Crystallization and preliminary X-ray crystallographic analysis of Z-ring-associated protein (ZapD) from *Escherichia coli*

Sang Hyeon Son^a and Hyung Ho Lee^{b*}

Received 7 November 2014

Accepted 4 January 2015

^aDepartment of Bio and Nano Chemistry, Kookmin University, Seoul 136-702, Republic of Korea, and ^bDepartment of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-742, Republic of Korea. *Correspondence e-mail: hyungholee@snu.ac.kr

Keywords: bacterial cytokinesis; FtsZ; ZapD.

Bacterial cytokinesis is accomplished by the Z-ring, which is a polymeric structure that includes the tubulin homologue FtsZ at the division site. ZapD, a Z-ring-associated protein, directly binds to FtsZ and stabilizes the polymerization of FtsZ to form a stable Z-ring during cytokinesis. Structural analysis of ZapD from *Escherichia coli* was performed to investigate the mechanism of ZapD-mediated FtsZ stabilization and polymerization. ZapD was crystallized using a reservoir solution consisting of 1.5 M lithium sulfate, 0.1 M HEPES pH 7.8, 2% (v/v) polyethylene glycol 400. X-ray diffraction data were collected to 2.95 Å resolution. The crystals belonged to the hexagonal space group $P6_4$, with unit-cell parameters $a = b = 109.5$, $c = 106.7$ Å, $\gamma = 120.0^\circ$. Two monomers were present in the asymmetric unit, resulting in a crystal volume per protein mass (V_M) of $3.25 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 62.17%.

1. Introduction

Cytokinesis is an essential process for separating a mother cell into two daughter cells in prokaryotes and eukaryotes. Bacterial cytokinesis is processed by the divisome, which is orchestrated by the tubulin homologue, the cytoskeletal protein FtsZ and various other regulators (Adams & Errington, 2009). Assembly of the divisome is initiated by the polymerization of FtsZ into a Z-ring along the midline of cells (Goehring & Beckwith, 2005). FtsZ is only conserved in bacteria and serves as a scaffold protein and also as a GTPase (de Boer *et al.*, 1992). In eukaryotes, the subunits of the endosomal sorting complex required for transport III (ESCRT-III) system, which are only conserved in eukaryotes, have membrane-scission activity (Wollert *et al.*, 2009). Perturbation of the assembly or disassembly dynamics of FtsZ has been recognized as a potential mechanism for antibiotic target attack (Sass & Brötz-Oesterhelt, 2013). FtsZ is composed of two domains and includes a conserved GTPase domain at the N-terminus. In the presence of magnesium ion, GTP binding initiates FtsZ polymerization, while GTP hydrolysis simultaneously regulates the Z-ring (Mukherjee & Lutkenhaus, 1998; Löwe, 1998; Löwe & Amos, 1998). The FtsZ-regulatory proteins, which include the Z-ring-associated protein family, which function in stabilizing the FtsZ protofilament, bundling separate protofilaments and preventing depolymerization of FtsZ by GTP hydrolysis, are involved in early FtsZ assembly (Durand-Heredia *et al.*, 2012). Several

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Table 1

Macromolecule-production information.

Source organism	<i>E. coli</i> strain B/REL606
DNA source	Genomic DNA
Expression vector	pRSFDuet-1
Expression host	<i>E. coli</i> BL21 (DE3)
UniProt accession No.	C6UM64
Complete amino-acid sequence of the construct produced for ZapD _{1–247} †	MSYHHHHHHDDYDIPPTTENLYFQGAMGSMQTQVL- FEHPLNEKMRTWLRIFELIQLTVNLPIDVHA- GALHFFRNVSSELLDVFERGEVRETELLKELDRQ- QRKLQWIGVPGVDQSRIEALIQQLKAAGSVL- ISAPRIGQFLREDRLIALVRQLSIPGGCCSF- DLPTLHIWHLHPQAQRDSQVETWIASLNPLTQ- ALTMVLDLIRQSAPFRKQTSNLNGFYQDNGGDA- DLLRLNLSLDSQLYPQISGHKSRFAIRFMPLD- TENGQVPERLDFELACC
Complete amino-acid sequence of the construct produced for ZapD _{2–247} †	MGSSHHHHHSQDPENLYFQGQTQVLFEHPLNEK- MRTWLRIFELIQLTVNLPIDVHAGALHFFRN- VSELLDVFERGEVRETELLKELDRQQRKLQWIG- VPGVDQSRIEALIQQLKAAGSVLISAPRIGQ- FLREDRLIALVRQLSIPGGCCSFDLPTLHIW- HLHPQAQRDSQVETWIASLNPLTQALTMVLDL- IRQSAPFRKQTSNLNGFYQDNGGADLLRLNLS- LDSQLYPQISGHKSRFAIRFMPLDTENGQVPE- RLDFELACC

† The non-native tags including the His₆ tag and TEV recognition site are underlined.

FtsZ-regulatory proteins involved in the assembly or disassembly of FtsZ and the spatiotemporal control of bacterial cytokinesis have been characterized (Kirkpatrick & Viollier, 2011).

Z-ring-associated protein D (ZapD) has been identified as a member of the divisome which directly interacts with FtsZ and promotes bundling of the protofilament (Durand-Heredia *et al.*, 2012). Interestingly, GTP hydrolysis of FtsZ is under the control of ZapD, which indicates its role in the stabilization and the promotion of FtsZ into a Z-ring at the cell midline (Durand-Heredia *et al.*, 2012). However, the molecular architecture of ZapD and the elements that are responsible for promoting FtsZ assembly are not yet known. In order to facilitate molecular understanding of the role of ZapD in FtsZ assembly, *Escherichia coli* ZapD was overexpressed, purified and crystallized. Its crystallization conditions, X-ray crystallographic data and preliminary structural determination are reported in this study.

2. Materials and methods

2.1. Protein expression and purification

The *E. coli* ZapD_{1–247} and ZapD_{2–247} genes were amplified by polymerase chain reaction (PCR) from the genomic DNA. The amplified DNAs were digested using BamHI and XhoI, and were then introduced into BamHI/XhoI-digested pHis-parallel2 (Sheffield *et al.*, 1999) and pRSFDuet-1 (Novagen) expression vectors, respectively (Table 1). The cloned plasmids were transformed into *E. coli* BL21 (DE3) pLysS expression host cells. Cells were grown at 310 K to an OD₆₀₀ of 0.5–0.8 in Luria–Bertani medium containing 100 µg ml^{−1} ampicillin and 70 µg ml^{−1} chloramphenicol. Protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 298 K for 20 h. The cells were harvested

Table 2

Crystallization.

Method	Vapour diffusion
Plate type	Sitting drop
Temperature (K)	298
Protein concentration (mg ml ^{−1})	18.7
Buffer composition of protein solution	20 mM Tris–HCl pH 8.0, 200 mM NaCl
Composition of reservoir solution	1.5 M lithium sulfate, 0.1 M HEPES pH 7.8, 2% (v/v) polyethylene glycol 400
Additives for crystallization	110 mM FOS-choline, 40% pentaerythritol ethoxylate or 30% MPD
Volume and ratio of drop	0.9 µl (1:1 ratio of protein and reservoir solutions) and 0.2 µl additive
Volume of reservoir (µl)	70

by centrifugation at 5000 rev min^{−1} (Vision V100004A rotor) for 25 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, 5 mM imidazole) containing 1 mM phenylmethylsulfonyl fluoride and was lysed using a microfluidizer (Microfluidics, USA). The crude cell extract was centrifuged at 12 000 rev min^{−1} (Vision V506CA rotor) for 1 h at 277 K and the resulting supernatant was applied onto an Ni-Sepharose affinity chromatography column (GE Healthcare) pre-equilibrated with the lysis buffer. The resin was washed with wash buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, 60 mM imidazole) and the fusion protein was eluted with elution buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, 300 mM imidazole). The eluate was cleaved with 200 µg His₆-tagged TEV protease and 5 mM β-mercaptoethanol for 16 h at 277 K. Further purification was performed by gel filtration on a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare) which was previously equilibrated with 20 mM Tris–HCl pH 8.0, 200 mM NaCl.

2.2. Crystallization

Crystallization experiments were carried out by the sitting-drop vapour-diffusion method at 298 K using 96-well plates (Greiner Bio-One) (Table 2). Crystals were obtained by mixing 0.9 µl protein solution with 0.9 µl reservoir solution consisting of 1.5 M lithium sulfate, 0.1 M HEPES pH 7.8, 2% (v/v) polyethylene glycol 400 and 0.2 µl 110 mM *n*-dodecylphosphocholine (FOS-choline), 40% pentaerythritol ethoxylate or 30% (±)-2-methyl-2,4-pentanediol (MPD) as an additive.

2.3. Data collection and processing

Crystals were flash-cooled in a liquid-nitrogen stream employing 2.0 M lithium sulfate and 5% polyethylene glycol 400 as a cryoprotectant. X-ray diffraction data were collected at 100 K at the BL-5C experimental station of the Pohang Accelerator Laboratory (PAL), Pohang, Republic of Korea using an ADSC Quantum 270 CCD detector (Area Detector Systems Corporation, Poway, California, USA). The wavelength of the synchrotron X-rays was 0.97952 Å and the crystal was rotated through a total of 150° with 1.0° oscillation range

per frame and 1 s exposure time. The raw data were processed and scaled using *HKL-2000* (Otwinowski & Minor, 1997).

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline 5C, PAL
Wavelength (Å)	0.97952
Temperature (K)	100
Detector	ADSC Quantum 270 CCD
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	1
Total rotation range (°)	150
Exposure time per image (s)	2
Space group	<i>P</i> 6 ₄
<i>a</i> , <i>b</i> , <i>c</i> (Å)	109.5, 109.5, 106.7
α , β , γ (°)	90.0, 90.0, 120.0
Mosaicity (°)	0.25
Resolution range (Å)	50.0–2.95 (3.00–2.95)
Total No. of reflections	138449
No. of unique reflections	15284
Completeness (%)	99.3 (100)
Multiplicity	9.1
$\langle I/\sigma(I) \rangle$	33.4 (4.7)
$R_{\text{r.i.m.}}^\dagger$	0.075 (0.535)
Overall <i>B</i> factor from Wilson plot (Å ²)	83.9

[†] The redundancy-independent merging *R* factor $R_{\text{r.i.m.}}$ was estimated by multiplying the conventional R_{merge} value by the factor $[N/(N-1)]^{1/2}$, where *N* is the data multiplicity.

2.4. Analytical gel filtration

The purified EcZapD_{1–247} was subjected to analytical gel-filtration chromatography on a Superdex 200 10/300 GL column with lysis buffer (20 mM Tris–HCl pH 8.0, 200 mM NaCl) at a constant flow rate of 0.5 ml min^{−1}. The standard curve was obtained using molecular-weight markers (Sigma). The Stokes radii of β -amylase, alcohol dehydrogenase, carbonic anhydrase and cytochrome *c* were calculated from the crystal structures of each protein (PDB entries 1fa2, 2hcy, 1v9e and 1hrc, respectively) using *HYDROPRO* (García de la Torre *et al.*, 2000).

3. Results and discussion

Purified *E. coli* ZapD was concentrated by ultrafiltration (3000 Da cutoff) to about 18.7 mg ml^{−1}, with a yield of ~19 mg per litre of culture (Fig. 1*a*). The protein concentration was estimated by measuring the absorbance at 280 nm employing a molar extinction coefficient of 25 230 M^{−1} cm^{−1} (calculated using *ProtParam*; <http://web.expasy.org/protparam/>). Crystals grew to maximum dimensions of 0.05 × 0.05 × 0.5 mm within several days (Fig. 2*a*). X-ray diffraction data were collected to 2.95 Å resolution and the space group was initially determined to be *P*6₂ or *P*6₄ based on systematic

Figure 1

(*a*) Coomassie Blue-stained SDS-PAGE of *E. coli* ZapD protein purified by size-exclusion chromatography. Lane *M*, molecular-weight marker (labelled in kDa); lanes 1–9, fractions of *E. coli* ZapD protein. (*b*) Analytical gel-filtration profile of *E. coli* ZapD_{1–247}. (*c*) Standard curve obtained using molecular-weight markers. The positions of the molecular-weight markers (β -amylase, alcohol dehydrogenase, carbonic anhydrase and cytochrome *c*) are indicated as black dots. The position of *E. coli* ZapD_{1–247} is marked by a red dot.

Figure 2
(a) ZapD crystal with dimensions of $0.05 \times 0.05 \times 0.5$ mm. The scale bar is 0.1 mm in length. (b) A diffraction image of the ZapD crystal.

absences and the symmetry of diffraction intensities (Fig. 2b). Table 3 summarizes the statistics of data collection. Two monomers were present in the asymmetric unit, resulting in a crystal volume per protein mass (V_M) of $3.25 \text{ \AA}^3 \text{ Da}^{-1}$ and a solvent content of 62.17%.

To resolve the enantiomorph ambiguity, structural determination of *E. coli* ZapD was performed using the molecular-replacement method. A sequence-based homology search of *E. coli* ZapD against the Protein Data Bank identified that *E. coli* ZapD has 42% sequence identity to VP2528 from *Vibrio parahaemolyticus* (PDB entry 2oez; Midwest Center for Structural Genomics, unpublished work). The dimer structure of VP2528 from *V. parahaemolyticus* was used as a search model for molecular replacement with *Phaser* (McCoy *et al.*, 2005). Initial rigid-body refinement with *REFMAC5* (Murshudov *et al.*, 2011) gave an R_{work} of $\sim 35\%$ and an R_{free} of $\sim 40\%$. The correct space group was determined to be $P6_4$ based on the map quality. The two subunits in the asymmetric unit associate to form a dimer. To analyze the quaternary structure of ZapD_{1–247} in solution, analytical gel filtration was

performed using a Superdex 200 10/300 GL column (Figs. 1b and 1c). The Stokes radius of *E. coli* ZapD_{1–247} was estimated to be 3.13 nm, which is highly similar to the calculated Stokes radius (3.65 nm) of the VP2528 dimer from *V. parahaemolyticus*. This result suggests that *E. coli* ZapD_{1–247} exists as a dimer in solution. Further model building and structural refinement will be reported in a separate paper.

Acknowledgements

The authors thank the staff of beamline BL-5C at Pohang Accelerator Laboratory for assistance during X-ray experiments. This study was supported by a grant from the National Research Foundation of Korea funded by the Korean government (2012K1A3A1A09033383 and 2013R1A1A1008195) and a grant from the Korea CCS R&D Center (KCRC; 2014M1A8A1049296) to HHL.

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