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Purification, crystallization and preliminary X-ray diffraction analysis of GatD, a glutamine amidotransferase-like protein from *Staphylococcus aureus* peptidoglycan

Amidation of peptidoglycan is an essential feature in *Staphylococcus aureus* that is necessary for resistance to β -lactams and lysozyme. GatD, a 27 kDa type I glutamine amidotransferase-like protein, together with MurT ligase, catalyses the amidation reaction of the glutamic acid residues of the peptidoglycan of *S. aureus*. The native and the selenomethionine-derivative proteins were crystallized using the sitting-drop vapour-diffusion method with polyethylene glycol, sodium acetate and calcium acetate. The crystals obtained diffracted beyond 1.85 and 2.25 Å, respectively, and belonged to space group $P2_12_12_1$. X-ray diffraction data sets were collected at Diamond Light Source (on beamlines I02 and I04) and were used to obtain initial phases.

1. Introduction

Peptidoglycan, the major component of the Gram-positive bacterial cell wall, is a polymer composed of glycan chains cross-linked by short peptides and is responsible for cell-shape maintenance and for counterbalancing turgor pressure (Höltje, 1998). The importance of peptidoglycan for cell survival and its exclusivity to the bacterial kingdom renders most enzymatic steps involved in its biosynthesis excellent targets for antimicrobial therapy.

Staphylococcus aureus, an opportunistic bacterium responsible for a wide range of infections (Klevens *et al.*, 2007; Boucher *et al.*, 2009), owes its success as a human pathogen mainly to its capacity to acquire antibiotic-resistance traits. Most genes involved in the peptidoglycan-biosynthesis pathway are intimately related to the mechanism of β -lactam resistance, enhancing their potential as targets for antimicrobial therapy (De Lencastre *et al.*, 1999).

The main biosynthesis of peptidoglycan is well understood (Veiga *et al.*, 2009; Scheffers & Pinho, 2005). Additional steps are responsible for secondary modifications to the main structure (Vollmer, 2008). In *S. aureus*, one such modification is the amidation of the γ -carboxyl group of the second residue of the stem peptide, D-isoglutamate, resulting in the formation of D-isoglutamine (Siewert & Strominger, 1968). Recently, the *murT-gatD* operon was identified as the genetic determinant of peptidoglycan amidation in *S. aureus*, which is found to be widespread among bacteria as a syntenic block, almost exclusively in Gram-positives. The impaired expression of this operon impacts bacterial growth, β -lactam resistance and intrinsic lysozyme resistance (Figueiredo *et al.*, 2012). Moreover, the two proteins physically interact and form a glutamine amidotransferase bi-enzymatic complex (Münch *et al.*, 2012).

We have cloned and expressed the GatD protein in *Escherichia coli* and purified and crystallized it. X-ray diffraction data were collected both from native as well as SeMet-containing protein and were used to obtain preliminary phases of the model.

2. Materials and methods

2.1. Cloning, overexpression and purification of GatD

The coding sequence of the *gatD* gene was amplified from *S. aureus* and cloned into the vector pOPINF using the In-Fusion method to

Table 1

Macromolecule-production information.

Source organism	<i>S. aureus</i> COL
DNA source	<i>S. aureus</i> COL
Forward primer	AAGTTCTGTTTCAGGGCCCGCATGAATTGACTATTATCAT- TTTATGTCAG
Reverse primer	ATGGTCTAGAAAGCTTTAACGAGATTTCTTCTGTCTATTG- CTC
Cloning vector	pOPINF
Expression vector	pOPINF
Expression host	<i>E. coli</i> strain Lemo21(DE3)
UniProt accession code	Q5HEN2
Complete amino-acid sequence of the construct produced	GPHELTIIYHFMSDKLNLYSDIGNIIALRQAKKRNIKVNVV- EINETEGITFDECDIFFIGGSDREQALATKELSKIKTP- LKEAIEDGMPGLTICGGYQFLGKKYITPDGTELEGLGIL- DFYTESKTNRLTGDIVIESDTFGTIVGFENHGGRTYHDF- GTLGHVTFGYGNDEDKKEGIHYKNLLGTYLHGPIPKN- YEITDYLLKACERKGIPEPKEIDNEAEIQAQVQLIDR- ANRQKKS

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	Greiner Bio-One
Temperature (K)	294
Protein concentration (mg ml ⁻¹)	45
Buffer composition of protein solution	20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM TCEP
Composition of reservoir solution	30% (w/v) PEG 400, 100 mM sodium acetate/ acetic acid pH 4.5, 200 mM calcium acetate
Volume and ratio of drop	200 nl; 1:1
Volume of reservoir (µl)	90

generate the construct OPPF12143 (Bird, 2011). The protein was produced in *E. coli* using the auto-induction method (Studier, 2005). Macromolecule production information is given in Table 1.

After 20 h of incubation, the cells were harvested and resuspended in lysis buffer: 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazole and 0.2% Tween 20 supplemented with protease inhibitors (Sigma) and 400 U ml⁻¹ DNase type I. The cells were lysed using a Basic-Z cell disruptor at 207 MPa and clarified by centrifugation at 30 000g for 30 min at 4°C. The supernatant was loaded onto a 5 ml HisTrap FF column (GE Healthcare) equilibrated with wash buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazole), extensively washed with this buffer and eluted with elution buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 500 mM imidazole). The sample was subsequently loaded onto a Superdex 200 HiLoad 16/60 column (GE

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

	SAD	Native
Diffraction source	I04, DLS	I02, DLS
Wavelength (Å)	0.9796	1.0000
Temperature (K)	100	100
Detector	ADSC Q315r	Pilatus 6M
Crystal-to-detector distance (mm)	375	407.4
Rotation range per image (°)	1.0	0.5
Total rotation range (°)	360	1200
Exposure time per image (s)	0.5	0.04
Space group	<i>P</i> ₂ ₁ ₂ ₁	<i>P</i> ₂ ₁ ₂ ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	48.28, 93.00, 109.30	48.61, 93.92, 110.08
α , β , γ (°)	90, 90, 90	90, 90, 90
Mosaicity (°)	0.387	0.141
Resolution range (Å)	47.12–2.25	36.43–1.85
Total No. of reflections	301512	839410
No. of unique reflections	24020	42987
Completeness (%)	98.7 (89.2)	97.9 (83.7)
Multiplicity	12.6 (6.7)	19.5 (10.1)
$\langle I/\sigma(I) \rangle$	16.5 (2.2)	20.7 (2.2)
<i>R</i> _{p.i.m.}	0.078 (0.477)	0.028 (0.313)
Overall <i>B</i> factor from Wilson plot (Å ²)	9.429	15.237
Anomalous completeness (%)	98.7 (89.2)	
Anomalous multiplicity	6.7 (3.4)	

Healthcare) equilibrated with gel-filtration buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM TCEP). Fractions containing GatD protein were pooled and the N-terminal hexahistidine tag was removed by cleavage with 3C protease. The mixture was then purified by reverse Ni-affinity chromatography. The protein was concentrated to 10, 20 and 45 mg ml⁻¹ in gel-filtration buffer for crystallization. The expressed protein differs from the native in its N-terminus, where the original M is substituted by GP.

A selenomethionine derivative was expressed using the Seleno-Methionine Expression Media kit (Molecular Dimensions) and the purification protocol was followed as described above.

2.2. Crystallization

Crystallization screens were performed at the Oxford Protein Production Facility (OPPF-UK) using a Cartesian instrument (Digilab MicroSys liquid-handling system). 100 nl GatD sample was mixed with 100 nl crystallization solution and equilibrated over 90 µl reservoir solution (see Table 2 for details). Crystals appeared in

Figure 1

GatD crystals obtained using the crystallization robot (native, left; SeMet derivative, right). The dimensions of the best crystal were 250 × 30 × 30 µm.

Figure 2
Representative diffraction patterns of the crystals (native, left; SeMet derivative, right). The resolution at the edge of the detector is 2.1 and 2.5 Å, respectively.

several conditions from the Emerald Wizard 1 and 2 crystallization screen (Rigaku Reagents). The best crystals (native and SeMet derivative) grew after 48 h (Fig. 1). The crystals were cryoprotected in 50% (v/v) PEG 400 and flash-cooled in liquid nitrogen prior to data collection.

2.3. Data collection and processing

Data were collected on beamlines I02 (on a Pilatus 6M detector) and I04 (on an ADSC Quantum Q315r detector) at the Diamond Light Source (DLS). Crystals of the native protein diffracted beyond 1.9 Å resolution and those of the SeMet-derivatized protein diffracted beyond 2.3 Å resolution (Fig. 2). The data were automatically processed using *xia2* (Winter, 2010). Data-collection and processing statistics are given in Table 3.

Initial phases were obtained by single-wavelength anomalous diffraction (SAD) using data collected from SeMet derivative at the Se edge peak.

3. Results and discussion

The recently identified MurT–GatD enzymatic complex represents an unexplored step as a potential antimicrobial target. MurT shares considerable similarity with the sequence of the Mur ligases of *S. aureus*, which are cytoplasmic enzymes that are responsible for the sequential addition of amino-acid residues to the growing muropeptide stem.

GatD shows similarity to the glutamine amidotransferases (GATases), with glutamine amide-transfer activity to a wide variety of substrates (Massière & Badet-Denisot, 1998). Typically, GATases catalyze two distinct reactions: the glutaminase reaction, in which glutamine is converted into ammonia and glutamate, and the synthase reaction, in which ammonia is transferred to an acceptor substrate. These two reactions occur at distinct active sites, which may sit on the same polypeptide chain or on independent protein subunits. GatD

protein corresponds to a glutaminase subunit, most probably being responsible for the production of ammonia from glutamine.

In order to determine the structure of *S. aureus* GatD protein, the encoding region of the *gatD* gene was cloned into pOPINF plasmid and expressed in *E. coli* Lemo21(DE3) as an N-terminal His-tag fusion. The purity of the recombinant protein was estimated by SDS-PAGE, which showed a single band corresponding to a molecular weight of 27 kDa.

The crystallization trials were performed at a high-throughput crystallization facility. Several crystallization hits were obtained using the Emerald Wizard 1 and 2 screens from Rigaku Reagents.

Diffraction data were collected on I02 and I04 at DLS to a resolution beyond 1.9 Å. Initial phases were obtained by single-wavelength anomalous diffraction (SAD) using data collected from SeMet derivatives at the Se edge peak.

The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 48.29$, $b = 93.00$, $c = 109.31$ Å.

The structure of GatD, together with complete biochemical studies, will provide important insights into the molecular basis of the mechanism responsible for the amidation of the glutamic acid residues of the peptidoglycan of *S. aureus*.

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