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Crystallization and preliminary X-ray analysis of a UDP-MurNAc-tripeptide D-alanyl-D-alanine-adding enzyme (PaMurF) from *Pseudomonas aeruginosa*

The ATP-dependent UDP-MurNAc-tripeptide:D-Ala-D-Ala ligase MurF catalyses the last step in the cytoplasmic phase of peptidoglycan biosynthesis, which is critical in the formation of the bacterial cell wall and in the recycling of peptidoglycan intermediates. In this study, the crystallization of MurF from the Gram-negative pathogen *Pseudomonas aeruginosa* in the presence of its UDP-MurNAc-tripeptide substrate is reported. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 57.81$, $b = 87.29$, $c = 92.61$ Å, and data were collected to 1.92 Å resolution, allowing study of the enzyme in the substrate-liganded form for the first time.

1. Introduction

Peptidoglycan is a unique and essential cell-wall component of virtually all bacteria, and its biosynthesis is the target of a large number of antimicrobial compounds that have had profound utility in human healthcare for over 70 years. The search for novel antimicrobial drugs in the face of rising levels of antibiotic resistance is a critical issue for industry and academia, prompting a search for, and a re-evaluation of, known targets (Payne *et al.*, 2007; Wright, 2012; Silver, 2011).

The penultimate stage of the cytoplasmic phase of peptidoglycan biosynthesis (Barreteau *et al.*, 2008) involves the formation of a UDP-*N*-acetylmuramic acid-tripeptide, which is the substrate for the UDP-MurNAc-tripeptide:D-Ala-D-Ala ligase activity of MurF. The MurF enzyme is the fourth in a series of enzymes in the cytoplasmic phase of the pathway (MurC–F), whose common catalytic mechanism involves the production of an amide bond *via* ATP-dependent acyl phosphate formation, creating a tetrahedral intermediate, which is then attacked by an incoming amino acid or dipeptide (Smith, 2006; Falk *et al.*, 1996). These enzymes have a shared ATP-binding site architecture and catalytic mechanism and share structural properties, rendering them interesting candidates for multiple targeting by novel antimicrobials (Shanmugam & Natarajan, 2012). MurF is distinguished from the other Mur ligases by the stereochemical selection of a D-alanyl-D-alanine dipeptide substrate as the incoming nucleophile rather than a single amino acid as used by MurC–E. In addition, MurF proteins from a number of Gram-positive bacterial strains, including enterococcal species, are endowed with sufficiently relaxed specificity for the dipeptide substrate to allow the incorporation of other D-alanyl-D-lactate/serine ligands, as required for the glycopeptide (vancomycin) resistance mechanism (Healy *et al.*, 2000). MurF enzymes from several different species (Patin *et al.*, 2010, 2012; Duncan *et al.*, 1990) have been characterized and used as a basis for the design of screens (Schouten *et al.*, 2006; Baum *et al.*, 2006), resulting in the discovery of small-molecule inhibitors (Baum *et al.*, 2006, 2007, 2009; El Zoeiby *et al.*, 2003; Gu *et al.*, 2004; Paradis-Bleau *et al.*, 2008; Sova *et al.*, 2009; Taha *et al.*, 2008; Silver, 2013).

X-ray crystal structures of *Escherichia coli* MurF in the apo form (Yan *et al.*, 2000) and of *Streptococcus pneumoniae* MurF complexed with small-molecule inhibitors (Longenecker *et al.*, 2005) have been reported, but there are no structures available of complexes with

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natural substrates. Structures in the presence of natural substrates may be of great utility in the design of future substrate-mimetic compounds, as they will aid in the elucidation of the stereoselective mechanism of the enzyme with respect to the added dipeptide and the phosphoryl activation of the DAP or L-lysine UDP-*N*-acetylmuramic acid-tripeptide substrate. To this end, we have initiated a study of MurF with natural substrates, with the expectation that the resulting structures will advance this topic.

2. Materials and methods

2.1. Overproduction and purification of *Pseudomonas aeruginosa* MurF (PaMurF)

We used the plasmid pMON3009 (El Zoeiby *et al.*, 2001) containing the *P. aeruginosa* *murF* gene with a noncleavable C-terminal histidine tag. The tag resulted in a single mutation at the C-terminus (H458Q owing to cloning into the *SacI* restriction site of the pET30b vector) and consisted of SSVDKLAAALEHHHHHH. The plasmid was transformed into *E. coli* BL21 (λ DE3) Star pRosetta cells. A colony selected for kanamycin and chloramphenicol resistance was used to inoculate 15 ml Luria broth (LB) starter culture with the addition of 50 mg l⁻¹ kanamycin and 34 mg l⁻¹ chloramphenicol. The starter culture was grown overnight at 310 K and 180 rev min⁻¹. Large-scale cultures were carried out in 21 flasks containing 1 l LB medium supplemented with the required antibiotics. The flasks containing the LB were inoculated with 15 ml starter culture and grown at 310 K and 180 rev min⁻¹ until an OD₆₀₀ of 0.5 was reached, at which point isopropyl β -D-1-thiogalactopyranoside was added to a final concentration of 1 mM. After 6 h of induction at 310 K, the cells were pelleted by centrifugation at 4000g for 20 min and resuspended in 40 ml buffer A (20 mM Tris pH 7.9, 500 mM NaCl, 5 mM imidazole). The cells were subsequently sonicated on ice by 6 $\times$ 15 s bursts with an interval of 15 s between each burst. The resulting lysate was cleared of cell debris by centrifugation at 48 000g for 45 min and the supernatant was applied onto a 5 ml HisTrap High Performance column (GE Healthcare) equilibrated with buffer A. The column was washed extensively with buffer A and the protein was eluted with buffer B (20 mM Tris pH 7.9, 500 mM NaCl, 250 mM imidazole) over a 60 ml gradient. The fractions containing PaMurF were confirmed by

Figure 1
Coomassie-stained SDS-PAGE (12%) of purified PaMurF used for crystallization. Left lane, molecular-mass marker (labelled in kDa); right lane, 20 μ g sample of purified PaMurF.

SDS-PAGE analysis, pooled and concentrated using a Vivaspin 20 centrifugal concentrator. The resulting concentrated sample was applied onto a Superdex 75 10/300 GL (GE Healthcare) column equilibrated in 100 mM HEPES pH 7.5, 20 mM MgCl₂, 200 mM NaCl, 6 mM DTT. The fractions containing PaMurF were pooled, concentrated to 2.5 ml and buffer-exchanged into crystallization buffer (50 mM HEPES pH 7.5, 150 mM KCl, 10 mM KCl) using a PD10 column (GE Healthcare). The protein solution was then concentrated to 12 mg ml⁻¹ for crystallization experiments (Fig. 1).

2.2. Crystallization

Crystallization experiments were performed by vapour diffusion at 291 K. Screening was carried out at a protein concentration of 12 mg ml⁻¹ with a tenfold molar excess of the substrate UDP-MurNAc-tripeptide-DAP (Lloyd *et al.*, 2008; Bugg *et al.*, 2011; Clarke *et al.*, 2009) in 96-well format using MRC 2 Well Crystallization Plates. The three commercially available screens that were set up were PACT Premier, JCSG-*plus* and Morpheus (all from Molecular Dimensions Ltd). Drops were dispensed with the aid of a Honeybee 963 robot (Digilab) to give a protein:mother liquor ratio of 1:1 and a final volume of 400 nl. Each well contained 54 μ l reservoir solution. Diffraction-quality crystals were obtained from JCSG-*plus* condition A5, with the reservoir solution consisting of 0.2 M magnesium formate, 20% PEG 3350 (Fig. 2). The crystals always formed in clusters, but single needles could be separated when pressure was applied to the centre of the cluster. Although the needles were thin

Figure 2
Crystals of PaMurF in the presence of UDP-MurNAc-tripeptide-DAP grown from the JCSG-*plus* screen (Molecular Dimensions) that were used for data collection. The mother liquor consisted of 0.2 M magnesium formate, 20% PEG 3350.

Figure 3
The crystal of PaMurF in the presence of UDP-MurNAc-tripeptide-DAP that was used for data collection.

Table 1

Data-collection and processing statistics for PaMurF–UDP–MurNAc–tripeptide–DAP.

Values in parentheses are for the highest resolution shell.

Diffraction source	I04-1, Diamond Light Source
Wavelength (Å)	0.920
Detector	MAR Mosaic 300 mm CCD
Data-processing software	xia2 v.0.3.4.0
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 57.81$, $b = 87.29$, $c = 92.61$
Molecules per asymmetric unit	1
Matthews coefficient (Å ³ Da ^{−1})	2.49
Solvent content (%)	50.55
Resolution range (Å)	49.04–1.92 (1.97–1.92)
Total observations	168745
Unique observations	36216
Multiplicity	4.7 (4.5)
Average $I/\sigma(I)$	13.2 (2.4)
$R_{\text{merge}}^{\dagger}$ (%)	0.087 (0.587)
Completeness (%)	99.3 (99.8)
Wilson B value (Å ²)	21.5

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl .

and long, they were not overly fragile and were easy to manipulate. Fig. 3 shows the crystal that was used for data collection.

2.3. Data collection

A single crystal of the PaMurF complex was separated from the needle cluster and flash-cooled in liquid nitrogen using reservoir solution containing 20% glycerol as a cryoprotectant. X-ray diffraction data were collected on beamline I04-1 at Diamond Light Source, UK at 100 K. Diffraction images were measured using a MAR Mosaic 300 mm CCD detector. Data were processed automatically using xia2 (Winter, 2010). Data-collection and processing statistics are shown in Table 1.

3. Results and discussion

PaMurF has been overexpressed, purified and potentially crystallized in the presence of its substrate UDP–MurNAc–tripeptide–DAP. The crystal diffracted to 1.92 Å resolution. One molecule is predicted to be present in the asymmetric unit, which gives a Matthews coefficient of 2.5 Å³ Da^{−1} and a solvent content of 51%. Structure solution is under way using the *E. coli* apo structure (PDB entry 1gg4; Yan *et al.*, 2000) for molecular replacement. The resulting high-resolution structure has the potential to provide insight into substrate binding and to indicate whether there are any domain rearrangements in the binding process, as has been implied in studies of other Mur ligase enzymes (Smith, 2006).

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