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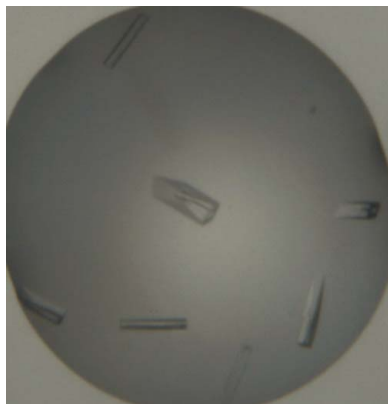
Crystallization and preliminary X-ray diffraction analysis of L,L-diaminopimelate aminotransferase (DapL) from *Chlamydomonas reinhardtii*

In the anabolic synthesis of diaminopimelate and lysine in plants and in some bacteria, the enzyme L,L-diaminopimelate aminotransferase (DapL; EC 2.6.1.83) catalyzes the conversion of tetrahydrodipicolinic acid (THDPA) to L,L-diaminopimelate, bypassing the DapD, DapC and DapE enzymatic steps in the bacterial acyl pathways. Here, the cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of DapL from the alga *Chlamydomonas reinhardtii* are presented. Protein crystals were grown in conditions containing 25% (w/v) PEG 3350 and 200 mM lithium sulfate and initially diffracted to ~ 1.35 Å resolution. They belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 58.9$, $b = 91.8$, $c = 162.9$ Å. The data were processed to 1.55 Å resolution with an R_{merge} of 0.081, an $R_{\text{p.i.m.}}$ of 0.044, an $R_{\text{r.i.m.}}$ of 0.093 and a V_M of $2.28 \text{ \AA}^3 \text{ Da}^{-1}$.

1. Introduction

The essential amino acid lysine (Lys) is synthesized *via* two routes. One pathway uses the intermediate α -amino adipic acid (AAA) and occurs in yeast, fungi and several archaeal species (Nishida *et al.*, 1999; Velasco *et al.*, 2002). The other pathway utilizes the intermediate *meso*-diaminopimelate (*m*-DAP). This pathway is present in most bacterial species and photosynthetic cohorts. We recently discovered a variant of the DAP pathway by the identification and characterization of the enzyme L,L-diaminopimelate aminotransferase (DapL; EC 2.6.1.83) from the organism *Arabidopsis thaliana* (Hudson *et al.*, 2006) and subsequently from a number of bacterial species, including some that are deemed to be pathogenic (McCoy *et al.*, 2006; Hudson *et al.*, 2008; Liu *et al.*, 2010).

There are four variants of the DAP/Lys pathway and all share the same enzymatic steps for the synthesis of tetrahydrodipicolinic acid (THDPA) from aspartate (Hudson *et al.*, 2006). What makes these anabolic pathways unique is the synthesis of *m*-DAP from tetrahydrodipicolinic acid (THDPA). Most bacteria employ the use of acetylated or succinylated (acyl) intermediates to convert THDPA to L,L-DAP using the enzymes DapD, DapC and DapE (Fig. 1). Another variant of the pathways utilizes the enzyme *m*-DAP dehydrogenase (Ddh), which converts THDPA to *m*-DAP in a single step, bypassing the DapD, DapC, DapE and DapF enzymatic reactions that are present in the acyl pathways (Fig. 1). The recently discovered DapL pathway synthesizes L,L-DAP from THDPA using the amino acid glutamate as the amino donor and THDPA as the amino acceptor in one step, bypassing the DapD, DapC and DapE steps that are present in the bacterial acyl pathways (Fig. 1; Hudson *et al.*, 2006). The conversion of *m*-DAP to lysine, catalyzed by the enzyme *m*-DAP decarboxylase (LysA), is the ultimate step in the pathway. This enzymatic reaction is common to all four variants. Given that animals do not possess the machinery necessary for the *de novo* biosynthesis of lysine, the enzymes associated with this pathway are attractive targets for the development of antibiotics, herbicides and algacides. Accordingly, we have been engaged in study of the structure and function of enzymes of lysine biosynthesis from a variety of pathogens (Dobson *et al.*, 2004, 2005; Boughton *et al.*, 2008; Burgess, Dobson, Bailey *et al.*, 2008; Griffin *et al.*, 2008; Domigan *et al.*, 2009).



Recently, the macromolecular structure of DapL from *Arabidopsis* was solved *via* X-ray crystallography (Watanabe *et al.*, 2007, 2008). These structures underpinned the rational design of a range of inhibitors of the DapL enzyme, which have been developed to bind the active site (Fan *et al.*, 2010).

We are interested in studying the macromolecular structure of DapL from the alga *Chlamydomonas reinhardtii*, which is a critical step in our effort to rationally design novel algaecides. Here, we present the cloning, recombinant expression, purification, crystallization and preliminary crystallographic studies of DapL from *C. reinhardtii*.

2. Materials and methods

2.1. *Chlamydomonas* growth, total RNA isolation and cDNA synthesis

C. reinhardtii strain CC-1690 was obtained from the Chlamydomonas Culture Collection (<http://www.chlamy.org/>) and was grown in Tris–acetate–phosphate (TAP) medium. The strain was grown with a 16 h light and 8 h dark period for 7 d. The temperature was 297 K during the light period and 293 K during the dark period. The light intensity was approximately $120 \mu\text{E m}^{-2} \text{s}^{-1}$.

Total RNA was isolated from *C. reinhardtii* using the RNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) using the manufacturer's protocol. cDNA was synthesized in a reaction containing 1 μl oligo(dT)_{12–18} primer, 5 μg total RNA, 1 μl 10 mM dNTP mix and DEPC-treated water up to 13 μl . The mixture was incubated at 338 K

for 5 min, followed by incubation on ice for 5 min. The Reverse Transcription System Kit (Promega, Madison, Wisconsin, USA) was used to synthesize cDNA following the manufacturer's protocol.

2.2. Amplification and cloning of the *C. reinhardtii* dapL cDNA

The full-length cDNA annotated by the locus tag CHLRE-DRAFT_129557 was amplified by PCR. The cDNA was amplified using 12 pmol forward and reverse primers, 1 mM MgSO₄, 0.5 mM of each of the four deoxynucleotide triphosphates, 2 μl cDNA product and 1 U Platinum Pfx DNA polymerase (Invitrogen Corporation, Carlsbad, California, USA) using the following PCR conditions: one cycle at 367 K for 2 min followed by 30 cycles of 367 K for 15 s, 333 K for 30 s and 345 K for 2 min. The forward and reverse primers used to amplify the gene were 5'-CCCCCGAATTCATGCAGCTCAACGTGCGGTCCACCGCCAGC-3' and 5'-CCCCCAAGCTTCTAGT-TACGCTTGCCGTAGGCCTCCTTAAA-3'. The bold sequences represent the restriction-enzyme sites used in the cloning of the cDNA, while the italicized sequences represent initiation and termination codons. For cloning, the PCR fragment was digested with *Eco*RI and *Hind*III and ligated into the plasmid pET30a (EMD Biosciences, Gibbstown, New Jersey, USA) to produce the plasmid pET30a-Cr-DapL. The recombinant protein derived from this plasmid carries a hexahistidine and S-TAG epitope derived from the pET30a plasmid at the amino terminus. To confirm the fidelity of the PCR reaction, the PCR fragment was sequenced from the pET30a construct using the T7 promoter primer 5'-TAATACGACTCACT-ATAGGG-3' and the T7 terminator primer 5'-TATGCTAGTTA-

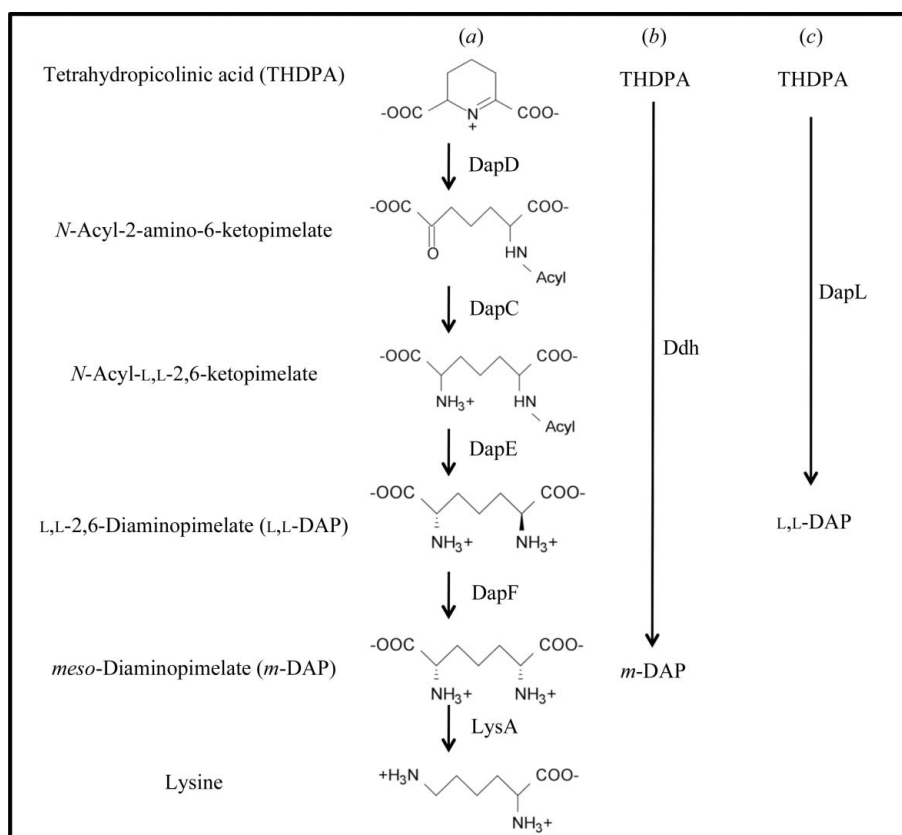


Figure 1

Lysine anabolic pathways as denoted by the acyl pathways (a), the dehydrogenase pathway (b) and the L,L-diaminopimelate aminotransferase pathway (c). The abbreviations of the enzymes are as follows: tetrahydrodipicolinate acylase, DapD; acyl-amino-ketopimelate aminotransferase, DapC; acyl-ketopimelate deacylase, DapF; diaminopimelate epimerase, DapE; diaminopimelate decarboxylase, LysA; *m*-diaminopimelate dehydrogenase, Ddh; L,L-diaminopimelate aminotransferase, DapL.

TTGCTCAG-3' and was compared with the published sequence deposited in the public database (<http://www.chlamy.org/cgi-bin/webblast.pl>).

2.3. Expression and purification

The plasmid pET30a-Cr-DapL was transformed into *Escherichia coli* BL21-CodonPlus-RIPL strain (Agilent Technologies, La Jolla, California, USA). For protein expression and purification, the strain was grown in LB broth containing 50 $\mu\text{g ml}^{-1}$ kanamycin and 34 $\mu\text{g ml}^{-1}$ chloramphenicol at 310 K to an OD_{600} of 0.5. Protein expression was induced with 0.5 mM IPTG for 4 h at 298 K. The cells were lysed by sonication in a solution consisting of 50 mM sodium phosphate pH 8.0 and 300 mM NaCl. The soluble extract was incubated with 1.5 ml Talon Metal Affinity Resin (Clontech, Mountain View, California, USA) for 30 min at 277 K. The resin was washed three times with sonication buffer containing 10 mM imidazole pH 8.0 and the enzyme was eluted with sonication buffer containing 250 mM imidazole. The pure protein was concentrated in an Amicon Ultra 10 kDa molecular-weight cutoff spin-filter unit, replacing the elution buffer with 100 mM HEPES containing 1 mM DTT and 2 mM EDTA pH 7.6 for storage. Prior to crystallization, the purified protein was subjected to size-exclusion chromatography on an S200 column pre-equilibrated with 20 mM Tris-HCl, 5 mM DTT, 2 mM EDTA pH 7.8 to remove any precipitated protein. The protein was then concentrated with an Amicon Ultra 10 kDa molecular-weight cutoff spin-filter unit.

2.4. Crystallization

Crystallization screens were conducted as described previously (Atkinson *et al.*, 2009; Burgess, Dobson, Dogovski *et al.*, 2008; Dobson *et al.*, 2008). Protein crystallization experiments were performed at the Bio21 Collaborative Crystallization Centre (C³; CSIRO, Parkville, Melbourne, Australia) using the PACT Suite and JCSG+ Suite crystal screens at 281 and 293 K (Newman *et al.*, 2005, 2008). The screens were set up using the sitting-drop vapour-diffusion method. The crystals shown in Fig. 3(a) were obtained at 293 K from a 300 nl drop formed from 150 nl protein solution (8.6 mg ml⁻¹ in 20 mM Tris-HCl, 5 mM DTT, 2 mM EDTA pH 7.8) and 150 nl reservoir solution [JCSG+ condition H9; 200 mM lithium sulfate,

25% (w/v) polyethylene glycol 3350, 100 mM bis-tris propane pH 5.5 including 0.02% (w/v) sodium azide].

2.5. Data collection and processing

For X-ray data collection, the crystal was briefly soaked in cryo-protectant solution consisting of reservoir solution made up to 20% (v/v) glycerol and flash-cooled in liquid nitrogen. Intensity data were collected at 110 K at the Australian Synchrotron (MX2 beamline). Data were collected in 0.5° rotations over 110° using an ADSC Q315r CCD positioned 120 mm from the crystal with an exposure time of 2.5 s and 80% attenuation. The diffraction data were processed using the programs *MOSFLM* (Leslie, 1992) and *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

C. reinhardtii DapL was successfully cloned, expressed and purified to homogeneity by a two-step purification protocol involving binding to Talon Metal Affinity Resin. The purity of the enzyme was assessed by SDS-PAGE (Fig. 2) and the enzyme activity was measured using the DapL quantitative forward and reverse assays (Hudson *et al.*, 2006). The specific activity of the enzyme in the forward direction (THDPA to L,L-DAP) was 0.7 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein and that in the reverse direction (L,L-DAP to THDPA) was 12.0 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein.

Purified DapL protein was screened for crystallization using the JCSG+ and PACT screens, but only afforded crystals in the following condition: 200 mM lithium sulfate, 25% (w/v) polyethylene glycol 3350, 100 mM bis-tris propane pH 5.5 (JCSG+ condition H9). The crystals in Fig. 3(a) appeared after 5 d and continued to grow over a further 5 d. The larger crystals appeared to develop imperfections in crystal morphology.

The crystal showed excellent diffraction (to $\sim 1.35 \text{ \AA}$ resolution) and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 58.9$, $b = 91.8$, $c = 162.9 \text{ \AA}$. However, the very intense beam on MX2 resulted in a significant loss of resolution, even after 20° of data had been collected, presumably owing to radiation damage. Nonetheless, the data were scaled to 1.55 \AA resolution with reasonable completeness (data-collection statistics are summarized in Table 1). The axial reflections showed systematic absences that were consistent

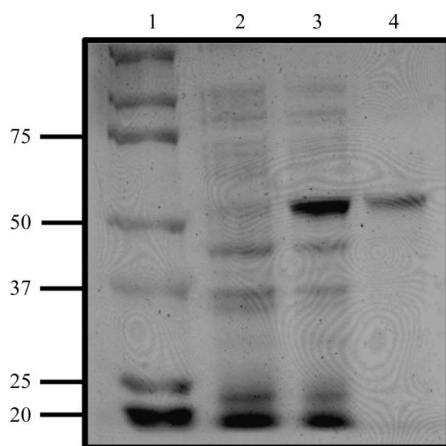


Figure 2
SDS-PAGE analysis of purified recombinant DapL. Lane 1, protein markers (kDa). Lane 2, 10 μg uninduced soluble protein extract. Lane 3, 10 μg induced soluble extract. Lane 4, 1.0 μg purified recombinant DapL protein. The proteins were resolved on an SDS-PAGE gel containing 10% (w/v) acrylamide and the gel was stained using Coomassie Blue.



Figure 3
Crystals of DapL grown in JCSG+ condition H9. The scale bar indicates 0.2 mm.

Table 1

X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell. The Matthews coefficient and the estimate of the solvent content are based on a molecular weight of 97 661 Da in the asymmetric unit (two molecules of DapL of 48 830 Da each).

Wavelength (Å)	0.95369
No. of images	220
Oscillation (°)	0.5
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 58.9, b = 91.8, c = 162.9$
Resolution (Å)	35.35–1.55 (1.63–1.55)
Average mosaicity (°)	0.27
Observed reflections	492985
Unique reflections	124970
Completeness (%)	97.3 (94.3)
$R_{\text{merge}}^{\dagger}$	0.081 (0.494)
$R_{\text{p.i.m.}}^{\ddagger}$	0.044 (0.283)
$R_{\text{r.i.m.}}$	0.093 (0.573)
Mean $I/\sigma(I)$	10.6 (2.7)
Multiplicity	3.9 (3.9)
Wilson B value (Å ²)	13.6
Estimated molecules per asymmetric unit	2
V_M (Å ³ Da ⁻¹)	2.28
Solvent content (%)	48

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)} \quad \ddagger R_{\text{p.i.m.}} = \frac{\sum_{hkl} [1/(N-1)]^{1/2} \times \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

with three twofold screw axes. The R_{merge} was 0.081, $R_{\text{p.i.m.}}$ was 0.044, $R_{\text{r.i.m.}}$ was 0.093, V_M was 2.28 Å³ Da⁻¹ and the estimated solvent content was 48% given two molecules in the asymmetric unit. The crystals shown in Fig. 3(a) could also be produced under the same conditions but at 281 K, although they grew noticeably more slowly. Diffraction data were also collected from these crystals, but the resolution was reduced: the best resolution was ~1.9 Å. We anticipate that the structure of DapL from *C. reinhardtii* will provide insight into the design of novel algaecides.

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