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# Crystallization and preliminary crystallographic analysis of the type III secretion translocator chaperone SicA from *Salmonella enterica*

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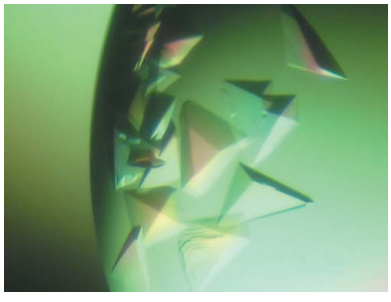
SicA is a member of the class II chaperones in type III secretion systems which bind to the pore-forming translocators in the bacterial cytoplasm and prevent them from premature association and degradation. In this study, SicA from *Salmonella enterica* serovar Typhimurium was overexpressed, purified and crystallized using PEG 8000 as the precipitant. X-ray diffraction data were collected using synchrotron radiation and processed at 3.5 Å resolution. The crystal belonged to the monoclinic space group *C*2, with unit-cell parameters $a = 180.4$, $b = 94.1$, $c = 131.8$ Å, $\beta = 130.9^\circ$. There may be eight monomers in the crystallographic asymmetric unit, corresponding to a $V_M$ of 2.52 Å$^3$ Da$^{-1}$ and a solvent content of 51.1%. This suggests an oligomerization state that differs from those of previously reported type III secretion chaperones.

## 1. Introduction

*Salmonella* species (*S. enterica*, *S. enteritidis*, *S. typhi* and *S. paratyphi*) are Gram-negative motile bacteria that cause various diseases in humans such as gastroenteritis and systemic disease (Tauxe & Pavia, 1998). The *Salmonella* pathogenicity island 1 (SPI-1) locus of *S. enterica* encodes a specialized protein-secretion system, termed the type III secretion system (T3SS), consisting of a needle-like structure that crosses both the inner and outer bacterial membranes and extends up to 50 nm from the cell surface (Galan, 2001; Galan & Collmer, 1999). The T3SS is responsible for the delivery of bacterial effector proteins into the host cell, subverting host cellular processes and enabling bacterial invasion of epithelial cells (Mota & Cornelis, 2005; Tucker & Galan, 2000; Cornelis, 2006). *S. enterica* also contains the *Salmonella* pathogenicity island 2 (SPI-2), which encodes a second distinct T3SS that is required for intracellular replication of the pathogen (Waterman & Holden, 2003; Fass & Groisman, 2009).

The type III secretion systems are complex molecular devices that are composed of over 20 different proteins that play structural, effector and regulatory roles (Cornelis, 2006). The SicA protein from *S. enterica* serovar Typhimurium is a member of the class II T3SS-associated chaperones and is encoded in SPI-1 immediately upstream of the T3SS-secreted proteins SipB and SipC, two translocator proteins that are thought to form a pore in the host plasma membrane for translocation of T3SS effector proteins (Tucker & Galan, 2000). SicA contains 165 amino-acid residues with a molecular mass of 19 200 Da. SicA is capable of binding to both SipB and SipC, preventing their degradation and premature molecular association (Tucker & Galan, 2000). In addition to its chaperone function, SicA has been shown to play a regulatory role in the synthesis of effector proteins (Tucker & Galan, 2000; Darwin & Miller, 2000, 2001). SicA is homologous to IpgC from *Shigella flexneri*, SycD from *Yersinia pestis* and PcrH from *Pseudomonas aeruginosa*, which have similar chaperone-like functions in the respective type III secretion systems. IpgC, SycD and PcrH proteins all form dimers in solution and their structures reveal a tetratricopeptide-like (TPR-like) fold that is responsible for protein–protein interaction (Büttner *et al.*, 2008; Lunelli *et al.*, 2009; Job *et al.*, 2010).



To understand the functions and underlying molecular mechanism of this important class of T3SS chaperones, we have started to analyze the structure of SicA from *Salmonella enterica*. In this study, we have overexpressed, purified and crystallized SicA. Preliminary X-ray crystallographic analysis suggested that there are eight monomers in the crystallographic asymmetric unit, indicating a unique oligomerization state that is distinct from those previously reported for T3SS chaperones.

## 2. Experimental procedures

### 2.1. Protein expression and purification

The full-length SicA gene (NP_461807) was amplified *via* PCR using the *S. enterica* subsp. *enterica* serovar Typhimurium strain LT2 genomic DNA as a template. The sequences of the forward and reverse oligonucleotide primers, which were designed from the published genome sequence (McClelland *et al.*, 2001), were 5′-GGAATTC**CATATG**GATTATCAAAATAATGTCAGC-3′ (bases in bold indicate the *Nde*I digestion site) and 5′-CCG**CTCGAG**-TTATTCCTTTTCTTGTTCACT-3′ (bases in bold indicate the *Xho*I digestion site), respectively. The amplified DNA was then inserted into the *Nde*I/*Xho*I-digested expression vector pET-28a (Novagen) to produce recombinant SicA with a hexahistidine tag and a thrombin cleavage site at the N-terminus (**M**GSSHHHHHHSSGLVPRGS-H**M**).

Next, SicA was overexpressed in *Escherichia coli* BL21 (DE3) cells. To accomplish this, the cells were grown at 310 K to an $OD_{600}$ of 0.6 in Luria–Bertani medium containing 30 µg ml$^{-1}$ kanamycin. Protein expression was then induced *via* the addition of 1.0 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG), after which cell growth was continued at 295 K overnight. The cells were then harvested by 30 min centrifugation at 4200*g* and 277 K. The cell pellet was resuspended in ice-cold lysis buffer *A* (20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 2 m*M* 2-mercaptoethanol) and the cells were disrupted using a French press. The crude cell extract was centrifuged for 40 min at 26 940*g* at 277 K. The recombinant protein in the supernatant was purified *via* three chromatographic steps. The supernatant was loaded onto a HisTrap FF (GE Healthcare) column, washed and gradient-eluted with buffer *A* plus 1 *M* imidazole (buffer *B*).

The fractions containing SicA were pooled and dialyzed against 20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl and 1 m*M* DTT (buffer *C*). The dialyzed protein sample was loaded onto a HiTrap Q FF (GE Healthcare) column and gradient-eluted with buffer *C* plus 1 *M* NaCl (buffer *D*). The major fractions containing SicA were then loaded onto a size-exclusion chromatography column (Sephacryl S-300 26/60, bed volume 320 ml; GE Healthcare) and eluted with 20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl and 2 m*M* DTT (buffer *E*) at a flow rate of 1 ml min$^{-1}$. The homogeneity of the purified protein was assessed by SDS–PAGE, after which the protein was concentrated to a final concentration of 20 mg ml$^{-1}$ using a Centri-Prep centrifugal filter (Amicon Millipore) with buffer *E*.

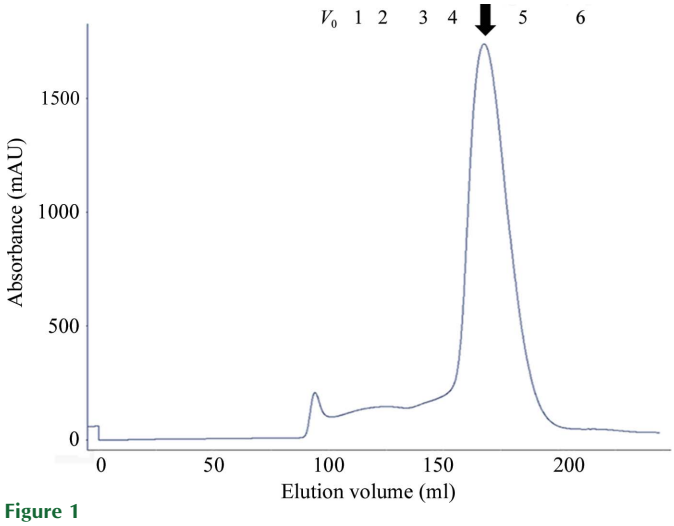



**Figure 1**
Size-exclusion chromatogram obtained using a Sephacryl 26/60 S-300 column (GE Healthcare) showing a major peak at 167 ml (arrow) corresponding to an approximate molecular mass of 94 kDa that suggests SicA to be a tetramer. The column was calibrated using the molecular-weight markers in the MWGF1000 Kit (Sigma) following the procedure suggested by the manufacturer. $V_0$ indicates the void volume; 1 (thyroglobulin, 669 kDa), 2 (apoferritin, 443 kDa), 3 (amylase, 200 kDa), 4 (alcohol dehydrogenase, 150 kDa), 5 (BSA, 66 kDa) and 6 (carbonic anhydrase, 29 kDa) represent the standard molecular weights used for comparison with the SicA protein.

### 2.2. Crystallization and data collection

Crystallization screening was conducted at 293 K in 96-well sitting-drop Intelli-Plates (Hampton Research) using approximately 300 conditions and a ratio of 0.5 µl precipitant solution to 0.5 µl protein solution equilibrated over 100 µl well solution. Crystallization trials were conducted using screening kits from Hampton Research and Emerald BioSystems. Initially, microcrystals were observed from Hampton Research Crystal Screen condition No. 36 [0.1 *M* Tris–HCl pH 8.5, 8%(*w*/*v*) PEG 8000]. This crystallization condition was further optimized by the hanging-drop vapour-diffusion method using 24-well VDX plates (Hampton Research). The solutions used for the optimized crystallization conditions were prepared by mixing 1.2 µl protein solution with 1.2 µl reservoir solution [0.1 *M* Tris–HCl pH 8.4, 10%(*w*/*v*) PEG 8000]. Each hanging drop was positioned over 1 ml reservoir solution. Crystals were cryoprotected by soaking for 5 s in a cryoprotectant solution consisting of 0.1 *M* Tris–HCl pH 8.4, 10%(*w*/*v*) PEG 8000 and 25%(*w*/*v*) xylitol and flash-frozen in liquid nitrogen. They were then mounted on the goniometer in a stream of cold nitrogen at 100 K. X-ray diffraction data were collected using an ADSC Quantum CCD 315 detector on beamline 11-1 at Stanford Synchrotron Radiation Lightsource (SSRL), USA. The crystal was oscillated by 1.0° per frame over a total range of 360° at a wavelength of 1.0000 Å and a crystal-to-detector distance maintained at 400 mm. X-ray diffraction data were collected to 3.5 Å resolution. Data were integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

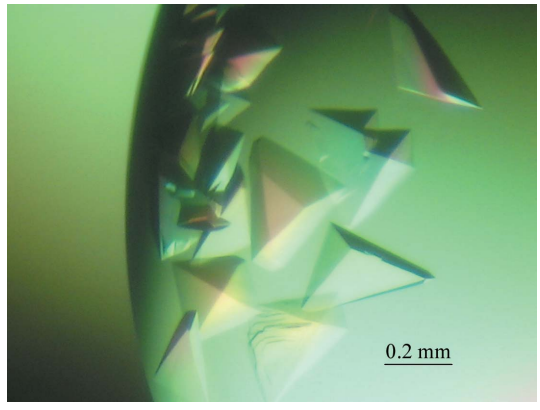



**Figure 2**
Crystals of SicA were grown in 0.1 *M* Tris–HCl pH 8.4, 10%(*w*/*v*) PEG 8000. The average dimensions of the crystals of SicA protein were approximately 0.4 × 0.2 × 0.2 mm.

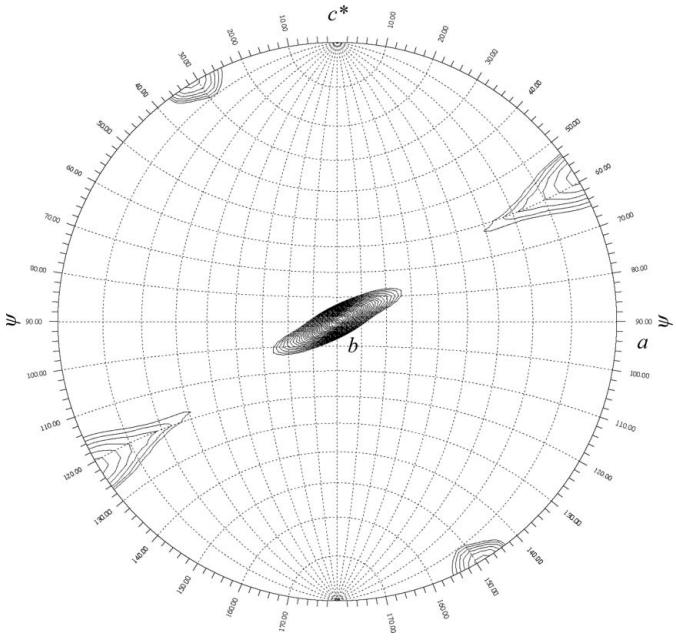



**Figure 3**
A $\kappa = 180^\circ$ section of the self-rotation function calculated from the data set using a radius of integration of 15.0 Å and data in the resolution range 8.0–3.5 Å. The strongest peak on the *b* axis arises from the space-group symmetry (*C*2). The major peak on the circumference at ∼32° from the *a* axis in the $a \times c$ plane indicates the presence of a noncrystallographic twofold axis. Latitude ($\varphi$ angle) and longitude ($\psi$ angle) grid lines are drawn at 10° intervals. This figure was produced using the program *GLRF*.

## 3. Results and discussion

### 3.1. Protein expression, purification and crystallization

SicA was overexpressed in *E. coli* in soluble form with a yield of ∼35 mg near-homogeneous protein per litre of culture. Based on the results of Sephacryl 300 26/60 gel filtration, the molecular weight of the protein was approximately 94 kDa, indicating a tetramer (Fig. 1). This oligomerization state is distinct from those previous reported for IpgC, SycD and PcrH, which all form dimers in solution (Büttner *et al.*, 2008; Lunelli *et al.*, 2009; Job *et al.*, 2010). Initially, very irregular microcrystals were observed; however, after optimization pyramid-shaped crystals ($0.4 \times 0.2 \times 0.2$ mm) were observed within 3 d when a reservoir solution consisting of 0.1 *M* Tris–HCl pH 8.4, 10%(*w*/*v*) PEG 8000 was used (Fig. 2).

### 3.2. Data collection and preliminary X-ray diffraction analysis

Flash-cooled SicA crystals diffracted to beyond 3.5 Å resolution using 25%(*w*/*v*) xylitol as a cryoprotectant. Autoindexing was conducted using *DENZO* and the results indicated that the crystals belonged to the monoclinic space group *C*2 on the basis of systematic absences, with unit-cell parameters $a = 180.4$, $b = 94.1$, $c = 131.8$ Å, $\beta = 130.9^\circ$. These results indicate that eight monomers are likely to be present in the asymmetric unit, which has a corresponding calculated Matthews coefficient ($V_M$) of 2.52 Å$^3$ Da$^{-1}$ and a solvent content of 51.1% (Matthews, 1968). Data-collection statistics are provided in Table 1. A self-rotation function calculated using *GLRF* (Tong & Rossmann, 1997) indicates the presence of noncrystallographic twofold symmetry in the asymmetric unit (Fig. 3). We did not observe any higher symmetry axis (*i.e.* threefold, fourfold, fivefold or sixfold symmetry) during self-rotation function calculation. SicA shares 60% and 24% sequence identity with IpgC and SycD, respectively. The molecular-replacement method was first attempted using the crystal structures of IpgC from *Shigella flexneri* (PDB entries 3gyz and 3gz1; Lunelli *et al.*, 2009) and SycD from *Y. pestis* (PDB entries 2vgx and 2vgy; Büttner *et al.*, 2008) as initial models. However, even after using both monomeric and dimeric structures as the initial model, none of our attempts gave rise to a clear solution. Crystals of selenomethionine-substituted SicA are in preparation, which will be used to solve the phase problem.

**Table 1**
Data-collection statistics for SicA.

Values in parentheses are for the outermost resolution shell.

| | |
|---|---|
| Space group | *C*2 |
| Unit-cell parameters (Å, °) | $a = 180.4$, $b = 94.1$, $c = 131.8$, $\beta = 130.9$ |
| Resolution (Å) | 50–3.50 (3.63–3.50) |
| No. of unique reflections | 20676 (1884) |
| Multiplicity | 4.6 (3.1) |
| Completeness (%) | 97.4 (89.3) |
| $R_{\rm merge}$† (%) | 7.8 (43.2) |
| Average $I/\sigma(I)$ | 25.5 (2.6) |

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

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