Structure of Flagellar Motor Proteins in Complex Allows for Insights into Motor Structure and Switching

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Background: The flagellar motor generates bidirectional rotation using the proteins FliG, FliM, and CheY and membrane-bound stator complexes.

Results: The structures of portions of FliG and FliM in complex allow for insights into switching and structure.

Conclusion: FliGMC and FliMIN in a 1:1 complex may form the C-ring of the motor.

Significance: Understanding motor structure is key to understanding its mechanism.

The flagellar motor is a rotary nano-turbine that drives motile bacteria. The cytoplasmic ring (C-ring) of the motor interacts with the stator to generate torque in clockwise and counterclockwise directions. The C-ring is composed of three proteins, FliM, FliN, and FliG. Together they form the “switch complex” and regulate switching and torque generation. Here we report the crystal structure of the middle domain of FliM in complex with the middle and C-terminal domains of FliG that shows the interaction surface and orientations of the proteins. In the complex, FliG assumes a compact conformation in which the middle and C-terminal domains (FliGMC) collapse and stack together similarly to the recently published structure of a mutant of FliG from Thermotoga maritima (see Fig. 1A). FliGMC assumes a compact conformation similar to the recently published PDB 3SOH structure from the Namba laboratory (9). Our structure also contains an apparent intramolecular ARM stack as well as the same interface between FliG and FliM as seen in the 3SOH structure. Our structure was able to be modeled into the three-dimensional cryo-tomography reconstructions of the motor provided by DeRosier laboratory, allowing for a proposal of the three-dimensional arrangement of the motor structure to be formed.

EXPERIMENTAL PROCEDURES

FliG (residues 115–327) and FliM (residues 46–230) were cloned into the pJY5 vector. Proteins were expressed using BL-21 Rosetta cells. The cell pellets were suspended in 25 mM Tris, pH 7.5, 400 mM NaCl, 10 mM imidazole, 1 mM EDTA and lysed with a French pressure cell. The lysate was heat shocked at 80 °C for 15 min followed by centrifugation at 13 rpm in a Beck-
RESULTS AND DISCUSSION

The FliG_{MC}-FliM complex (Fig. 1A) was generated by mixing truncated forms of FliG (residues 115–327) and FliM (residues 46–230) in an equimolar ratio. Optimized crystals displayed diffraction with Bragg spacing of 1.9 Å. The structure was solved by molecular replacement using the previously solved structures of FliG (PDB 1LKV) and FliM (PDB 2HP7) as search models (7, 14). The complex was refined to a resolution of 1.9 Å with an R_{free} of 24.7% and an R_{work} of 21.7%. Complete refinement statistics are shown in Table 1. The overall architecture of the complex is cylindrical with a vertical height of \( \sim 100 \) Å and a diameter of \( \sim 25 \) Å, with a contact surface area between FliM and FliG of 703 Å² (15, 16). Four of the C-terminal residues of FliM and seven residues of a loop in FliG were not observed in the electron density and were presumed disordered. The unstructured region of FliG does not contain conserved residues, suggesting that this is a variable region among species. Furthermore, the missing residues precede the conserved Gly-Gly hinge located between the domains of FliG and may be unstructured due to the inherent flexibility in this region of the protein (17).

Upon binding to FliG_{MC}, FliM does not undergo any significant conformational changes, with an r.m.s. deviation of 0.6 Å as compared with the FliM 2.0 Å resolution structure (PDB 2HP7) or the 3.5 Å resolution structure of FliM bound to FliG both previously solved by the Blair and Crane laboratories (7, 18). FliM consists of three β-strands (β1–β3) and three α-helices (α1–α3) that form a pseudo-symmetric α/β/α three-layered sandwich. The highly conserved GGXG sequence motif, located in the loop between helices α3–α1, is involved in FliG binding and confirmed by both the 3SOH and this structure (6, 19). In the 2HP7 structure, the two glycine residues of the GGXG motif are resolved, but the following four residues (PGEN) are disordered (7). Upon binding to FliG_{MC}, the loop in FliM surrounding the GGXG motif becomes ordered and stabilized and is observable in both the 3SOH and this structure. The FliG_{MC}/FliM interaction surface is positioned directly above the secondary binding site of CheY located on FliM that was identified by NMR experiments (20). When the response regulator CheY is phosphorylated (CheY-P), it is able to bind to FliM to signal the switch in rotation direction. The proximity of the CheY-P binding site to the FliG_{MC}/FliM interface could explain how the switch signal is propagated from FliM through FliG toward the MotAB complex.

FliG_{MC} in complex with FliM is predominantly α-helical consisting of 14 helices arranged in an extended structure (Fig. 1A). A right-handed super-helix is generated from the stacking

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**TABLE 1**

| Crystal 1 |
|---|
| **Data collection** |
| Space group | C21 |
| Cell dimensions (Å) | \( a, b, c \) |
| \( a, b, c \) | 125.96, 45.72, 98.84 |
| Resolution (Å) | 50–1.93 (2.0–1.93) |
| \( R_{work} \) or \( R_{free} \) | 0.076 (0.44) |
| \( I/\sigma(I) \) | 17.3 (1.65) |
| Completeness (%) | 99.5 (99.8) |
| Redundancy | 5.3 (4.6) |

*Highest resolution shell is shown in parentheses.*

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**FIGURE 1. The FliG_{MC}-FliM complex.** A, the FliG_{MC}-FliM complex. B, expanded view of the ARM stacking region. A green dashed line is shown for the residues that could not be modeled in the structure. C, expanded view of the FliG_{MC} and FliM binding interface. Hydrogen bonding residues and resulting hydrogen bonds are shown in magenta. The black residues are the buried hydrophobic residues at the binding interface. D, the FliG_{MC} structure sequence. Helices are underlined, and the disorder residues are marked with a strikethrough.
of pseudo-armadillo repeats from helices 1–4 (ARM$_{M}$) and helices 6–8 (ARM$_{C}$) (Fig. 1B), mimicking the stacking observed in eukaryotic ARM motifs (21, 22). The ARMM/ARM$_{C}$ stacking results in the burial of 14 hydrophobic residues forming a contact area of 745 Å$^2$ (15, 16). Helix$_{MC}$ lies below ARM$_{M}$ and interacts with helices 1–2 of FliG$_{MC}$ as well as parts of FliM$_{M}$. The FliM$_{M}$ loop between helices α1′ and α3 and residues within the helices themselves interact with ARM$_{M}$ and Helix$_{MC}$ of FliG, forming a network of bonding interactions. Fig. 1C shows the packing of hydrophobic residues and hydrogen bonding between the two proteins. The C-terminal domain (Helices$_{C1–6}$) is located above the intramolecular ARM stack, the ARM domain structures do not change significantly whether the domains come from the same molecule or from adjacent molecules. The crystal packing of FliG structures 1LK$\nu$ and 3HJ$L$ show ARM stacking between ARMM and ARM$_{C}$ of symmetry-related copies of FliG (Fig. 3, A and D) (14, 23). This results in an ARM$_{C}$-ARM$_{M}$-1 Stack in the co-crystal, the presence of FliM apparently drove the ARM domains to stack within a single FliG$_{MC}$ protomer (Fig. 3A). The intramolecular ARM stacking is also assumed in the 3AJC structure (Fig. 3B), although the residues connecting the domains are not observed (9). We propose that ARM stacking is driven by the requirement to bury the hydrophobic residues that comprise the ARM domains, and the intramolecular stack is driven by the binding to FliM as seen in the complex. Determining whether the ARM stacking is intra- or intermolecular in the C-ring requires further experimentation, but in the co-crystal, the presence of FliM apparently drove the ARM domains to stack within a single FliG$_{MC}$ monomer, suggesting that the in vivo interaction could be intramolecular.

The intramolecular ARM stacking we observed in the crystal may represent a preassembly conformation of FliG. In complex with FliM, FliG in the intramolecular stacking configuration may be a precursor building block of the C-ring, and as they are assembled into the motor, FliG may undergo a transformation to the intermolecular stacking configuration. The in vivo stacking may be represented by either the stacking we observed in the co-complex structure or the intermolecular stacking observed in the other FliG structures. If the intermolecular stacking proposal were correct, the rearrangement from cytoplasmic intramolecular stacking to the motor assembled intermolecular stacking would require the 745 Å$^2$ of contact surface between the ARM domains to be broken and reassembled during the transition. This transition would have to overcome the energetics of exposing the 14 buried hydrophobic residues to reassemble the ARM stacks in the motor in the exact way they were previously, but now in the intermolecular stacked arrangement. Further experimentation on intact isolated motors will be necessary to unambiguously determine the arrangement of FliG inside the C-ring.

FliG and FliM have been extensively studied by mutational analysis and biophysical techniques to determine the interac-
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FliG$_{M}$ contains a conserved EHPQ motif that when mutated disrupts the binding to FliM$_{M}$. These same residues were shown to be in contact in the FliG$_{M}$-FliM$_{M}$ co-crystal (PDB 3SOH) and in the complex presented here. The interaction surface between FliM$_{M}$ and FliG$_{M}$ has also been analyzed by NMR and chemical cross-linking. In NMR experiments, the resonances assigned to the EHP(Q/R) motif broaden when labeled FliG$_{M}$ is titrated with FliM$_{M}$, suggesting that the surfaces seen in the crystal interact in solution as well (20). Cross-linking experiments were designed from the 3SOH structure and showed that FliG$_{M}$ is able to cross-link to FliM$_{M}$ in vivo, confirming that these faces are in contact in the assembled motor.

Blair and co-workers (7, 18) have established that mutations in the conserved hydrophobic patch in ARM$_{C}$ also affect binding to FliM and have performed chemical cross-linking of intact cells to determine whether FliG$_{C}$ is in contact with FliM$_{M}$ in vivo (6, 19). Their cross-linking results show that the hydrophobic patch of FliG$_{C}$ binds to the GGXG surface on FliM$_{M}$ as FliG$_{M}$. In previous work (20), we used NMR to monitor the interactions of FliG$_{C}$ and FliM$_{M}$. This showed chemical shifts in the resonances corresponding to the Helices$_{C1-6}$ region of FliG$_{C}$ and not in the ARM$_{C}$ region, suggesting that the cross-linking results may not be present in solution. Composition gradient multilangle light scattering was also performed to measure the affinity of FliG$_{C}$ and FliM$_{M}$, resulting in a $K_{d}$ of 580 $\mu$M. This low affinity suggests that the interaction would probably not survive EM extraction. A model for the C-ring presented by Blair and co-workers (7, 18) imagines that a portion of the FliG molecules in the C-ring interacts with FliM$_{M}$ via the FliGM domain only. DeRosier and co-workers (27) suggest that this unusual arrangement allows for matching the 26–34-fold stoichiometry difference inferred from averaging of the EM reconstructions of the flagellar motor. The yields of the cross-links they observe between these domains are relatively low, suggesting that these interactions may reflect the behavior of a subpopulation of both FliM and FliG.

The FliG$_{MC}$-FliM$_{M}$ complex was modeled into the high-resolution maps of the motor derived from three-dimensional electron micrograph reconstructions of a clockwise locked motor from Salmonella typhimurium (27, 28). The C-ring from the three-dimensional reconstructions consists of two parts, an inner lobe with 26-fold symmetry and an outer lobe with 34-fold symmetry. The inner lobe of the C-ring disappears in three-dimensional reconstructions of a motor deleted for FliG$_{N}$ (29). This suggests that FliG$_{N}$ is only present in the inner lobe and that the outer lobe contains FliG$_{MC}$-FliM$_{M}$ and FliN that is mirrored by our construction of the C-ring.

The outer lobe is divided into three parts consisting of an upper, middle, and lower section. The upper section faces the membrane and is bridged to the middle section by a thin strip of density. Below the middle section is a ring that is believed to contain the Flit tetramer and the C terminus of FliM. The FliG$_{MC}$-FliM$_{M}$ complex structure was docked into the density by the program VEDA with a correlation coefficient of 66.6 and an R-factor of 58.0. The alignment positioned the ridge of charged residues in FliG$_{C}$ toward the membrane in position to interact with the MotAB membrane channel (Fig. 4A). FliG$_{M}$ aligns nicely into density between the upper and middle sections, interacting with both FliM$_{M}$ and FliG. The orientation of FliM$_{M}$ in the middle lobe places the FliM$_{M}$-FliM$_{M}$ subunit interfaces in close proximity, as identified previously by disulfide cross-linking experiments (7). Once we were satisfied with the position of our complex, the FliN tetramer was docked into the lower section of density. An expanded view of the docking is shown in Fig. 4C. The orientation of FliN to FliM was estimated from cross-linking experimental data and then refined by the VEDA fitting macro. Our x-ray structure and EM model fitting places the Flit$_{C}$ domain, FliM, and FliN subunits similarly to the model proposed by Lee et al. (15) without invoking the crystal packing ARM$_{C}$-ARM$_{M}$,1 notion they proposed. Furthermore, we observed a partial melting of Helix$_{MC}$, suggesting that Helix$_{NM}$ might be destabilized as well in the complex. This has major implications with regard to some of the rigid body movements proposed for these proteins during the flagellar motions (9, 18, 23).
The symmetry of the C-ring observed in the three-dimen-
sional reconstructions shows a shift from 34-fold to 26-fold
when going from the outer to inner rings, respectively (Fig. 4B).
The modeling of the C-ring presented here shows FlIG and
FlIM in a 1:1 stoichiometry, which implies that the mismatch in
the symmetry is managed in the connection from the outer
to inner rings. We postulate that only 26 out of the 34 of the FlI\_N
domains are able to bind to the 26 FlIF copies located in the
inner ring. The eight unbound FlI\_N domains would most
likely be located unbound inside the inner space of the C-ring
and would not participate in torque transfer from the outer
to inner rings. This would allow for the motor to reconcile the
symmetry mismatch in the simplest method possible. The sym-
metry mismatch may be a dynamic part of motor assembly.
The three-dimensional reconstructions showed that the C-ring var-
ies between 31-, 33-, and 34-fold symmetries, and the free FlI\_N
domains may explain the ability of the C-ring to assume differ-
cent copy numbers by displacing FlIG-FlIM complexes when
necessary.

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