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Expression and crystallization of SeDsbA, SeDsbL and SeSrgA from *Salmonella enterica* serovar Typhimurium

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Pathogens require protein-folding enzymes to produce functional virulence determinants. These foldases include the Dsb family of proteins, which catalyze oxidative folding in bacteria. Bacterial disulfide catalytic processes have been well characterized in *Escherichia coli* K-12 and these mechanisms have been extrapolated to other organisms. However, recent research indicates that the K-12 complement of Dsb proteins is not common to all bacteria. Importantly, many pathogenic bacteria have an extended arsenal of Dsb catalysts that is linked to their virulence. To help to elucidate the process of oxidative folding in pathogens containing a wide repertoire of Dsb proteins, *Salmonella enterica* serovar Typhimurium has been focused on. This Gram-negative bacterium contains three DsbA proteins: SeDsbA, SeDsbL and SeSrgA. Here, the expression, purification, crystallization and preliminary diffraction analysis of these three proteins are reported. SeDsbA, SeDsbL and SeSrgA crystals diffracted to resolution limits of 1.55, 1.57 and 2.6 Å and belonged to space groups $P2_1$, $P2_12_12$ and $C2$, respectively.

1. Introduction

Organisms have evolved complex machineries to support disulfide catalysis in proteins, a process called oxidative protein folding. The disulfide-bond (Dsb) proteins catalyze the formation of disulfide bonds during protein folding in bacteria (Kadokura *et al.*, 2003; Masip *et al.*, 2004). *Escherichia coli* K-12 contains two pathways to regulate the oxidative-folding process: in the oxidative pathway DsbA and DsbB (Bardwell *et al.*, 1991, 1993; Akiyama *et al.*, 1992) introduce disulfide bonds into target proteins and in the isomerase pathway DsbC, DsbG and DsbD (Rietsch *et al.*, 1996) catalyze the reshuffling of incorrect disulfide bonds.

The Dsb systems described for *E. coli* K-12 are not completely conserved in bacteria (Dutton *et al.*, 2008; Heras *et al.*, 2009); many Gram-negative pathogens encode an extended arsenal of Dsb proteins which may assist in the production of virulence factors (Heras *et al.*, 2009; Sinha *et al.*, 2004). For example, *Neisseria meningitidis*, the causative agent of meningitis and meningococcal septicaemia, encodes three different DsbA-like proteins: NmDsbA1 and NmDsbA2 are lipoproteins anchored to the inner membrane, while NmDsbA3 is a soluble periplasmic protein (Sinha *et al.*, 2004; Tinsley *et al.*, 2004). Two of these proteins, NmDsbA1 and NmDsbA3, have been structurally characterized and the disulfide-oxidation mechanism in this pathogen has been investigated (Vivian *et al.*, 2008, 2009; Lafaye *et al.*, 2009). Multiple Dsb proteins are also found in uropathogenic *E. coli* (UPEC), which contains a DsbL/DsbI pair in addition to the oxidase and isomerase pathways found in the prototypic K-12 (Grimshaw *et al.*, 2008; Totsika *et al.*, 2009).

The mechanisms for disulfide catalysis in Gram-negative pathogens with an extended collection of Dsb proteins mostly remain uncharacterized. To further investigate oxidative-folding processes in these organisms we have focused on *Salmonella enterica* serovar Typhimurium, which encodes the standard DsbA–DsbB and DsbC–DsbD systems as well as the DsbL/DsbI redox pair and a virulence plasmid-encoded DsbA-like protein (termed SeSrgA). The three *Salmonella* DsbA paralogues, SeDsbA, SeDsbL and SeSrgA, share low sequence

identity with each other (between 18 and 34%), which might result in different redox function and substrate specificities. Here, we report the crystallization and initial diffraction data for these three DsbA-like oxidoreductases.

2. Material and methods

2.1. Cloning, expression and protein purification

The coding sequences for SeDsbA (Locus Tag SL3945), SeDsbL (Locus Tag SL3166) and SeSrgA (Locus Tag SLP1_0094) were amplified by PCR from *S. enterica* serovar Typhimurium strain SL1344 genomic DNA. All three genes were amplified using the Elongase enzyme mix (Invitrogen, Carlsbad, California, USA) and a protocol that involved an initial denaturation step (3 min at 368 K) followed by 35 cycles of 368 K for 30 s, 323 K for 30 s and 341 K for 2 min. The primers for the PCR reactions were designed to amplify only the mature forms of the proteins (lacking the signal peptide) containing ligation-independent cloning (LIC) overhangs (Table 1).

The amplified genes were subsequently inserted into a modified version of pET21a vector, which encodes an N-terminal His_6 tag followed by the tobacco etch virus (TEV) protease cleavage site using LIC cloning (Donnelly *et al.*, 2006). This construct allows the removal of the tagged signal upon expression and metal-affinity purification. Three additional residues are introduced at the N-terminus of the proteins (*i.e.* SNA) after TEV cleavage.

For crystallization purposes, all three *Salmonella* proteins were expressed in *E. coli* BL21 (DE3) pLysS cells (Invitrogen, Carlsbad, California, USA) using autoinduction (Studier, 2005). Briefly, cells harbouring pET/LIC-SeDsbA, pET/LIC-SeDsbL or pET/LIC-SeSrgA 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 and 34 µg ml^{-1} chloramphenicol.

The cells were then harvested by centrifugation and resuspended in 25 m*M* Tris pH 7, 150 m*M* NaCl, 0.5% Triton X-100, Protease Inhibitor Cocktail 3 (Astral Scientific Pty Ltd, Caringbah, NSW 2229, Australia) and DNAse (Sigma–Aldrich, St Louis, Misssouri, USA) and lysed by sonication (using a Sonifier 250, Branson Ultrasonic Corporation, Danbury, Connecticut, USA). Cell debris was removed by centrifugation at 41 400*g* for 30 min and histidine-tagged proteins were purified using PrepEase His-tagged protein purification resin (USB Corporation, Cleveland, Ohio, USA; 2 g per 100 ml of lysate). The proteins bound to the resin were washed extensively with buffer *A* (25 m*M* Tris–HCl pH 7, 0.5 *M* NaCl, 20 m*M* imidazole) using gravity flow and eluted in buffer *B* (25 m*M* Tris–HCl pH 7, 150 m*M* NaCl, 300 m*M* imidazole). The purified proteins were then cleaved using recombinant His-tagged TEV protease purified to homogeneity (Blommel & Fox, 2007). The protease was added to the Dsb protein samples (0.2 mg of TEV per 10 mg of protein) and dialyzed for 48 h at 277 K against buffer *C* (25 m*M* HEPES–NaOH pH 7, 150 m*M* NaCl). After removing the His-tagged TEV protease from the dialysed samples by batch treatment with PrepEase Ni^{2+}-affinity resin, the proteins were further purified by gel-filtration chromatography (ÄKTA, GE Healthcare, Piscataway, New Jersey, USA) using a Superdex S-75 (GE Healthcare, Piscataway, New Jersey, USA) column pre-equilibrated in buffer *C*. SeDsbA was purified to homogeneity (Fig. 1) by ion-exchange chromatography on a Mono Q 5/50 GL (GE Healthcare, Piscataway, New Jersey, USA) using buffer *D* consisting of 25 m*M* HEPES–NaOH pH 7.5, 50 m*M* NaCl and buffer *E* consisting of 25 m*M* HEPES–NaOH pH 7.5, 500 m*M* NaCl (gradient of 0–60% buffer *E* over 60 min). SeDsbL and SeSrgA were further purified by cation-exchange chromatography (Mono S 5/50 GL column, GE Healthcare, Piscataway, New Jersey, USA) using buffer *F* consisting of 25 m*M* HEPES–NaOH pH 6.7, 50 m*M* NaCl and buffer *G* consisting of 25 m*M* HEPES–NaOH pH 6.7, 500 m*M* NaCl (gradient of 0–60% buffer *G* over 60 min; Fig. 1).

All proteins were oxidized by the addition of 1.7 m*M* copper(II)-1,10-phenanthroline (1 h, 277 K) and then equilibrated in buffer *F* using a PD-10 column (GE Healthcare, Piscataway, New Jersey, USA).

SeDsbA, SeDsbL and SeSrgA protein samples were concentrated to 82, 29 and 20 mg ml^{-1}, respectively, in buffer *F* using Amicon Ultra centrifugal filter devices (10 kDa cutoff; Millipore, Billerica, Massachusetts, USA). Protein concentration was measured at 280 nm using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, Delaware, USA) and the purity was assessed by SDS–PAGE analysis and mass spectrometry.

Table 1
Primers used for the amplification of *SedsbA*, *SedsbL* and *SesrgA*.

Nucleotides in bold represent the ligation-independent cloning (LIC) overhangs.

Primer (5′–3′)		Protein
TACTTCCAATCCAATGCGGCACAGATCAG	Forward	SeDsbA
TTATCCACTTCCAATGTTATTTTTTATCAACC	Reverse	SeDsbA
TACTTCCAATCCAATGCGTTTACCGAGGGCAC	Forward	SeDsbL
TTATCCACTTCCAATGTTATTTTTTAGTGGCCAGTTC	Reverse	SeDsbL
TACTTCCAATCCAATGCGCAGGAGTGGGAG	Forward	SeSrgA
TTATCCACTTCCAATGTCAGTCTGCATCAGGG	Reverse	SeSrgA

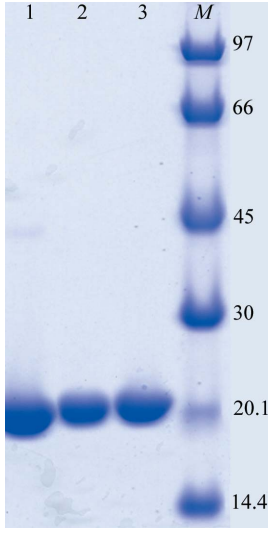


Figure 1
SDS–PAGE analysis of SeDsbA, SeDsbL and SeSrgA. Proteins were analyzed on NuPAGE Novex 4–12% bis-tris gels run using NuPAGE MES SDS running buffer (Invitrogen, Carlsbad, California, USA) and stained with Coomassie Blue. Lane 1, purified SeSrgA; lane 2, purified SeDsbL; lane 3, purified SeDsbA; lane *M*, molecular-weight markers (Low Molecular Weight Calibration Kit for Electrophoresis, GE Healthcare, Piscataway, New Jersey, USA; labelled in kDa).

2.2. Crystallization

Initial vapour-diffusion crystallization experiments were performed in 96-well hanging-drop plates (96-well tissue-culture plates sourced from Lomb Scientific Pty Ltd, Taren Point, NSW, Australia) using ViewDrop II plate seals (TTP LabTech Ltd, Melbourn, UK) and commercial crystallization screens including PEG/Ion, PEG/Ion 2, Index Screen (Hampton Research, Aliso Viejo, California, USA), PACT Premier, JCSG-*plus* (Molecular Dimensions Limited, New-

market, UK), Precipitant Synergy (Jena Bioscience, Jena, Germany) and an in-house malonate grid screen pH 5–7 and 0.2–3.4 *M*. The crystallization plates contained 85 µl of the crystallization solutions placed using a Biomek 2000 Laboratory Automation workstation (Beckman Coulter Inc., Brea, California, USA). Crystallization drops consisting of 100 nl protein solution and 100 nl reservoir solution solution were prepared using a Mosquito robot (TTP LabTech Ltd, Melbourn, UK). The plates were incubated at 293 K and monitored and scored using a Rock Imager and *Rock Maker* system (Formulatrix Inc,. Waltham, Massachusetts, USA).

SeDsbA crystals grew in six different conditions consisting of (i) 25%(*w*/*v*) polyethylene glycol (PEG) 3350 and 100 m*M* Tris pH 8.5; (ii) 20%(*w*/*v*) PEG 8000 and 100 m*M* *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) pH 9.5; (iii) 20%(*w*/*v*) PEG 3350, 100 m*M* bis-tris propane pH 7.5, 200 m*M* sodium formate; (iv) 20%(*w*/*v*) PEG 3350, 100 m*M* bis-tris propane pH 8.5, 200 m*M* sodium nitrate; (v) 20%(*w*/*v*) PEG 3350, 100 m*M* bis-tris propane pH 8.5, 200 m*M* sodium formate and (vi) 20%(*w*/*v*) PEG 3350, 100 m*M* bis-tris propane pH 8.5, 200 m*M* sodium acetate. Crystals of SeDsbL were observed in two conditions: (i) 1.6 *M* trisodium citrate dihydrate pH 6.5 and (ii) 2.9 *M* sodium malonate pH 7.0. SeSrgA crystals grew in 1.1 *M* sodium malonate, 0.5%(*w*/*v*) Jeffamine ED-2001, 100 m*M* HEPES pH 7. Crystal optimization experiments were carried out at 293 K in 24-well hanging-drop VDXm plates using 18 mm siliconized cover slips (Hampton Research, Aliso Viejo, California, USA), a reservoir volume of 500 µl and a drop size of 2 µl. Given the good quality and size of the SeDsbA crystals obtained in the initial screens, we only replicated these conditions in the 24-well plates; a total of about six droplets produced enough crystals for SeDsbA diffraction experiments which grew overnight in 20%(*w*/*v*) PEG 3350, 100 m*M* bis-tris propane pH 8.5, 200 m*M* sodium acetate. SeDsbL crystals were optimized by setting up a 24-well plate screening different concentrations of sodium malonate (3.2–2.6 *M*) and pH (pH 6.8–7.4). Each cover slip held two 2 µl drops with two different ratios of protein to well solution (1:1 and 1.2:0.8). SeDsbL crystals grew in 2 d in most conditions. For SeSrgA we initially set up a grid screen based on the initial hit, in which the concentrations of sodium malonate and Jeffamine were varied from 0.9 to 1.4 *M* and from 0.3% to 1%, respectively. Crystals only appeared in the conditions with the highest sodium malonate concentration, while changing the Jeffamine concentration did not have any noticeable effect. To further optimize SeSrgA crystals we then set up a 24-well plate in which we modified the sodium malonate concentration (from 1.25 to 1.5 *M* in increments of 0.05 *M*) and tested four different pH values (6, 6.25, 6.5 and 7). Each cover slip contained two drops with two different ratios of protein to well solution (1:1 and 0.75:1.25). Diffraction-quality crystals of SeSrgA grew within 6–10 d in conditions consisting of 1.25–1.5 *M* sodium malonate and with pH values between 6 and 6.5.

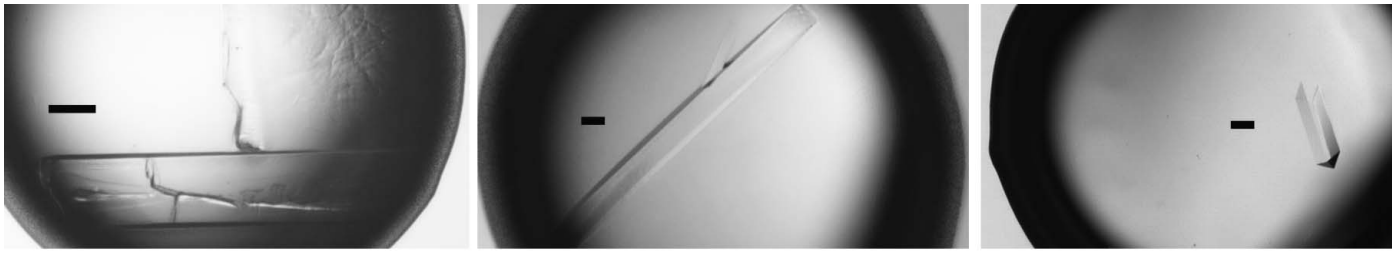
2.3. X-ray diffraction data measurement

X-ray diffraction data for SeDsbA and SeDsbL crystals were collected on the high-throughput protein crystallography beamline (MX1 beamline 3BM1) at the Australian Synchrotron using an ADSC Quantum 210r CCD detector. Crystals of the two proteins were cryoprotected by soaking them for 2 min in mother liquor supplemented with PEG 400 (the reservoir solution was diluted with PEG 400 to obtain a final PEG concentration of 20%). 360 and 180 images were collected for SeDsbA and SeDsbL, respectively, with an oscillation angle of 1° per image under cryogenic conditions (100 K) and an exposure time of 1 s. Diffraction data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

SeSrgA diffraction images were measured in-house using a Rigaku FR-E copper rotating-anode generator operating at 45 kV and 45 mA with Osmic Confocal Vari-Max HF optics at a wavelength of 1.5418 Å (Rigaku Americas, The Woodlands, Texas, USA). Reflections were measured using a Rigaku Saturn 944 CCD area detector (Rigaku Americas, The Woodlands, Texas, USA). A Cryo Industries CryoCool LN_2 (Cryo Industries, Manchester, New Hampshire, USA) was used to keep the SeSrgA crystals at 100 K during data collection. Prior to data collection, SeSrgA crystals were transferred into cryoprotectant solution consisting of mother liquor supplemented with PEG 400 (as described for SeDsbA and SeDsbL). For diffraction experiments we used a crystal-to-detector distance of 70 mm and oscillation images were collected every 0.5° over a total of 180° with an exposure time of 15 s. Diffraction data were processed using *CrystalClear* v.1.4 (Rigaku Americas, The Woodlands, Texas, USA).

3. Results and discussion

Three genes (*SedsbA*, *SedsbL* and *SesrgA*) encoding homologues of the thiol-disulfide oxidoreductase DsbA in *S. enterica* serovar Typhimurium SL1344 were cloned into pET21a-LIC vector and expressed in BL21 (DE3) pLysS cells. Three consecutive chromatography steps (affinity, size-exclusion and ion-exchange chromatography) were used to purify the recombinant SeDsbA, SeDsbL and SeSrgA proteins to homogeneity for crystallization trials. Crystals of SeDsbA, SeDsbL and SeSrgA were observed under different crystallization conditions, which yielded well diffracting single crystals upon optimization. SeDsbA crystals grew using a reservoir solution consisting of 20%(*w*/*v*) PEG 3350, 100 m*M* bis-tris propane pH 8.5, 200 m*M* sodium acetate, SeDsbL crystallized in 2.6–3.4 *M* sodium malonate pH 7.0–7.4 and single crystals of SeSrgA were obtained in 1.45 *M* sodium malonate, 0.5%(*w*/*v*) Jeffamine ED-2001, 100 m*M* HEPES pH 7 (Fig. 2). SeDsbA, SeDsbL and SeSrgA crystals diffracted to resolution limits of 1.55, 1.57 and 2.6 Å and belonged to space groups $P2_1$, $P2_12_12$ and $C2$, respectively. Assuming that the



(*a*) (*b*) (*c*)

Figure 2
Crystals of (*a*) SeDsbA, (*b*) SeDsbL and (*c*) SeSrgA obtained using the hanging-drop vapour-diffusion method. The crystals reached typical dimensions of 0.16 × 0.05 × 0.8, 0.1 × 0.05 × 1.3 and 0.1 × 0.1 × 0.4 mm, respectively. The scale bars represent 0.1 mm.

Table 2
Summary of X-ray data-measurement and processing statistics.

Values in parentheses are for the highest resolution shell.

	SeDsbA	SeDsbL	SeSrgA
Wavelength (Å)	0.956667	0.956667	1.5418
Temperature (K)	100	100	100
Space group	$P2_1$	$P2_12_12$	$C2$
Unit-cell parameters (Å, °)	a = 37.3, b = 61.7, c = 42.8, $\alpha = \gamma = 90$, β = 101.3	a = 85.6, b = 46.6, c = 60.2, $\alpha = \beta = \gamma = 90$	a = 186.3, b = 80.3, c = 105.5, $\alpha = \gamma = 90$, β = 124.3
Anticipated solvent content (%)	49.2	53.9	62.3
Resolution range	50.0–1.55 (1.58–1.55)	50.0–1.57 (1.60–1.57)	36.4–2.6 (2.7–2.6)
No. of observations	192033	246716	134920
No. of unique reflections	26275 (1039)	34491 (1693)	37521 (3734)
Redundancy	7.3 (6.6)	7.2 (7.0)	3.6 (3.4)
Completeness (%)	94.2 (75.7)	99.9 (100)	99.8 (99.4)
$R_{\rm merge}$†	0.082 (0.217)	0.066 (0.459)	0.12 (0.425)
$I/\sigma(I)$	19.5 (8.5)	29.6 (4.1)	6.1 (2.0)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the measured intensity of each individual reflection and $\langle I(hkl)\rangle$ is the mean value of the intensity.

crystals contain one molecule per asymmetric unit for SeDsbA and SeDsbL and five molecules per asymmetric unit for SeSrgA, the Matthews coefficients ($V_{\rm M}$) are 2.4, 2.7 and 3.3 Å^3 Da^{-1}, respectively, and the corresponding solvent contents are 49.2, 53.9 and 62.3% (Matthews, 1968). Statistics for the diffraction data are provided in Table 2.

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