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Cloning to crystallization of dihydrodipicolinate synthase from the intracellular pathogen *Legionella pneumophila*

Dihydrodipicolinate synthase (DHDPS) catalyses the rate-limiting step in the biosynthesis of *meso*-diaminopimelate and lysine. Here, the cloning, expression, purification and crystallization of DHDPS from the intracellular pathogen *Legionella pneumophila* are described. Crystals grown in the presence of high-molecular-weight PEG precipitant and magnesium chloride were found to diffract beyond 1.65 Å resolution. The crystal lattice belonged to the hexagonal space group $P6_122$, with unit-cell parameters $a = b = 89.31$, $c = 290.18$ Å, and contained two molecules in the asymmetric unit. The crystal structure was determined by molecular replacement using a single chain of *Pseudomonas aeruginosa* DHDPS as the search model.

1. Introduction

Dihydrodipicolinate synthase (DHDPS) functions as a key enzyme in the diaminopimelate (DAP) pathway of bacteria, plants and some fungi (Hutton *et al.*, 2007; Dogovski *et al.*, 2009, 2012). In bacteria, the pathway yields two metabolites important to bacterial survival (Kobayashi *et al.*, 2003), namely *meso*-diaminopimelate (*meso*-DAP) and lysine. *meso*-DAP is a key component of the peptidoglycan layer in the bacterial cell wall and lysine is a fundamental building block of housekeeping proteins and bacterial virulence factors (Schleifer & Kandler, 1972; Dogovski *et al.*, 2009, 2012). As a consequence, enzymes of the DAP pathway are promising antibiotic targets (Hutton *et al.*, 2007; Dogovski *et al.*, 2009, 2012). In addition, lysine is one of the essential amino acids in the human diet and as such offers a potential avenue for the design of inhibitors to this pathway with low host toxicity (Hutton *et al.*, 2007; Dogovski *et al.*, 2009, 2012).

The first committed step in the synthesis of *meso*-DAP and lysine involves aldol condensation of pyruvate and (*S*)-aspartate semialdehyde (ASA) to form (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinic acid (Blickling, Renner *et al.*, 1997; Hutton *et al.*, 2007; Dogovski *et al.*, 2009, 2012). This rate-limiting step is catalysed by the enzyme DHDPS. In all Gram-negative pathogens (Dobson, Devenish *et al.*, 2005; Phenix & Palmer, 2008; Devenish *et al.*, 2009; Kaur *et al.*, 2011) and plants (Matthews & Widholm, 1979; Wallsgrove & Mazelis, 1980; Kumpaisal *et al.*, 1987; Dereppe *et al.*, 1992; Blickling, Beisel *et al.*, 1997; Griffin *et al.*, 2012; Atkinson *et al.*, 2013) studied to date, lysine acts as a feedback inhibitor to DHDPS.

The structure of DHDPS has been investigated in several bacteria including *Agrobacterium tumefaciens* (Atkinson *et al.*, 2012), *Bacillus anthracis* (Blagova *et al.*, 2006; Voss *et al.*, 2009, 2010), *Clostridium botulinum* (Atkinson *et al.*, 2009), *Corynebacterium glutamicum* (Rice *et al.*, 2008), *Escherichia coli* (Mirwaldt *et al.*, 1995; Dobson, Griffin *et al.*, 2005), *Hahella chejuensis* (Kang *et al.*, 2010), *Methanococcus jannaschii* (Padmanabhan *et al.*, 2009), *Mycobacterium tuberculosis* (Kefala *et al.*, 2008; Evans *et al.*, 2011), *Neisseria meningitidis* (Devenish *et al.*, 2009), *Pseudomonas aeruginosa* (Kaur *et al.*, 2011), *Shewanella benthica* (Wubben *et al.*, 2010), *Staphylococcus aureus* (Burgess *et al.*, 2008; Girish *et al.*, 2008), *Streptococcus pneumoniae* (Sibarani *et al.*, 2010) and *Thermotoga maritima* (Pearce *et al.*, 2006). The enzyme typically forms a homotetramer that is described as a dimer of dimers. In this arrangement, two monomers associate to

create a dimer at the 'tight-dimer' interface (Perugini *et al.*, 2005; Griffin *et al.*, 2008, 2010). Two such dimers then unite to create a tetramer *via* interactions at the 'weak-dimer' interface where weaker bonding interactions exist (Perugini *et al.*, 2005; Griffin *et al.*, 2008, 2010). Recently, homodimers and dimeric mutants of DHDPS have also surfaced that exhibit high levels of activity (Burgess *et al.*, 2008; Girish *et al.*, 2008; Wubben *et al.*, 2010; Evans *et al.*, 2011; Kaur *et al.*, 2011).

Despite different quaternary states, the DHDPS monomer remains a $(\beta/\alpha)_8$ -barrel in which the active site is located central to the barrel (Mirwaldt *et al.*, 1995). This gives rise to four active sites in a tetrameric DHDPS or two active sites in a dimeric DHDPS (Mirwaldt *et al.*, 1995). Adjacent to the active sites is a cleft at the tight-dimer interface to which lysine binds in order to inhibit the enzyme (Dobson, Griffin *et al.*, 2005; Kaur *et al.*, 2011; Griffin *et al.*, 2012; Atkinson *et al.*, 2013).

Whilst DHDPS has been extensively studied in extracellular bacterial species, there have been no published data on DHDPS from intracellular bacteria. Investigating DHDPS from an intracellular species will provide greater insight into the structure, function and inhibition of this enzyme. Furthermore, it will enable discovery of inhibitors appropriate for penetration into host cells.

To develop our understanding in this area, the work outlined here describes the cloning, expression, purification, crystallization and preliminary X-ray crystallographic data of DHDPS from the intracellular human pathogen *Legionella pneumophila* (*Lp*-DHDPS). *L. pneumophila* is a Gram-negative bacterium that invades and replicates within a broad range of hosts extending from protozoa to human alveolar macrophages (Rowbotham, 1980; Fields, 1996). Upon entering the human body, it causes the often fatal pneumonia called Legionnaires' disease. Its major source of carbon and energy come from amino acids scavenged from the host cell (Warren & Miller, 1979; George *et al.*, 1980; Tesh & Miller, 1981; Tesh *et al.*, 1983; Sauer *et al.*, 2005). Interestingly, DAP pathway genes have been annotated and shown to be expressed in *L. pneumophila* (Brüggemann *et al.*, 2006; Faucher *et al.*, 2011), suggesting that the pathway is important for survival in this bacterium.

Previous attempts at crystallizing the *apo* form of this enzyme resulted in crystals with poor diffraction. Reports of the pyruvate-stabilization phenomenon (Blickling, Renner *et al.*, 1997; Burgess *et al.*, 2008; Voss *et al.*, 2010; Atkinson *et al.*, 2009) prompted cocrystallization of *Lp*-DHDPS with pyruvate. Diffraction was found to significantly improve with the substrate-bound form of the enzyme.

2. Materials and methods

2.1. Cloning the gene encoding dihydrodipicolinate synthase from *L. pneumophila*

The *dapA* gene, which encodes DHDPS, was PCR-amplified using *L. pneumophila* genomic DNA and the primer pair F1 (5'-GCTGATAGAGATTCCGGTTG-3') and R1 (5'-GCTTCGCCAT-TACGGCTCTG-3'). The amplified product was cloned into the pCR-Blunt II-TOPO shuttle vector (Invitrogen) to create the plasmid pTS01. *dapA* was subsequently amplified from pTS01 using the primer pair F2 (5'-CATATGTTTCAGTGAAGTATAGTA-3') and R2 (5'-GGATCCTTATATCAACTCTAAATTCTTC-3'). The design of these primers allowed the incorporation of *Nde*I and *Bam*HI restriction sites upstream and downstream, respectively, of the open reading frame. The resulting product was ligated between *Nde*I and *Bam*HI restriction sites of pET11a (Novagen) to create the expression vector pET-LpS. The identity of the *dapA* gene from

L. pneumophila was confirmed by restriction analysis and dideoxy-nucleotide sequencing.

2.2. Expression and purification of *L. pneumophila* DHDPS

E. coli BL21-DE3 cells (Stratagene) harbouring pET-LpS were cultivated in 1 l Luria broth containing 75 $\mu\text{g ml}^{-1}$ ampicillin. Cells were incubated at 310 K with shaking at 180 oscillations per minute (OPM) to an OD_{600 nm} of 0.4 and were subsequently transferred to 289 K until the cultures attained an OD_{600 nm} of 0.7. Cells were treated with 1.0 mM isopropyl β -D-1-thiogalactopyranoside to induce recombinant protein production at 289 K and were subsequently harvested 16 h post-induction by centrifugation (10 000g at 277 K for 20 min). Cell pellets were resuspended in 11 ml buffer A (20 mM Tris, 5 mM pyruvate pH 7.5) and then lysed using a Misonix S-4000 sonicator (20 s bursts at 40 μ followed by 40 s rest periods for 10 min). The cell lysate was centrifuged (20 000g at 277 K for 20 min) to pellet cellular debris and the resulting supernatant was further clarified by filtration using a 0.45 μm syringe filter (Millipore).

Recombinant *Lp*-DHDPS was purified using a two-step strategy that involved anion-exchange and hydrophobic interaction chromatography. Briefly, lysate was applied onto a Q Sepharose column (bed volume 60 ml; 26 mm diameter $\times$ 10 cm length; GE Healthcare) pre-equilibrated with buffer A at 277 K. The column was washed in buffer A at 3 ml min⁻¹ until a steady absorbance baseline ($A_{280\text{ nm}}$) was achieved. To elute protein, a two-step gradient was applied. The first involved a gradient of 100–40% buffer A:0–60% buffer B (20 mM Tris, 5 mM pyruvate, 1 M NaCl pH 7.5) over 10 column volumes (CV). Secondly, a gradient of 40–0% buffer A:60–100% buffer B over 1 CV was applied. *Lp*-DHDPS eluted at approximately 0.3 M NaCl. Peak fractions were pooled and prior to performing hydrophobic interaction chromatography, ammonium sulfate was added to a final concentration of 1 M. Pooled fractions were subsequently injected onto a Phenyl Sepharose column (bed volume 60 ml; 26 mm diameter $\times$ 10 cm length; GE Healthcare) pre-equilibrated with buffer C (20 mM Tris, 5 mM pyruvate, 1 M ammonium sulfate pH 7.5) at 277 K. The column was washed in buffer C at 3 ml min⁻¹ until a steady absorbance baseline ($A_{280\text{ nm}}$) was attained. A two-step gradient allowed further purification of *Lp*-DHDPS. Firstly, a gradient of 100–50% buffer C:0–50% buffer A was applied over 2 CV. This was followed by a gradient of 50–0% buffer C:50–100% buffer A over 8 CV. Peak fractions were pooled and dialysed in buffer D (20 mM Tris, 5 mM pyruvate, 150 mM NaCl pH 7.5) overnight. Thereafter, the dialysed sample was separated into 1.5 ml aliquots and flash-cooled before storage at 193 K. Prior to crystallization trials, protein was thawed overnight at 277 K and concentrated using a 10 000 MWCO Amicon filter (Millipore). Size-exclusion chromatography was performed in buffer D using a Superose 12 column (bed volume 24 ml; 10 mm diameter $\times$ 30 cm length; GE Healthcare) to remove aggregates, followed again by concentrating the protein using a 10 000 MWCO Amicon filter (Millipore) for crystallization. Protein samples were quantified by UV-Vis spectrophotometry using an extinction coefficient at 280 nm of 19 940 M⁻¹ cm⁻¹.

2.3. Mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was employed to accurately determine the mass of recombinant *Lp*-DHDPS. Protein was prepared at a concentration of 0.15 mg ml⁻¹ in 20 mM Tris pH 7.5. Mass data were collected using a microTOF-Q mass spectrometer (Bruker Daltonics, Germany) coupled to an UltiMate 3000 RSLCnano system (Dionex, Holland) as described previously (Brand *et al.*, 2012). Data were annotated and deconvoluted.

luted using the *DataAnalysis* program (Version 4.0, Bruker Daltonics).

2.4. Protein crystallization

Initial trials were performed at the CSIRO node of the Bio21 Collaborative Crystallization Centre (C3; <http://www.csiro.au/c3/>) using the PACT Suite and JCSG+ Suite screens (Qiagen; Newman *et al.*, 2005). 96-well plates were established at 281 and 293 K using the sitting-drop vapour-diffusion method. Each well contained 150 nl reservoir solution and 150 nl recombinant enzyme solution (10 mg ml⁻¹) with pyruvate (5 mM final concentration). Conditions yielding promising crystals were replicated on a larger scale and further screened using the hanging-drop vapour-diffusion method. Drops contained 2 µl protein solution and 2 µl reservoir solution. Each drop varied in pH, polyethylene glycol (PEG) concentration or protein concentration and plates were incubated at 281 and 293 K. The best diffracting crystal was produced at 281 K in reservoir solution consisting of 0.2 M magnesium chloride, 10% (w/v) PEG 8000, 0.1 M Tris chloride pH 10 with protein concentration at 11 mg ml⁻¹.

2.5. Data collection and processing

To prepare the crystal for X-ray data collection, it was briefly soaked in reservoir solution containing 20% (v/v) 2-methyl-2,4-pentanediol and directly flash-cooled in liquid nitrogen. Intensity data were collected at 110 K using the MX2 Micro Crystallography 03ID1 source and the *Blu-Ice* control software (McPhillips *et al.*, 2002) at the Australian Synchrotron. Data were collected as one 360° pass with 0.5° oscillations using an ADSC Quantum 315r detector positioned 200 mm from the crystal. The exposure time was 1 s with 80% attenuation. Indexing and integration of the data were performed using the program *iMOSFLM* (Battye *et al.*, 2011). *POINTLESS* (Evans, 2006) from the *CCP4* program suite (Winn *et al.*, 2011) was used to verify the space group. Scaling, data reduction and truncation of the data were performed using the program *SCALA* (Evans, 2006) also from the *CCP4* suite.

Figure 1
SDS-PAGE analyses of recombinant *Lp*-DHDPS (molecular mass 31 581 Da). Lane 1, molecular-mass markers (kDa); lane 2, cell lysate (post-induction); lane 3, post-anion-exchange chromatography; lane 4, 2 µg protein post-hydrophobic interaction chromatography.

Table 1

X-ray data-collection and processing statistics.

Values in parentheses are for the outermost resolution bin.

Wavelength (Å)	0.9537
No. of images	129
Oscillations (°)	0.5
Space group	<i>P</i> 6 ₁ 22
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 89.31, <i>c</i> = 290.18, α = 90.0, β = 90.0, γ = 120.0
Resolution (Å)	36.27–1.65 (1.74–1.65)
Observed reflections	376126 (58890)
Unique reflections	81908 (11872)
Completeness (%)	98.9 (99.8)
<i>R</i> _{merge} [†]	0.108 (0.343)
<i>R</i> _{r.i.m.} [‡]	0.122 (0.384)
<i>R</i> _{p.i.m.} [§]	0.055 (0.169)
Mean <i>I</i> /σ(<i>I</i>)	9.1 (4.2)
Multiplicity	4.6 (5.0)
Wilson <i>B</i> value (Å ²)	11.76
Molecules per asymmetric unit	2
<i>V</i> _M (Å ³ Da ⁻¹)	2.64
Solvent content (%)	53.52

[†] $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. [‡] $R_{\text{r.i.m.}} = \frac{\sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. [§] $R_{\text{p.i.m.}} = \frac{\sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the *i*th intensity measurement of reflection *hkl*, $\langle I(hkl) \rangle$ is its average and *N*(*hkl*) is the redundancy of a given reflection.

3. Results and discussion

Recombinant *Lp*-DHDPS was expressed and purified as outlined in §2. Lane 2 in Fig. 1 represents crude lysate before injection onto a Q Sepharose column. Recombinant *Lp*-DHDPS is indistinguishable in this sample owing to a high content of cellular proteins. However, removal of such proteins upon anion-exchange reveals the presence of a densely stained band at an apparent molecular weight of 31 kDa (Fig. 1, lane 3). Remaining contaminants were removed after hydrophobic interaction chromatography (Fig. 1, lane 4) to yield approximately 6 mg of protein per litre of culture and >95% purity as estimated by SDS-PAGE. Mass spectrometry confirmed the identity of this sample to be *Lp*-DHDPS, with a major peak existing at 31 580.4 Da in the deconvoluted mass spectrum (Fig. 2). This is

Figure 2
Electrospray ionization mass spectrometry analysis of purified recombinant *Lp*-DHDPS. The deconvoluted mass spectrum reveals a predominant peak at 31 580.4 Da consistent with the expected mass of *Lp*-DHDPS. Inset: raw mass-to-charge data showing multiple charge states of the protein.

(a)

(b)

Figure 3

Crystallization and diffraction of recombinant *Lp*-DHDPS with pyruvate. (a) Crystals were of a similar morphology and size. (b) X-ray diffraction frame from one of the cryocooled crystals from (a). Diffraction at the edge of the detector was 1.46 Å resolution.

consistent with the expected molecular mass of *Lp*-DHDPS from the amino-acid sequence.

Crystallization screens were then performed with an initial protein concentration of 10 mg ml⁻¹ that yielded crystals in several conditions from the PACT and JCSG+ screens. Crystal growth was typically observed after 3 d and frequently in conditions with PEG and chloride salt. Promising conditions were replicated in-house and produced crystals ranging between 0.05 and 0.40 mm in the longest dimension.

Favourable diffraction data to beyond 1.65 Å resolution (Fig. 3b) were collected on a crystal from reservoir solution consisting of 0.2 M magnesium chloride, 10% (w/v) PEG 8000, 0.1 M Tris chloride pH 10 (Fig. 3a). Integration and scaling of the data by *XDS* (Kabsch, 2010) and *iMOSFLM* indicated that the crystal lattice belonged to space group *P*6₁22, with unit-cell parameters *a* = *b* = 89.31, *c* = 290.18 Å,

$\alpha = \beta = 90.0^\circ$, $\gamma = 120.0^\circ$. The Matthews coefficient (Matthews, 1968) was 2.64 Å³ Da⁻¹, with an estimated solvent content of 53.52% for two monomers in the asymmetric unit. Only the first 129 images (64.5°) out of 720 were processed and scaled given that the remaining images contained significant radiation damage. Scaling and merging of the crystallographic data resulted in an overall *R*_{merge} of 0.108 with an *R*_{merge} of 0.343 in the highest resolution shell. Complete data-collection and processing statistics are summarized in Table 1. The structure of *Lp*-DHDPS was determined by molecular replacement using chain A of *P. aeruginosa* DHDPS (PDB entry 3qze; R. Schnell, T. Sandalova & G. Schneider, unpublished work) as the search model, which shares 54% sequence identity with *Lp*-DHDPS. The structure was solved using *Phaser* (McCoy *et al.*, 2007) employing two copies of the *P. aeruginosa* DHDPS model. The solution gave a translation-function *Z* score of 29.9 with a final log-likelihood gain of 1400. The first round of refinement gave an *R*_{free} of 29.1% and an *R* factor of 26.9%. Further model building and refinement of the structure are in progress.

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