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Purification, crystallization and preliminary X-ray crystallographic analysis of diaminopimelate epimerase from *Acinetobacter baumannii*

The *meso* isomer of diaminopimelate (*meso*-DAP) is a biosynthetic precursor of L-lysine in bacteria and plants, and is a key component of the peptidoglycan layer in the cell walls of Gram-negative and some Gram-positive bacteria. Diaminopimelate epimerase (DapF) is a pyridoxal-5'-phosphate-independent racemase which catalyses the interconversion of (6*S*,2*S*)-2,6-diaminopimelic acid (LL-DAP) and *meso*-DAP. In this study, DapF from *Acinetobacter baumannii* was overexpressed in *Escherichia coli* strain SoluBL21, purified and crystallized using a vapour-diffusion method. A native crystal diffracted to a resolution of 1.9 Å and belonged to space group $P3_1$ or $P3_2$, with unit-cell parameters $a = b = 74.91$, $c = 113.35$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. There were two molecules in the asymmetric unit.

1. Introduction

Acinetobacter baumannii is an opportunistic and nosocomial pathogen that causes a broad spectrum of human infections, including bacteraemia, pneumonia, meningitis, endocarditis, osteomyelitis, wound infections and urinary-tract infections (Dijkshoorn *et al.*, 2007; Kwon *et al.*, 2009). A cell wall composed of peptidoglycan (PGN) provides the first line of defence against antibiotics in this pathogen.

PGN is a mesh-like polymer made up of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) disaccharide units and pentapeptide, which is attached to NAM, and plays a crucial role in the structural integrity of cell walls (Cho *et al.*, 2007; Dramsi *et al.*, 2008; Vollmer *et al.*, 2008; Meroueh *et al.*, 2006). A unique amino acid, (2*R*,6*S*)-2,6-diaminopimelic acid (*meso*-DAP), in the pentapeptide provides the attachment site for the inner or outer membrane to PGN. *meso*-DAP is also a biosynthetic precursor of L-lysine in bacteria and plants (Wiseman & Nichols, 1984; Cox *et al.*, 2000).

Diaminopimelate epimerase (DapF) catalyses the interconversion of (6*S*,2*S*)-2,6-diaminopimelic acid (LL-DAP) and *meso*-DAP, and is a member of the pyridoxal-5'-phosphate-independent racemase family of enzymes (Baumann *et al.*, 1988; Cox *et al.*, 2000; Hor *et al.*, 2010; Pillai *et al.*, 2006, 2009). In the periplasm of Gram-negative bacteria, *meso*-DAP of PGN interacts noncovalently with OmpA-like domain proteins such as OmpA (Park *et al.*, 2012), a peptidoglycan-associated lipoprotein (Cascales & Lloubès, 2004; Parsons *et al.*, 2006) and MotB (De Mot & Vanderleyden, 1994; Roujeinikova, 2008; Kojima *et al.*, 2009). In addition, *meso*-DAP is of interest in terms of the human defence system against pathogens, as bacterial peptidoglycan fragments containing *meso*-DAP trigger a Nod1-mediated immune response to fight infection (Uehara *et al.*, 2006).

Here, we report the cloning, expression, purification, crystallization and preliminary X-ray crystallographic analysis of DAP epimerase from *A. baumannii*.

2. Materials and methods

2.1. Cloning and overexpression

Full-length *A. baumannii* DapF (AbDapF; residues 1–287; molecular weight 31.1 kDa) was amplified using genomic DNA from *Acinetobacter* 19606 with the primers 5'-ACTCATATGTTATTA-GAATTTACC-3' and 5'-CCGCTCGAGTTAACCTTGGAATA-ACG-3'. To generate the recombinant protein, the PCR product was

cloned into the *NdeI/XhoI*-digested expression vector pET-28a(+) (Novagen) to yield an open reading frame consisting of an N-terminal His₆ tag, a thrombin cleavage site and AbDapF. The resulting plasmid was transformed into the expression host *Escherichia coli* SoluBL21 (Genlantis), and bacterial cells were grown at 310 K in Luria–Bertani (LB) medium containing 50 µg ml⁻¹ kanamycin until the OD₆₀₀ reached 0.5–0.6. Expression of recombinant AbDapF was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside at 310 K for 3 h. The bacterial cells were harvested by centrifugation at 3800g for 30 min at 277 K.

2.2. Purification

The cells were harvested by centrifugation (3800g for 30 min at 277 K) and the cell pellet was resuspended in lysis buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride). The resuspended cells were disrupted by sonication (Cole–Parmer Instruments, USA) on ice and the supernatant, which contained the target protein, was separated from the crude lysate by centrifugation (36 000g for 45 min at 277 K). The supernatant was loaded onto a 5 ml HisTrap FF column (GE Healthcare) pre-equilibrated with buffer A (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 2 mM DTT). The protein was eluted with a linear gradient of 0–500 mM imidazole in buffer B (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 2 mM DTT, 500 mM imidazole) at a flow rate of 2 ml min⁻¹. To remove the His₆ tag, the eluted fraction was reacted with thrombin overnight by dialysis in buffer A at 293 K. The protein was purified again to remove the cleaved His₆ tag on a 5 ml HisTrap FF column pre-equilibrated with buffer A. The flowthrough fractions were concentrated by filtration using an Amicon Ultra 10K cutoff filter (Millipore). In the final step, the concentrated AbDapF protein was further purified using a HiLoad 16/60 Superdex 75 prep-grade gel-permeation chromatography column (GE Healthcare) equilibrated with buffer C (20 mM HEPES pH 7.0, 100 mM NaCl, 5 mM DTT) at a flow rate of

1 ml min⁻¹. The purity of the AbDapF protein was confirmed by SDS–PAGE and the concentration was adjusted to ~14 mg ml⁻¹ prior to crystallization screening.

2.3. Crystallization and X-ray data collection

Initial crystallization screening was performed using the sitting-drop vapour-diffusion method with The Stura Footprint Combination, MIDAS and PGA Screens (Molecular Dimensions, Altamonte Springs, USA), Crystal Screen HT, PEGRx HT (Hampton Research, Aliso Viejo, USA), CP-Custom-III and CP-Custom-IV (Axygen, Union City, California, USA) in 96-well plates using a high-throughput nanolitre Mosquito crystallization robot (TTP LabTech, Melbourn, Hertfordshire, England).

Sitting drops were produced by mixing equal volumes (200 nl each) of protein solution and reservoir solution. After several hits had been obtained from the initial screening, optimization of the crystallization conditions was performed. The final crystallization condition was optimized from condition H4 of The Stura Footprint Combination screen using the hanging-drop vapour-diffusion method. 1 µl protein solution consisting of 14 mg ml⁻¹ protein in buffer C was mixed with 1 µl reservoir buffer and equilibrated by vapour diffusion against the reservoir solution. AbDapF crystals were obtained using a reservoir buffer consisting of 2 M ammonium sulfate, 0.1 M sodium HEPES pH 7.5, 4.9–5.1% PEG 400. A single crystal of suitable dimensions was transferred into reservoir solution supplemented with 25% (v/v) PEG 400 as a cryoprotectant and mounted on a CryoLoop (Hampton Research, Aliso Viejo, California, USA) for diffraction studies under cryogenic conditions.

X-ray diffraction tests were performed on native crystals using a MicroMax-007 HF rotating-anode X-ray generator and an R-Axis IV⁺⁺ imaging-plate area detector (Rigaku Americas, Texas, USA). The final diffraction data were collected on the BL-1A beamline at the Photon Factory, Tsukuba, Japan and 360 frames of diffraction images were recorded on a PILATUS 2M detector (DECTRIS Ltd, Switzerland) with an oscillation angle of 0.5° and a crystal-to-detector distance of 164 mm. The diffraction images were integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997).

3. Results and discussion

Full-length AbDapF (residues 1–287; molecular weight 31.1 kDa) was successfully overexpressed in *E. coli* SoluBL21 cells and purified with 95% purity using a nickel-affinity column and subsequent size-exclusion chromatography (Fig. 1). AbDapF was eluted in the 65 ml

Figure 1
Purification of recombinant AbDapF. The results of the purification steps were detected on an SDS–PAGE gel. (a) Lane M, molecular-weight markers (labelled in kDa); lane 1, crude cell lysate; lane 2, pellet; lane 3, supernatant; lanes 4–5, flowthrough; lanes 6–7, washing fractions; lanes 8–9, fractions eluted from a HisTrap FF column. (b) Lane M, molecular-weight markers (labelled in kDa); lanes 1–7, fractions eluted from a HiLoad 16/60 Superdex 75 size-exclusion column.

Figure 2
A crystal of DapF from *A. baumannii*.

Figure 3
X-ray diffraction image of AbDapF.

Table 1

Data-collection and processing statistics for AbDapF.

Values in parentheses are for the highest resolution shell.

Beamline	BL-1A, Photon Factory
Detector	PILATUS 2M
Temperature (K)	95.0
Wavelength (Å)	1.1000
Resolution range (Å)	50–1.9 (1.97–1.90)
Space group	$P3_1$ or $P3_2$
Unit-cell parameters (Å, °)	$a = b = 74.91$, $c = 113.35$, $\alpha = \beta = 90$, $\gamma = 120$
$R_{\text{merge}}^{\dagger}$	0.067 (0.489)
Total reflections	288066
Unique reflections	56343
Completeness (%)	100 (100)
Multiplicity	5.1 (4.9)
Mean $I/\sigma(I)$	33.5 (3.0)

$$^{\dagger} R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

fraction from a HiLoad 16/60 Superdex 75 prep-grade gel-permeation chromatography column, corresponding to about 31 kDa. This result means that AbDapF is a monomer in solution. Initial screening of the crystallization conditions was performed by the sitting-drop vapour-diffusion method using commercially available screening kits.

AbDapF crystals were obtained from an optimized condition using the hanging-drop vapour-diffusion method. The crystals grew in clusters, and a single crystal was detached prior to data collection (Fig. 2). The native AbDapF crystal diffracted to 1.9 Å resolution using synchrotron radiation (Fig. 3). Upon processing the X-ray diffraction data, the native AbDapF crystal belonged to space group $P3_1$ or $P3_2$, with unit-cell parameters $a = b = 74.91$, $c = 113.35$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The Matthews coefficient was $2.93 \text{ Å}^3 \text{ Da}^{-1}$ and the solvent content was 58.1% (Matthews, 1968), suggesting that the asymmetric unit contained two AbDapF molecules. Data-collection and processing statistics are given in Table 1.

The structures of DapF have been studied owing to their interest in antibacterial drug development. Currently, structures of DapF from *Haemophilus influenza* (Cirilli *et al.*, 1998; Lloyd *et al.*, 2004; Pillai *et al.*, 2006, 2007; 53% amino-acid sequence identity and 69% conservation compared with AbDapF), *Bacillus anthracis* (PDB entry 3edn; Center for Structural Genomics of Infectious Diseases, unpublished work; 35% identity and 56% conservation) and *Mycobacterium tuberculosis* (Usha *et al.*, 2009; 28% identity and 40% conservation) are available in the Protein Data Bank (PDB). The high-resolution structure of AbDapF will be invaluable in understanding substrate binding and in the development of novel antibiotics. Structure determination of AbDapF by molecular replacement is in progress using the structure of *H. influenza* DapF (PDB entry 1gqz; Lloyd *et al.*, 2004) as a model.

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