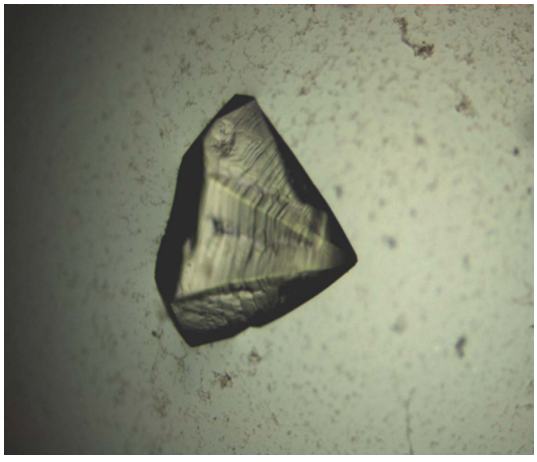


Figure 2
Crystal of Rv3290c (MtLAT) grown in 0.1 *M* trisodium citrate dihydrate pH 5.7, 0.2 *M* ammonium acetate and 25% PEG 4000.

3. Results

MtLAT was cloned in vector pET23a (Invitrogen) with a C-terminal hexahistidine tag and was purified by Ni^{2+}-IDA affinity column chromatography. The recombinant protein is expressed in a soluble form in *E. coli* strain C41(DE3). Prior to crystallization, the protein was concentrated to ∼30 mg ml^{-1}. The purified protein gave a single band corresponding to a molecular weight of 50 kDa on SDS–PAGE. Size-exclusion chromatography indicates that MtLAT is dimeric in nature. An activity assay of the enzyme clearly shows that it exhibits lysine aminotransferase activity. A total of 275 297 measured reflections were merged into 42 553 unique reflections with an R_{merge} of 12%. A sequence alignment with known structures showed that the sequences of γ-aminobutyric acid aminotransferase from pig (Storici *et al.*, 2004; PDB code 1ohv), human ornithine aminotransferase (Shen *et al.*, 1998; PDB code 1oat) and γ-aminobutyric acid aminotransferase from *E. coli* (Liu *et al.*, 2004; PDB code 1sf2) have a nearly identical level of sequence similarity. In our search to find a suitable model for molecular replacement, these three structures were superposed using *PROFIT* (http://www.bioinf.org.uk/software/profit) and parts of 1ohv that structurally align well with 1oat and 1sf2 were identified visually. Structurally unaligned regions of 1ohv (about 50 residues) were deleted from the search model. In the search model, non-identical amino acids were replaced by alanine. Molecular-replacement calculations were carried out using *AMoRe* (Navaza, 1994). A clear solution with good packing was obtained with a correlation coefficient of 56.2% and an R factor of 47.8% using data between 15 and 3 Å. Phase improvement and model building are presently in progress, as is crystallization of protein–substrate complexes.

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