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Assembly States of FliM and FliG within the Flagellar Switch Complex

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Abstract

At the base of the bacterial flagella a cytoplasmic rotor (the C-ring) generates torque and reverses rotation sense in response to stimuli. The bulk of the C-ring forms from many copies of the proteins FliG, FliM, and FliN, which together constitute the switch complex. To help resolve outstanding issues regarding C-ring architecture, interactions between FliM and FliG from *Thermotoga maritima* have been investigated with x-ray crystallography and pulsed dipolar electron spin resonance spectroscopy (PDS). A new crystal structure of an 11-unit FliG:FliM complex produces a large arc with a curvature consistent with the dimensions of the C-ring. Previously determined structures along with this new structure provided a basis to test switch complex assembly models. PDS combined with mutational studies and targeted cross-linking reveal that FliM and FliG interact through their middle domains to form both parallel and antiparallel arrangements in solution. Residue substitutions at predicted interfaces disrupt higher-order complexes that are primarily mediated by contacts between the C-terminal domain of FliG and the middle domain of a neighboring FliG molecule. Spin separations among multi-labeled component proteins fit to a self-consistent model that agrees well with electron microscopy images of the C-ring. An activated form of the response regulator CheY destabilizes the parallel arrangement of FliM molecules to perturb FliG alignment in a process that may reflect the onset of rotation switching. This data suggest a model of C-ring assembly in which intermolecular contacts among FliG domains provide a template for FliM assembly and cooperative transitions.

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Keywords

chemotaxis; signal transduction; flagella motor; x-ray crystallography; disulphide cross-linking; protein-protein interactions; cooperativity

Introduction

Many types of bacteria control their movement by switching the sense of flagellar rotation between clockwise (CW) and counter clockwise (CCW). Great strides have been made in understanding how the flagella motor functions, yet detailed information on how the molecular components assemble is not complete.^{1; 2; 3} The bacterial flagellum consists of an export apparatus, a reversible rotary motor, a universal joint, and filament (Fig. 1A).^{1; 2; 3} The core rotor structure at the flagella base (the switch complex) is composed of many copies of three conserved multi-domain proteins: FliG, FliM, and FliN (Fig. 1).^{4; 5; 6} The switch complex interacts directly with the transmembrane proton channels and is essential for torque generation, rotational switching and flagellar assembly. FliG (which has three domains, Fig. 1B) lies closest to the membrane and functions directly in torque generation. FliM (also composed of three domains, Fig. 1B) sits in the center of the rotor and interacts with the signaling protein phosphorylated CheY (CheY-P). FliN resides at the cytoplasmic end of the rotor and is essential for flagellar export, assembly and possibly switching (Fig. 1).

Rotation of the flagella involves the movement of the rotor with respect to the stator. The membrane-embedded stator, embedded in the membrane, is an oligomer composed of four MotA and two MotB subunits, which together act as proton channels and actuators for the rotor.^{7; 8; 9; 10; 11} The FliG C-terminal domain (FliG_C) contains conserved charged residues situated on an α -helix that interacts with MotA.^{11; 12; 13} FliG has two other conserved patches of residues for binding FliM: an EHPQR motif in the middle domain (FliG_M) and a conserved hydrophobic patch along the C-terminal domain.¹⁴ A Gly-Gly linker joining FliG_M to FliG_C confers flexibility to the molecule that is important for rotation and switching.^{15; 16}

The FliM amino terminal domain (FliM_N) binds to CheY-P¹⁷, the response regulator of intracellular chemotaxis signaling.^{18; 19; 20; 21} In *E. coli*, CheY-P binds to the rotor to change its rotation sense from CCW to CW. CCW rotation causes cells to swim smoothly, whereas CW rotation causes cells to tumble and reorient.^{1; 22; 23} Binding of CheY-P to FliM (and possibly FliN) promotes a conformational change in FliG that rearranges the FliG-MotA interface.^{19; 21; 24; 25; 26; 27} FliM sits directly below FliG in the C-ring and interacts with FliG through the conserved GGXG motif in the middle domain (FliM_M).^{20; 28; 29; 30; 31} The FliM C-terminal domain (FliM_C) interacts with FliN and together they form the lower part of the C-ring.^{32; 33} Some bacteria such as *Thermotogae* and *Bacilli*, contain FliY, which is FliN fused to an additional CheY-P phosphatase domain.^{34; 35; 36; 37; 38; 39} The FliG N-terminal domain (FliG_N) interacts with FliF in the smaller, membrane-situated MS-ring (membrane and supramembranous ring).⁴⁰ Fusions of

FliF with truncated versions of FliG produce assembled rotors with altered EM density at the top of the C-ring.⁴¹

Structures of major portions of the switch complex proteins together with biochemical assays has led to several models for C-ring assembly.^{12; 15; 16; 19; 28; 29; 30; 32; 42; 43; 44; 45} Electron microscopy (EM) reconstructions of the intact flagellar rotor from *Salmonella typhimurium*, and *Borrelia burgdorferi* provide an overview of the rotor architecture.^{41; 46; 47} Electron cryotomography of flagella from many different organisms has revealed core conserved features, but also striking diversity in overall structure.⁴⁸ These images combined with protein binding assays, targeted cross-linking and knowledge of the component structures indicate the general positions of the rotor proteins.^{14; 19; 30; 42; 49} However, the domain arrangements within the switch complex components are somewhat ambiguous and as such, different models have been suggested.^{14; 30; 47} Several structures of FliG_M in complex with FliM_M display a similar interaction between the EHPQR motif of FliG and the GGPG motif of FliM_M.^{28; 30; 31} In contrast, there are substantial differences in the arrangements of FliG_M and FliG_C found in various crystal structures.^{15; 16; 25; 28; 44} Although all contain the same FliG_M:FliG_C association somewhere in the crystal lattice, this interaction can be either intra or inter-molecular. In the structure between FliM_M and both middle and C-terminal FliG domains (FliG_{MC}), the FliG_C domain associates closely with the FliG_M domain, however the linker between them is not well ordered.³¹ Nonetheless, biochemical data suggests that *E. coli* FliG_C also interacts with FliM_M, an observation that leads to a mixed interaction model for the C-ring wherein some FliM_M units bind FliG_M and others bind FliG_C.^{30; 50} This latter arrangement can explain the rotor stoichiometry mismatch between 26 FliG copies and 34 FliM copies^{14; 19; 47} approximately 1 out of 3 FliG molecules binds two FliM units, with one FliM binding to FliG_M and the other binding to FliG_C.^{30; 50}

Here, we report the crystal structure of FliM_M:FliG_M from *Thermotoga maritima* in a new packing arrangement that produces a large arc consistent with the dimension of the C-ring. We evaluate the prevalence of this assembly state against other models through targeted cross-linking, multi-angle light scattering (MALS) and site-directed spin labeling (SDSL)^{51; 52; 53} combined with PDS.^{54; 55; 56} Cross-linking and MALS find evidence for heterotetrameric assemblies of FliG and FliM that involve both parallel and anti-parallel arrangements of the FliM subunits. The PDS data confirms the crystallographic heterodimeric interaction between the FliG and FliM middle domains, but also supports higher-order assemblies mediated by contacts between FliG_M and FliG_C of an adjacent molecule. Residue substitutions at the predicted FliG:FliM and FliG:FliG interfaces of the parallel arrangement perturbs the PDS signals by disrupting the heterotetramers. A set of closely related models consistent with this new information and known electron microscopy images of the C-ring are then globally fit to PDS distant restraints derived from the multi-spin systems. The determined interaction between FliG_M and the FliG_{C+1} produces a chain-like structure in the C-ring capable of explaining the high degree of cooperativity observed upon switching.^{57; 58; 59; 60} Activated phosphono CheY (CheY-pP) destabilizes the interface between aligned FliM modules, and in the absence of the membrane assemblies, promotes the antiparallel arrangement observed in the new crystal structure.

Results

Structure of the FliG_M:FliM_M binary complex

A new structure of *T. maritima* FliG_M:FliM_M was determined to 4.3 Å from crystals of a large unit cell that contained 11 copies of the complex per asymmetric unit. Molecular Replacement (MR) was successful due to the availability of a high quality search model (PDB 3SOH) and crystals of large solvent content (Table 1). At this resolution, each FliG_M:FliM_M unit is nearly identical to that of the previously determined structure of the same complex,⁵⁰ with contact mediated through the GGPG motif of FliM and EHPQR motif of FliG. The FliM_M molecules are arranged in an antiparallel fashion involving interactions of the long helices, α1 and α1' in each molecule (Fig. 2A). The analogous contact is also seen in the crystal structures of the FliM homolog CheC.⁶¹ Of the 11 unique FliM_M:FliG_M molecules, 7 subunits generate a curved arc, with dimensions consistent with that of the C-ring (Fig. 2B). By extrapolating this arrangement, five equivalent 7-subunit segments generate a closed ring with a diameter of ~ 43 nm and composition of 35 FliM units (Fig. 2C). These values are consistent with the 45 nm diameter of the C-ring observed in EM images of the flagellar rotor from *Salmonella*⁴⁷ and match the expected FliM stoichiometry, which can vary between 32 to 37.^{47; 62} However, the antiparallel arrangement of the FliM subunits in the current structures alternates their polarity relative to the ring axis, and hence half of the FliG subunits would point toward the membrane and half toward the cytoplasm. This anti-parallel arrangement is inconsistent with our current understanding of FliG interactions within the rotor, where all FliG subunits interact with the stator and FliM has 35-fold rotation symmetry.^{6; 12; 27} Furthermore, cross-linking analysis of FliM *in vivo*¹⁹ associates the α1-α1' side of FliM, with the opposite side of the adjacent subunit to generate a back-to-front, repeating chain, not the antiparallel front-to-front and back-to-back arrangement in the structure (Fig. 2A). Nonetheless, the unusual nature of the crystal packing and its correspondence to C-ring dimensions encouraged us to further investigate interactions of the subunits in solution.

Possible arrangements of FliM and FliG in the C-ring

Taking the available FliM:FliG crystal structures and data based on previous cross-linking experiments^{19; 30; 42} we built four models for parallel and antiparallel arrangements of the FliM:FliG oligomer (Fig. 3A) The first model contains the antiparallel interaction of FliM based on the crystal structure described above. The second model is based on the FliM_M:FliG_{MC} co-crystal structure (PDB 4FHR) with an assumed intramolecular contact between FliG_M and FliG_C.³¹ In this crystal structure, the electron density for the loop connecting the two FliG domains (residues 185 to 196) is not apparent in the structure. The third model is based on the FliG_{MC} structure (PDB 3AJC).⁴⁴ In this structure, the connection between FliG residues 185 and 196 is ambiguous because of discontinuous electron density, but here we assume an intermolecular contact to distinguish model III from model II. Beneath the FliG_M molecule, the FliM_M subunits align in a parallel arrangement. The fourth model is based on the proposal that 26 copies of FliM_M interact with FliG_C and remaining 8 copies of FliM bind to FliG_M.⁵⁰ This model explains the symmetry mismatch between the MS and C-ring and the stoichiometry mismatch between FliG and FliM. The

heterodimer associations in models II, III, and IV are generated based on cross-linking experiments on FliM_M^{19; 30} (Fig. 3).

Cross-linking experiments

With models I-IV as reference, cross-linking of proteins with engineered Cys residues were performed in an effort to distinguish between the parallel (models II-IV) versus antiparallel (model I) arrangements of the FliM:FliG heterotetramer in solution. Unmodified FliM did not show any cross-linked products in the presence or absence of FliG (data not shown). Targeted cross-linking was performed on single or double Cys-substituted FliM in the presence of FliG, using Cys positions that were previously identified to be cross-linked¹⁹ (Fig. 4). We also tested FliM residue 164, which was predicted to cross-link only in the antiparallel arrangement (model I). As previously reported for the *E. coli* proteins,¹⁹ FliM containing the cysteine pairs (64/185 and 57/185) cross-linked efficiently, yielding dimers and multimers in the presence of FliG that were consistent with a back-to-front chain of parallel FliM subunits. Cross-linking was also observed with the single cysteine FliM substitutions (57, 64, and 164) in the presence of FliG under identical experimental conditions. However, the single Cys substitutions only produced dimers and no higher order species, as expected by the formation of symmetric disulfides. The observed cross-linking of the single Cys variants at positions 64 and 164, would be most consistent with an antiparallel arrangement of FliM (as found in the current crystal structure, Fig. 4C). The Cys 185 variant did not cross-link at all, as predicted from the C_β-C_β separation in either arrangement. Although both arrangements disfavor cross-linking from position 57, substantial dimer formation was observed, which may owe to elevated reactivity of Cys 57 (Fig. 4C). Overall, the cross-linking results provide strong evidence for a parallel arrangement of FliM subunits in the presence of FliG, but also reveal contributions from anti-parallel dimers. Clearly, the antiparallel configuration must form in solution to some extent, because it crystallizes. Most important is that the cross-linking data also finds evidence for the parallel arrangement, which is much more consistent with what is known about rotor assembly. Regardless, cross-linking data alone cannot evaluate the relative contributions of these solution assemblies because products accumulate over time and also depend on Cys reactivity in a given environment.

Does FliG_C also bind to FliM_M in *T. maritima*?

Although all known crystal structures demonstrate interactions between FliG_M and FliM_M, prior mutational and cross-linking experiments on the *E. coli* proteins implicated a conserved hydrophobic patch of FliG_C for binding FliM.^{14; 30; 50} NMR experiments also suggest that CheY or CheY-P lead to altered interactions between FliM_M and FliG_C.⁶³ To probe interactions between FliG_C and FliM_M in *Thermotoga maritima* we performed pull-down assays between FliM_{NM} and two FliG_C variants: FliG₂₀₄₋₃₃₅ and FliG₁₉₅₋₃₃₅, which includes a conserved GG motif of the interdomain linker. No interaction was observed in the absence or presence of CheY or CheY-P (SI Fig. 1). The inability of FliG_C to interact with FliM_M suggests that model IV is absent in solution, at least under these conditions. To further investigate the FliG:FliM arrangement, PDS was conducted on spin labeled FliM and FliG, individually and in complex.

Spin-labeling and PDS

PDS measures distances between specifically placed nitroxide spin-labels through their magnetic dipolar coupling.^{64; 65; 66; 67; 68} The PDS technique known as double electron electron resonance (DEER) provides the long-range distance restraints (<90 Å) necessary to map the architecture of multicomponent complexes.^{64; 65; 66; 67; 68; 69} For labeling sites, we substituted six residues on FliM_M (E60, R64, D121, M131, R141, and S167) and four residues on FliG (K160, K174, L208, and E305) to cysteine and reacted them with MTSSL ((1-Oxyl-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-methane sulfonate) to produce the nitroxide side chain known as R1 (Fig. 3B). FliM has one native, buried cysteine (Cys 214), which was not reactive to spin label (< 2 % labeling). Experiments on singly labeled proteins with unlabeled partners were performed before investigating complexes with both components labeled. Under single-label conditions, a spin-spin separation detectable by PDS will arise only if the FliM:FliG heterodimer associates into a heterotetramer or larger species (SI Fig 2A).

Spin labels on FliG_M

Two surface-exposed residues in FliG_M (K160 and K174) were chosen as spin-labeling sites such that the parallel and antiparallel arrangements of the FliM:FliG complex would yield different distances (Fig. 3A). The distance distribution data of FliG 160-R1 with unlabeled FliM produces a distance of about 47 Å, close to that expected for FliG associated through a parallel arrangement of FliM (models II, III, and IV) but too short for the distance expected for an antiparallel FliM arrangement (model I) (Table 2, SI Fig. 3). FliG 174-R1 produced a very wide distance distribution with $R_{\max}$ of 32 Å, which is also close to the distance expected for a parallel FliM arrangement (Table 2). Thus, the parallel arrangement is likely present in solution; however, contribution from other species cannot be ruled out because the broad distribution for FliG 174-R1 could result from a mixture of distances from two or more different arrangements.

Spin labels on FliM_M

PDS was performed with spin-labels on four sites of FliM_M. Three sites, E60, R64, and S167 rise along the helices $\alpha 1$ and $\alpha 1'$ and the fourth site, D121, resides on $\alpha 3$, closer to the center of the molecule (Fig. 3B). All sites produce very weak dipolar signals with FliM alone, which indicates that the protein is primarily monomeric in the absence of FliG. Addition of a FliG protein that contains both the middle and C-terminal domain are necessary to produce a strong dipolar signal indicative of FliM oligomerization (as shown by the time domain signal of FliM 60-R1 (Fig. 5A)). Thus, FliG_{MC} associates the FliM molecules, but either FliG_M or FliG_C alone, does not. These findings are consistent with model III, model IV or both (Fig. 5B).

When FliM_M is in complex with unlabeled FliG_{MC}, the spin labels on FliM 60-R1, 64-R1 and 167-R1 are predicted to yield short spin-label separations (< 25 Å) for the anti-parallel arrangement, but larger separations for the parallel arrangement (~ 40 Å). Spin-label conformational variability can add ± 7 Å to C_{β} - C_{β} distances.^{54; 69; 70} The observed distributions for FliM 60-R1, 64-R1, and 167-R1 are broad and somewhat bimodal, with peaks at about 32 Å and 45 Å (Table 2). Thus, these distributions suggest contributions from

multiple species, which could include both parallel and anti-parallel arrangements, but may also derive from a predominantly parallel arrangement broadened by flexible domain linkages and spin labels. Given the weight of the longer distances, a predominant antiparallel arrangement seems unlikely. The spin label on FliM 121-R1 again gave a bimodal distance distribution with $R_{\max}$ values of 44 and 58 Å, which agrees with the antiparallel arrangement, but could also reflect substantial contribution from the parallel arrangement (Table 2, SI Fig. 3). Taken together, these data indicate that FliG_{MC} drives FliM oligomerization and finds evidence for a parallel configuration, but they do not conclusively define the FliM arrangement (SI Fig. 2A).

Inter-protein interactions from spin labels on FliM_M and FliG_M

PDS data from FliM labeled at D121 and FliG at K160 exhibited two distinct distance contributions: a short component at ~20 Å and a longer one at ~47 Å. The short distance can be attributed to the FliM_M:FliG_M intermolecular distance (m-g) in the crystallographic heterodimer (Table 2). The longer distance of ~47 Å can be explained by a mixture of signals from the FliG_M:FliG_M distance (g-g'), the FliM_M:FliM_M distance (m-m') and the cross FliG_M:FliM_M distance (g-m', from different heterodimers within a tetramer). The FliM 60-R1 and FliG 160-R1 spin pair produce a wide distribution that can be assigned to overlapping spin separations from m-m (32 Å), g-g (48 Å) and m-g (37 Å). FliM 167-R1 and FliG 160-R1 produced a wide bimodal distribution spanning about 40 Å, with a peak at ~34 Å and another at ~43.7 Å. For the FliM 60-R1 and FliG 174-R1 spin pair, a very wide distribution spanning ~40 Å corresponds to the g-m' cross heterodimer distance, with another peak at ~44 Å possibly representing a conformational variant of this separation. A broad peak at ~30 Å corresponds to overlapping separations from m-m', g-g', and m-g spin sites (Table 2). In summary, the inter-protein distance restraints reflect the m-g crystallographic dimer contact but also indicate higher-order complexes. The close spin-spin separations predicted by the antiparallel arrangement of FliM are not well represented, and thus this data is more consistent with a parallel arrangement, where the spin-separations are broadened by flexibility within the oligomer.

Inter-protein interactions from spin labels on FliM_M and FliG_C

Based on previous cross-linking data⁵⁰ spin-labeled FliM (131-R1 and 141-R1); and FliG (208-R1) were tested for dipolar interactions indicative of a short distance across the FliM_M:FliG_C contact, but none was apparent. We do note that labeling FliM_M at M131 disrupts the FliM_M:FliG_M interaction, and similarly could have perturbed a FliM_M:FliG_C contact. Thus, we also investigated the interaction between FliG_C and FliM_{NM} by placing spin-labels on FliG_C (E305) and FliM (E60, D121, and S167). For all of the spin pairs, dipolar interactions were weak and the resulting distributions were broad, inconsistent with a specific FliG_C:FliM_M contact. This experiment further confirmed the results of the pull-down assay (SI Fig 1).

Disrupting subunit interactions by residue substitution

Owing to the difficulty of interpreting the multi-spin distributions we sought to examine the effect of residue substitutions within potential interfaces involved in oligomerization.

Residue changes in certain positions of *E. coli* FliG are known to disrupt FliG:FliM interactions.¹⁴ Thus, we investigated the effects of the corresponding substitutions in the *T. maritima* proteins (residues 129, 204, 227) on the PDS distributions (Figs. 5A, 6, and 7). As expected, substitution of FliG Q129 to W in the EHPQR motif caused loss of the m-g heterodimer distances (and broadening of the distribution) reported both by the FliG 160-R1 and FliM 121-R1 spin pair (Fig. 6A) as well as by the FliG 160-R1 and FliM 60-R1 spin pair (Fig. 6B). Thus, the Q129W substitution weakens the m-g interaction. In contrast the dipolar signals corresponding to g-g' separation remained intact, which confirms that FliG oligomerization does not involve this interface (Fig. 6).

In contrast, substitutions in the hydrophobic patch of FliG_C (I204W and L227W, *T. maritima* numbering) previously found to lessen interactions between FliG and FliM in *E. coli*,³⁰ did not abrogate the interactions between FliG:FliM as above (reported by the FliG 160-R1:FliM 60-R1 pairs), but did lessen signals due to the g-g' separation (reported by the FliG 160-R1:FliG 160-R1 spin pairs; Fig. 7). The I204W and L227W substitutions also produce an overall drop in signal amplitude, which suggests that these changes affect the overall stability of the complex (Fig. 7). A contact between FliG_C and FliG_M is found in the crystal structure of a CW-locked FliG PEV (PDB 3AJC) variant from *T. maritima*,⁴⁴ and in crystal structures of FliG_{MC} (PDB 1LKV), *Helicobacter pylori* FliG_{MC} (PDB 3USW) and *Aquifex aeolicus* FliG_{FL} (PDB 3HJL), although in the former case there is some ambiguity as to whether this interaction is intermolecular or intramolecular. These mutagenesis results indicate an intermolecular contact between FliG_C to FliG_M (model III) that mediates formation of higher order FliG:FliM complexes.

MALS experiments on spin-labeled FliG_{MC} show that the native protein dimerizes but the spin-labeled FliG_{MC} L227W and I204W variants did not form larger oligomeric states (SI Fig. 2B). Although, the FliG_C hydrophobic patch is critical for oligomerization; substitutions therein do not affect signals arising from the m-g crystallographic heterodimer. Thus, an intermolecular FliG_C:FliG_{M+1} interaction mediates the higher order associations. An antiparallel FliM arrangement (model I) or an intramolecular FliG_M and FliG_C interaction (model II) precludes a FliG_C:FliG_{M+1} interaction, but the parallel arrangement of model III accommodates such a state.

Model evaluation

To consolidate the data we performed parameter fits to the PDS distance distributions assuming Gaussian functions of varying widths for each individual spin-separation. The primary goal here was to evaluate agreement of the experimental data as a whole to various models of FliM-FliG assembly. Although these fits involve a relatively large number of parameters, the models are the simplest representation of the information reported by the experiments and as shown below, this procedure is able to discriminate closely related assembly states. For example, as the major species in solution model III provides the best fit to the available data (Fig. 8). According to model III, PDS experiments with spin-labels on FliM and FliG generate five distinct spin-spin separations upon further oligomerization, which based on MALS experiments we take to be primarily tetramerization (Fig. 8A, SI Fig. 2A). Spin labels on FliG alone in the presence of FliM assign the FliG:FliG homodimer

distance (g-g'). Similarly, spin labels on FliM in the presence of unlabeled FliG assign the FliM_M:FliM_M homodimer distance (m-m'). The FliM-FliG interdimer distances are generally unique and conform to expectations from the crystal structure (m-g). The other heterodimer in the tetramer (dimer of dimers) would yield the same distance m-g (= m'-g'). Thus, m-g contributes twice to the weighting scheme (See eqn (1) in Materials and Methods). We also allow for asymmetry in the assembly by fitting two distinct intermolecular distances m-g' and g-m' in the dimer-of-dimers, which were initially assigned as unique peaks in the distributions distinct from those assigned above. Gaussian fittings were performed following an established procedure developed by Georgieva *et al.*⁶⁹ (See Materials and Methods for details). For fitting purposes we did not include distances greater than 60 Å, given that the time durations used for most of the DEER experiments limit accuracy at these longer distances. This excluded distances from FliG 160-R1:FliM 60-R1 (68 Å), FliG 160-R1:FliM 167-R1 (71 Å), and FliG 174-R1:FliG 305-R1 (61 Å). In the case of bimodal distributions (e.g. FliG 60-R and FliM 121-R) the closer spin separation was chosen for fitting. The fraction of interacting spins was measured for each single labeled protein in the presence of unlabeled partner. Gaussian values obtained from the single-label control experiments were held constant for refinements of the multi-spin distributions to reduce the number of free parameters.

We considered several closely related orientations of subunits in the general arrangement of model III (SI Fig. 4) that were consistent with the EM density of the *Salmonella* rotor (Fig. 9). FliM_M was placed in the center of the C-ring in the electron density map, with FliG_M interacting directly above it through the crystallographically defined interface. FliG_C was positioned at the top of the ring for interaction with MotA. Four separate fittings were evaluated against the PDS data that varied based on the spacing and tilt of the FliM:FliM dimer and the arrangement of the FliG_C head. (SI Fig. 4). In the latter case, crystal structures of FliG_C that have different orientations of the head domain that contains the MotA-interacting charged helix were used as templates. The linker connecting FliG_M and FliG_C was modeled as an extended chain, whose length was consistent with the spacing between FliG_C and FliG_{M+1}. This arrangement also directs the N-terminus of the FliG_M toward the inner MS-ring where FliG_N interacts with FliF.⁴⁰ Overall, the results obtained from the Gaussian fits (Fig. 8 B-F) matched well with the experimental distance distribution data with the adjusted R² parameter for each distance distribution ranging from 0.89 – 0.97. Nevertheless, the four models were clearly distinguished, with the more structurally similar ones giving closer agreement (SI Fig. 5). Starting parameters that varied by several Å in Gaussian mean or width generally converged to the same solution. Similar analysis for the anti-parallel arrangement produced considerably worse fits (SI Fig. 5). Small discrepancies between the calculated and experimental curves may stem from heterogeneity in the samples, minor inaccuracy in baseline corrections and some non-Gaussian behavior in the distance distributions, which could arise as a result of preferred spin-label orientations in a given environment.⁵⁵ The time domain DEER data could also be reasonably reconstructed from the experimental and Gaussian fit P(r) distributions, with some deviations arising again from baseline inaccuracy and the ability of the maximum likelihood treatment to filter minor contributions from heterogeneity or measurement error (SI Fig. 6).

From the fits we extracted 18 distances that were in good overall agreement with the C_β separations from the model, yielding an rmsd. of 6.4 Å in the best case (Fig. 8G, SI Table 1). For comparison, in monomeric T4 Lysozyme, 20 different distance measurements between spin-labeled residues on the same monomer yielded an rmsd. of 6.9 Å,⁷¹ thus, the agreement is within the limit expected from uncertainties in the nitroxide positions. The shorter g-g' versus m-m' values indicate greater conformational variability about the FliM units than the FliG units,¹⁹ which is not surprising considering that the FliG units mediate the oligomerization. In the best model, FliG_C assumes the conformation found in 3AJC (PDB code) with the charged helix axis aligned roughly along the radius of the C-ring.

The experimental distances which show the largest discrepancy with respect to the refined model involve the FliG:FliG juxtaposition. FliG_M 160-R1 alone yielded a bimodal spin-separation that peaked at ~45 Å and ~58 Å. Distances obtained from ESR experiments are typically longer than the C_β distances by 5-7 Å^{54; 69; 70} and thus, the ~45 Å distance may be in keeping with parallel arrangement. The larger distance may be due to conformational flexibility at this region of the molecule that extends the distance distribution. Furthermore, position 160 resides at the interface of FliG_C:FliG_M (PDB 3AJC), where its aliphatic side chain participates in a hydrophobic interface. Addition of spin label to this interface may perturb the interaction between FliG_C and FliG_{M+1} and thereby produce P(r) broadening and increased distances between neighboring FliG_M domains. An antiparallel arrangement should give distances of ~70 Å, but no long distances were observed, even with a DEER acquisition time increased to 5 µsec and samples prepared in D₂O to increase spin dephasing times (SI Fig. 3).

Effects of CheY-P on the FliM:FliG complex

PDS experiments were used to probe whether the individual domains change juxtaposition when CheY-pP (phosphono CheY-a stabilized, activated mimic of CheY⁷²) binds to the amino terminus of FliM (FliM_N).³⁸ Indeed, spin reporters on FliM_M show changes upon addition of CheY-pP to the FliM_M:FliG_{MC} complex. A broad weak m-m' signal with FliM 60-R1 and unlabeled FliG_{MC} converts to a sharp peak at ~30 Å upon addition of CheY-pP (Fig. 10A). The narrowed distribution could represent enhanced FliM dimerization, rigidification of the association, or orientation selection effects of the spin-label. All possibilities reflect the ability of CheY-pP to alter the FliM:FliM interface despite FliM_N being the primary binding site. A variant form of unphosphorylated CheY (D10K, F101W; *Thermotoga* numbering) locked in the activated state⁷³ produced similar effects on the 60-R1 distribution as CheY-pP (SI Fig. 7). Other FliM spin label sites also responded to CheY-pP. For FliM 64-R1, CheY-pP promotes a strong signal at short distance (~20 Å), although in this case a longer separation is also enhanced at ~45 Å (Fig. 10B). In this case, the two spin-separations likely represent mixtures of assembly states. For 167-R1 on FliM α1' (adjacent to site 60) CheY-pP enhanced short separations at ~20 Å, but to a lesser extent than for 60-R1 (Fig. 10C). For FliM 121-R1 with unlabeled FliG_{MC} we did not observe any shift in distances, although the original peak slightly sharpens in presence of CheY-pP (Fig. 10D). The short FliM:FliM distances (~20 Å) from spin-labels on the α1-α1' helices are consistent with an antiparallel arrangement of FliM. Thus, CheY-pP destabilizes the parallel, aligned interaction of FliM in model III, which then may favor the otherwise minority

antiparallel FliM arrangement. No CheY-pP effect is seen in the absence of the FliM N-terminal target sequence.³⁸ Importantly, the interaction between CheY-pP and FliM must be relayed to FliG because CheY-pP binding disrupts the parallel FliM arrangement templated by the FliG_M:FliG_{C+1} contact.

Discussion

Through combining data from PDS, cross-linking, MALS, and crystallographic data we have investigated the interactions between the flagellar switch complex proteins FliG and FliM. We find strong evidence for the anticipated FliM_M:FliG_M heterodimer, but also detect higher order assemblies that include a parallel back-to-front arrangement of the FliM_M units. These oligomeric structures are mediated through interaction between FliG_C and FliG_{M+1}, as observed in known crystal structures of FliG.^{15; 16; 25}

Parallel arrangement of FliM and FliG mediated by the FliG_C:FliG_{M+1} interaction

Although our new crystal structure of the FliG:FliM complex produces a compelling packing arrangement, the relevance to C-ring assembly is questionable. Extrapolation of the crystallographic arc structure generates a ring of the correct size (diameter ~43 nm), but the FliG arrangement does not meet with expectations of C-ring assembly. Furthermore, alternating FliG molecules would point upward and downward with respect to the stator, thereby rendering a subset of the FliG subunits inaccessible to MotA and another subset inaccessible to FliF.^{27; 74} Furthermore, the antiparallel arrangement of FliM and FliG found in this structure is unlikely to be the major species in solution (see below). Nonetheless, this structure provided a means to interpret the response of the FliM:FliG complex to CheY-pP, which appears to promote the antiparallel arrangement.

Several lines of evidence rule against a major contribution from the antiparallel configuration in the absence of CheY-P: 1) FliG_{MC} dimerizes on its own, producing very similar PDS signals whether or not FliM is present, yet FliM would mediate the key contacts for an antiparallel configuration; 2) FliM only oligomerizes in the presence of FliG_{MC}; again, this would be unexpected from an antiparallel configuration that depends primarily on FliM contacts; and mutations at the FliG_M-FliG_C interface disrupt the oligomer. Hence, FliG_M-FliG_C contacts, which rely on a parallel assembly, largely mediate oligomerization. Out of the three parallel arrangements tested (models II-IV), model IV was ruled out based on the inability of pull-down assays and PDS experiments to detect an interaction between FliG_C and FliM_M and the finding that FliG_C rather interacts with FliG_M. Interface disruption experiments demonstrated the importance of the contact between FliG_C and FliG_{M+1} for heterotetramer assembly, thereby favoring model III over model II. Repetition of this key linchpin between FliG_C and FliG_{M+1}, into successive subunits will polymerize the FliG molecules into a one dimensional array (SI Fig 2B, Fig 7). The FliG_C:FliG_{M+1} interaction was observed previously in the crystal structures of *A. aeolicus* full-length FliG, *T. maritima* FliG_{MC}, *H. pylori* FliG_{MC} and possibly the CW locked *T. maritima* FliG_{MC} (PEV) (PDB 3HJL, 1LKV, 3USW, and 3AJC respectively).^{15; 16; 25; 44} FliM_M then associates with the EHPQR motif of FliG_M to assemble FliM in an aligned back-to-front arrangement beneath FliG. Switching of the flagella is highly cooperative.^{57; 58; 59} Coupling of overlapping FliG

units could propagate CheY-P induced perturbations in the lower part of the C-ring to neighboring FliG subunits.

Interaction between FliG_C and FliM_M

The relative positioning of FliG_M and FliG_C in the upper region of the switch complex is an issue of considerable debate.³⁰ The general position of FliM in the middle of the switch is relatively well established and the crystallographically defined FliM_M:FliG_M contact is without dispute; however, there is also biochemical and genetic evidence for interactions between FliG_C and FliM_M in *E. coli*.^{30; 50} This led to the proposal that FliM_M may interact with both FliG_M and FliG_C, the former contact favoring tilted FliM_M subunits.^{30; 50} This arrangement explains the stoichiometry mismatch between FliG and FliM, electron density distributions within the inner part of the C-ring, and also better accounts for EM images of a FliF:FliG fusion variant.^{30; 41; 50} Nonetheless, we could not find evidence for direct interactions between *T. maritima* FliM_M and FliG_C. Rather, we find that FliG_C largely interacts with FliG_M as seen in several crystal lattices (PDB codes: 1LKV, 3HJL, 3AJC, 3USW). There is only one current structure that contains both FliM_M and FliG_MC and indeed FliG_M intersperses FliM_M and FliG_C.³¹ Solution state NMR studies detected only weak interaction between FliM and FliG_C.⁶³ Furthermore, a recent study of *Helicobacter pylori* switch proteins show no evidence for interaction between FliM and FliG_C.²⁸ Notably, the experiments that demonstrate the FliG_C:FliM_M interaction were performed on intact rotors within cells.^{30; 50} Thus, constraints of the rotor may favor the FliG_C:FliM_M interaction. It is also possible that this interaction differs between the rotors of thermophiles and enteric bacteria. Nevertheless, the prevalence of the FliG_C:FliG_M contact in crystalline and soluble proteins from several sources suggest that this is indeed a biologically relevant contact. The preference for a FliG_C:FliG_{M+1} arrangement, as observed here, has compelling functional utility because FliG subunits polymerized in this manner could impart cooperativity to switching, as well as provide a stable ringed template for FliM.

Stoichiometry mismatch between FliG and FliM

According to the EM images of *Salmonella typhimurium* flagella motors, the C-ring and MS-ring has ~34-fold and 26-fold symmetry, respectively. Biochemical analyses and stoichiometries are consistent with this symmetry mismatch between FliM and FliG.⁵⁰ Recent photobleaching experiments confirm these subunit ratios, but interestingly find that the number of subunits is quite variable and appears to change with the functional state of the rotor.^{75; 76; 77} From a spatial perspective, the smaller circumference of the MS-ring compared to the C-ring would necessarily accommodate fewer copies of FliG (26) than FliM (34). The stoichiometry mismatch could be explained if the C-ring is “gated” such that all FliM_M sites are not occupied by FliG_M. Due to the interdigitated stacking of the FliG molecules, a FliG vacancy would position the preceding FliG_C above FliM_M. This FliG_{C-1} may shift down to interact with FliM_M, thereby generating the interaction observed in *E. coli* cells (SI Fig. 8). If the vacant FliG molecules were randomly distributed throughout the rotor, such a gap at the FliG_C, or FliG_M position would be averaged out in the EM reconstructions. It should be noted that the stepping frequency of the motor corresponds to ~1/26th of a full rotation, although there is some uncertainty as to the uniformity of the step size.^{78; 79} FliG contributes to both the 26-fold symmetric inner C-ring and 34-fold

symmetric outer C-ring.⁴⁷ Presumably, the inner symmetry determines the step size, but the FliG C-terminal domains, which interact with the stator, reside at positions that give the symmetry of the outer ring.⁴⁷ Thus, if the step size is truly regular, it is difficult to understand how torque is generated by interactions involving the stator and FliG_C alone.

Activated CheY interactions with FliM

The chemotaxis signal is highly amplified within the switch⁸⁰. The structural basis for this amplification is not clear. Studies have shown switching to be a highly cooperative event having a Hill coefficient of 10 or 20.^{57; 58; 60; 81} However, FRET results show that binding of CheY-P to FliM is about fivefold less cooperative than switching⁵⁸. In this study, we probe primary interactions between CheY-pP and FliM that may manifest during the early stages of switching.

CW and CCW-biasing mutations localize to different regions of residues at the FliM:FliM interface.^{19; 20} Thus, rotation switching likely involves rearrangements at this interface induced by CheY-P. PDS experiments on spin-labeled residues at the FliM interface (E60, R64, and S167) with CheY-pP show changes in the solution FliG:FliM higher-order complex when CheY-pP binds FliM_N. In particular, short separations consistent with the FliM:FliM antiparallel arrangement arise when CheY-pP binds. This is not to say that CheY-P causes FliM to flip over in rotors. Indeed, this seems unlikely. We believe that the antiparallel state results because CheY-P destabilizes the parallel association in the less constrained solution complexes. Although the antiparallel FliM arrangement is a minority conformer in solution, disfavoring the parallel assembly increases its population. With constraints provided in the assembled rotors, destabilization of the m-m' interface may produce a more modest change in their juxtaposition. Importantly, FliG mediates the parallel FliM assembly; hence CheY-P binding to FliM_N must influence the interactions of FliG_C with FliG_{M+1} or FliG_M with FliM_M. In either case, alteration in the conformation of FliG_C relative to the stator is a likely consequence. In *E. coli*, cross-linking between the switch components and a CheY variant fused to FliM_N detected interactions between CheY-P and FliN in the lower part of the switch⁸². Our experiments do not contain FliN, yet show perturbations to the m-m' interface in keeping with the sensitivity of these regions to mutants that affect switching²⁰. Unfortunately, we have not been able to reconstitute *T. maritima* FliY(N) with FliM and FliG (or versions thereof) to test interactions of CheY-pP with FliY(N). It is possible that CheY-P binds at the interface of FliM and FliN, with partial recognition sites supplied from both proteins. It is also possible that switch complexes from diverse species produce different interactions with CheY-P. Indeed, "FliN" from *T. maritima* is fused to an additional phosphatase domain to comprise FliY.³⁸

In conclusion, we identified a highly coupled interaction modes of FliM and FliG using a combination of cross-linking, pull-down assays, MALS, PDS, and crystallography. Activated CheY directly perturbs a parallel alignment of FliM subunits. The flagella switch proteins have provided a test bed to develop PDS for application to multi-component protein complexes. That said, we acknowledge that our conclusions are drawn from measurements of soluble, truncated proteins. Although the state-of-affairs could be different in the assembled flagella motor, our models do agree well with EM reconstructions and a host of

biochemical, genetic and structural studies on the switch complex. Here we have established a base line of distance restraints and models that can be evaluated in more complex assemblies. These metrics may prove useful benchmarks for mapping detailed interactions within intact flagella by PDS or other methods.

Materials and methods

Cloning mutagenesis and protein expression

The genes encoding *T. maritima* FliG_{MC} (residues 116-335), FliG_M (residues 117-195), FliM_{NM} (residues 1-249), FliM_M (residues 46-242), and *T. maritima* CheY were PCR cloned from *T. maritima* genomic DNA (obtained from the American Type Culture Collection) into the vector pET28a (Novagen) and expressed with a His₆-tag in *E. coli* strain BL21-DE3. Point mutations to introduce cysteine residues on FliG_{MC} (K160, K174, L208, E305) and FliM_{NM} (E60, R64, D121, M131, R141, S167) and tryptophan residues on FliG_{MC} (Q129, I204, L227) were performed by QuikChange (Stratagene) or overlap extension. Mutations were confirmed by sequencing. *E. coli* cultures transformed with expression vectors were grown overnight at 25 °C after induction with 100 μM IPTG at the optical density of 0.6. Cells were collected by centrifugation, frozen and stored at -80°C. Frozen cells were thawed and resuspended in lysis buffer (25 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM imidazole). Cells were sonicated and centrifuged at 22000 rpm for 1 hr at 4 °C. Protein samples were purified by Ni-NTA affinity chromatography and further purified by size exclusion chromatography (Superdex 75; Pharmacia Biotech) after cleaving the His₆-tag with thrombin.

Spin labeling

E. coli cultures expressing cysteine variants were processed as described above. Cell lysates were applied to the Ni-NTA column. 5-10 mM MTSSL nitroxide spin-label (Toronto research, Toronto, ON) was added to the column, incubated at room temperature for 4 hrs and then overnight at 4 °C. Samples were eluted after a subsequent overnight incubation with thrombin to remove the His₆-tag. Proteins were further purified on a size-exclusion column (Superdex 75; Pharmacia Biotech) and concentrated in GF buffer (50 mM TRIS, pH=7.5, 150 mM NaCl).

Crystallization

Crystals of the FliM_M:FliG_M complex were obtained by vapor diffusion at room temperature from a 2 μL drop containing 1 μL of well solution (0.1M imidazole, pH 6.5, 1.0 M sodium acetate trihydrate; Hampton Research) and 1 μL of a mixture of two proteins FliM_M, and FliG_M (~ 20 mg/ml in GF buffer). Crystals were observed in five days. Crystals were optimized by varying precipitant concentration and pH of buffer to obtain better diffraction quality crystals, with the optimized condition being: 0.1M imidazole, pH 6.5, 1.2 M sodium acetate trihydrate.

Data collection, structure determination and refinement

Data was collected at Cornell High Energy Synchrotron Source (CHESS) station A1 using the ADSC Quantum 210 CCD. Crystals were briefly soaked in 30 % glycerol before

mounting for x-ray exposure. Data was scaled by HKL2000⁸³ and the structure of the complex was obtained by PHENIX AutoMR⁸⁴ with PDB 3SOH (FliM_M:FliG_M structure) as a search model. The asymmetric unit was comprised of 11 FliM_M:FliG_M subunits. Electron density beyond residue 187 for FliG and residue 228 for FliM was not discernible. For further refinement, FliG was truncated to residue 187 and FliM was truncated to 228. Given the low resolution, only rigid body, group B-factor, and limited positional refinement were performed in PHENIX.⁸⁴

Disulfide cross-linking studies

Cross-linking studies on FliM and FliG were performed according to Bass, et al.⁸⁵ with minor modifications. Copper-phenanthroline (Cu-phen) was used as the initiator. Concentrated proteins in GF buffer were diluted with disulfide reaction buffer, as described previously.⁸⁵ Final concentration of each protein was kept at 6 μ M with the Cu-phen concentration at 2 mM. The volume for each reaction was kept constant at 10 μ L. 8 μ L of the reaction was quenched with equal volume of 2x SDS with 10 mM imidazole after 1 hr of incubation. 15 μ L of this mixture was run on the SDS-PAGE gel after heat treatment at 90 °C for 2 minutes. For each sample, a control at the zero time point was collected and quenched before the addition of the Cu-phen.

Pull-down assays

Assays were carried out in binding buffer (25 mM HEPES, pH= 7.5, 500 mM NaCl, and 50mM Imidazole). Proteins were incubated in 40 μ L Ni-NTA with the binding buffer for 30 minutes at room temperature. The beads were washed with binding buffer thrice and once with binding buffer containing 1% Triton X-100 to minimize non-specific binding. 2x SDS loading dye was added to the resin and boiled for 5 minutes at 90 °C, centrifuged at 13,000 rpm for 5 minutes. The supernatant was used for SDS-PAGE analysis. To demonstrate the binding of various constructs of FliG (75 μ M) to FliM (100 μ M) in absence or presence of CheY/CheY-P (100 μ M), pull-down assays were performed with His-tagged proteins as described previously⁸⁶ with minor modifications. For samples which required phosphorylation of CheY, 20 mM acetyl phosphate (Sigma-Aldrich) in the presence of 20 mM MgCl₂ was added for incubation and wash steps to the binding buffer to ensure complete phosphorylation of CheY.

Phosphono CheY generation

T. maritima phosphono-CheY was prepared and characterized in a similar manner to phosphono-CheY from *E. coli*, as previously described.⁷² Briefly, D54C/C81A CheY was reacted with 120 mM phosphonomethyltrifluoromethanesulfonate and 125 mM CaCl₂ in 125 mM BES/125 mM CHES buffer, pH 8.25. Phosphono-CheY was purified from unmodified CheY by cation-exchange HPLC using a linear gradient of LiCl in a buffer of lithium acetate. RP-HPLC was used to assess the purity of the CEX fractions. A complete description of the synthesis, purification, and characterization of phosphono-CheY will appear elsewhere.

Sample preparation for PDS

Spin-labeled proteins as well as unlabeled proteins were aliquoted in small volumes and stored at -80°C after flash freezing. The samples were prepared by incubating FliG_{MC} (50 μM) and FliM_{NM} (50 μM) (labeled or unlabeled) for 30 minutes on ice in GF buffer with 40% glycerol before flash-freezing them in liquid N_2 . For PDS measurements in presence of CheY-pP, 50 μM of CheY-pP was added to the mixture and incubated as described above.

PDS Measurements

Four pulse DEER experiment were conducted at 60 K on a 17.3 GHz FT ESR spectrometer, which is modified to perform PDS experiments.^{54; 87; 88} The baseline used for data processing was approximated by a linear polynomial. Distance distributions of spin separations within the sample were calculated by the Tikhonov method⁸⁹ and refined by the Maximum Entropy Regularization Method (MEM).⁹⁰

Gaussian fitting

For quantitative analysis we modeled the PDS-derived distance distributions as sums of five Gaussian functions representing the five distances. The probability of spin separation is then defined as:

$$P(r) = S \left\{ \left(\frac{f_1^2}{\sigma_1} \right) G_1 + \left(\frac{f_2^2}{\sigma_2} \right) G_2 + \left(\frac{2f_1 f_2}{\sigma_3} \right) G_3 + \left(\frac{f_1 f_2}{\sigma_4} \right) G_4 + \left(\frac{f_1 f_2}{\sigma_5} \right) G_5 \right\} \quad (1)$$

where S is a normalization factor, G_1 , G_2 , G_3 , G_4 , and G_5 are Gaussian means, σ_1 , σ_2 , σ_3 , σ_4 , and σ_5 are standard deviations for the five distinct distance distributions respectively and f_1 and f_2 are spin labeling efficiencies for each site. Non-linear curve-fitting was used to optimize the free parameters in eqn (1) against each of the five experimental multi-spin distance distributions using a strategy developed by Georgieva et al.⁶⁹ The C_β - C_β distances from the model and distances from control experiments were used as initial values for Gaussian means that were updated in subsequent iterations. For the fitting process, the standard deviations were constrained to be constant except for one spin (FliG 174-R1) that had a very wide distribution. For distance distributions from structured residues a 6 width serves as a practical upper limit⁶⁷. A modified eqn (1) was used (see eqn (2)) to model the anti-parallel arrangement (model I), which generates four unique distances (m - m' , g - g' , m - g , and m - g') on heterotetramer formation.

$$P(r) = S \left\{ \left(\frac{f_1^2}{\sigma_1} \right) G_1 + \left(\frac{f_2^2}{\sigma_2} \right) G_2 + \left(\frac{2f_1 f_2}{\sigma_3} \right) G_3 + \left(\frac{2f_1 f_2}{\sigma_4} \right) G_4 \right\} \quad (2)$$

Gaussian fitting of distance distributions followed the procedure of Georgieva et al., using Origin Lab software.⁶⁹

Assessment of spin labeling occupancy

The modulation depth for N-coupled spins represents the fraction of A spins effected by B-spin pumping and is given by $y = 1 - (1 - p)^N$. For a pair of spins, modulation depth becomes p provided there is 100% spin labeling. For incomplete spin labeling the modulation depth is a function of spin labeling occupancy (f), $(p, f) = 1 - (1 - fp)^N$. The pulse sequence used to calculate the spin labeling occupancy had pulse widths of 16 ns - 32 ns and a pump pulse of 32 ns. For the 17.3 GHz spectrometer, p is approximately 0.23 for the above mentioned pulse sequence. The spin labeling occupancy was hence calculated from the modulation depth of the control two-label experiments.

Multi-angle light scattering

Size-exclusion chromatography coupled with multi-angle light scattering was used to study the molar mass of the various protein fragments. Proteins (1.5 mg / mL) were run at room temperature on an SEC column (WTC050N5 - Wyatt) pre-equilibrated with GF buffer. For the FliM:FliG complex, individual components were mixed and incubated for 30 minutes. Analysis and molecular weight determination was carried out with Wyatt technologies ASTRA. Bovine serum albumin (Sigma) was used as a control for data quality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- FliG and FliM are major components of the bacterial flagellar rotor.
- Pulsed dipolar ESR spectroscopy (PDS) and crystallography define complexes formed by FliG:FliM.
- FliG assembles by intermolecular stacking and mediates FliM organization.
- The phosphorylated CheY signal destabilizes the parallel FliM alignment templated by FliG.
- Intermolecular FliG contacts may contribute to the ultrasensitive flagella switch.

Figure 1. Schematic representation of flagellar motor

(A) The general positioning of the switch complex components FliG, FliM, and FliN, and the stator unit based on various biochemical and electron microscopic analyses (adapted and modified from 3). Binding of the phosphorylated response regulator CheY-P to the switch complex changes the direction of flagellar rotation between clockwise (CW) and counter clockwise (CCW). (B) Schematic representation of different rotor proteins FliG, FliM, and FliY (FliN in *E. coli*) from *T. maritima*. Arrows indicate interactions among components. Similar colored box denotes homology among domains. Domain fragments used in this study are designated by *Thermotoga* numbering below the box.

Figure 2. Antiparallel arrangement of FliM_M:FliG_M forms a large arc in crystals

(A) Ribbon representation of the heterotetramer formed by FliM_M (tan) and FliG_M (cyan) in the crystal structure shown in (B). (B) Ribbon representation of a portion of the asymmetric unit containing seven FliM_M:FliG_M heterodimers in an alternating antiparallel assembly. Top view (below) shows that seven units produce an arc. (C) Extrapolation of the contacts found in one segment to produce a closed ring structure from five segments (or 35 copies of FliG_M:FliM_M).

Figure 3. Possible arrangements of FliM and FliG in the C-ring and spin label positions on FliM and FliG

Model I is an antiparallel dimer of the heterodimer of FliM:FliG as observed in the structure mentioned above (PDB 4QRM). Model II is a parallel back-to-front arrangement of the heterodimer of FliM_M:FliG_{MC} (PDB 4FHR). Model III is based on the FliG_{MC} crystal structure (PDB 3AJC), with a parallel arrangement of FliM beneath the FliG units. Model IV involves one FliG binding two parallel FliM molecules that tilt relative to each other.⁹¹

Figure 4. Cross-linking studies on FliM in presence of FliG

(A) Positions of cysteine residues on FliM_M used for cross-linking studies. (B) SDS PAGE gel showing results from cross-linking reactions involving Cys residues at 64 (lane 1 and 2), 64/185 (lane 3 and 4), 57 (lane 5 and 6), 57/185 (lane 7 and 8), 164 (lane 9 and 10), and 185 (lane 11 and 12). The first lane of each pair shows the reaction without the addition of initiator, whereas the second lane shows product after 1 hr of incubation without the initiator. Multimers are generated only for a parallel arrangement indicated by asterisk (C) the distances in Å separating C_β atoms expected for a parallel or antiparallel arrangement of FliM in presence of FliG.

Figure 5. FliG dependent oligomerization of FliM

(A) Ribbon representation of FliM_M (tan) and FliG_{MC} (aqua) indicating the positions of the spin labels used in this study (grey spheres). The conserved GGXG motif of FliM (dotted lines) is disordered in the crystal structure of FliM_M alone (PDB 2HP7). The stacked middle and C-terminal domains of FliG_{MC} (PDB 4FHR) shown with the EHPQR motif that mediates contacts with FliM (side chain denoted as sticks) and residue substitutions used to test interfaces (stars). (B) Time domain data of FliM 60-R1 showing increase in FliM dimerization in presence of both the domains of FliG, but not FliG_M or FliG_C. (C) Two possible arrangements of FliM and FliG based on the observations from the time domain signal.

Figure 6. A residue substitution in the FliM_M:FliG_M interface disrupts a subset of intermolecular spin-spin dipolar signals

(A) Time domain data (left) and corresponding distance distribution (right) for the FliG 160-R1:FliM 121-R1 spin pair without (orange) and with (green) the FliG Q129W substitution. Absence of the short distance component (20 Å, red dotted line) upon substituting the key interface residue FliG Q129W when compared to the WT indicates that the substitution disrupts the FliG_M:FliM_M intermolecular heterodimer, while leaving the FliG:FliG homodimer intact. (B) Time domain data (left) and corresponding distance distribution (right) for the FliG 160-R1:FliM 60-R1 spin pair without (orange) and with (green) the Q129W substitution. Loss in amplitude at distance 40 Å (red dotted line) due to disruption of the FliG_M:FliM_M heterodimer. Schematic at right illustrates the consequences of the Q129W variant on the tetrameric complex of FliM (tan) and FliG (blue). All distance distribution signals are scaled to a common maximum value for ease of comparison. Inset shows the same PDS data without scaling.

Figure 7. Residue substitutions in the FliG_C hydrophobic patch leaves the FliM:FlhG heterodimer intact, but disrupts higher-order assembly

Time domain data (left) and corresponding distance distributions (right) for spin labels at FliG 160-R1:FlhM 60-R1 spin pair (orange), (A) FliG L227W 160-R1:FlhM 60-R1 (red) and (B) FliG I204W 160-R1:FlhM 60-R1 (blue) spin pair. A loss in a peak at distance 48 Å (indicated by red dotted line) due to the mutation is attributed to the FliG_M:FliG_M homodimer distance. The cartoon on the right illustrates the disruption of the FliM (tan) and FliG (blue) tetrameric complex after introduction of the mutation (I204W in blue, L227W in red) in FliG_C. All distance distribution signals are scaled to a common value for ease of comparison. Inset shows the same PDS data without scaling.

Figure 8. Global agreement of distance distribution data

(A) Ribbon representation of the FliM_M:FliG_{MC} model used in data fitting. The dark blue color indicates the interaction between FliG_M domain and FliG_C domain of the neighboring molecule. Grey spheres indicate the position of the spin labels and dotted lines indicate the distances generated upon tetramer formation. Distance distributions of spin pairs (B) FliG 160-R1:FliM 60-R1, (C) FliG 160-R1:FliM 167-R1, (D) FliG 160-R1:FliM 121-R1, (E) FliG 174-R1:FliM 60-R1, and (F) FliG 174-R1:FliG 305-R1. Good agreement is found between the experimental (orange curve) and summed envelope from the four or five fitted Gaussians (black curve). Gaussian functions modeled the distances g-g' (solid beige), m-m' (solid green), m-g = m'-g' (solid blue), m-g' (solid pink) and m'-g (solid violet) for all five PDS experiments. The arrows on top indicate the C_β-C_β distances as measured from model III. For (B), (C), and (F) the distance distribution is fitted to four Gaussian functions instead of five. For (F) solid beige represents the FliG 305-R1:FliG 305-R1 distance, solid green represents the FliG 174-R1:FliG 174-R1 distance, solid blue represents the FliG 305-R1:FliG 174-R1 intramolecular distance, and solid pink represents intermolecular distance between the two sites. (G) Distances obtained from the Gaussian fits of PDS data versus those measured from the best fit model III.

Figure 9. The FliM:FliG_{MC} complex superimposed in the electron density map of the CW locked flagellar motor from *Salmonella*

An expanded view of a portion of the C-ring shows the best PDS fit model of the higher order protein complex relative to the electron density. Note that the C-terminal head region of FliG_C is oriented such that the charged helix (highlighted in yellow) is aligned roughly along the rotor radius.

Figure 10. Changes in FliM spin distributions with CheY-pP

Distance distributions for spin labels at (A) FliM 60-R1:FliG, (B) FliM 64-R1:FliG, (C) FliM 167-R1:FliG and (D) FliM 121-R1:FliG are shown in the absence (orange) and presence of CheY-pP (purple). Distance distributions are normalized for ease of comparison.

Table 1

Data collection, phasing, and refinement statistics

Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell (Å)	a = 105.4, b = 216.3, c = 262.2
Resolution range (Å)	50 – 4.3 (4.46– 4.3) *
$\dagger R_{\text{merge}}$	0.105 (0.389) *
I/ σ I	11.7 (3.0) *
Completeness (%)	98.8 (98.7) *
<u>Refinement</u>	
No. of reflections	36914
R work/R free	0.21/0.29
<u>No. of atoms</u>	
Residues	183 (#46–228) for each chain of FliM, 75 (#113 –187) for each chain of FliG
<u>Geometry (rmsd)</u>	
Bond lengths (Å)	0.005
Bond angles (°)	0.87
<u>Ramachandran Plot (%)</u>	
Favored	89.8
Allowed	7.8
<u>Mean B values (Å²)</u>	
Overall	167.6

* Highest resolution range for compiling statistics

$$\dagger R_{\text{merge}} = \sum \sum_i |I_i - \langle I \rangle| / \sum \sum_i I_i$$

Table 2

Model predicted C β -C β distances and PDS separations (see SI Fig. 3 and Fig. 8).

	Antiparallel	Parallel			PDS distances (Å)
	Model I distances (Å)	Model II distances (Å)	Model III distances (Å)	Model IV distances (Å)	
FliM _M -FliM _M					
60-60	25	31.9	39.2	31.3	30 [*] ; 46
64-64	11.9	32.0	38.9	31.2	20; 47 [*]
121-121	46.5	31.2	39.8	30.9	44; 58 [*]
167-167	20.4	34.6	37.3	33.9	30, 21-38; 48 [*]
FliG _M -FliG _M					
160-160	67.2	31.3	39.4	NR	47; 58 [*]
174-174	39.5	35.5	36.3	NR	32, broad
FliM _M -FliG _M					
60-160	40.6, 30.6	40.8, 56.1	40.3, 41.8, 68.1	42.3, 47.3	31; 35, 43 [*]
121-160	19.8, 53	20.8, 42	20.4, 43.5, 45.5	24.0, 42.9	20; 47 [*]
167-160	44.5, 27.4	44.8, 60.9	44.8, 43.6, 70.9	47.4, 50.6	31, 30-36; 44 [*]
60-174	35.6, 22.6	35.9, 52.3	35.9, 42.5, 60.1	34.1, 47.1	31, 25-35 [*] ; 44

For PDS signals that exhibit broad distributions, a distance range is reported. Semicolons separate multiple maxima within the same distribution. The spin-label linkers generate ± 7 Å uncertainty in the C β -C β distances. (NR: not relevant)

* In cases where the observed PDS signal consists of several related peaks, multiple R_{max} values are provided and the dominant peak is starred.