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Cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of LysA (Rv1293) from *Mycobacterium tuberculosis*

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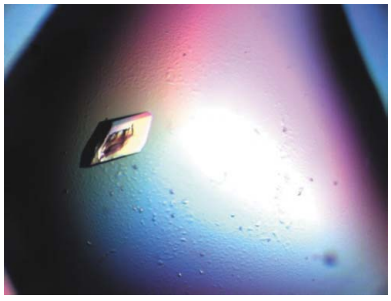

Diaminopimelate decarboxylase from *Mycobacterium tuberculosis* (LysA, DAPDC, Rv1293) has been cloned and heterologously expressed in *Escherichia coli*, purified using standard chromatographic techniques and crystallized. Preliminary diffraction data analysis suggests the presence of a homotetramer in the asymmetric unit.

1. Introduction

Recently, the enzymes of the lysine-biosynthetic pathway have drawn considerable attention as potential targets for new antibacterial drugs and herbicides (Hutton *et al.*, 2003). In both plants and bacteria, the lysine-biosynthetic pathway starts from L-aspartate and leads to L-lysine in nine enzymatically catalysed steps. The final step in this pathway is carried out by the enzyme diaminopimelate decarboxylase (LysA, DAPDC), which converts *meso*-diaminopimelate (DAP) to lysine in a PLP-dependent decarboxylation reaction. The crystal structures of DAPDC from *Escherichia coli* in complex with PLP and with PLP and lysine have been determined (Momany *et al.*, 2002; V. Levdikov, L. Blagova, N. Bose & C. Momany, unpublished results; PDB entries 1knw and 1ko0), as have the crystal structures of the enzyme from *Methanococcus jannaschii* in complex with a substrate analogue as well as in complex with PLP and lysine (Ray *et al.*, 2002; PDB entries 1twi and 1tuf). More recently, the crystal structure of the *Mycobacterium tuberculosis* enzyme has been determined in complex with lysine as well as in complex with both PLP and lysine (Gokulan *et al.*, 2003; PDB entries 1hkv and 1hkw). The biologically active unit of DAPDC appears to be a homodimer consisting of two domains per subunit. For the enzyme from *M. tuberculosis*, domain 1 is a $(\beta/\alpha)_8$-barrel consisting of residues 48–308, whereas domain 2 is a β-sandwich consisting of residues 1–47 and 309–446 (Gokulan *et al.*, 2003). The two active sites per dimer are located at the subunit interface and are made up from residues of both domains. In the case of the DAPDC–PLP complex, PLP is covalently attached to the amino group of Lys73 (*M. tuberculosis* DAPDC numbering). All of the DAPDC structures reported so far are structures of the enzyme in complex with either the cofactor or a substrate analogue or the product lysine, meaning that the structure of the free enzyme, required for a more complete structural analysis, has still to be elucidated. Here, we report the crystallization of the apoenzyme of *M. tuberculosis* DAPDC in a new crystal form and preliminary X-ray diffraction investigation of this crystal form.

2. Experimental methods

2.1. Cloning

Genomic DNA from the H37Rv strain of *M. tuberculosis* was used as the template for the polymerase chain reaction. The following oligonucleotides (Invitrogen) were used as forward and reverse primers, respectively: 5′-GGGG**CATATG**GCTGTGAACGAGCTG-CTGCACTTAGCGCCGAATGTGTGG-3′ and 5′-GGGG**AAG-CTT**ACCTCGTGGTACACCCCTCACTTCCAAACTCAGCAAA-TCGTCGACCGTCTCCC-3′. In the forward primer the GCT triplet coding for Ala (underlined) was introduced as the second codon to

increase the efficiency of expression (Looman *et al.*, 1987). In the reverse primer a thrombin-cleavage site (underlined) was introduced. The amplified fragment containing 5′-*Nde*I and 3′-*Hin*dIII restriction sites (in bold) was cloned into the pCR-BluntII-TOPO vector (Invitrogen). After sequence confirmation, the insert was subcloned into the pET22b expression vector (Novagen), which adds a His_6 tag to the expressed recombinant protein. Both the pCR-BluntII and the final pET22b constructs were sequenced to confirm the cloning of the *lysA* gene sequence.

2.2. Expression and purification

The recombinant plasmid was used to transform *E. coli* BL21 Star (DE3) pRARE cells. These cells were prepared by transforming BL21 Star (DE3) cells (Invitrogen) with the pRARE plasmid isolated from the Rosetta strain (Novagen). Cells from an overnight 5 ml preculture were grown in LB broth medium containing chloramphenicol (30 µg ml^{-1}) and ampicillin (50 µg ml^{-1}) at a temperature of 310 K and shaken at 200 rev min^{-1}. The culture was induced with 0.25 m*M* isopropyl *β*-D-thiogalactopyranoside (IPTG) at an OD_{600} of approximately 0.6 at 293 K. Following induction, the culture was incubated for about 15 h at 293 K and 220 rev min^{-1} and then harvested. The cells were frozen and stored at 193 K until further processing. 1 g of cell pellet was dissolved in 10 ml buffer *A* [20 m*M* Tris pH 8.0, 250 m*M* NaCl, 10 m*M* imidazole, 5%(*v*/*v*) glycerol], 3 m*M* *β*-mercaptoethanol (*β*-ME) and one Complete Mini EDTA-free Protease Inhibitor Cocktail tablet (Roche) per 30 ml and then lysed by sonication for 3 × 5 min in 0.4 s pulses at 277 K. The cell debris was pelleted by centrifugation for 60 min at 277 K and 20 000 rev min^{-1}. The crude lysate was filtered through a 0.22 µm membrane and loaded onto a 5 ml Hi-Trap Chelating HP column charged and equilibrated with Ni^{2+} and buffer *A*, respectively. In order to remove unbound proteins, the column was first washed with five column volumes of buffer *A*, then with five column volumes of buffer *B* [20 m*M* Tris pH 8.0, 1 *M* NaCl, 10 m*M* imidazole, 5%(*v*/*v*) glycerol, 2 m*M* *β*-ME] and finally with three column volumes of buffer *C* [20 m*M* Tris pH 8.0, 250 m*M* NaCl, 50 m*M* imidazole, 5%(*v*/*v*) glycerol, 2 m*M* *β*-ME]. The protein was eluted by running a linear gradient from 50 to 800 m*M* imidazole (in buffer *C*). The major peak fractions were pooled and dialyzed against buffer *D* [20 m*M* Tris pH 8.0, 250 m*M* NaCl, 5%(*v*/*v*) glycerol, 5 m*M* EDTA, 5 m*M* *β*-ME]. The protein was subsequently purified by gel filtration (Superdex 200, 16/60) using buffer *E* [20 m*M* Tris pH 8.0, 250 m*M* NaCl, 5%(*v*/*v*) glycerol, 5 m*M* DTT] for both equilibration and elution. The protein eluted with an apparent molecular weight of approximately 100 kDa, which is consistent with a homodimer. The peak fractions were analyzed by SDS–PAGE, pooled and concentrated to 6 mg ml^{-1}. Both columns used for purification were supplied by Amersham Pharmacia Biotech. SDS–PAGE and dynamic light scattering were used to assess the chemical and conformational purity of the protein, respectively.

2.3. Crystallization

Recombinant DAPDC with an additional Ala residue in position 2 and the C-terminal extension GVPRGKLAAALEHHHHHH was crystallized using the hanging-drop vapour-diffusion method (2 µl protein solution and 2 µl reservoir solution) in the presence of 20–23%(*w*/*v*) polyethylene glycol monomethylether 5000, 0.1 *M* MES pH 6.1–6.6 and 60 m*M* ammonium sulfate. Crystals appeared within a week and grew to a maximum size of 300 × 300 × 300 µm. They diffract X-rays to about 2.2 Å resolution.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

No. of crystals	1
Wavelength (Å)	0.8126
Crystal-to-detector distance (mm)	210
Rotation range per image (°)	0.5
Total rotation range (°)	200
Resolution range (Å)	99.0–2.33 (2.37–2.33)
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 75.78, b = 106.88, c = 121.93, β = 104.99
Mosaicity (°)	0.9
Total No. of reflections	321789
Unique reflections	80203
Redundancy	4.0
$I/\sigma(I)$	17.8 (2.3)
Completeness (%)	99.9 (99.9)
R_{merge} (%)	7.7 (62.3)
$R_{r.i.m.}$ (%)	8.8 (72.1)
$R_{p.i.m.}$ (%)	4.4 (35.9)
Overall B factor from Wilson plot (Å^2)	42.5
Optical resolution (Å)	1.77

2.4. Diffraction data collection and processing

For data collection, a crystal was quickly immersed in reservoir solution containing 20%(*v*/*v*) MPD and flash-cooled to 100 K in a nitrogen stream. Diffraction data were then collected on the X11 beamline (EMBL Hamburg, Germany) using a MAR CCD detector. The data were indexed and integrated using *DENZO* (Otwinowski & Minor, 1997) and scaled using *SCALEPACK* (Otwinowski & Minor, 1997). The redundancy-independent merging *R* factor $R_{r.i.m.}$ as well as the precision-indicating merging *R* factor $R_{p.i.m.}$ (Weiss, 2001) were calculated using the program *RMERGE* (available from http://www.embl-hamburg.de/~msweiss/projects/msw_qual.html or from MSW upon request). The relevant data-collection and processing parameters are given in Table 1. Intensities were converted to structure-factor amplitudes using the program *TRUNCATE* (French & Wilson, 1978; Collaborative Computational Project, Number 4, 1994). The optical resolution of the data set was calculated using the program *SFCHECK* (Vaguine *et al.*, 1999). Calculation of the self-rotation function was carried out using the program *MOLREP* (Collaborative Computational Project, Number 4, 1994) and structure-factor amplitudes to a maximum resolution of 4.0 Å.

3. Results and discussion

A single crystal of LysA from *M. tuberculosis* grown under the conditions described above is shown in Fig. 1. The presence of lysine

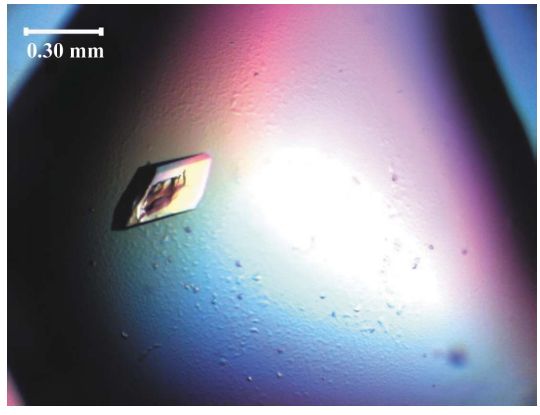

Figure 1
Crystal of LysA from *M. tuberculosis*.

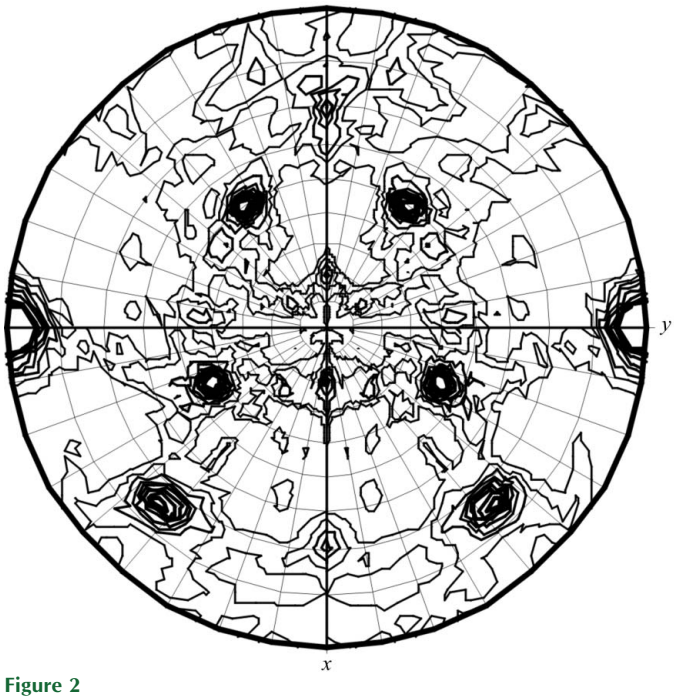

Figure 2
Self-rotation function, $\kappa = 180^\circ$ section. The figure was produced using the program *MOLREP* (Collaborative Computational Project, Number 4, 1994).

was not required for successful LysA crystallization, which is in contrast to the findings reported by Gokulan *et al.* (2003), who only managed to crystallize LysA in the presence of lysine. Moreover, if crystallization was attempted in the presence of lysine, only poorly diffracting crystals ($d_{\rm min} = 6$ Å or less) were obtained. Despite the fact that the crystallization conditions resemble very much those reported by Gokulan *et al.* (2003), the space group and unit-cell parameters are very different and seemingly unrelated (Table 1). The reason for this is probably the longer C-terminal extension and the extra Ala residue at position 2 of the polypeptide chain construct used in this study. The crystals also do not exhibit a distinctly yellow colour; it is therefore likely that the apoprotein has been crystallized.

Based on the molecular weight of the polypeptide and the volume of the unit cell, it is most likely that four molecules are present in the asymmetric unit. This would correspond to a Matthews parameter of 2.5 Å^3 Da^{-1} and a solvent content of 51%. Since the biological unit of LysA is a dimer, it may therefore be expected that the four molecules in the asymmetric unit occur as two homodimers. However, the self-rotation revealed the presence of three distinct peaks on the $\kappa = 180^\circ$ section (Fig. 2). This is inconsistent with two separate homodimers, but suggests that a homotetramer of D_2 symmetry is present in the asymmetric unit. Since the protein eluted as an apparent dimer from the gel-filtration column, it may be that the homotetramer is really a dimer of dimers and that the formation of the tetramer is a consequence of the crystallization conditions. The exact nature and architecture of the LysA tetramer present in this crystal form will have to await the determination of the three-dimensional structure.

4. Conclusion

The protein LysA from *M. tuberculosis* has been heterologously expressed in *E. coli* and purified to homogeneity. Crystals grown in the absence of substrate or cofactor are colourless and appear to contain one homotetramer of LysA in the asymmetric unit.

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