The structure of the flagellar motor protein complex PomAB: Implications for the torque-generating conformation

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ABSTRACT

The bacterial flagellar motor is driven by an ion flux through a channel called MotAB in Escherichia coli or Salmonella and PomAB in Vibrio alginolyticus. PomAB is composed of two transmembrane (TM) components, PomA and PomB, and converts a sodium ion flux to rotation of the flagellum. Its homolog, MotAB, utilizes protons instead of sodium ions. PomB/MotB has a peptidoglycan (PG)-binding motif in the periplasmic domain, allowing it to function as the stator by being anchored to the PG layer. To generate torque, PomAB/MotAB is thought to undergo a conformational change triggered by the ion flux and to interact directly with FliG, a component of the rotor. Here, we present the first three-dimensional structure of this torque-generating stator unit analyzed by electron microscopy. The structure of PomAB revealed two arm domains, which contain the PG-binding site, connected to a large base made of the TM and cytoplasmic domains. The arms lean downward to the membrane surface, likely representing a “plugged” conformation, which would prevent ions leaking through the channel. We propose a model for how PomAB units placed around the flagellar basal body to function as torque generators.
There are many essential biological processes coupled to ion potentials across the membrane. These include ATP synthesis by the $F_{1}F_{0}$-ATPase and rotation of the bacterial flagellar motor. In *Escherichia coli* or *Salmonella*, an inner membrane complex composed of two components, MotA and MotB (9, 50, 8, 62), converts proton flux into rotation of the flagellum to power bacterial motility (2, 25, 52). The MotAB complex also functions as a stator by being anchored to the peptidoglycan (PG) layer of the cell through a binding motif in the periplasmic domain of MotB (10, 23). Other channel complexes, ExbBD and TolQR, have weak sequence similarity to MotAB, but utilize the proton motive force to power very different physiological functions (14, 24). ExbBD is involved in active transport of iron siderophores and vitamin B$_{12}$ through the outer membrane (24), whereas TolQR is known to maintain the integrity of the outer membrane (14). Both complexes need other subunits, TonB for ExbBD or TolAB and Pal for TolQR, to bridge the periplasmic space.

Some bacteria, such as alkalophilic *Bacillus* and *Vibrio* species, use an electrochemical potential of sodium ions to drive the flagellar motor (59, 39). In *Vibrio* species, which normally have only one polar flagellum, the four proteins, PomA, PomB, MotX, and MotY, are necessary to generate torque (59, 39). The maximum rotational speed of the sodium-driven motor in *V. alginolyticus* is ~1,700 Hz (37), whereas the maximum speed of the proton-driven motor in *E. coli* is ~300 Hz (7, 35). PomA and PomB are closely related to MotA and MotB, respectively, whereas paralogs of MotX and MotY (40, 41, 44) are not found in *E. coli*. Although the functions of MotX and MotY are not yet clear, it has been shown that they form a ring structure, called the T ring, that protrudes into the periplasmic space (Fig. 1), where it could interact with PomAB (51, 20). Another ring
structure, named the H ring, has recently been identified around the LP ring. The H ring is necessary for T ring formation (Fig. 1; 53).

PomA and B are thought to form a sodium channel complex. MotAB from *E. coli* can drive the polar flagellum of *V. cholerae* (15) and *V. alginolyticus* (1), using the proton motive force. Also, a complex of PomA and a chimera protein made of PomB and MotB segments can convert a proton-driven motor into a sodium-driven one (1). Hence, PomAB or MotAB determine the ion specificity of the motor, and the two types of motors most likely share a similar mechanism for ion-driven torque generation. Fig. 1 shows a schematic drawing that compares the proton-type motor of *E. coli* and *Salmonella* with the sodium-type motor of *Vibrio*.

Despite the importance of its function, structural information for the intact torque-generation unit is limited, although cross-linking experiments (3, 4) proposed an arrangement for the transmembrane (TM) helices. Recently, tomographic reconstructions of the spirochete flagella have reveled the motor structures *in situ* (30, 33, 43). However, these reconstructions, at 70 – 35 Å, resolution gave little information about the molecular boundary and shape of the torque-generating unit (30, 33, 43). Here, we report the first three-dimensional structure reconstructed from molecular images of isolated PomAB by electron microscopy (EM), single-particle analysis, and EM tomography.

**MATERIALS AND METHODS**
Samples preparation and electron microscopy

PomA/PomB-(His)_6 and PomA/PomBΔC-(His)_6 (56) were overproduced in E. coli BL21. The plasmids carrying these genes with a sequence for a hexahistidine tag fused to the C-terminus were kindly provided by Toshiharu Yakushi, Yamaguchi University. The genes were expressed under the control of the tac promoter. We purified the PomAB complex as described previously (58), with some modifications. Here, Cymal-5 (Anatrace, Maumee, OH) was used instead of CHAPS to extract proteins from the membrane fraction.

A few μl of ~0.03 mg/ml purified protein solution was applied onto a carbon-coated grid. It was washed with deionized water 5 times and negatively stained with 2% uranyl acetate. We examined the sample grids with an FEI Tecnai T20 electron microscope (FEI, Hillsboro, OR) with a LaB₆ gun operated at an accelerating voltage of 120 kV. Images were recorded on a 4k × 4k slow-scan CCD (SSCCD) camera (UltraScan 4000, GATAN, Pleasanton, CA) at a final magnification of ~110,000 × and at defocus settings of 5,700 ~ 21,000 Å. The magnification was calibrated from negatively stained catalase crystals. We collected tilt series of negatively stained wild-type PomAB complexes on the SSCCD at the same magnification and at a defocus level of ~30,000 Å by using the UCSF tomo software (61). A constant tilt increment of 3 or 4 or 5° was applied for each tilt series over a tilt range of ±60°. Tilt series were also acquired by SerialEM (38) with a JEOL-2100 (JEOL, Tokyo, Japan). We examined frozen-hydrated samples with a JEM-3200SFC electron microscope (JEOL, Tokyo, Japan) operated at 200 kV with a specimen temperature of ~50 K. Zero-energy loss images were recorded at defocus settings of 50,000 ~ 80,000 Å by selecting only electrons with an energy loss less than 10 eV.
Image analysis

For single-particle analysis of negatively stained particles, we first reduced image frames by a factor of 2. The phase reversal due to the contrast transfer function (CTF) was corrected by taking the astigmatism into account with a modified version of CTFCORRECT from the TOMOCTF package (11). The EMAN software suite (36) was used for the following single-particle analysis. We manually picked up individual molecular images with BOXER by applying a $9 \times 9$ median filter to enhance the visibility of the particles. After low-pass filtered, three-dimensional maps were constructed from those images by using STARTNRCLASSES and STARTANY with two-fold symmetry enforced. The structures were refined by REFINE with PC clusters. For the wild-type PomAB complex, the three-dimensional map obtained was used as a starting model for higher-resolution refinement, with no filter applied. The total number of molecular images included in the three-dimensional reconstruction was 5,428 for the wild type, after a few dozen of iterations starting from 7,018 images, and 2,254 for the deletion mutant (PomABΔC), starting from 2,838. The numbers of averaged molecules were doubled by two-fold averaging. The Fourier-shell correlation (FSC) was calculated between two volumes, each generated from half the data set. The resolution was taken to be the spatial frequency at which the FSC drops below 0.5 and was measured to be 21.3 Å for the wild type and 30.1 Å for the deletion mutant (Supplementary Fig. 1). A full CTF correction produced a map with the identical structural features, although the map appeared to be at lower resolution.
For electron tomography of negatively stained PomAB, each tilt series was aligned by using IMOD (31) after tilt angles were determined by using PRIISM (6). Individual particles in the tilt series were cut out with BSHOW from the Bsoft suite (17) and molecular tomograms were reconstructed by weighted back-projection in a similar way described by Iwasaki et al. (21). Then, the tomograms were aligned without Fourier terms inside the missing wedge, using a modified version of BFIND (17). A total of 19 molecular tomograms were averaged (Fig. 3B).

Map interpretation

We extracted the conserved region of the PG-binding domain from the atomic model of MotB-C from H. pylori (PDB accession code: 3CYP) (48). The model was manually docked into the density map with a graphics program O (22). To build a model in a conformation aligned to interact with the PG layer, we cut out the two arm domains with XDISPMSK (57) and manually adjusted by using CHIMERA (46). The rotation and translation matrix thus obtained was converted using a handmade program (CHIMERA2OMAT) and applied to the density maps of the arm domain by MRCRTR. These programs are available on request. A two-dimensional average of the hook-basal body (HBB) complex (54) was kindly provided by David J. DeRosier, Brandeis University, and was cylindrically averaged around the rotational axis of the flagellum to produce a three-dimensional volume. We placed the modeled PomAB complexes manually around the HBB volume. Molecular structures presented in Figs. 3 - 5 and Supplementary Fig. 1 were all prepared with CHIMERA (46).
RESULTS

Characterization of the PomAB complex

We used tac-based expression plasmids in *E. coli* to overexpress the PomAB complex. PomAB was well represented in the membrane fraction and did not significantly slow cell growth after induction by IPTG. Because previous samples in CHAPS detergent aggregated, based on observation by EM (58), we screened more than 20 different detergents to find one that minimized aggregation. Cymal-5 was found to solubilize the complex from the membrane without disassociation of PomA and PomB. A modified purification protocol using Cymal-5 reproducibly generated mono-dispersed particles.

Several studies suggest that the stoichiometry of the flagellar stator unit is \((\text{PomA})_4(\text{PomB})_2 / (\text{MotA})_4(\text{MotB})_2\) (3, 26, 49). The relative amount of PomA and PomB on a SDS-PAGE gel appeared to be consistent with. The molecular weight of \((\text{PomA})_4(\text{PomB})_2\) is \(\approx 180\) kDa. After gel filtration of the solubilized complex, a single peak was seen at \(200 \sim 250\) kDa as calibrated using soluble globular proteins as standards. This is less than half that observed with the complex solubilized by CHAPS (\(\sim 550\) kDa) (58) or sucrose monolaurate (\(\sim 900\) kDa) (55). Considering that detergent micelles are probably bound to the complex, the molecular mass calculated from gel filtration is consistent with the particles being complete PomAB complexes. The relatively small size of the complex makes it suitable for single-particle analysis of negatively stained images. Fig. 2A shows typical EM images of negatively stained particles. We also examined frozen-hydrated samples by cryo-electron microscopy (cryo-EM). Most of particles were absorbed onto carbon film on EM grids, but particles embedded in ice resemble negatively stained ones (cf. Fig. 2A and B).
Three-dimensional structure

We analyzed the three-dimensional structure of PomAB by electron tomography and single-particle analysis of negatively stained particles. We collected tilt series of molecules and carried out tomographic reconstructions (Supplementary Fig. 1). Individual molecular tomograms showed dimeric structures (Supplementary Fig. 1A). Hence, two-fold averaging was applied for single-particle analysis. We reconstructed an initial three-dimensional map from two-dimensional class averages by the common-line method implemented in the EMAN suite (36) and used it as the reference for structure refinement. Fig. 3 shows the three-dimensional structure calculated from ~ 5,500 images after iterative refinement. Representative raw images show characteristic shapes similar to corresponding class averages and reprojections of the three-dimensional map (Fig. 2C). The density map looks similar to the average of individual tomograms (cf. Supplementary Fig. 1B and Fig. 3A). The resemblance of these two independent reconstructions by tomography and single-particle analysis gives confidence in the reconstruction shown in Fig. 3. The resolution of the single-particle reconstruction was measured to be ~ 21 Å by the Fourier-shell correlation (Supplementary Fig. 2).

The structure is composed of two arm-like domains and an inverted pyramidal base domain (Fig. 3). The base domain is ~ 60 Å tall and could be divided into 4 subdomains (Fig. 3C). The arm domains contact each other in the middle of the arms and lean back toward the base domain (Fig. 3).

Identification of the peptidoglycan-binding domain
We then examined a mutant stator complex lacking the C-terminal 120 residues of PomB, which includes the PG-binding motif (PomABΔC) (56). This mutant complex was purified by the same procedure used for the wild-type complex, and similarly mono-dispersed particles were observed. We reconstructed the three-dimensional map from ~2,250 molecular images. Although the structure of the mutant complex retains the same base domain as the wild type, it lacks the arm domains (Fig. 3D), indicating that the arms contain the PG-binding domain.

Interpretation of the map

The PG-binding domain shares sequence similarity with other PG-associated proteins (16, 27, 29, 45, 48). X-ray crystallography and NMR spectroscopy revealed that the core structure of the PG domain is composed of 3 α-helices, a 4-stranded β-sheet and loops connecting these elements (16, 27, 29, 45, 48). We docked an atomic model of the PG-binding domain from the *Helicobacter pylori* MotB C-terminal structure (MotB-C) (48) into the arm domain of the EM density map (Fig. 4). The docked model consists of ~110 amino acids, which is nearly equal to the deleted region in the PomB mutant protein (56). The arm domain is the same size as the atomic model of the PG-binding domain, which fits well into the EM density except for slight mismatches in loop regions. *H. pylori* MotB-C forms a dimer in the crystal. Although the arm domains in the EM density make a dimer, the crystal structure requires shifting the two subunits to achieve the best fit to the EM density (Fig. 4).

The base domain should contain the TM region and the cytoplasmic domain. Each of the 4 subdomains in the TM region should include one PomA monomer (Fig. 3C),
indicating that the stoichiometry of PomA and PomB in the density map is consistent with the previous reports (3, 26, 49). Unfortunately, the resolution of the map is insufficient to interpret more structural details in the base domain. Bound detergent might impair the resolution of the base domain to some degree. There appears to be a cavity in the TM region. In general, it is difficult to analyze the TM region from negatively stained particles due to uneven staining, flattening, and other complicating factors. We cannot rule out that artifacts arising from negative staining and solubilization with detergent may affect the structure, although the cavity seen in the base domain is also visible in cryo images (Fig. 2B).

DISCUSSION

Here, we report the first three-dimensional structure for the flagellar torque-generation unit. The structure was determined by single-particle analysis and corroborated by electron tomography. The structure can be summarized as a pyramidal base with four visible domains topped with two extended arm-like structures.

The orientation of the arm domains, which contain the PG-binding domains of PomB, relative to the cytoplasmic membrane surface is incompatible with binding to the PG layer. Thus, the isolated stator complex is likely to be somewhat different from the stator when it is bound to PG. The periplasmic domain of MotB is thought to reach back toward the membrane surface and plug the channel before the stator complex assumes its correct position relative to the MS ring and C rings (Fig. 1) (19). The structure of PomAB described here is probably in this plugged-channel conformation.
When the stator is correctly placed around the rotor, Hosking et al. (19) proposed that the plug domain of MotB should stand upright, perpendicular to the membrane, to achieve the ion-conducting state. They found that forming a disulfide cross-link between short segments just after the MotB TM helix in E. coli locks the stator in an unplugged, proton-conducting form (19). We previously observed that PomAB reconstituted into liposomes exposed a 60 ~ 70 Å long domain that protruded from the liposome (58). To modify our structure to simulate the PG-bound state, we separated the arm domains from the base structure and rotated each of the arm domains so that the PG-binding site, which is located in a loop between α-helix 2 and β-strand 2 (48) (green residues in Fig. 5A), faces the PG layer (up in Fig. 5A). The positions of residues responsible for PG binding are well conserved relative to the periplasmic domain structure of Pal with a bound PG precursor (45). In our modified model, the arm domain extends ~ 60 Å from the cytoplasmic membrane surface. Our previous cryo-EM observations showed a much smaller number of images in this conformation than were expected from the concentration of PomAB in the dialysis button (58). They probably represent the unplugged-channel conformation or an intermediate conformation extending toward the PG layer (see below), which may appear stochastically even when PomAB is not assembled around the rotor.

Single-molecule fluorescence microscopy demonstrated a quick turnover of motor-associated MotAB with its membrane pool (32), indicating that the binding of MotAB/PomAB to the PG layer is not tight. Also, a PG-binding assay found no MotB-C dimer in the PG-associated fraction (28), suggesting that anchoring to the PG layer requires conformational changes in the MotB dimer. The disposition of the arm in our
model is at least consistent with the possibility of dynamic association and dissociation of PomAB/MotAB with the PG layer around the flagellar rotor (28, 32).

The inner surface of the PG layer is more than 100 Å from the outer surface of the cytoplasmic membrane (12, 54). The arms in our model (Figs. 5A and B) are obviously not long enough to reach the PG layer. In addition to the PG-binding domain, there are another ~160 amino acids in the periplasmic portion of PomB. The stalk and shoulder of the arm (arrows in Figs. 4A and B) probably correspond to those residues, and they might be able to stretch to the PG layer. If so, our model (Fig. 5A) and the complexes previously observed with liposomes (58) would represent an intermediate conformation between the plugged and the PG-bound states. In this scenario, most of the residues forming the stalk and shoulder would need to be extended to reach the PG layer.

However, deletion of 50–90 amino acids in the MotB periplasmic region following the TM segment did not fully eliminate motility (42), implying that it is unlikely that this entire region must stretch out to contact the PG layer.

A recent study used disulphide cross-linking to show that MotB comes into at least occasional direct contact with FlgI, which forms the P ring (18). The P ring and the L ring form a bushing that allows the flagellar rod to pass through the PG layer and the outer membrane, respectively (Fig. 1). The efficiency of MotB-FlgI cross-linking was affected by the protonophore CCCP (18), which may indicate that the structure of the periplasmic domain of MotB changes in response to the proton motive force. It has been speculated that drastic structural changes in the periplasmic region of MotB are required to reach the PG layer and also to activate the proton channel (29). The sodium motive force may
change the structure of the PomB periplasmic domain, and it has been observed that sodium ions are essential for assembly of the PomAB stator complex into the flagellar motor (13).

The other possibility is that the PG layer may be distorted around the flagellar motor in a way that makes contact with the PomAB/MotAB PG-binding domain possible. The formation of the P ring may push the PG layer aside and downward toward the cytoplasmic membrane and make the periplasmic space smaller (left side in Fig. 5B).

When the structures of the spirochete flagella were analyzed in situ (30, 33, 43), the density maps resolved a collar bridging the inner membrane and the PG layer. The mass of the collar was too large to contain only the stator complex, and most of the density may consist of unknown proteins (33) and/or PG. We do not know if a corresponding collar exists in the E. coli or Salmonella flagella because these bacteria are too thick for cryo-electron tomography in situ. This scenario, however, would match nicely with the idea that the plug region stands upright (right half in Fig. 5B). Indeed Vibrio species possess MotX and Y, which protrude just below the H and P rings (Fig. 1; 20, 51, 53) into the periplasmic space, where they are suggested to interact with the PomAB complex.

MotY has a PG-binding motif (27). We propose that MotY holds the PG layer close to PomB so that PomB is able to reach the PG. In E. coli or Salmonella, part of FlgI may project to the periplasmic space and interact with MotB (18).

The switch complex is made of three proteins, FliM, FliN and the C-terminal domain of FliG, and these three form the C ring (Fig. 1). The switch complex is required for torque generation and switching of direction of the motor rotation (34).
The flagellar hook-basal body (HBB) complex has previously been isolated from *Salmonella* and analyzed by cryo-EM (12, 54). A three-dimensional volume of the HBB was calculated by cylindrically averaging a two-dimensional image (54) around the flagellar axis of the HBB (see MATERIALS and METHODS). This reconstruction includes the C ring, but not the stator complex. Based on the HBB reconstruction and the crystal structures of the *Thermotoga maritima* FliG C-terminal and middle domains, Brown et al. (5) proposed a model in which important charged residues of FliG are aligned on the membrane-facing surface (the upper side in Fig. 5B) of the C ring so as to interact with the cytoplasmic loop of MotA (or PomA). We placed our modeled PomAB stator complexes around the MS ring of the HBB and above the C ring (Figs. 5B and C). The radius of the C ring at the membrane-facing side is ~200 Å, and is ~70 Å larger than that of the MS ring. The deck of the C ring has sufficient width to accommodate one PomAB unit (Fig. 5C). The maximum number of MotAB/PomAB stators around one flagellar motor in *E. coli* has been estimated to be at least 11 (47). The model shown in Figs. 5B and C can accommodate about this number of complexes around the MS ring, but little space remains for additional complexes unless some stator are tilted relative to the MS ring.
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Figure legends

Fig. 1. A schematic diagram of two types of flagellar motors. A hypothetical model for the proton-driven motor of *E. coli* or *Salmonella* (left) and the sodium-driven motor of *Vibrio* (right). The sodium-driven polar flagellum of *Vibrio* is sheathed. The energy source for flagellar motor rotation is provided by an electrochemical potential gradient across the inner membrane. The functional units of the stator are thought to be \((\text{MotA})_4(\text{MotB})_2\) and \((\text{PomA})_4(\text{PomB})_2\) (3, 26, 49). IM, inner membrane; PG, peptidoglycan layer; OM, outer membrane.

Fig. 2. EM images of PomAB. A. Images of negatively stained PomAB. B. Frozen-hydrated molecules. The particles show similar dimensions and features to those shown in A. C. Selected raw images of particles (upper row) aligned with their respective classes (middle row) in the same column, and corresponding reprojections (lower row) of the three-dimensional volume shown in Fig. 3. The bar in A corresponds to 100 Å.

Fig. 3. Three-dimensional structure of PomAB constructed from single-particle analysis. A. Viewed parallel to the membrane plane. Top and bottom correspond to the periplasmic and cytoplasmic sides of the membrane, respectively. B. As in A, but rotated by 90° around the vertical (two-fold) axis. C. Viewed from the periplasm. Subdomains are labeled 1 to 4. D. Three-dimensional structure of a PomAB mutant with the PG-binding domain deleted (PomABΔC; 56) shown in solid blue. Magenta nets correspond to the solid density of the wild-type PomAB in A – C. Arrows in A and C indicate the shoulder of one arm domain. A contour level of the solid densities in blue is \(\sim 1.8 \sigma\) in A – D and that of the nets in cyan are \(\sim 1.5 \sigma\) in A – C. The counter level of the
nets in A – C corresponds to ~ 100% volume recovery based on a molecular weight of ~ 180 kDa for the complex. The bar represents 25 Å.

**Fig. 4.** Docking of atomic models into the PomAB map shown in Figs. 3A - C. A. Viewed from the same direction as in Fig. 3A. B. Rotated by 90º as in Fig. 3B. C. Viewed as in Fig. 3C. Two atomic models of the core of the MotB-C PG-binding domain from *H. pylori* (PDB accession code: 3CYP) (48) are shown in red and blue. They were docked into the arm domains. Arrows in A and B indicate the stalk and shoulder of the arm (see text), and the horizontal bars in A refer to the TM region. The bar in C represents 25 Å.

**Fig. 5.** Models of PomAB anchored to the PG layer in position for torque generation. A. The two arm domains are modelled to face to the PG layer. For clarity, the counter level of the map is the same as for the solid density in Figs. 3 and 4. The atomic model of the MotB-C core region (48) is displayed for one of the arms. Residues involved in glycan binding (48) are displayed in green. B., C. Eleven of the PomAB models shown in A are placed around a cylindrical average of the hook-basal body (HBB) complex (54). Viewed parallel to the membrane in B and viewed from the periplasm in C. Part of the PG layer supported by MotXY in *Vibrio* protrudes into the periplasmic space so that the arm of PomAB can be anchored to it (left side in B). When the plug region of PomB/MotB stands upright from the membrane, the PG-binding domains can reach even a smaller protrusion from the PG layer over a gap indicated with horizontal lines (right
side). IM, inner membrane; PG, peptidoglycan layer; OM, outer membrane. The bar represents 100 Å.
Fig. 1
Fig. 2
Fig. 3