Conformational change in the periplasmic region of the flagellar stator coupled with the assembly around the rotor

Shiwei Zhu1,a,1, Masato Takao1,a,1, Na Li2,a,2, Mayuko Sakuma3, Yuuki Nishino3, Michio Homma3, Seiji Kojima2,a,3, and Katsumi Imada2,b,3

1Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-Ku, Nagoya 464-8602, Japan; and 2Department of Macromolecular Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

Edited by Steven M. Block, Stanford University, Stanford, CA, and approved August 1, 2014 (received for review December 30, 2013)

The torque of the bacterial flagellum is generated by the rotor-stator interaction coupled with the ion flow through the channel in the stator. Anchoring the stator unit to the peptidoglycan layer with proper orientation around the rotor is believed to be essential for smooth rotation of the flagellar motor. The stator unit of the sodium-driven flagellar motor of Vibrio composes of PomA and PomB, and is thought to be fixed to the peptidoglycan layer and the T-ring by the C-terminal periplasmic region of PomB. Here, we report the crystal structure of a C-terminal fragment of PomB (PomBz2) at 2.0-Å resolution, and the structure suggests a conformational change in the N-terminal region of PomBz2 for anchoring the stator. On the basis of the structure, we designed double-Cys replaced mutants of PomB for in vivo disulfide cross-linking experiments and examined their motility. The motility can be controlled reproducibly by reducing reagent. The results of these experiments suggest that the N-terminal disordered region (121–153) and following the N-terminal two-thirds of α1(154-164) in PomBz2 changes its conformation to form a functional stator around the rotor. The cross-linking did not affect the localization of the stator nor the ion conductivity, suggesting that the conformational change occurs in the final step of the stator assembly around the rotor.

ion-driven motor | peptidoglycan binding | Vibrio alginolyticus | X-ray crystallography

Motility is a fundamental function for bacterial survival in environmental change. The bacterial flagellum is a large filamentous macromolecular assembly for motility. A reversible motor, which is embedded at the base of each filament and powered by the electrochemical gradient of the coupling ion (H+ or Na+), rotates the filament like a screw to drive the cell toward a favorable environment (1–3).

The torque generation unit of the flagellar motor consists of a rotor and stators. A dozen stators surround the rotor and work together, although a single stator can generate torque. Stepwise drops and restorations of the rotational speed have been reported, suggesting that each stator is able to associate into or dissociate from the functioning motor (4–6). The fluorescent photo-bleaching study has revealed a rapid turnover of the stator in the rotating motor (7). The rotor is made up of the MS-ring and the C-ring. The C-ring is formed just beneath the MS-ring and is essential for torque generation and switching the direction of rotation.

The stator is a hetero-hexameric complex of two membrane proteins, A and B, with a stoichiometry of A2B2 (8, 9). The stator consists of MotA and MotB for the H+-driven motor of Escherichia coli and Salmonella enterica, and PomA and PomB for the Na+-driven motor of Vibrio and Shewanella spp. (10). The torque is generated by the interaction between rotor protein FIIG in C-ring and the stator A subunit coupled with the ion flow through the channel in the stator (1). The stator B subunit consists of a short cytoplasmic segment, a single transmembrane helix and a C-terminal periplasmic region (11) (Fig. L4 and Fig. SL4). The transmembrane helix has an aspartate residue essential for ion translocation across the cytoplasmic membrane, and forms an ion channel with the transmembrane region of the A subunits (12–14). The periplasmic region of the B subunit (Bc) shows a sequence similarity to OmpA-like proteins and contains a putative peptidoglycan binding motif (PGB motif), which is thought to anchor the stator to the peptidoglycan layer (PG layer) (15). Many nonmotile mutation sites were found within or contiguous to the PGB motif, suggesting that proper anchoring of the stator to the PG layer is important to the motor function (16, 17). Ion-conductivity of the stator is coupled to its installation in the motor. Overproduction of the stator does not arrest cell growth, indicating that the ion-conductivity of the stator is activated only when it is installed around the rotor (13). A short segment following the transmembrane region of the B subunit, called the plug, is thought to suppress the ion leakage until the stator is incorporated into the motor (18, 19). However, it is not clear how the activation of the ion channel is coupled to the stator incorporation.

A systematic deletion study of the periplasmic region of MotB demonstrated that a MotB mutant with the deletion of residues 51–100 still forms a stable active stator. Moreover, another MotB mutant with the deletion of residues 51–110 and 271–309 is also functional, although unstable (20), indicating that the essential periplasmic region for motor function is only the residues 111–270, named periplasmic region essential for motility (PEM).

**Significance**

Stator is the energy-converting membrane protein complex in the flagellar motor. Its ion-conducting activity is only activated when incorporated into the motor, but the mechanism for assembly-coupled activation remains a mystery. In this study, we solved the structure of a C-terminal fragment of the sodium-driven stator protein PomB (PomBz2), the region responsible for anchoring the stator unit, at 2.0-Å resolution. In vivo disulfide cross-linking studies of PomB double-Cys mutants and their motility assay suggested that the N-terminal region of PomBz2 changes its conformation, which is expected for MotB, the counterpart of PomB in the proton-driven Salmonella motor, in the final step of the stator assembly around the rotor.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3WPW and 3WPX).

*Present address: Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN 55455.

1To whom correspondence may be addressed. Email: kimada@chem.sci.osaka-u.ac.jp or z47616@bc.nagoya-u.ac.jp.

2Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-Ku, Nagoya 464-8602, Japan; and 3Department of Macromolecular Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan.
that covers the PEM has been solved (21). St-MotBC forms a homo-
dimer, the subunit of which is composed of a single OmpA-like
domain, which contains the putative PGB site, with N-terminal long
extension of MotBC is expected to anchor the stator
and motility assay, revealed novel insights into the molecular
mechanism of the stator assembly and activation of the sodium-
driven flagellar motor.

Results

Structure of PomBC. PomBC4 and PomBC5 were crystallized in the
same space group with almost the same cell dimensions (Table S2).
The two structures are basically identical, and the residues before 154 and after 305 are invisible in the density maps of both crystals. The structures of PomBC4 and PomBC5 were determined by 2.3-Å and 2.0-Å resolution, respectively. We therefore mainly describe the structure of PomBC5 in this section.

PomBC consists of a single OmpA-like domain composed of three α-helices (α3, α4, and α5) and five β-strands (β1, β2, β3, β4, and β5) with the N-terminal long and short α-helices (α1 and α2). Although the overall structure of PomBC shows remarkable similarity to that of St-MotBC, marked differences are found in the N-terminal helices (α1 and α2), the loop connecting β3 and α4 (the β3-α4 loop), a short helix between β4 and β5 (as), and the C-terminal region (Fig. 1 B and C). α1 of St-MotBC is projected from the core domain, whereas α1 of PomBC is 10 residues shorter than that of St-MotBC and interacts with the core
domain throughout its entire length. The orientation of α2 of PomBC differs from that of St-MotBC, and hence the angle formed by α1 and α2 is sharp in St-MotBC but obtuse in PomBC. It should be noted that α1 and α2 are not present in MotBC of Helicobacter
pylori (Hp-MotBC) (31). The conformation of the β3-α4 loop is quite different from St-MotBC. This loop is thought to be included in the putative PG-binding site (21), because the corre-
sponding loop of peptidoglycan-associated lipoprotein from Haemophilus influenza (Hi-Pal) interacts with the peptidoglycan
precursor (32). The loop conformations of the five independent structures of St-MotBC are different each other, suggesting that the loop is highly flexible (21). Thus, the conformational difference of the loop of PomBC does not reflect the intrinsic property of PomBC, but reflects the flexibility of this region. PomBC has a short helix between β4 and β5. This helix is not present in St-MotBC and Vibrio MotY, but Hp-MotBC and some other OmpA-like proteins, such as Hi-Pal (32) and Pal from E. coli (Ec-Pal; PDB ID code 1OAP), have it. The C-terminal residues following β5 are disordered in PomBC. This region is folded into an α-helix in Hp-MotBC and St-MotBC, but deletion of the helix of St-MotBC has no effect on Salmonella motility (20). Thus, the structural difference is thought to be irrelevant to the stator function.

PomBC is known to form a dimer in solution (19). The asymmetric unit of the crystal contains a PomBC dimer, whose subunits are related by a pseudo twofold symmetry. The subunit arrangement of the dimer resembles that of the St-MotBC dimer. The subunit interface is formed by α4 and β4. The interaction between the two β4 strands creates an intersubunit β-sheet, and the side chain interactions between the two α4 helices stabilize the dimer (Fig. S1 B). These structural features are similar to St-MotBC; however, the residues involved in the subunit
interaction are not conserved. Charged residues mainly contribute to the interaction in St-MotBC, but the hydrogen bonding network between hydrophilic neutral amino acid residues or between those and acidic or basic residues contribute to the interaction in PomBC (Fig. S1 B and C). Moreover, other parts of the mole-
cule, such as the β3-α4 loop and the α4-β4 loop, are also involved in the interaction. Thus, the dimeric structure of PomBC seems to be stable, but not that of St-MotBC.

Cross-Linking Between α1 and the Core Domain Inhibits the Motility. The structural similarity between PomBC and St-MotBC suggests that a conformational change of the N-terminal helical region of PomBC is required to anchor the stator. To detect the confor-
mational change, we adopted an in vivo disulfide cross-linking approach. On the basis of the PomBC structure, we selected

![Fig. 1. Structure of the C-terminal fragment of PomB. (A) Schematic representation of the full-length PomB, PomBΔL, and the PomB variants used for crystallization. PomBΔL is the smallest construct that forms a stable functional stator unit derived from a systematic deletion experiment (19). The PEM region is highlighted in blue. Asp-24, which is essential for ion translocation across the cytoplasmic membrane, is shown as a solid red circle. (B) Ribbon representation of PomBC5 dimer. One subunit is color coded from blue to red from the N to the C terminus, and the other is painted magenta. (C) Structure of Salmonella MotBC2 dimer (PDB ID code 2ZVY) (21). The two subunits are colored in cyan and magenta.](image)
seven pairs of residues that would be possible to form a disulfide bridge between α1 and the core domain when they were substituted to cysteine (Fig. 2A). To avoid interference of natural cysteine residues in PomB, we used a mutant PomB whose all three cysteine residues (Cys-8, Cys-10, and Cys-31) were replaced with alanine (Cys-less PomB) (33) for the cross-linking experiments. This mutation slightly affects the motor function, but not drastically, compared with the wild-type (Fig. 2B).

The Vibrio cells (ΔpomAB), transformed with plasmid pBAD33 encoding PomA and Cys-less PomB with the pair of cysteine substitution, were inoculated onto the soft-agar plates. The cells producing PomB(M157C-I186C), PomB(L160C-I186C), PomB(L164C-L217C), or PomB(168C-Q213C) did not show any motility for 5.5-h incubation (Fig. 2B). We observed a small and significant number of motile cells for PomB(L160C-I186C) and PomB(L168C-Q213C), respectively, but no motile cells for PomB(M157C-I186C) and PomB(164C-L217C) in liquid under optical microscope (Table S3). These results suggest that the four double-cysteine mutations influence the motor function, probably as a result of forming a disulfide bridge (Fig. 2A). The remaining three mutants, PomB(I164C-V179C), PomB(L168C-I177C), and PomB(L160C-V221C), show comparable motility ring size to wild-type Cys-less PomB (Fig. 2B), indicating that these mutations did not affect the cell motility. Single cysteine substitutions of the residues that are selected for the double-cysteine mutation experiments did not change the motility on the soft-agar plate, suggesting that the cysteine replacement itself does not affect the stator function (Fig. S2A). We also confirmed that all of the mutations did not change the expression level of the stator proteins in the host cell (Fig. S2B).

**Motility of the Double-Cysteine Mutants Depends on Reducing Reagent.** We found that the four double-cysteine mutants lost motility on soft-agar plate. If the loss of motility is because of the disulfide bridge formation between the cysteine pairs, the motility would be restored by reduction of the disulfide bond. Then we added DTT, which is frequently used to reduce the disulfide bonds in proteins, into the soft-agar plate and observed motility of the cells. Addition of 5 mM DTT did not change the motility of the motile mutants and the wild-type Cys-less PomB. However, the nonmotile mutants, PomB(M157C-I186C), PomB(I164C-L217C), PomB(L160C-I186C), and PomB(L168C-Q213C), showed motility rings almost the same size as the wild-type Cys-less PomB (Fig. 2C). Although the wild-type cells with DTT showed a smaller motility ring than that without DTT, the motile fraction of wild-type cells was independent of the presence of DTT (Fig. 2C and Fig. S3B). These results suggest that the loss of motility of the mutants is because of the disulfide bridge formation between α1 and the core domain, and breakage of the disulfide bond by the reducing reagent restores the motility.

DTT-dependent motility of the mutant cells is also detected in liquid. The cells were incubated in TMN buffer (50 mM Tris-HCl pH7.5, 5 mM glucose, 5 mM MgCl2, and 500 mM NaCl) with or without 1 mM DTT, and were observed under a dark-field optical microscope. The motile fractions of the cells producing PomB(M157C-I186C) or PomB(I164C-L217C) increased over time and reached to 50%, which is almost the same ratio as the wild-type Cys-less cells, after 40-min incubation with DTT (Fig. 2D). The swimming speed of the mutants recovered more than 80% of the wild-type after 30-min incubation (Fig. S3A). The DTT-dependent restoration of motility was also observed for PomB(M157C-I186C) or PomB(I164C-L217C) cells treated with 1 mg/mL kanamycin for 20 min, indicating that the motility recovery is not because of installation of newly synthesized stator proteins into the motor (Fig. S3C).

The recovered motility was lost again by removing DTT. The mutant cells after incubation in TMN buffer containing DTT for 30 min were harvested and moved into TMN buffer without DTT. The motile fraction was gradually reduced over time and the motility was completely lost after 60-min incubation. The motility was recovered by adding DTT again, so this DTT-dependent motility is highly reproducible (Fig. 2E). In contrast, the motile fraction of the wild-type Cys-less cells was not affected by the presence of DTT (Fig. 2E and Fig. S3F). These results support the finding that the disulfide bridge formation between α1 and the core domain of PomB inhibits the motility.

**Detection of Disulfide Bridge Formation in the Double-Cysteine Mutants.** To confirm the disulfide bridge formation in the PomB mutants, we detected free cysteine by modifying the thiol group with biotin maleimide. After incubation in buffer with 1 mM DTT or without DTT for 35 min at 30 °C, the cells were treated with biotin maleimide and lysed. Then PomB was isolated from the solubilized cell membrane by immunoprecipitation with protein A-conjugated beads and the anti-PomB antibodies. The amount of free cysteine in PomB was estimated by the level of the biotinylated PomB labeled with streptavidin-HRP. More free cysteine was detected when they were treated with DTT.
with DTT than without DTT for all of the double-cysine mutants except for PomB(L160C-V221C), which shows similar strong bands both with and without DTT (Fig. 3A). Thus, the disulfide bridge is surely formed in the mutants except for PomB (L160C-V221C), and is reduced by addition of DTT. PomB (M157C-I186C), PomB(I164C-V179C), PomB(I164C-L217C), and PomB(L168C-I177C) indicated no or a faint band without DTT, suggesting that the disulfide bridge is fully formed in these mutants. We further confirmed the disulfide bridge formation of these four mutants by biotin-maleimide labeling in an in vitro method (Fig. 3B). It should be noted that a disulfide bridge is formed not only in the nonmotile mutants but also in the motile mutants (Figs. 2B and 3B). In contrast, PomB(L160C-I186C) and PomB(L168C-Q213C) show clear bands under without DTT condition (Fig. 3A), indicating a certain amount of PomB was not cross-linked in those mutants. This result is consistent with the fact that motile cells were observed in liquid for these mutants (Table S3).

Cross-Linking Does Not Affect the Stator Assembly Around the Rotor.
The stator of Vibrio is known to assemble around the rotor in a sodium-dependent manner (30). To assess the effect of disulfide cross-linking on the stator assembly around the rotor, we fused a GFP tag to the N terminus of PomB(M157C-I186C), PomB(I164C-L217C), or wild-type Cys-less PomB, and observed the cells expressing each of the GFP-tagged PomB mutants by fluorescence microscopy. In the TMN medium containing 500 mM NaCl, fluorescent dots were localized to the cell poles in both of the two mutants and the wild-type Cys-less cells, suggesting that the disulfide bridge formation of the PomB mutants does not affect the assembly of the stator (Fig. 4A and C). Without sodium ions (TMK medium containing 500 mM KCl), however, the polar localization of stator is drastically decreased in all cells (Fig. 4B and C). These results indicate the dynamic assembly of the mutant stator is still dependent upon the sodium ion, having a same character as the wild-type (30). Thus, we exclude the possibility that the loss of motility of the cross-linked mutants is caused by inhibition of the stator assembly around the rotor. It should be noted that the localization of the wild-type Cys-less or double-Cys mutant stators was not observed in the motY deletion strain (Fig. S4A), indicating that the cross-linked stator associates with the T-ring.

**Fig. 3.** Detection of the intramolecular disulfide bridge in PomB by thiol modification with biotin maleimide. (A) In vivo labeling of PomB with free cysteine. PomB labeled with biotin maleimide was detected by streptavidin-HRP (Upper), and total amount of PomB in the samples was detected by anti-PomB antibody (Lower). (B) In vitro labeling to confirm in vivo labeling results. PomB labeled with biotin maleimide (Upper) and total amount of PomB in the samples (Lower) are shown. The bands corresponding to PomB are indicated by arrowheads.

**Fig. 4.** Sodium-dependent localization of the stators with the PomB mutations. (A) Representative fluorescent microscopic images of the GFP-fused stator complex in the presence of NaCl. The fluorescent foci at the cell pole indicate the assembly of the stators around the rotor. (B) Representative fluorescent microscopic images of the GFP-fused stator complex in the absence of NaCl. Magnification: A and B, 100×. (C) Polar localization of the stator was evaluated by counting the number of the cells with polar fluorescent foci in the observed field. Results in the presence or absence of NaCl were shown as open and closed bars, respectively. All experiments were repeated three times, and average values with SD are shown in C.

Cross-Linking Does Not Inhibit the Ion Flow. Because the cross-linking did not affect stator assembly, we suspected that the cross-linking inhibits the ion conductivity of the stator channel. PomB/MotB has a periplasmic short segment called a “plug” just at the C terminal to their single transmembrane region (Fig. 1A and Fig. S4A). We previously showed that overproduction of the plug-deleted PomA/PomB complex inhibits cell growth (19), and this effect was more severely observed in E. coli than in Vibrio cells (34). We also showed that growth inhibition was correlated with the massive influx of sodium ion through the overproduced mutant stators (34); thus, we can evaluate the ion-conducting activity of the stator by this simple assay. To analyze the cross-linking effect on the ion conductivity, we performed the growth assay of E. coli cells expressing PomA and PomB mutants. When the PomB that lacks the plug region (Δ41–60 of PomB) was overexpressed with PomA, cells showed strong growth inhibition, regardless of the presence of additional M157C-I186C mutation (Fig. S4B). This finding suggests that the cross-link at position 157 and 186 of PomB did not disturb the ion-conductivity of the stator.

**Discussion**

The stator of the sodium-driven polar flagellar motor assembles around the rotor with proper orientation through the interaction between PomBC and the T-ring (25, 28, 29). Thus, PomBC is expected to have a specific structure for the interaction with the T-ring. The overall structure of PomBC, however, is similar to that of St-MotBC, and no such extra structure was found. The electrostatic surface potential indicates that the N-terminal helical region of PomBC is negatively charged, whereas St-MotBC has no special charge distribution on its corresponding region (Fig. S5A and B). We therefore substituted the negatively charged residues of α1...
and α2 to positively charged residues (E156K, E158K, E162K, E169K, and E173K) (Fig. S5C). These substitutions, however, did not affect motility of the mutant cells, even when all of the five residues were replaced at a time (Fig. S5D). So, where is the interaction site for the T-ring? The most plausible candidate is the N-terminal disordered region of PomB3, which is included in the PEM of PomB (19). A recent deletion study of PomB revealed that the PEM of PomB is about 30 residues (121–153) longer in the N-terminal side than that of MotB (19). These residues show no sequence similarity with MotB (Fig. S1A) and are indispensable for Vibrio motility. Thus, it is possible that the N-terminal disordered region is responsible for interaction with the T-ring.

When the stator assembly is completed, the Vibrio stator should interact with both the T-ring and the PG layer. Recent single-particle analysis of the purified PomA/PomB complex revealed that the isolated stator showed a compact conformation (35). We fitted the atomic model of PomB3 into the cryo-electron microscopic density of the PomA/B complex and compared it with the electron micrographic image of the Vibrio basal body (Fig. 5A and B). We found that the stator in this conformation was unlikely to reach the T-ring and the PG layer; therefore, a large conformational change is expected for the periplasmic region of PomB to fix the stator. In our previous study, we solved the structure of St-MotBC, which fully covers the T-ring, and suggested that a large conformational change of the N-terminal region of the PEM is required for anchoring the stator to the PG layer and stator activation (21). The crystal structure of PomB2 is similar to that of St-MotBC, suggesting that the Vibrio PomA/B stator shares the common activation mechanism to the Salmonella MotA/B stator. This fact led us to test the conformational change by intramolecular cross-linking between α1 and the PGB core of PomB. Among seven pairs of double-Cys replacements in PomB6 (one in the α1 helix and the other in the core domain), four pairs (M157C-I186C, L160C-I186C, L164C-I217C, and I168C-Q213C) lost motility on a soft-agar plate (Fig. 2B), and the motility was restored by adding DTT (Fig. 2C). Such a reversible inhibition of motility supports our conformational-change model. However, assay of free cysteine residues revealed that the motile mutants (I164C-V179C and L168C-I177C) also form a disulfide bridge (Fig. 3). This result seems to be inconsistent with the model; thus, careful interpretation is needed for the mutation experiments.

PomB(M157C-I186C) and PomB(I164C-L217C) mutant cells completely lost motility and the disulfide bridge was almost perfectly formed. In contrast, the PomB(I164C-V179C) and the PomB(L168C-I177C) mutant cells showed normal motility, although the disulfide bridge was also almost completely formed. The PomB(I160C-I186C) mutant did not show motility on a soft-agar plate, but a small number of cells were motile in liquid. The disulfide formation assay revealed that a certain amount of PomB(L160C-I186C) were not cross-linked. Thus, only the no-cross-linked stators are engaged in torque generation, and so such cells were thought to be motile. The PomB(L160C-V221C) mutant cells show normal swimming behavior. This finding is consistent with the fact that no cross-link was observed for this mutant. The PomB(L168C-Q213C) mutant did not show motility on a soft-agar plate, but the percentage of the swimming cells in liquid medium was comparable to the control cells (wild-type Cys-less). This type of behavior is often seen in chemotactic mutants or flagellar switching mutants. The motility on a soft-agar plate was fully recovered by addition of DTT. Thus, the disulfide bridge formation between 168 and 213 probably caused a defect not in torque generation but in the rotational switching of the motor. Taking these results together, we find that intramolecular cross-link at 157 or 160 inhibits the motility, but at 168 does not. This finding suggests a possibility that the N-terminal two-thirds of α1 changes its conformation to activate the motor but the C-terminal one-third of α1 retains the helix structure. Cross-link at 164 gave contradictory results on motility depending on its cross-link partner. The PomB(I164C-V179C) mutant is motile, whereas the PomB(I164C-L217C) mutant is not. Leu-217 is in α3, which interacts with the N-terminal region of α1. The disulfide formation stabilizes the interaction between α1 and α3, resulting in hindering the conformational change of the N-terminal region of α1. In contrast, Val-179 is in β1, which has interaction to the C-terminal region of α1 but no interaction to the N-terminal region. Thus, disulfide formation does not affect the N-terminal conformational change of α1.

As discussed above, our cross-linking experiments of PomB suggest that the N-terminal two-thirds of α1 (154–164) may change its conformation. However, if all of the residues in this region (11 residues) adopt a fully extended conformation, it becomes longer by only about 22 Å, which is insufficient to reach the PG layer if the distance between the inner membrane and the PG layer of Vibrio is same as that of Salmonella (about 100 Å). The PEM of PomB begins from Asp-121 and the N-terminal region of the PEM (121–153) is disordered before incorporation into the motor (Left). During the assembly into the motor, the disordered region changes its conformation to interact with the T-ring (Center), and then the N-terminal two-thirds of α1 changes its conformation to an extended form to anchor the PG layer (Right).

**Figure 5.** A model of a conformational change in PomB coupled with the stator assembly around the rotor. (A) The atomic model of PomB3 is fitted into the cryo-electron microscopic density of the PomA/B complex. (B) The PomA/PomB complex is placed on the electron micrographic image of the basal body of V. alginolyticus. Possible locations of inner membrane (IM), outer membrane (OM), and the peptidoglycan layer (PG) are indicated. (Scale bar, 50 Å.) (C) A plausible model for the sequential conformational changes of PomB. The N-terminal PEM (121–153) is disordered before incorporation into the motor (Left). During the assembly into the motor, the disordered region changes its conformation to interact with the T-ring (Center), and then the N-terminal two-thirds of α1 changes its conformation to an extended form to anchor the PG layer (Right).
cross-link interferes with the second conformational change, which may be required for interaction with the PG layer or transition to the active conformation to generate torque.

It should be noted that an alternative explanation without conformational change in the N-terminal helix α1 is also possible, because the disulfides that eliminate function might do so by locking the stator into a conformation that is nonfunctional for reasons unrelated to conformational change, installation, or ion conduction. A specific example might be a structural distortion that alters the orientation of the stator in relation to the rotor. Additionally, we do not have detailed information of the distance between the inner membrane and the PG layer in the _Viribrio algihnuticus_ cell. Although the distance from the M-ring to the L-ring is almost the same in the _Viribrio_ and _Salmonella_ basal bodies, it may be possible that the distance is shorter than that observed in the _E. coli_ and _Salmonella_. If so, a large conformational change may not be needed to reach the PG layer. In any case, the cross-link would lock the stator into a particular conformation that still allows installation but is not compatible with function.

On the basis of this study and together with our previous results, we propose a model for activation mechanism of the _Viribrio_ sodium-driven motor (Fig. S6). The stator diffusing in the cell membrane is in an inactive state. When the stator reaches around the outer ring, a protein interacts with the T-ring. This interaction triggers opening of a “plug,” allowing sodium ion to translocate into the channel of the stator. The sodium flow may induce the binding of PomB to the T-ring. This step probably includes a conformational change of the disordered N-terminal region of the PEm. After that, the N-terminal two-thirds of α1 changes its conformation to an extended form to anchor to the PG layer. Localization of the stator is sodium-dependent and no stator localization was observed in the PomB D24N mutant (30), suggesting that the stator localization requires sodium binding to Asp-24, which is in the transmembrane helix of PomB. Thus, the plug opening should occur in the early stage of the stator assembly process. The binding of PomB to the T-ring may follow or coincide with this event. Without binding to the T-ring, the stator is not stably anchored and easy to disassemble from the motor. The conformational change of α1 is the final step of the stator assembly, because the stator of the PomB (M157C-I186C) mutant can localize around the rotor and shows ion-conductivity. With this conformational change, the stator is firmly fixed to the PG layer, with proper orientation allowing the rotation of the motor. Direct observation of the conformational changes might be the next issue to address to answer the detailed mechanism of stator assembly around the rotor.

Materials and Methods

Bacterial strains and plasmids used in this study are listed in Table S1. PomBα4 and PomBα5 were expressed and purified as described previously (19) with slight modification. Diffraction data were collected at beamlines BL32XU and BL41XU in Spring-8 (Hiyama, Japan). The statistics of the diffraction data and refinements are summarized in Table S2. Localization of the stator was observed as described previously (30). Cell growth was monitored as described previously with slight modification (34). Full methods are provided in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank the beamline staff of Spring-8 for technical assistance with data collection; S. Tatematsu for technical assistance in protein purification; and K. Yonekura for providing the cryo-electron microscopy map of the PomB/A complex. S.Z. was partly supported by Integrative Graduate Education and Research program of Nagoya University. This work was supported by Japan Society for the Promotion of Science KAKENHI Grants 23247024, 24117004 (to M.H.), 24657087 (to S.K.), and 18074006 and 23115008 (to K.I.).