Crystallization and preliminary X-ray analysis of the periplasmic domain of FliP, an integral membrane component of the bacterial flagellar type III protein-export apparatus

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The bacterial flagellar proteins are transported *via* a specific export apparatus to the distal end of the growing structure for their self-assembly. FliP is an essential membrane component of the export apparatus. FliP has an N-terminal signal peptide and is predicted to have four transmembrane (TM) helices and a periplasmic domain (FliP_p) between TM-2 and TM-3. In this study, FliP_p from *Thermotoga maritima* (TmFliP_p) and its selenomethionine derivative (SeMet-TmFliP_p) were purified and crystallized. TmFliP_p formed a homotetramer in solution. Crystals of TmFliP_p and SeMet-TmFliP_p were obtained by the hanging-drop vapour-diffusion technique with 2-methyl-2,4-pentanediol as a precipitant. These two crystals grew in the hexagonal space group *P*6₂22 or *P*6₄22, with unit-cell parameters *a* = *b* = 114.9, *c* = 193.8 Å. X-ray diffraction data were collected from crystals of TmFliP_p and SeMet-TmFliP_p to 2.4 and 2.8 Å resolution, respectively.

1. Introduction

Many bacteria swim in liquid environments by rotating flagella, each of which consists of at least three parts: the basal body, the hook and the filament. Flagellar assembly begins with the basal body, followed by the hook and finally the filament. Most flagellar proteins are transported *via* the flagellar type III export apparatus to the distal end of the growing structure. The export apparatus consists of a membrane-embedded export gate consisting of FlhA, FlhB, FliO, FliP, FliQ and FliR, and a cytoplasmic ATPase complex consisting of FliH, FliI and FliJ. The export apparatus is located within the central pore of the basal body MS ring formed by 26 copies of FliF, FliG, FliM and FliN, which form the C ring on the cytoplasmic face of the MS ring, are required for efficient and rapid protein export. These proteins are highly homologous to those of the type III secretion systems of animal- and plant-pathogenic Gram-negative bacteria, which directly inject virulence factors into their host cells (Macnab, 2004; Minamino & Namba, 2004; Minamino *et al.*, 2008; Minamino, 2013). Interestingly, the atomic structures of FliI and FliJ look similar to the α/β and γ subunits of F₀F₁-ATP synthase, respectively, suggesting an evolutionary relationship between the type III protein-export system and F₀F₁-ATP synthase (Imada *et al.*, 2007; Ibuki *et al.*, 2011; Kishikawa *et al.*, 2013).

FlhA forms a nonameric ring structure in the export apparatus and requires FliO, FliP, FliQ and FliR for its oligomerization and subsequent assembly into the MS ring (Abrusci *et al.*, 2013; Morimoto *et al.*, 2014). The C-terminal cytoplasmic domain of FlhA (FlhA_C) has been visualized at the centre of the cavity within the C ring (Abrusci *et al.*, 2013; Kawamoto *et al.*, 2013). FlhA_C and the C-terminal cytoplasmic domain of FlhB (FlhB_C) provide binding sites for the ATPase complex, flagellar chaperones and export substrates (Bange *et al.*, 2010; Kinoshita *et al.*, 2013; Minamino & Macnab, 2000; Minamino *et al.*, 2003, 2008, 2011). Both FlhA_C and FlhB_C act as an export switch to coordinate flagellar protein export with assembly (Hirano *et al.*, 2009; Kinoshita *et al.*, 2013). A specific interaction of FliJ with FlhA brought about by FliH and FliI allows the export gate to utilize only the electric potential difference part ($\Delta\psi$) of the proton motive force across the cytoplasmic membrane to drive protein translocation (Minamino & Namba, 2008; Paul *et al.*, 2008; Minamino *et al.*, 2011;

Ibuki *et al.*, 2013). Although crystal structures of FlhA_C and FlhB_C have been solved by X-ray crystallography (Bange *et al.*, 2010; Meshcheryakov *et al.*, 2013; Moore & Jia, 2010; Saijo-Hamano *et al.*, 2010), it remains unknown how the export gate recognizes export substrates according to flagellar assembly and transports them in a $\Delta\psi$ -dependent manner.

FliP is a 25 kDa polytopic cytoplasmic membrane protein with a cleavable signal peptide at its N-terminus (Malakooti *et al.*, 1994; Ohnishi *et al.*, 1997). The Sec translocase and the YidC insertase mediate membrane insertion and topogenesis of FliP, and the signal peptide of FliP is cleaved during its membrane insertion, producing its mature form with a molecular weight of 23 kDa (Malakooti *et al.*, 1994; Pradel *et al.*, 2004, 2005; Ohnishi *et al.*, 1997). The mature form of FliP is predicted to have four TM (transmembrane) helices and a periplasmic domain (FliP_P) between TM-2 and TM-3. About four copies of FliP are estimated to be associated with the MS ring (Fan *et al.*, 1997). FliP requires FliO for its protein stability in the cytoplasmic membrane (Barker *et al.*, 2010). It has been reported that FliP interacts with FlhA (McMurry *et al.*, 2004). These observations suggest that FliP forms a complex along with FliO and FlhA in the export apparatus. However, it remains unknown how FliP acts during flagellar protein export. In order to understand the role of FliP in the protein-export process, the atomic structure of FliP is essential. In this paper, we describe the expression, purification and crystallization of FliP_P from the hyperthermophilic bacterium *Thermotoga maritima*.

2. Materials and methods

2.1. Protein expression and purification

A DNA fragment encoding FliP_P (TmFliP_P) consisting of residues 110–188 of *T. maritima* FliP (UniProt ID Q9WZG2) was generated by PCR with the plasmid pGS1-*TmfliP* (Gene Design Inc.) as a template and 5'-GGAATTCATATGTACAACAATGCCATACGCGG-3' and 5'-CGCGGATCCTCATTTGAAGGCAACTTCAGTTTC-3', containing *Nde*I and *Bam*HI restriction sites, respectively, as primers. The amplified DNA fragment was purified using QIAquick PCR purification kits (Qiagen). The purified PCR product and the pET-15b vector were digested with *Nde*I and *Bam*HI (New England Biolabs), gel-purified and ligated. The recombinant plasmids were isolated using QIAprep Spin Miniprep kits (Qiagen). The cloned DNA fragment was sequenced with a 3130 Genetic Analyzer (Applied Biosystems).

A 30 ml overnight culture of *Escherichia coli* strain BL21 (DE3) (Novagen) harbouring pKY045, which encodes TmFliP_P with an N-terminal hexahistidine tag (His₆) followed by a thrombin site (His₆-TmFliP_P; MGSS-6×His-SSGLVPRGSH-FliP) on pET-15b, was inoculated into 2.5 l LB medium (10 g l⁻¹ Bacto tryptone, 5 g l⁻¹ yeast extract, 10 g l⁻¹ NaCl) containing 50 µg ml⁻¹ ampicillin. Cells were grown at 303 K until the culture density had reached an OD₆₀₀ of 0.4. Expression of His₆-TmFliP_P was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM, and the culture was incubated for a further 6 h. The cells were harvested by centrifugation (6400g, 10 min, 277 K) and stored at 193 K. The cells were thawed, suspended in buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl) containing 20 mM imidazole and sonicated (Astrason model XL2020 sonicator, Misonix Inc.). The cell lysate was centrifuged (110 000g, 40 min, 277 K) to remove cell debris. The supernatant was loaded onto Ni-NTA agarose resin (Qiagen) equilibrated with buffer A containing 20 mM imidazole. The protein was then eluted with a 50–300 mM imidazole gradient in buffer A and fractions

containing His₆-TmFliP_P were collected. The His₆ tag was removed by adding 50 units of thrombin (GE Healthcare) to the His₆-TmFliP_P solution. The reaction mixture was dialyzed overnight at 277 K against buffer A with 200 mM imidazole followed by buffer A with 100 mM imidazole and finally buffer A with 20 mM imidazole. TmFliP_P was purified using a HisTrap HP 5 ml column (GE Healthcare) to remove the N-terminally His-tagged polypeptide, noncleaved His₆-TmFliP_P and thrombin. The eluted fractions containing TmFliP_P were concentrated using a Vivaspin 20 (30 000 MWCO, Sartorius), dialyzed overnight against buffer B (10 mM sodium phosphate pH 7.0, 100 mM NaCl) and loaded onto a HiLoad Superdex 75 (26/60) column (GE Healthcare) equilibrated with buffer B. Fractions containing TmFliP_P were collected. The purity of the product was examined by SDS-PAGE and MALDI-TOF mass spectrometry (Voyager DE Pro, Applied Biosystems).

To purify a selenomethionine (SeMet) derivative of TmFliP_P (SeMet-TmFliP_P) for phase determination, we used *E. coli* B834 (DE3) strain (Novagen) as a host. Cells grown overnight in LB medium containing 50 mg l⁻¹ ampicillin at 303 K were harvested by centrifugation (5000g, 5 min, 277 K), washed and suspended in 0.5% (w/v) NaCl. The cells were inoculated into 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 grown at 310 K until the cell density reached an OD₆₀₀ of 0.4. Expression of SeMet-labelled His₆-TmFliP_P was induced with 0.4 mM IPTG and the culture was continued for a further 4 h. The cells were harvested by centrifugation (6400g, 10 min, 277 K) and stored at 190 K. SeMet-TmFliP_P was purified similarly to native TmFliP_P. Incorporation of SeMet into TmFliP_P was confirmed by MALDI-TOF mass spectrometry.

2.2. Analytical ultracentrifugation

Analytical ultracentrifugation was carried out using a Beckman Optima XL-A analytical ultracentrifuge with an An-60 Ti rotor. The

Figure 5 Sedimentation-equilibrium analysis of TmFliP_P. The initial protein concentration was 0.85 mg ml⁻¹. Open circles are data points and the solid line is a model fit. Data points are fitted to a single-species model with a molecular mass of 39.7 kDa. Measurements were taken at 277 K.

protein samples were extensively dialyzed against buffer *B* prior to analytical ultracentrifugation. Sedimentation-equilibrium measurements were performed at 277 K at speeds of 22 000, 24 000 and 26 000 rev min^{-1} on a sample at an initial concentration of 0.85 mg ml^{-1} using a two-channel charcoal-filled epon centrepiece and quartz windows. Scans were collected at a wavelength of 280 nm at a spacing of 0.001 cm in step mode with 20 averages per step. The equilibrium of the system was judged by superimposition of the three scans. Sedimentation-equilibrium data of TmFliP_P, the partial specific volume $\bar{v}$ of which was calculated to be 0.736 ml g^{-1} from the amino-acid composition of the protein, were analyzed to obtain the average molecular weight using the Optima XL-A/XLI software v.04.

(a)

(b)

2.3. Crystallization

Purified TmFliP_P was concentrated and passed through a 0.20 μm Millex-LG syringe filter (Millipore). The protein concentration was estimated based on an A_{280} of 0.47 for a 1 mg ml^{-1} solution. Initial crystallization screening of TmFliP_P was performed at 277 and 293 K by the sitting-drop vapour-diffusion technique using NeXtal Evolution plates (Qiagen) and the following screening kits: Wizard Classic 1 and 2, Wizard Cryo 1 and 2 (Rigaku), Crystal Screen and Crystal Screen 2 (Hampton Research). Each drop was prepared by mixing 1.0 μl protein solution (20 mg ml^{-1} TmFliP_P, 10 mM sodium phosphate pH 7.0, 100 mM NaCl) with 1.0 μl reservoir solution and was equilibrated against 60 μl reservoir solution. The crystallization conditions were optimized by varying the precipitant concentration, pH and additives using the hanging-drop method with VDX plates (Hampton Research). Finally, crystals suitable for X-ray analysis were obtained from drops prepared by mixing 1.5 μl protein solution (5 mg ml^{-1}) with 1.5 μl reservoir solution consisting of 0.1 M phosphate-citrate pH 4.4, 36% 2-methyl-2,4-pentanediol (MPD) at 277 K within a week.

2.4. Data collection and processing

All X-ray diffraction data were collected on beamline BL41XU at SPring-8, Harima, Japan. Crystals were mounted in nylon CryoLoops (Hampton Research). Because the concentration of MPD in the crystallization drops was high enough for cryoprotection, the crystals were directly transferred into liquid nitrogen for freezing. The diffraction data were recorded on an MX225HE CCD detector (Rayonix) at 100 K using a nitrogen-gas flow to reduce radiation damage. The diffraction data were indexed, integrated and scaled using *iMosflm* (Battye *et al.*, 2011) and *SCALA* (Evans, 2006) from the *CCP4* program suite (Winn *et al.*, 2011). The statistics of data collection are summarized in Table 1.

3. Results and discussion

To carry out high-resolution structural analysis of TmFliP, we first focused on FliP_P because overexpression of full-length FliP totally

(c)

Figure 2

(a) Crystals of native TmFliP_P. Crystals obtained using 36% MPD, 0.1 M phosphate-citrate pH 4.4. (b) Diffraction image of the TmFliP_P crystal grown from the optimized condition. A typical X-ray pattern of the TmFliP_P crystal collected on beamline BL41XU at SPring-8. The inset shows a close-up view of the diffraction image. (c) Bijvoet difference Patterson map at $w = 0.33$ Harker section calculated from the TmFliP_P SeMet-derivative data at 3.5 Å resolution. The contour lines are drawn from 2.0σ to 6.0σ with an increment of 0.5σ .

Table 1
Summary of the data statistics.

Values in parentheses are for the highest resolution shell.

	Native	SeMet derivative
Space group	<i>P</i> 6 ₂ 22 or <i>P</i> 6 ₄ 22	<i>P</i> 6 ₂ 22 or <i>P</i> 6 ₄ 22
Unit-cell parameters		
<i>a</i> = <i>b</i> (Å)	114.9	115.3
<i>c</i> (Å)	193.8	193.6
$\alpha = \beta$ (°)	90	90
γ (°)	120	120
Wavelength (Å)	1.0	0.9791
Resolution (Å)	43.6–2.4 (2.53–2.40)	43–2.8 (2.95–2.80)
No. of observed reflections	214552	257930
No. of unique reflections	30262	18573
<i>R</i> _{merge} [†]	0.088 (0.357)	0.097 (0.23)
<i>R</i> _{ano} [‡]		0.053 (0.12)
Mean <i>I</i> /σ(<i>I</i>)	13.5 (5.5)	17.0 (5.4)
Completeness (%)	99.9 (100.0)	95.1 (67.6)
Multiplicity	7.1 (7.2)	7.5 (2.4)

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl* and $\langle I(hkl) \rangle$ is the weighted average intensity for all *i* observations of reflection *hkl*. [‡] $R_{\text{ano}} = \sum_{hkl} |\langle I(hkl+) \rangle - \langle I(hkl-) \rangle| / \sum_{hkl} [\langle I(hkl+) \rangle + \langle I(hkl-) \rangle]$, where $\langle I(hkl+) \rangle$ and $\langle I(hkl-) \rangle$ correspond to the average intensities of each Friedel pair for reflection *hkl*.

inhibited the growth of *E. coli* cells (data not shown). A topological model of FliP derived from *Salmonella enterica* serovar Typhimurium (UniProt ID P54700) has been proposed (Ohnishi *et al.*, 1997). Sequence alignment between TmFliP and *Salmonella* FliP allowed us to identify residues 110–188 in TmFliP as TmFliP_P. *E. coli* BL21(DE3) cells were transformed with pKY045, which encodes His₆-TmFliP_P on pET-15b, and cell lysates were prepared from the resulting transformants. The soluble fraction containing His₆-TmFliP_P was loaded onto an Ni-NTA column and purified. Because His₆-TmFliP_P tended to form aggregates on rapid removal of imidazole during dialysis, imidazole was slowly removed by stepwise dialysis. After removal of the His₆ tag by thrombin, TmFliP_P was purified by Ni-affinity chromatography followed by size-exclusion chromatography. The elution profile from size-exclusion chromatography indicated that the purified TmFliP_P is in an oligomeric state (data not shown). To determine the oligomeric state, we carried out sedimentation-equilibrium analytical ultracentrifugation. A single-species model with a molecular weight of 39.7 kDa produced the best fit (Fig. 1). Because the deduced molecular weight of TmFliP_P is 9553.9 Da, this indicates that TmFliP_P forms a homotetramer in solution. However, some of the tetramers dissociated into dimers a few days later. To stabilize the tetramer, we varied the pH and the NaCl concentration and found that the tetramer is quite stable at a pH below 7.0. This is consistent with our observation that no crystals grew from a purified TmFliP_P sample stored at pH 8.0. As a result, TmFliP_P crystals were successfully grown from a solution consisting of 0.1 M phosphate-citrate pH 4.4, 36% MPD at 277 K (Fig. 2*a*). The TmFliP_P crystals diffracted to 2.4 Å resolution (Fig. 2*b*) and belonged to the hexagonal space group *P*6₂22 or *P*6₄22, with unit-cell parameters *a* = *b* = 114.9, *c* = 193.8 Å, $\alpha = \beta = 90.0^\circ$, $\gamma = 120.0^\circ$ (Table 1). Calculation of the Matthews coefficient (*V*_M; Matthews, 1968) suggests that the asymmetric unit contains between six and 11 molecules with solvent contents of between 62% (*V*_M = 3.24 Å³ Da^{−1}) and 31% (*V*_M = 1.77 Å³ Da^{−1}).

SeMet-TmFliP_P crystals were obtained under the same condition as the native TmFliP_P crystals. The shape and size of the SeMet-TmFliP_P crystals were almost the same as those of the native crystals. The SeMet-TmFliP_P crystals diffracted to 2.8 Å resolution. The unit-cell parameters of the derivative crystals (*a* = *b* = 115.3, *c* = 193.6 Å, $\alpha = \beta = 90.0^\circ$, $\gamma = 120.0^\circ$) were almost the same as those of the

native crystals (Table 1). Anomalous dispersion difference Patterson maps of the SeMet derivative showed significant peaks on the Harker section (Fig. 2*c*), suggesting the usefulness of these data for phasing by the SAD method.

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