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Crystallization and preliminary X-ray crystallographic analysis of *Salmonella* Typhimurium CueP

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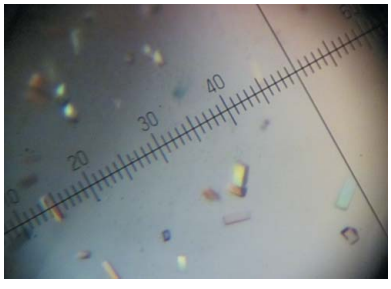

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) can survive in the phagosome of macrophages, causing serious medical and veterinary problems. CueP is uniquely found in *S.* Typhimurium and has been characterized as a major periplasmic copper-binding protein. Although *cueP* has been identified as being responsible for the copper resistance of the bacterium *in vivo*, the biochemical role and three-dimensional structure of CueP remain unknown. In this study, CueP from *S.* Typhimurium was overexpressed and the recombinant protein was purified using Ni–NTA affinity, anion-exchange and gel-filtration chromatographies. The purified CueP protein was crystallized using the vapour-diffusion method. A diffraction data set was collected to 2.5 Å resolution at 100 K. The crystal belonged to space group $P2_12_12_1$. To obtain initial phases, selenomethionyl-substituted protein was overproduced and purified. Optimization of crystallization conditions for the selenomethionyl-substituted protein is in progress.

1. Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) causes *Salmonella* gastroenteritis in animals including humans, leading to substantial morbidity and mortality and a considerable burden of disease globally (Coburn *et al.*, 2007). *Salmonella* can survive in macrophage phagosomes, which is critical for the virulence of the bacteria during systemic disease (Fields *et al.*, 1986). Since macrophages can kill the invading bacteria in the phagosomes by generating reactive oxygen species (ROS) such as hydrogen peroxide, the ability of the bacteria to survive in the phagosomes is associated with efficient scavenging of ROS (Fang, 2004).

The transition metal copper is essential for every living cell as a key metal ion in electron-transport proteins (Nelson, 1999). However, the cuprous copper ion (Cu^+) can generate highly toxic hydroxyl radicals by reacting with hydrogen peroxide (Macomber *et al.*, 2007). To prevent the toxic effect of the cuprous ion, most Gram-negative bacteria possess the periplasmic enzyme CueO, which catalyzes the oxidation of cuprous ions (Grass & Rensing, 2001), and the copper-ion exporter CusABFC, which exports copper ions across the outer membrane (Franke *et al.*, 2003; Outten *et al.*, 2001; Long *et al.*, 2010; Xu *et al.*, 2009; Su *et al.*, 2009).

S. Typhimurium has a unique defence system against copper toxicity: it has the CueP protein instead of the exporter CusABFC (Osman *et al.*, 2010). CueP is a dominant periplasmic copper protein in *S.* Typhimurium and transcription of the *cueP* gene is under the control of the copper-responsive regulator CueR (Pontel & Soncini, 2009). CueP has a role in copper resistance of *S.* Typhimurium *in vivo*, particularly in the absence of oxygen, suggesting that CueP may be a functional substitute for the copper exporter CusABFC (Osman *et al.*, 2010). However, the biochemical role of CueP with respect to copper resistance remains unknown. To date, no crystal structure of CueP or its homologues has been determined. In this study, we report the crystallization and preliminary X-ray analysis of the mature form of CueP from *S.* Typhimurium. The CueP structure could provide insight into the molecular role of CueP in the copper resistance of *S.* Typhimurium.

2. Materials and methods

2.1. DNA construction, protein expression and protein purification

A DNA fragment encoding *S.* Typhimurium CueP (residues 22–180; accession No. NC_003197; locus tag STM3650) was amplified from the genomic DNA of *S.* Typhimurium using the polymerase chain reaction. The DNA fragment was inserted into the *Nco*I and *Xho*I sites of the pPROEX-HTA vector (Invitrogen, USA). The resulting protein contained three additional amino acids (Gly-His-Met) between the TEV protease cleavage site and the mature protein as a cloning artifact. Recombinant CueP protein was expressed in *Escherichia coli* BL21 (DE3) using 1.5 l LB medium supplemented with 50 µg ml^{-1} ampicillin at 310 K until the OD_{600} reached 0.6–0.8. Protein expression was induced by adding 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at 303 K. The cells were harvested by centrifugation at 5000*g* for 15 min at 277 K. The harvested cells were suspended in 60 ml lysis buffer consisting of 20 m*M* Tris pH 8.0, 150 m*M* NaCl and 2 m*M* *β*-mercaptoethanol. The soluble lysate was centrifuged at 5000*g* for 30 min and the supernatant was mixed with Ni–NTA affinity resin (Qiagen, Netherlands) that had been pre-incubated with lysis buffer; the mixture was then stirred for 1 h at 277 K. After the slurry had been loaded into a column, unbound proteins were washed off with lysis buffer supplemented with 20 m*M* imidazole. Recombinant CueP was eluted with 30 ml lysis buffer supplemented with 250 m*M* imidazole. Fractions containing CueP were pooled and 5 m*M* $NiCl_2$ and 5 m*M* *β*-mercaptoethanol were added to the protein sample. The hexahistidine tag was then cleaved using recombinant TEV protease by incubation at room temperature overnight. The CueP protein was diluted threefold with 20 m*M* Tris pH 8.0 buffer and then loaded onto a HiTrap Q column (GE Healthcare, USA). The protein was eluted from the column using a linear gradient of 0–1 *M* NaCl in lysis buffer. The CueP-containing fractions were concentrated using a Centriprep (GE Healthcare, USA) and the CueP protein was further purified using HiLoad Superdex 200 (GE Healthcare, USA) pre-equilibrated with lysis buffer. The purified protein was concentrated to 10 mg ml^{-1} in 20 m*M* Tris buffer pH 8.0 containing 150 m*M* NaCl and 2 m*M* *β*-mercaptoethanol and stored frozen at 193 K until use.

2.2. Crystallization and data collection

Initial crystallization of CueP was performed with commercially available screening solutions (Hampton Research, USA) in droplets consisting of 0.5 µl protein solution and 0.5 µl of each precipitation solution using the microbatch method at various temperatures (277, 287 and 295 K). Very small rod-shaped crystals of recombinant CueP protein were obtained at 287 K using a precipitation solution consisting of 0.1 *M* sodium acetate pH 4.6 and 2.0 *M* sodium chloride, while only clusters of needle-shaped crystals could be obtained at the other temperatures. The crystallization conditions were optimized to produce rod-like single crystals (0.05 × 0.01 × 0.01 mm) using the hanging-drop vapour-diffusion method at 287 K in 24-well plates with the initial precipitation solution (Fig. 1). To grow crystals to maximum size, the droplets were equilibrated against 1 ml precipitant solution using the hanging-drop vapour-diffusion method at 287 K for a month. X-ray diffraction data were collected from the crystals using an ADSC Q310 CCD detector on beamline 4A of Pohang Light Source (PLS), Republic of Korea.

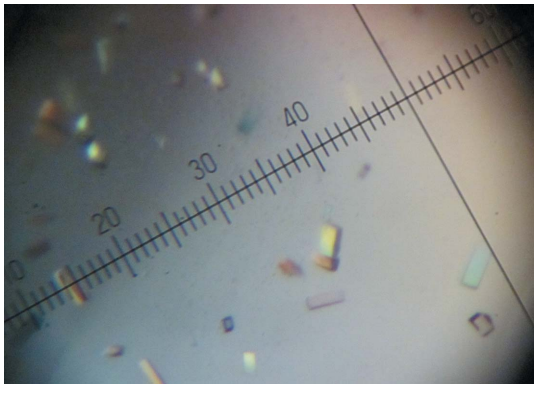

Figure 1
Crystals of *S.* Typhimurium CueP. Approximate dimensions are 0.05 × 0.01 × 0.01 mm.

Table 1
Diffraction statistics.

(*a*) Data collection. Values in parentheses are for the highest resolution shell.

X-ray source	Beamline 4A, PLS
Wavelength (Å)	1.000
Resolution (Å)	50–2.5 (2.54–2.50)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 58.5, b = 102.1, c = 114.0
Completeness (%)	96.0 (91.3)
R_{merge}† (%)	12.7 (29.9)
Multiplicity	4.7 (3.2)
Average $I/\sigma(I)$	10.1 (2.8)

(*b*) Cell-content analysis.

No. of molecules in asymmetric unit	3	4	5
V_M (Å^3 Da^{-1})	3.21	2.40	1.92
Solvent content (%)	61.7	48.9	36.1

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the observed intensity and the mean intensity of related reflections, respectively.

3. Results and discussion

To express the protein in the bacterial cytoplasm, we removed the signal sequence (residues 1–21) for periplasmic secretion from the full-length protein (residues 1–180) when we generated the DNA construct for protein expression. Crystals suitable for data collection were obtained in droplets consisting of 1 µl protein solution (10 mg ml^{-1} protein in 20 m*M* Tris pH 8.0 buffer containing 150 m*M* NaCl and 2 m*M* *β*-mercaptoethanol) and 1 µl of a precipitant solution consisting of 0.1 *M* sodium acetate pH 4.6 and 2.0 *M* sodium chloride (Fig. 1). For X-ray data collection, a single crystal was soaked overnight in a cryoprotectant solution consisting of 0.08 *M* sodium acetate pH 4.6, 1.6 *M* sodium chloride and 25%(*v*/*v*) glycerol. A set of 360 images (0–360°) was obtained using a 1° oscillation width and 5 s exposure time at 1.00 Å and 100 K. The data collected were processed and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997).

Based on the diffraction data, the crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 58.5, b = 102.1, c = 114.0 Å. In particular, analysis of the diffraction along the h, k and l axes clearly demonstrated that all of the axes of the crystal are screw axes not rotational axes. The diffraction data set had a resolution range of 50–2.5 Å with 96.0% completeness and an R_{merge} of 12.7%. Since the self-rotation function from the data set did not give any clue to the number of molecules per asymmetric unit, we were only able to predict the putative number of molecules in the asymmetric unit from the calculated solvent content. Candidates for the number of molecules per asymmetric unit and the corresponding Matthews coeffi-

cients (Matthews, 1968) and solvent contents were calculated as listed in Table 1.

In order to solve the structure of CueP, we intend to obtain experimental phases by the multiple-wavelength anomalous diffraction (MAD) approach using selenomethione (SeMet) incorporated crystals because of the high methionine frequency in the protein (three methionine residues in 158 residues). We overproduced the CueP protein in the presence of SeMet and purified SeMet-substituted CueP protein according to the protocol established for the native protein. This SeMet-substituted CueP crystallized under the same conditions as the native protein. However, the crystals were too small to obtain experimental phases by the multiple-wavelength anomalous diffraction (MAD) approach. Optimization of the crystallization conditions is in progress in order to obtain better diffracting SeMet-incorporated CueP crystals.

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