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Expression, purification, crystallization and preliminary X-ray diffraction analysis of a core fragment of FlgG, a bacterial flagellar rod protein

FlgG is a bacterial flagellar rod protein and constructs the distal rod connecting to the hook. FlgG of *Salmonella enterica* serovar Typhimurium is a 260-amino-acid protein composed of a folded core region and N- and C-terminal regions that are unfolded in solution. A core fragment of FlgG (FlgG_{47–227}) was expressed, purified and crystallized. Crystals of native and SeMet-labelled FlgG_{47–227} were obtained by the sitting-drop vapour-diffusion technique with PEG MME 2000 as precipitant. The native crystal belonged to the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 47.78$, $b = 68.94$, $c = 110.57$ Å. The SeMet crystal also belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 47.53$, $b = 67.04$, $c = 110.27$ Å.

1. Introduction

The bacterial flagellum is a long filamentous organelle for bacterial motility. It consists of a basal body, a short curved segment called the hook and a long helical filament. The basal body is a reversible rotary motor and generates torque. The hook is a universal joint that transmits the motor torque to the long helical propeller in its different orientations. The filament is a thin helical structure that typically grows to around 15 µm in length and rapidly rotates as a propeller for cell locomotion in viscous environments (Berg & Anderson, 1973; Berg, 2003; Macnab, 2003). The flagellar rod at the centre of the basal body is a competent drive shaft that transmits torque through the hook to the filament to propel bacterial locomotion. In *Salmonella*, the rod is composed of FlgB, FlgC, FlgF and FlgG proteins (Homma *et al.*, 1990; Kubori *et al.*, 1992). FlgG (260 amino acids, 27.8 kDa) forms the distal part of the rod (distal rod), which is directly connected to the hook, and the other three proteins are believed to constitute the cell-proximal portion (Okino *et al.*, 1989; Kubori *et al.*, 1992).

It has been thought that the rod is a helical assembly of these rod proteins forming a continuous axial structure with its 11 protofilaments extending to the hook and the filament, the structures of which have been well characterized in atomic detail (Yonekura *et al.*, 2003; Fujii *et al.*, 2009; Maki-Yonekura *et al.*, 2010). The distal rod is surrounded by the P and L rings, which together act as a bushing embedded in the peptidoglycan layer and outer membrane, respectively. The distal rod freely rotates inside the P and L rings, which have rotational symmetry, and the symmetry mismatch between the rod and the rings may be important to achieve smooth rotation with low friction (Khan *et al.*, 1985; Akiba *et al.*, 1991). To elucidate the mechanism of the distal rod function, high-resolution structural information of FlgG from X-ray crystallographic study is essential. We purified full-length *Salmonella* FlgG expressed in *Escherichia coli* cells and characterized it. Limited proteolysis of FlgG in solution showed that it consists of a compact core and unfolded N/C-terminal regions (Saijo-Hamano *et al.*, 2004). FlgG tends to form β -amyloid-like fibrils through the interactions of the unfolded regions under most crystallization conditions (Saijo-Hamano *et al.*, 2004). Therefore, we removed the unfolded N- and C-terminal regions for crystallization. This strategy had previously worked well for the crystallization of FlgC (flagellin) and FlgE (hook protein) (Samatey *et al.*, 2000, 2001; Samatey, Matsunami, Imada, Nagashima & Namba, 2004; Samatey, Matsunami, Imada, Nagashima, Shaikh *et al.*, 2004).

Here, we report the expression, purification, crystallization and preliminary X-ray crystallographic study of a core fragment of FlgG (FlgG_{47–227}).

2. Materials and methods

2.1. Expression of FlgG_{47–227}

The gene coding for residues 47–227 of FlgG (FlgG_{47–227}) from *Salmonella enterica* serovar Typhimurium strain SJW1103 (Yamaguchi *et al.*, 1984; GenBank accession No. X52094) was PCR-amplified with specific primers (5'-GGAATTCATATG-CAGACCATCCGCCAGCCG-3' and 5'-ACGAATTCCTTACGCTTACGCCACGTTAACGTTTCGACG-3'; the restriction-enzyme sites are shown in bold) and then cloned into the *NdeI*–*EcoRI* site of pET-23b (Novagen) to create pHMK542. The *NdeI*–*BlnI* fragment from pHMK542 was subcloned into the same site of pET-14b and pET-15b (Novagen) to create pHMK543 and pHMK544, respectively.

The plasmid pHMK543 was transformed into *E. coli* strain BL21 Star (DE3) (Invitrogen). The transformant was grown in 2×YT medium supplemented with 50 mg l^{−1} ampicillin at 300 K for 20–24 h. FlgG_{47–227} with an N-terminal His₆ tag and a thrombin cleavage site from pET-14b (His₆-FlgG_{47–227}) was expressed in the cells without isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. The cells were harvested by centrifugation and stored at 190 K.

The plasmid pHMK544 was transformed into *E. coli* strain B834 (DE3) (Novagen). The transformant was grown in LB medium supplemented with 50 mg l^{−1} ampicillin at 300 K overnight. The cells were harvested by centrifugation and washed with 1% (w/v) NaCl. This cell suspension was diluted to an OD_{600 nm} of 0.3 in selenomethionine (SeMet) minimal medium [1 g l^{−1} NH₄Cl, 3 g l^{−1} KH₂PO₄, 8 g l^{−1} Na₂HPO₄·12H₂O, 20 g l^{−1} glucose, 0.6 g l^{−1} MgSO₄·7H₂O, 10 mg l^{−1} Fe₂(SO₄)₃, 10 mg l^{−1} thiamine and 50 mg l^{−1} L-SeMet; Guerrero *et al.*, 2001; Sakane, 2008] supplemented with 50 mg l^{−1} ampicillin and 10 ml l^{−1} Kao and Michayluk Vitamin Solution (Sigma). The cells were grown in SeMet minimal medium at 300 K to an OD_{600 nm} of 0.8 and expression of SeMet-labelled His₆-FlgG_{47–227} was then induced with 1 mM IPTG for 16 h. The cells were harvested by centrifugation (10 000g, 10 min, 277 K) and stored at 190 K.

2.2. Protein purification

To purify native FlgG_{47–227}, the frozen BL21 Star (DE3) cells were suspended in solution I (50 mM Tris–HCl pH 8.0, 300 mM NaCl, 20 mM imidazole–HCl pH 8.0) and sonicated on ice. The cell lysate

was centrifuged (100 000g, 30 min, 277 K) and His₆-FlgG_{47–227} in the soluble fraction was purified by affinity chromatography using a HisPrep FF 16/10 column (GE Healthcare). The HisPrep column was equilibrated with solution I and the bound proteins were eluted with a linear gradient of 20–300 mM imidazole–HCl pH 8.0. A main fraction containing His₆-FlgG_{47–227} was dialysed against solution II (50 mM Tris–HCl pH 8.0, 20 mM imidazole–HCl pH 8.0) at 277 K for 2 h using a cellulose tubular membrane with MWCO 3500 (Orange Scientific) with stirring and was then incubated overnight at 277 K with one unit of thrombin protease (GE Healthcare) per mg of His₆-FlgG_{47–227} to remove the His₆ tag. The digestion mixture was then loaded onto the HisPrep column and eluted with solution II. FlgG_{47–227} recovered from the flowthrough fraction was further purified by anion-exchange chromatography using a RESOURCE Q (6 ml) column (GE Healthcare). The column was equilibrated with 50 mM Tris–HCl pH 8.0 and eluted with a linear gradient of 0–300 mM NaCl. After the anion-exchange chromatography, the main fraction was dialysed against 50 mM Tris–HCl pH 8.0, and purified again with the RESOURCE Q column to completely remove thrombin and some degradation products of FlgG_{47–227}. SeMet-labelled FlgG_{47–227} was purified from the frozen B834 (DE3) cells using the same protocol as that described above for native FlgG_{47–227}. The purity of the protein samples was examined by SDS–PAGE, gel-filtration chromatography using a Superdex 200 10/300 GL column (GE Healthcare) and MALDI-TOF mass spectrometry using a Voyager DE Pro (Applied Biosystems). Protein samples were dialysed extensively against 50 mM Tris–HCl pH 8.0 using the cellulose tubular membrane with MWCO 3500 and were concentrated to 50 mg ml^{−1} using a Vivaspin 20 MWCO 5K (Sartorius Stedim Biotech) (7000g, 277 K). Protein concentration was estimated by assuming an Abs_{280 nm} of 0.310 for a 1 mg ml^{−1} solution.

2.3. Crystallization

We used NeXtal Evolution μplates (Qiagen) for the sitting-drop vapour-diffusion method and VDX plates (Hampton Research) for the hanging-drop vapour-diffusion method. Initial crystallization screening was performed by the sitting-drop vapour-diffusion method using a Phoenix crystallization robot (Art Robbins Instruments) and the following screening kits: Wizard I, II and III (Emerald BioStructures), Cryo I and II (Emerald BioStructures), and Crystal Screen and Crystal Screen 2 (Hampton Research). Sitting drops consisting of 1 μl protein solution (3, 10, 30 and 50 mg ml^{−1}) and 1 μl crystallization solution were equilibrated against 60 μl reservoir solution at 277, 285 and 293 K. The crystallization conditions were optimized manually using the Optimize-polymers reagents (Hampton Research) by the sitting-drop and hanging-drop methods. Sitting and hanging drops consisting of 1 μl protein solution (30 mg ml^{−1}) and 1 μl crystallization solution were equilibrated against 60 μl and 0.6 ml reservoir solutions, respectively. The best rod-shaped crystals were obtained from sitting drops with a reservoir solution consisting of 28–30% PEG MME 2000, 0.2 M ammonium sulfate, 0.1 M sodium acetate pH 4.6 at 277 K after 4–5 months (Fig. 1). Crystals of SeMet-labelled FlgG_{47–227} were obtained under the same conditions as used for the native crystals after 2 months.

2.4. X-ray data collection and processing

Crystals (approximate dimensions of 0.01 × 0.01 × 0.2 mm) were mounted in nylon Cryo-Loops (Hampton Research) with the mother liquor containing 10% (v/v) MPD as a cryoprotectant and were cooled in liquid nitrogen prior to X-ray experiments. Diffraction data collection was performed on the BL41XU beamline at SPring-8,

Figure 1
Crystals of FlgG_{47–227}. The scale bar is 0.1 mm.

Table 1Summary of the data statistics for FlgG_{47–227}.

Values in parentheses are for the outermost resolution shell.

	Native	SeMet derivative
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å, °)	$a = 47.78, b = 68.94,$ $c = 110.57,$ $\alpha = \beta = \gamma = 90$	$a = 47.53, b = 67.04,$ $c = 110.27,$ $\alpha = \beta = \gamma = 90$
Wavelength (Å)	1.00000	0.97900
Resolution (Å)	34.47–2.00 (2.11–2.00)	36.76–2.00 (2.11–2.00)
Observed reflections	209521 (30046)	165577 (22553)
Unique reflections	25189 (3587)	24009 (3421)
Mean $I/\sigma(I)$	21.9 (7.3)	10.8 (4.0)
Completeness (%)	99.2 (98.8)	98.1 (97.5)
Multiplicity	8.3 (8.4)	6.9 (6.6)
$R_{\text{merge}}^{\dagger}$ (%)	6.5 (29.0)	10.6 (38.9)
$R_{\text{meas}}^{\ddagger}$ (%)	6.9 (30.9)	11.5 (42.1)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is an individual intensity measurement and $\langle I(hkl) \rangle$ is the average intensity for this reflection. $\ddagger R_{\text{meas}} = \sum_{hkl} [N(hkl) / (N(hkl) - 1)]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is an individual intensity measurement, $\langle I(hkl) \rangle$ is the average intensity for this reflection and $N(hkl)$ is the multiplicity (Diederichs & Karplus, 1997).

Harima, Japan. Diffraction data were recorded on a MAR 225HE CCD detector (Rayonix) at 100 K using a nitrogen-gas flow to reduce the radiation damage. Each of the full data sets was obtained from a single crystal. The diffraction data were processed using the *iMOSFLM* X-ray data processing package (Battye *et al.*, 2011) and were scaled using *SCALA* (Evans, 2006) from the *CCP4* program suite (Winn *et al.*, 2011). The data-collection statistics are summarized in Table 1.

3. Results

Our previous limited-proteolysis experiment showed that the N-terminal 42 residues (Met1–Glu42) and the C-terminal 31 residues (Leu230–Leu260) are highly sensitive to proteases, indicating dis-ordering of these regions. The limited proteolysis also showed that the most rigid core of FlgG is the central 137 residues (Gly84–Glu220) and that the central 150 residues (Leu80–Glu229) and 171 residues (Gln59–Glu229) are semi-stable fragments (Saijo-Hamano *et al.*, 2004). Therefore, we started crystallization screening with FlgG_{80–229} and FlgG_{59–229}, but they yielded neither crystals nor precipitates. Therefore, we constructed a new FlgG fragment based on secondary-structure prediction by the program *PHD* (Rost & Sander, 1993). The terminal residues Met1–Tyr46 and Glu228–Leu260, which contain the regions sensitive to proteases, were predicted to be α -helices. Since the highly disordered terminal regions of FlgC and FlgE in solution were predicted to be α -helices, Met1–Tyr46 and Glu228–Leu260 of FlgG are also thought to be highly disordered in solution. Therefore, we prepared a fragment of FlgG_{47–227} as a new crystallization target.

After thrombin treatment to remove the His₆ tag, a small number of unexpected proteolytic fragments of FlgG_{47–227} were produced owing to incorrect cleavage by thrombin. The amounts of these fragments were very small and they were not detected by SDS–PAGE. We only found these fragments by MALDI–TOF mass spectrometry. We found that contamination by these fragments disturbed the growth of single crystals. Therefore, we purified the digestion mixture by using an anion-exchange column twice to completely remove the unexpected proteolytic products. This was essential for producing high-quality crystals suitable for X-ray analysis.

In the initial crystallization screening, FlgG_{47–227} yielded neither crystals nor precipitate even after 4 months, as was the case for FlgG_{80–229} and FlgG_{59–229}. However, a year and a half later various needle-shaped polycrystals appeared in drops with 30 mg ml^{−1} FlgG_{47–227} under nine different conditions containing PEG or PEG MME at 277 K. The crystallization conditions were optimized by varying the precipitant concentration, pH and additives using the sitting-drop and hanging-drop methods. After 6 months, rod-shaped single crystals of FlgG_{47–227} appeared only in the sitting drops at pH 4.5–8.5 containing 28–36% (w/v) PEG 4000 or 24–36% (w/v) PEG MME 2000 as precipitant and ammonium or lithium salts as additives. In hanging drops, FlgG_{47–227} only formed polycrystals under the same condition. To reduce the time required for crystallization, streak-seeding was also attempted but did not work well. The quality of the crystals was checked by X-ray diffraction, and the crystals grown from the solution containing PEG MME 2000 and ammonium sulfate gave significantly better diffraction patterns than the others. The condition was further optimized, and the best crystals were obtained from the condition described in §2.3.

Both the native and SeMet-derivative crystals diffracted to 2.0 Å resolution and belonged to the primitive orthorhombic space group $P2_12_12_1$ (Fig. 2). The unit-cell parameters of the native crystal were $a = 47.78, b = 68.94, c = 110.57$ Å and those of the SeMet derivative were $a = 47.53, b = 67.04, c = 110.27$ Å. The diffraction data statistics of these crystals are summarized in Table 1. Assuming the presence of two molecules in the asymmetric unit, the Matthews coefficient (V_M ; Matthews, 1968) was estimated to be 2.37 Å³ Da^{−1}, corresponding to a solvent content of 48.1%. The self-rotation function, however, showed no significant peaks other than those from the crystallographic symmetry, implying that the local symmetry involves translation or that the local twofold axis is parallel to one of the crystallographic twofold screw axes. Model building and structure refinement are currently under way.

Figure 2

X-ray diffraction image of the native FlgG_{47–227} crystal collected on SPring-8 beamline BL41XU. The red circle indicates a resolution of 2.0 Å. The inset shows a magnified view of the diffraction image.

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