Expression, purification and crystallization of a membrane-associated, catalytically active type I signal peptidase from *Staphylococcus aureus*

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Staphylococcus aureus infections are becoming increasingly difficult to treat as they rapidly develop resistance to existing antibiotics. Bacterial type I signal peptidases are membrane-associated, cell-surface serine proteases with a unique catalytic mechanism that differs from that of eukaryotic endoplasmic reticulum signal peptidases. They are thus potential antimicrobial targets. *S. aureus* has a catalytically active type I signal peptidase, SpsB, that is essential for cell viability. To elucidate its structure, the *spsB* gene from *S. aureus* Newman strain was cloned and overexpressed in *Escherichia coli*. After exploring many different protein-modification constructs, SpsB was expressed as a fusion protein with maltose-binding protein and crystallized by hanging-drop vapour diffusion. The crystals belonged to the monoclinic space group $P2_1$ and diffracted to 2.05 Å resolution. The crystal structure of SpsB is anticipated to provide structural insight into Gram-positive signal peptidases and to aid in the development of antibacterial agents that target type I signal peptidases.

1. Introduction

Bacterial proteins that are exported outside the cytoplasm are normally synthesized as pre-proteins with an N-terminal signal peptide that directs them to either the Sec or the Tat (twin-arginine translocation) protein-export pathway (Dalbey *et al.*, 2012; Auclair *et al.*, 2012). Both pathways rely on type I signal peptidases (SPase-I) to remove the signal peptide, a step essential for mature protein release and cell viability (Smitha Rao & Anné, 2011; Dalbey *et al.*, 2012; Auclair *et al.*, 2012). SPase-I enzymes are membrane-associated proteins that are recalcitrant to crystallization owing to their tendency to aggregate, their flexible nature and their transient interaction with substrate signal peptides. The first crystal structure of an SPase-I (LepB), from the Gram-negative *Escherichia coli*, was co-crystallized with a (5S)-penem inhibitor covalently bound to the nucleophile serine (Ser90) in the catalytic site (Paetzel *et al.*, 1998). This structure showed that LepB utilized a unique Ser/Lys catalytic dyad rather than the conventional Ser/His/Asp triad common in eukaryotic serine proteases. In both Gram-positive and Gram-negative prokaryotes, SPase-I enzymes are located on the extra-cellular cell surface, thus presenting a feasible antimicrobial target (Paetzel *et al.*, 2000; van Roosmalen *et al.*, 2004).

Crystal structures of LepB have aided in the development of three potential classes of inhibitors: a morpholino- β -sultam inhibitor (Luo *et al.*, 2009; Liu *et al.*, 2011), acrylomycins (Liu *et al.*, 2011) and lipoproteins (Paetzel *et al.*, 2004; Liu *et al.*, 2011). However, these LepB inhibitors show only limited inhibition of the *Staphylococcus aureus* SPase-I SpsB (Paetzel *et al.*, 1998; Smith *et al.*, 2010; Smith & Romesberg, 2012), which is suggestive of structural differences between Gram-positive and Gram-negative bacterial signal peptidases. Although SpsB and LepB are predicted to share a conserved catalytic apparatus, they share only 23% sequence identity (Cregg *et al.*, 1996) and the actual mechanism of signal-peptide binding remains

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DNA polymerase (iProof, Bio-Rad) was used for the PCR amplification of the pProEx-SpsB plasmid to produce a linearized PCR product with the desired mutation at the 5' end of the sense primer. The methylated parental template without the S36A mutation was then removed from the nonmethylated linear PCR product by *DpnI* digestion. Finally, the PCR product was re-circularized by intramolecular ligation. The resulting plasmid pProEx-SpsB S36A was transformed into *E. coli* DH5 α cells and sequence-verified.

2.1.4. Cloning of MBP-SpsB S36A fusion protein. An MBP-fusion vector was generated by inserting the gene for maltose-binding protein (MBP) between the His₆ tag and the rTEV (recombinant *Tobacco etch virus* protease) cleavage site of pProExHta (Fig. 1a). Firstly, inverse PCR using the primers MBPpProEx_F6 and MBPpProEx_R6 was used to introduce a sequence containing *NdeI* and *SmaI* restriction sites in the linker region between the N-terminal His₆-tag and rTEV sites of pProExHta. The MBP (residues Leu33–Asn393) was PCR-amplified from the pMAL-c5X vector (New England Biolab) using MBP_F7 and MBP_R7 primers, and the PCR product was cloned by standard cloning protocols into the newly generated *NdeI* and *SmaI* restriction sites of the modified vector. The resulting vector, pMBP-ProExHta, contains an N-terminal His₇-tagged MBP fusion protein followed by an rTEV cleavage site and the standard pProExHta multiple cloning site (MCS). The SpsB S36A gene was subcloned from pProEX-SpsB S36A with *KasI* and *EcoRI* restriction digestion into pMBP-ProExHta to produce a cleavable MBP-SpsB S36A fusion-protein construct.

To produce a noncleavable MBP-SpsB S36A fusion protein with a two-residue linker between the MBP and SpsB S36A (pMBP2-SpsB S36A), the rTEV recognition site was excised from the pProEX-SpsB S36A plasmid construct through *SmaI* and *EheI* restriction digestion followed by intramolecular blunt-end re-ligation. This construct was the template for inverse PCR to extend the linker region from two residues to three, four or five residues. Briefly, the template was linearized by inverse PCR with oligonucleotide primer pairs that both extended the linker sequence and re-introduced the *KasI* restriction-endonuclease site previously removed in the production of the two-residue linker construct. The methylated template DNA (pMBP2-SpsB S36A) was removed by *DpnI* digestion and the resulting linear PCR product was intramolecularly re-ligated to create plasmid constructs with a three-, four- or five-residue linker between the MBP and SpsB S36A. The forward and reverse primers pairs were MBPLink_F8 and MBPLink3_R8 with an –AGA– three-residue linker, MBPLink_F8 and MBPLink4_R9 with an –AGGA– four-residue linker and MBPLink_F8 and MBPLink5_R10 with an –AGSGA– five-residue linker (Table 1). These three constructs were designated pMBP3-SpsB S36A, pMBP4-SpsB S36A and pMBP5-SpsB S36A, respectively. Recombinant proteins were expressed and purified as described in §2.1.5.

2.1.5. Protein expression and purification of recombinant SpsB. *E. coli* BL21 (λ DE3) pRIL cells (Stratagene) harbouring recombinant protein constructs (Table 1) were grown in LB medium supplemented with appropriate antibiotics to a density of OD₆₀₀ = 0.5–0.6 at 310 K in an aerating shaker. Protein expression was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM and the cells were incubated for 3 h at 310 K. The cells were then pelleted at 4000g at 277 K for 20 min, snap-frozen and stored at 253 K.

Recombinant protein was purified from frozen cells, which were thawed and resuspended in lysis buffer [100 mM Tris–HCl pH 8.0, 500 mM NaCl, 5% (v/v) glycerol, 10 mM imidazole] with the addition of cOmplete Protease Inhibitor Cocktail EDTA-free tablets (Roche) and were lysed using a cell disruptor at 124 MPa (Constant Systems).

The insoluble protein fraction was removed by centrifugation (20 198g at 277 K for 30 min) and the soluble recombinant protein fraction was loaded onto a HiTrap Chelating 5 ml column (GE Healthcare) for purification by immobilized metal-affinity chromatography (IMAC). The recombinant protein was washed with wash buffer [50 mM Tris–HCl pH 8.0, 500 mM NaCl, 5% (v/v) glycerol, 20 mM imidazole] and eluted with a linear gradient to elution buffer (wash buffer + 500 mM imidazole). The eluted protein was concentrated and subjected to size-exclusion chromatography on a Superdex 200 10/300 column (GE Healthcare) equilibrated with crystallization buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl). For MBP-fused recombinant protein, an alternative crystallization buffer (10 mM Tris–HCl pH 8.0, 20 mM NaCl, 5 mM maltose) was used. The purity of the recombinant protein was analyzed by SDS–PAGE. Purified protein was either used immediately or snap-frozen in liquid nitrogen and stored at 193 K in crystallization buffer. Frozen protein samples were quickly thawed at 310 K and then transferred to ice prior to use.

For non-MBP-fused pProExHta constructs which have a removable His₆ tag, fractions from IMAC containing recombinant protein were dialyzed overnight against a 100 $\times$ volume of dialysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM β -mercaptoethanol) and the His₆ tag was concomitantly removed using recombinant TEV protease at a 1:50 ratio of rTEV to recombinant protein. Undigested protein and rTEV protease were removed by a second round of IMAC followed by size-exclusion chromatography as described above.

2.1.6. In vitro SpsB activity assay. An *S. aureus* signal peptide was fused to green fluorescent protein (SP-GFP) to provide a substrate to assess the catalytic activity of recombinant SpsB. The GFP construct used for this study was previously amplified from *Aequorea victoria* using primers GFP_F11 and GFP_R11, and cloned into pProExHta in the *NcoI* and *NotI* multiple cloning sites. Customized complementary 69 bp synthetic oligonucleotides (Invitrogen) encoding a signal-peptide sequence known to be cleaved by SpsB in *in vitro* assays (Bruton *et al.*, 2003; LTPTAKAASKIDD) were annealed by a temperature gradient from 368 to 293 K. The annealed product contained single-strand overhangs contemporary to *NcoI* restriction endonuclease digestion and was inserted at the N-terminus of GFP into the *NcoI* site to create pSP-GFP. The resulting constructs were sequenced to select the correct orientation of the signal peptide, and the recombinant protein SP-GFP was expressed and purified as described in §2.1.5.

Purified recombinant SpsB (0.01–1 μ g) and substrate SP-GFP (1 μ g) were mixed on ice in assay buffer (50 mM Tris–HCl pH 8.0) to a total volume of 20 μ l. Catalytic activity was assayed at 310 K overnight. The reaction was stopped by the addition of 5 $\times$ SDS–PAGE protein loading dye, boiled and kept on ice until analyzed by

Figure 2

Cleavage of SP-GFP substrate by recombinant SpsB. Lane 1, SpsB only (1 μ g); lane 2, SP-GFP substrate only (1 μ g); lanes 3–9, SP-GFP (1 μ g) with a dilution series of SpsB at 1, 0.5, 0.2, 0.1, 0.04, 0.02 and 0.01 μ g, respectively. The reaction was carried out overnight at 310 K prior to analysis by SDS–PAGE. Lane M contains molecular-mass marker (labelled in kDa).

Table 2

Crystallization of MBP3-SpsB S36A.

Method	Vapour diffusion with streak-seeding
Plate type	24-well plates
Temperature (K)	290
Protein concentration (mg ml ⁻¹)	2.5–5
Buffer composition of protein solution	10 mM Tris-HCl pH 8, 20 mM NaCl, 5 mM maltose
Composition of reservoir solution	12% PEG 8000, 20% ethylene glycol, 0.2 M amino-acids mix (0.2 M sodium L-glutamate, 0.2 M DL-alanine, 0.2 M glycine, 0.2 M DL-lysine, 0.2 M DL-serine), 100 mM Tris-HCl pH 8.5
Volume and ratio of drop	1 µl:1 µl
Volume of reservoir (µl)	500

SDS-PAGE. Cleavage activity by SpsB was detected by a reduction in the size of the SP-GFP substrate *via* removal of the signal peptide (Fig. 2).

2.2. Crystallization

Purified recombinant protein (MBP3-SpsB S36A) was concentrated *via* a Vivaspin concentrator (GE Healthcare; 4000g at 277 K) to approximately 20–50 mg ml⁻¹ as measured by molar absorptivity. Purified recombinant proteins were subjected to sitting-drop vapour-diffusion crystallization screening trials at 290 K using a locally compiled crystallization screen that has subsequently been modified to include conditions inspired by the Morpheus screen (Moreland *et al.*, 2005; Gorrec, 2009). Fine needle-shaped crystals were obtained by hanging-drop vapour diffusion in 24-well flat-bottom plates when 1 µl protein solution (10 mg ml⁻¹ in 10 mM Tris-HCl pH 8.0, 20 mM NaCl, 5 mM maltose) was mixed with an equal volume of well solution [10% PEG 8000, 20% ethylene glycol, 0.2 M amino-acids mix (0.2 M sodium L-glutamate, 0.2 M DL-alanine, 0.2 M glycine, 0.2 M DL-lysine, 0.2 M DL-serine), 100 mM Tris-HCl pH 8.5]. This was followed by grid-screen optimization around the same condition with various concentrations of protein (2–20 mg ml⁻¹) and PEG 8000 precipitant (8–16%). Similar clusters of needle-shaped crystals were obtained by spontaneous nucleation within 24 h with 10% PEG 8000 or higher and with a protein concentration of 5 mg ml⁻¹ or higher. These crystals were subsequently optimized by streak-seeding (Fig. 3). A single cluster of needle-shaped crystals were transferred to 1 µl well solution on a glass slide. A cat's whisker was gently passed across the surface of the needle cluster and then streaked into drops

Table 3

Data collection and processing for MBP3-SpsB S36A.

Values in parentheses are for the outer shell.

Diffraction source	Australian Synchrotron beamline MX1
Wavelength (Å)	0.95468
Temperature (K)	100
Detector	ADSC
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	1.0
Total rotation range (°)	360
Exposure time per image (s)	1.0
Space group	<i>P</i> 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 57.7, <i>b</i> = 63.6, <i>c</i> = 79.9, α = 90.0, β = 92.6, γ = 90.0
Mosaicity (°)	0.51
Resolution range (Å)	19.44–2.05 (2.16–2.05)
Total No. of reflections	275184 (39732)
No. of unique reflections	36318 (5227)
Completeness (%)	99.8 (99.7)
Multiplicity	7.6 (7.6)
$\langle I/\sigma(I) \rangle$	10.1 (1.4†)
<i>R</i> _{meas}	0.224 (1.51)
Overall <i>B</i> factor from Wilson plot (Å ²)	26

† The data limits were chosen according to Karplus & Diederichs (2012), with a *CC*_{1/2} of ~0.5 in the outer shell. Owing to a degree of anisotropy, the mean $\langle I/\sigma(I) \rangle$ in the outer shell falls below 2.0.

from the grid screen mentioned above, in which no crystals had formed after 24 h. The hanging drop was re-sealed after seeding and crystals grew within 7 d. The best crystals were obtained from multiple rounds of streak-seeding with a protein concentration of 2.5–5 mg ml⁻¹ mixed with an equal volume of well solution at 290 K (Table 2).

2.3. Data collection and processing

Crystals of recombinant MBP3-SpsB S36A protein were flash-cooled directly in liquid nitrogen. Diffraction data were collected on the MX1 beamline at the Australian synchrotron. Diffraction images were integrated using *XDS* (Kabsch, 2010), reindexed using *POINTLESS* (Evans, 2006) and scaled using *SCALA* (Evans, 2006). Data-collection statistics for MBP3-SpsB S36A are given in Table 3.

3. Results and discussion

Type I signal peptidases are membrane-associated proteins that tend to be recalcitrant to crystallization. In this study, we have

Figure 3

Needle-shaped crystals of MBP3-SpsB S36A with a three-residue linker from the initial screen (left) and optimized (right) in the presence of PEG 8000 as precipitant.

systematically engineered a series of constructs in an effort to crystallize SpsB, the only active SPase-I in *S. aureus*. The pProEx-SpsB construct was soluble as a recombinant His₆-tagged protein, but failed to crystallize even at a high protein concentration (up to 90 mg ml⁻¹). The recombinant protein was catalytically active and able to cleave the SP-GFP reporter protein, a signal-peptide GFP-fusion protein, thus indicating that the protein was correctly folded (Fig. 2). We also generated an inactive SpsB S36A mutant in which the catalytic Ser36 was mutated to alanine, abolishing activity, through concern that the previously reported intermolecular self-cleavage may effect the sample homogeneity and thus crystallization of the protein (Zheng *et al.*, 2002). Additional experiments using the SpsB S36A mutant alone and surface-entropy reduction also failed to give crystals (data not shown). Finally, N-terminal MBP-fusion constructs joined to SpsB by linkers of different lengths were constructed for carrier-driven crystallization. All five MBP-fusion constructs were subjected to crystallization trials, but only recombinant MBP3-SpsB S36A protein (with a three-residue linker; Fig. 1*b*) yielded crystals. The initial needle-shaped crystals (Fig. 3) grew in a condition consisting of 10% PEG 8000, 20% ethylene glycol, 0.2 M amino-acids mix (0.2 M sodium L-glutamate, 0.2 M DL-alanine, 0.2 M glycine, 0.2 M DL-lysine, 0.2 M DL-serine), 100 mM Tris-HCl pH 8.5. Crystal conditions were propagated using hanging-drop vapour diffusion, with the best crystals grown after multiple rounds of streak-seeding. Diffraction-quality crystals (Fig. 3) grew in 12% PEG 8000, 20% ethylene glycol, 0.2 M amino-acids mix, 100 mM Tris-HCl pH 8.5 mixed with an equal volume of protein solution at 2.5 mg ml⁻¹ 7 d after seeding. The crystal belonged to the monoclinic space group *P*2₁, with unit-cell parameters *a* = 57.7, *b* = 63.6, *c* = 79.9 Å, α = 90.0, β = 92.6, γ = 90.0°, and diffracted to 2.05 Å resolution. The calculated Matthews coefficient (Matthews, 1968) was 2.61 Å³ Da⁻¹, with a solvent content of 52.8%, which corresponds to the presence of one monomer in the asymmetric unit. Diffraction-quality crystals of SpsB were ultimately produced as a fusion protein with maltose-binding protein, which highlights the potential of carrier-driven crystallization

as a strategy for the crystallization of challenging membrane-associated proteins.

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