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Crystallization and preliminary X-ray analysis of the flagellar motor 'brake' molecule YcgR with c-di-GMP from *Escherichia coli*

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In *Escherichia coli* and *Salmonella enterica*, bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), a ubiquitous bacterial second-messenger molecule that participates in many cellular processes, can regulate flagellar motor speed and reduce cell swimming velocity by binding to the PilZ-containing protein YcgR. Here, the crystallization and preliminary X-ray crystallographic analysis of YcgR with c-di-GMP are reported. The crystals diffracted to 2.3 Å resolution and belonged to space group *R*3:*H*, with unit-cell parameters $a = b = 93.96$, $c = 109.61$ Å. The asymmetric unit appeared to contain one subunit with a Matthews coefficient of 3.21 Å^3 Da^{-1}. The results reported here provide a sound basis for solving the crystal structure of YcgR with c-di-GMP and revealing its structure–function relationship based on the three-dimensional structure.

1. Introduction

The mechanisms of how bacteria actively move and how they regulate their movement are of great interest. *Escherichia coli* and related bacteria swim through a liquid environment *via* the rotating flagella. The c-di-GMP [bis-(3′-5′)-cyclic dimeric guanosine monophosphate] binding protein YcgR (Ryjenkov *et al.*, 2006) has recently been identified as regulating the motor speed by binding the flagellar motor in *E. coli* and *Salmonella enterica* (Ko & Park, 2000; Wolfe & Visick, 2008). YcgR contains a PilZ domain and this domain was shown to be an effector of c-di-GMP by bioinformatic analysis (Amikam & Galperin, 2006) and by biochemical (Ryjenkov *et al.*, 2006; Merighi *et al.*, 2007; Pratt *et al.*, 2007) and structural studies (Benach *et al.*, 2007). c-di-GMP is a ubiquitous bacterial second-messenger molecule that participates in signal transduction of many cellular processes such as inhibiting motility, expressing virulence genes (Tamayo *et al.*, 2007), sessility, biofilm formation and cell cycle progression (Hengge, 2009). Intracellular concentration of c-di-GMP is controlled by diguanylate cyclases (DGCs) carrying GGDEF domains (Paul *et al.*, 2004; Chan *et al.*, 2004) and phosphodiesterases (PDEs) harbouring EAL (Tamayo *et al.*, 2005) or HD-GYP domains (Ryan *et al.*, 2006).

However, there are some questions about the detailed mechanisms of how the binding of YcgR to c-di-GMP can regulate flagellar motor speed. The flagellar motor is composed of a rotor located at the bottom of the basal body and a stator spanning the cytoplasmic membrane (Sowa & Berry, 2008). The rotor, also called the C-ring or switch complex, contains three subunits: FliG (26 copies), FliM (34 copies) and FliN (more than 100 copies) (Paul *et al.*, 2011). The stator consists of the membrane proteins MotA and MotB, in the form of a $MotA_4MotB_2$ complex (Kojima & Blair, 2004). The motor is driven by the proton flux through the channels composed of the stator, and the rotor protein FliG interacts with the stator protein MotA to generate torque depending on electrostatic interactions (Zhou *et al.*, 1998). There have been two reports describing the mechanisms of how c-di-GMP modulates bacterial motility through the target protein YcgR. According to Boehm and coworkers, YcgR interacts with MotA directly and this interaction correlates with the cellular concentration of c-di-GMP (Boehm *et al.*, 2010). On the other hand,

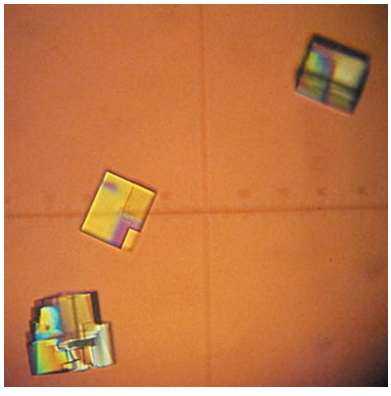

Table 1
Statistics of the X-ray data set for YcgR–c-di-GMP.

Values in parentheses are for the outermost shell.

Space group	*R*3:*H*
Unit-cell parameters (Å, °)	$a = b = 93.96$, $c = 109.61$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution (Å)	46.98–2.30 (2.42–2.30)
Wavelength (Å)	0.9791
Total reflections	86790 (12934)
Unique reflections	15963 (2375)
Completeness (%)	99.5 (100.0)
Multiplicity	5.4 (5.5)
Average $I/\sigma(I)$	15.0 (3.1)
$R_{\rm merge}$† (%)	5.4 (49.3)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean of the observations $I_i(hkl)$ of reflection *hkl*.

the studies of Paul and coworkers suggested that YcgR interacts with FliG and FliM, and the interactions become stronger in the presence of c-di-GMP (Paul *et al.*, 2010). Both of the studies demonstrated that the activated YcgR interfered in the electrostatic interaction between MotA and FliG (Armitage & Berry, 2010).

Currently, the model for inferring the function of YcgR is based on the crystal structure of YcgR-like protein VCA0042/PlzD from *Vibrio cholera* (Benach *et al.*, 2007; Paul *et al.*, 2010), but the sequence alignment showed a very low sequence similarity between VCA0042 and YcgR. Therefore determining the structure of YcgR is imperative for understanding the molecular mechanisms of how c-di-GMP slows down the bacterial swimming velocity by binding to YcgR. Here, we report the crystallization, diffraction data collection and preliminary crystallographic studies of YcgR with its ligand c-di-GMP. These results provide a foundation for solving the crystal structure of this complex.

2. Materials and methods

2.1. Cloning, expression and purification

The *ycgR* gene was amplified by PCR from *E. coli* MG1655 and cloned into pET-22b(+) plasmid (Novagen) between *Nde*I and *Xho*I restriction-enzyme sites. A six-histidine tag was fused to the C-terminus of recombinant YcgR. The correctness of the clone was confirmed by DNA sequencing. The plasmid was transformed into *E. coli* BL21 (DE3) host cells, which were grown on an LB plate containing 100 µg ml^{-1} ampicillin overnight at 310 K. A single colony was cultured in 60 ml LB medium overnight and then inoculated into 800 ml LB medium. When the OD_{600} reached 0.6–1.0, the cells were induced with 0.1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 h at 289 K to produce recombinant YcgR. The bacteria were harvested by centrifugation (Hitachi Himac CRT, R5S2 rotor) at 4670*g* for 30 min. The cell pellet was resuspended in lysis buffer consisting of 50 m*M* phosphate buffer pH 8.0, 300 m*M* NaCl, 10 m*M* imidazole, 5%(*v*/*v*) glycerol. The cells were lysed by sonication and the cell debris was removed from the lysate by centrifugation at 20 000*g* for 40 min at 277 K (Sigma 3k30, 12150 rotor). The clarified lysate (typically ∼40 ml) was loaded directly onto an Ni–NTA column (Novagen) pre-equilibrated with lysis buffer. The column was washed with wash buffer [50 m*M* phosphate buffer pH 8.0, 1 *M* NaCl, 20 m*M* imidazole, 5%(*v*/*v*) glycerol] to remove impurities. The target protein was eluted with elution buffer [50 m*M* phosphate buffer pH 8.0, 150 m*M* NaCl, 250 m*M* imidazole, 5%(*v*/*v*) glycerol] containing 250 m*M* imidazole. The fractions including YcgR were concentrated to 1 ml by ultrafiltration (Sartorius) at 277 K and further purified twice in tandem by size-exclusion chromatography using a Superdex 200 10/300 GL (GE Healthcare) gel-filtration column mounted on an ÄKTApurifier (Pharmacia Inc.) and equilibrated with 20 m*M* HEPES pH 7.5, 150 m*M* NaCl, 10%(*v*/*v*) glycerol, 2 m*M* DTT. The peak corresponding to YcgR monomers was collected, concentrated, flash-cooled in liquid nitrogen and stored at 193 K.

2.2. Crystallization

Initial crystallization screening was performed using hanging-drop vapour diffusion at 293 K by mixing 1 µl protein solution with an equal volume of reservoir liquor and suspending the drop over 0.43 ml reservoir liquor. Both YcgR and YcgR mixed with c-di-GMP (YcgR–c-di-GMP) were screened for crystallization conditions. YcgR was concentrated to about 17 mg ml^{-1} and c-di-GMP was added to the YcgR solution at a 3:1 ligand:protein molar ratio for YcgR–c-di-GMP. Commercial kits, including Index, PEGRx and PEG/Ion kits from Hampton Research (Jancarik & Kim, 1991), were used in initial screening. The crystals only appeared in the drops containing c-di-GMP, and YcgR did not form crystals in the absence of c-di-GMP. The original conditions for tiny crystals of YcgR–c-di-GMP were Index condition No. 71, which consists of 0.2 *M* sodium chloride, 0.1 *M* bis-tris pH 6.5, 25%(*w*/*v*) PEG 3350, and PEG/Ion 2 condition No. 26, which consists of 0.2 *M* sodium acetate trihydrate pH 7.0, 20%(*w*/*v*) PEG 3350. The crystallization optimizations for YcgR–c-di-GMP were performed by altering the type and concentration of

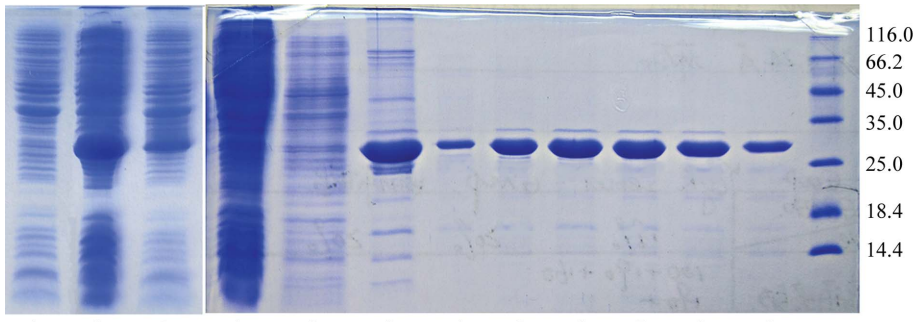

Figure 1
SDS–PAGE of purified YcgR. (*a*) Protein expression. Lane 1, cell lysate before induction; lane 2, cell lysate after induction; lane 3, soluble component of the cell lysate. (*b*) Protein purification. Lanes 1–3, unbound, washed and eluted component of Ni–NTA purification; lanes 4–9, purified YcgR after Superdex 200 10/300 GL gel-filtration column. Molecular-mass markers (in kDa) are in the lane at the far right of the figure.

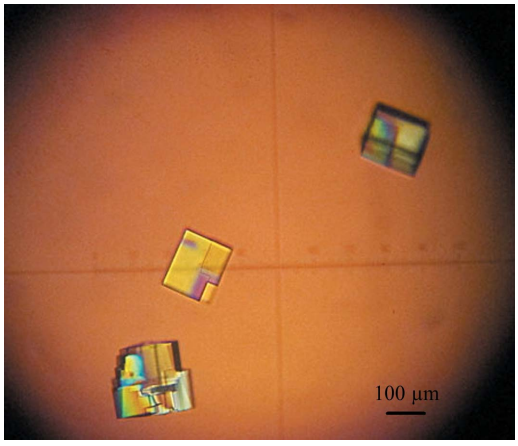

Figure 2
Crystals of YcgR–c-di-GMP. The crystals grew in about 3 d at 293 K using 0.2 *M* potassium thiocyanate, 0.1 *M* bis-tris pH 6.0, 20%(*w*/*v*) PEG 3350.

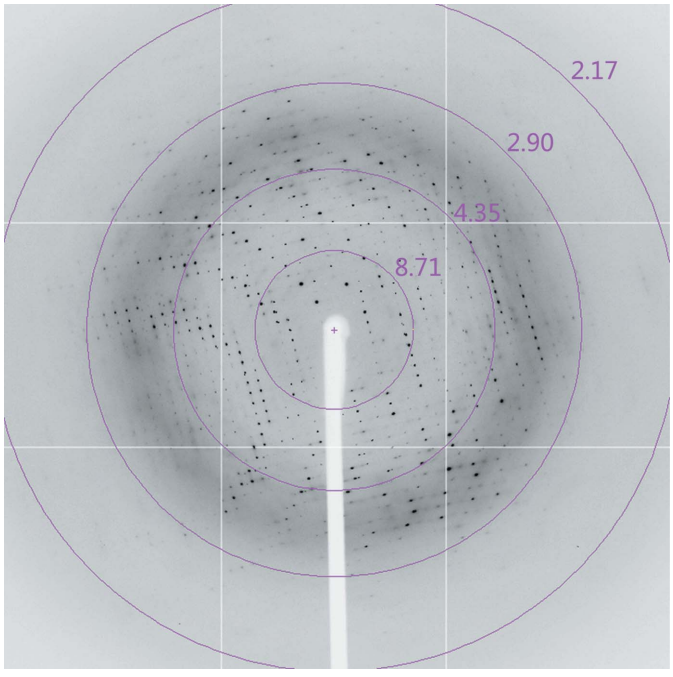

Figure 3
X-ray diffraction pattern of a YcgR–c-di-GMP crystal using a synchrotron source.

the precipitant (PEG), the pH and the salts. The best crystals were obtained in a condition consisting of 0.2 *M* potassium thiocyanate, 0.1 *M* bis-tris pH 6.0, 20%(*w*/*v*) PEG 3350.

2.3. Data collection and processing

The crystals were soaked in reservoir solution supplemented with 10%(*v*/*v*) glycerol for 1 min, followed by flash-cooling to 100 K. The data were collected using an ADSC Q315r CCD detector on beamline BL-17A at KEK (Photon Factory, Tsukuba, Japan) at a wavelength of 0.9791 Å. A total of 180 images were collected with 1° oscillation per image. The data were processed and scaled using *MOSFLM* v.7.0.4 (Battye *et al.*, 2011) and *SCALA* v.6.1 from the *CCP*4 program suite (Winn *et al.*, 2011).

3. Results

Monomeric YcgR was obtained using a Superdex 200 10/300 GL gel-filtration column. The purified protein was confirmed by SDS–PAGE (Fig. 1) and concentrated to 17 mg ml^{-1}.

In the preliminary crystallization screening, tiny polycrystalline crystals of YcgR–c-di-GMP were observed in several conditions from the Index and PEG/Ion kits. The conditions were optimized by varying parameters such as the pH, the type and concentration of precipitant and by the use of additives. Crystals suitable for diffraction experiments were obtained in 0.2 *M* potassium thiocyanate, 0.1 *M* bis-tris pH 6.0, 20%(*w*/*v*) PEG 3350 (Fig. 2).

The crystals diffracted to 2.3 Å resolution (Fig. 3) and belonged to the trigonal space group *R*3:*H*, with unit-cell parameters $a = b = 93.96$, $c = 109.61$ Å. Calculations revealed that there was one molecule in the asymmetric unit with a solvent content of 61.72% and a Matthews coefficient of 3.21 Å^3 Da^{-1} (Matthews, 1968). The data-collection statistics are shown in Table 1. Phasing, model building and refinement are in progress.

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