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Expression and crystallization of DsbA from *Staphylococcus aureus*

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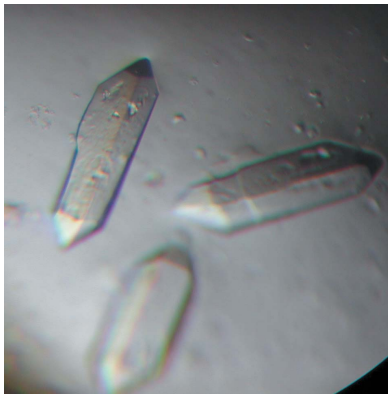

Bacterial Dsb proteins catalyse the *in vivo* formation of disulfide bonds, a critical step in the stability and activity of many proteins. Most studies on Dsb proteins have focused on Gram-negative bacteria and thus the process of oxidative folding in Gram-positive bacteria is poorly understood. To help elucidate this process in Gram-positive bacteria, DsbA from *Staphylococcus aureus* (SaDsbA) has been focused on. Here, the expression, purification, crystallization and preliminary diffraction analysis of SaDsbA are reported. SaDsbA crystals diffract to a resolution limit of 2.1 Å and belong to the hexagonal space group $P6_5$ or $P6_1$, with unit-cell parameters $a = b = 72.1$, $c = 92.1$ Å and one molecule in the asymmetric unit (64% solvent content).

1. Introduction

Disulfide bonds play a key role in the folding pathway of many proteins and cells have evolved complex machineries to promote disulfide catalysis. In the periplasmic space of Gram-negative bacteria, the disulfide-bond (Dsb) proteins regulate the formation and rearrangement of disulfide bonds during protein folding (Kadokura *et al.*, 2003; Masip *et al.*, 2004). *Escherichia coli* Dsb proteins form two pathways, the DsbA-DsbB pathway (Bardwell *et al.*, 1991, 1993), which rapidly introduces disulfide bonds into target proteins, and the DsbC/DsbG-DsbD pathway (Rietsch *et al.*, 1996), which catalyses the rearrangement of incorrect disulfide bonds.

Protein disulfide catalysis has been studied extensively in *E. coli*, but little information is available about this process in other organisms. For example, Gram-positive bacteria do not have a classic periplasmic compartment, but do contain an extraprotoplasmic compartment confined between the plasma membrane and the outer cell wall (Pooley *et al.*, 1996; Matias & Beveridge, 2006), and genomic sequence data indicates that they encode Dsb orthologues (Kouwen *et al.*, 2007). Moreover, functional Dsb homologues have been described in Gram-positive organisms, including *Bacillus brevis* (Ishihara *et al.*, 1995), *B. subtilis* (Erlendsson & Hederstedt, 2002; Dorenbos *et al.*, 2002; Sarvas *et al.*, 2004; Meima *et al.*, 2002; Stein, 2005) and *Mycobacterium tuberculosis* (Goulding *et al.*, 2004). However, the mechanisms of disulfide catalysis in Gram-positive bacteria remain mostly uncharacterized.

To investigate the oxidative folding of secreted proteins in Gram-positive bacteria, we focused on *Staphylococcus aureus*, a human pathogen that secretes disulfide-bond-containing virulence factors (Dziewanowska *et al.*, 1996). This organism encodes a DsbA homologue (SaDsbA), a 23 kDa lipoprotein that shares just 15% sequence identity with *E. coli* DsbA yet can functionally complement this protein (Dumoulin *et al.*, 2005). Here, we report the crystallization of SaDsbA, which to the best of our knowledge is the first Gram-positive DsbA to be crystallized.

2. Material and methods

2.1. Cloning, expression and protein purification

To prepare SaDsbA (AAG41993) for crystallization, a gene fragment encoding a soluble form of the protein (residues 6–181 of the

mature protein; Dumoulin *et al.*, 2005) was cloned into a pET21a vector (Novagen) using *Nhe*I and *Xho*I restriction sites. The cloning procedure introduced four additional residues: two at the N-terminus (Met-Ala) and two at the C-terminus (Leu-Glu). SaDsbA engineered with a C-terminal hexahistidine tag was expressed in BL21 (DE3) pLysS cells using autoinduction (Studier, 2005). Briefly, cells harbouring pET21a-SaDsbA were grown with agitation at 200 rev min^{-1} for 24 h at 303 K in minimal medium (Studier, 2005) supplemented with 100 µg ml^{-1} ampicillin.

The cells were then harvested by centrifugation and lysed in 50 m*M* Tris pH 7, 150 m*M* NaCl, 0.5% Triton-X, EDTA-free protease-inhibitor cocktail (Roche) and DNase. Cell debris was removed by centrifugation at 21 000*g* for 30 min. Histidine-tagged SaDsbA was purified using cobalt-chelate chromatography (BD TALON resin) and eluted with 50 m*M* Tris pH 7, 150 m*M* NaCl, 250 m*M* imidazole.

SaDsbA was oxidized by the addition of 1.7 m*M* copper(II) 1,10-phenanthroline (1 h, 277 K) and then equilibrated in 25 m*M* 4-(2-hydroxyethyl)-1-piperazine-*N*-2-ethanesulfonic acid (HEPES) pH 6.7, 150 m*M* NaCl using a PD-10 column (GE Healthcare). The protein was further purified by gel-filtration chromatography (ÄKTA, GE Healthcare, Piscataway, NJ, USA) using a Superdex S-200 column (GE Healthcare, Piscataway, NJ, USA) followed by ion-exchange chromatography on a Mono S 5/50GL column (GE Healthcare, Piscataway, NJ, USA).

Fractions containing SaDsbA were pooled and concentrated to 50 mg ml^{-1} in 20 m*M* HEPES buffer pH 6.7, 50 m*M* NaCl using Amicon Ultra centrifugal filter devices with 10 kDa cutoff (Millipore, Billerica, MA, USA). The protein concentration was measured at 280 nm (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and the purity was assessed by SDS–PAGE analysis and mass spectrometry.

LC/MS analysis of SaDsbA was undertaken using an Applied Biosystems/MDS SCIEX QSTAR Pulsar electrospray ionization quadrupole time-of-flight (ESI QqTOF) mass spectrometer equipped with an electrospray ionization source and linked to an upstream Agilent 1100 Series HPLC system.

Selenomethionine (SeMet) SaDsbA was produced using previously described methods (Heras *et al.*, 2003). Briefly, BL21(DE3) pLysS cells harbouring pET21a-SaDsbA plasmid were grown at 310 K in M63 minimal medium containing 50 µg ml^{-1} DL-selenomethionine. Cultures were induced with 0.5 m*M* IPTG (final concentration) and harvested 3 h post-induction. SeMet SaDsbA was extracted and purified as described above for native SaDsbA.

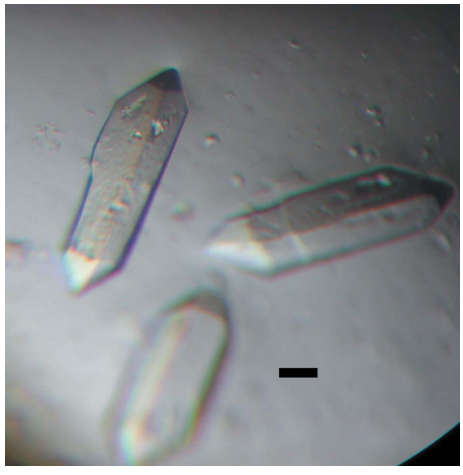

Figure 1
Crystals of SaDsbA obtained in 30% PEG 3350 using the hanging-drop vapour-diffusion method. The crystals reached typical dimensions of 0.2 × 0.15 × 0.6 mm. The scale bar corresponds to 0.1 mm.

Table 1
Summary of X-ray data-measurement and processing statistics.

Values in parentheses are for the highest resolution shell.

	Native SaDsbA	SeMet SaDsbA
Wavelength (Å)	1.5418	2.2909
Temperature (K)	100	100
Space group	$P6_5$ or $P6_1$	$P6_5$ or $P6_1$
Unit-cell parameters (Å, °)	$a = b = 72.1$, $c = 92.1$, $\alpha = \beta = 90$, $\gamma = 120$	$a = b = 72.0$, $c = 92.4$, $\alpha = \beta = 90$, $\gamma = 120$
Solvent content (%)	64	64
Resolution range	50.0–2.1 (2.18–2.10)	50.0–2.44 (2.53–2.44)
No. of observations	176568	130808
No. of unique reflections	15961 (1578)	10161 (947)
Redundancy	11.1 (10.8)	12.9 (10.4)
Completeness (%)	100 (100)	99.5 (94.5)
$R_{\rm merge}$†	0.051 (0.236)	0.081 (0.407)
$I/\sigma(I)$	44.6 (15.1)	31.3 (5.6)

† $R_{\rm merge} = \sum|I - \langle I\rangle|\sum\langle I\rangle$, where I is the intensity of each individual reflection.

2.2. Crystallization and diffraction data measurement

Initial crystallization trials were carried out using the sitting-drop vapour-diffusion method in 96-well plates (Greiner low profile with one and three drops per well) and commercial crystallization screens including Crystal Screens I and II, PEG/Ion Screen, Sodium Malonate Grid Screen (Hampton Research, San Diego, CA, USA), JB Screen 2, JB Screen 3, Precipitant Synergy Screen and Wizard I and II (Jena Bioscience, Jena, Germany). The sitting-drop crystallization trials were set up manually using a multi-channel pipette (Matrix Impact, Thermo Fisher Scientific, Waltham, MA, USA); 1 µl protein solution (50 mg ml^{-1} in 25 m*M* HEPES pH 6.7, 50 m*M* NaCl) and 1 µl precipitant solution were added together and then equilibrated against well solution (100 µl) in a temperature-controlled room at 293 K. The sitting-drop crystallization trials (∼800) failed to produce crystals. Further trials were carried out using a TOPAZ FID Crystallizer (TOPAZ Screening Chip 1.96) and OptiMix-1 and OptiMix-2 screens (Fluidigm Corporation, San Francisco, CA, USA). This system requires just 1 µl of protein to screen 96 conditions. Using this free-interface diffusion approach, crystals were observed in a condition containing only 25% polyethylene glycol of average molecular weight 3350 Da (PEG 3350). This condition was translated following the manufacturer's recommended procedure to 24-well hanging-drop vapour-diffusion experiments using VDXm plates and 18 mm siliconized cover slips (Hampton Research, San Diego, CA, USA). Higher concentrations of PEG 3350 were screened (25–35% PEG 3350 in water) and three different protein:precipitant ratios (1 µl:1 µl, 0.75 µl:1.25 µl and 0.5 µl:1.5 µl). The protein concentration used in the optimization screens was 50 mg ml^{-1} in 25 m*M* HEPES pH 6.7, 50 m*M* NaCl. Crystals of SeMet SaDsbA were grown essentially as described for the native protein.

2.3. X-ray diffraction

Diffraction data for native SaDsbA crystals were measured using a Rigaku FR-E copper rotating-anode generator operated at 45 kV and 45 mA with Osmic Confocal Max-Flux optics (either HiRes^2 or Maxscreen; Rigaku Americas, Houston, TX, USA). Reflections were measured with an R-AXIS IV^{++} imaging-plate area detector (Rigaku Americas, Houston, TX, USA). A Cryo Industries CryoCool LN2 (Cryo Industries, Manchester, NH, USA) was used to cool the crys-

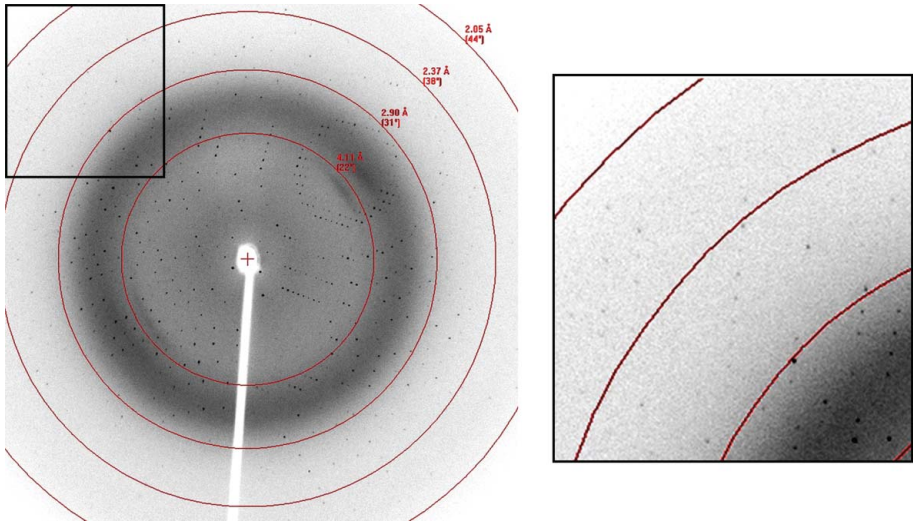

Figure 2
Diffraction from SaDsbA. 0.5° oscillation image of native SaDsbA (Cu $K\alpha$, wavelength = 1.5418 Å). An enlarged region is shown on the right. The crystals diffracted to 2.1 Å resolution.

tals during data measurement. Diffraction data for SeMet SaDsbA crystals were measured using X-rays from an RU-H2R-Cr generator with chromium Osmic MaxFlux optics and R-AXIS IV^{++} detector (Rigaku Americas, Houston, TX, USA). The crystal-to-detector distance was 150 mm (SaDsbA) or 102 mm (SeMet SaDsbA). Crystals were cryoprotected by soaking for 5 min in 30% PEG 3350 and data were processed using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

A standard vapour-diffusion approach using several commercial crystallization screens failed to generate SaDsbA crystals. However, using a free-interface diffusion crystallization chip we were able to grow small elongated rod-shaped crystals in condition No. 54 of the OptiMix-1 screen, which contains only 25% PEG 3350. This condition was successfully translated to 24-well hanging-drop vapour-diffusion experiments by screening higher concentrations of PEG 3350 and a range of protein:precipitant ratios. Single hexagonal rod-shaped crystals were obtained in 28–30% PEG 3350 in drops that had a protein-to-precipitant ratio of less than 1 (Fig. 1). We observed that crystals grown from freshly produced protein took twice as long to grow (∼10 d) than crystals obtained from protein stored for a few days at 277 K. Mass-spectrometric analysis of dissolved crystals indicated that the protein in the crystals corresponded to a degradation product in which the N-terminal four residues (MASA) and C-terminal seven residues (KLEHHHHHH) were removed. Fortuitously, most of these residues corresponded to non-native residues engineered into the construct.

These SaDsbA crystals diffract to 2.1 Å resolution on the high-brilliance FR-E generator (Fig. 2). They belong to the hexagonal space group $P6_5$ or $P6_1$, with unit-cell parameters $a = b = 72.1$, $c = 92.1$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. Assuming the crystals have one molecule per asymmetric unit, the Matthews coefficient (V_M) is 3.5 Å^3 Da^{-1} and the solvent content is 64% (Matthews, 1968); both are within the usual range for protein crystals. Statistics for the diffraction data are provided in Table 1.

Initial attempts to solve the structure by molecular replacement using *E. coli* DsbA as a model (Martin *et al.*, 1993) and either *CNS* (Brünger *et al.*, 1998) or *Phaser* (Storoni *et al.*, 2004) failed to produce a solution (15% sequence identity). We therefore produced SeMet SaDsbA crystals in order to attempt in-house SAD phasing using Cr $K\alpha$ radiation ($\lambda = 2.29$ Å). Measuring data at this wavelength doubles the anomalous signal of many elements, including sulfur ($\Delta f'' = 1.14$ e$^-$) and selenium ($\Delta f'' = 2.28$ e$^-$), compared with Cu $K\alpha$ radiation ($\lambda = 1.54$ Å; $\Delta f'' = 0.56$ and 1.14 e$^-$ for S and Se, respectively). SeMet SaDsbA crystals diffracted to the corner of the detector (2.44 Å resolution) on the RU-H2R generator, with unit-cell parameters $a = b = 72.0$, $c = 92.4$ Å, $\alpha = \beta = 90$, $\gamma = 120°$ (Table 1). However, the signal was weak for Se-SAD/Cr data (anomalous signal d''/σ at 3.5 Å of 0.98), so that the most likely route for phasing will be MAD or SAD data measurement at a synchrotron.

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