

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, we have investigated interactions between FliM and FliG from *Thermotoga maritima* with X-ray crystallography and pulsed dipolar ESR 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 higherorder 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 components fit a self-consistent model that agree 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. These 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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Introduction

Many types of bacteria control their movement by switching the sense of flagellar rotation between clockwise (CW) and counterclockwise (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 [1–3]. The bacterial flagellum consists of an export apparatus, a reversible rotary motor, a universal joint, and a filament (Fig. 1a) [1–3]. 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) [4–6]. 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 membraneembedded stator, is an oligomer composed of four MotA and two MotB subunits, which together act as proton channels and actuators for the rotor [7–11]. The FliG C-terminal domain (FliG_C) contains conserved charged residues situated on an α -helix that interacts with MotA [11–13]. FliG has two other

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Fig. 1. Schematic representation of flagellar motor. (a) The general positioning of the switch complex components FliG, FliM, and FliN, as well as 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 CW and 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.

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-P [17], the response regulator of intracellular chemotaxis signaling [18-21]. In Escherichia 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 [19,21,24-27]. FliM sits directly below FliG in the C-ring and interacts with FliG through the conserved GGXG motif in the middle domain (FliM_M) [20,28–31]. 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 [34-39]. The FliG N-terminal domain (FliG_N) interacts with FliF in the smaller, membrane-situated MSring (membrane and supramembranous ring) [40]. Fusions of FliF with truncated versions of FliG produce assembled rotors with altered electron microscopy (EM) density at the top of the C-ring [41].

Structures of major portions of the switch complex proteins together with biochemical assays have led researchers to several models for C-ring assembly [12,15,16,19,28–30,32,42–45]. EM reconstruc-

tions 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 not only 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 intramolecular or intermolecular. In the structure between FliM_M and both middle and Cterminal 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 suggest that E. coli $FliG_{C}$ also interacts with $FliM_{M}$, an observation that led us 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

Space group	P212121	
Unit cell parameters (Å) <i>a</i> <i>b</i> <i>c</i> Resolution range (Å) <i>R</i> _{merge} ^b <i>I/σI</i> Completeness (%)	105.4 216.3 262.2 50–4.3 (4.46–4.3) ^a 0.105 (0.389) ^a 11.7 (3.0) ^a 98.8 (98.7) ^a	
Refinement No. of reflections R _{work} /R _{free}	36,914 0.21/0.29	
<i>No. of atoms</i> Residues	183 (numbers 46–228) for each chain of FliM 75 (numbers 113–187) for each chain of FliG	
<i>Geometry (rmsd)</i> Bond lengths (Å) Bond angles (°)	0.005 0.87	
<i>Ramachandran plot (%)</i> Favored Allowed	89.8 7.8	
<i>Mean B values (Ų)</i> Overall	167.6	
^a Values in parentheses refe compiling statistics. ^b $R_{merge} = \sum \sum_i l_i - \langle l \rangle l / \sum_i l_i$	er to the highest-resolution range for	

Table 1. Data collection, phasing, and refinement statistics

1 out of 3 FliG molecules binds two FliM units, with one FliM binding to $FliG_M$ and the other one 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 relevance of this assembly state against other models through targeted cross-linking, multi-angle light scattering (MALS), and site-directed spin labeling [51-53] combined with pulsed dipolar ESR spectroscopy (PDS) [54-56]. Cross-linking and MALS find evidence for heterotetrameric assemblies of FliG and FliM that involve both parallel and antiparallel arrangements of the FliM subunits. The PDS data not only confirm the crystallographic heterodimeric interaction between the FliG and FliM middle domains but also support 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 perturb the PDS signals by disrupting the heterotetramers. A set of closely related models consistent with this new information and known EM images of the Cring 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 [57–60]. 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 resolution from crystals of a large unit cell that contained 11 copies of the complex per asymmetric unit. Molecular replacement was successful due to the availability of a highquality search model (PDB code 3SOH) and crystals of large solvent content (Table 1). At this resolution, each FliG_M:FliM_M unit is nearly identical with that of the previously determined structure of the same complex [30], with contact mediated through the GGPG motif of FliM and the EHPQR motif of FliG. The FliM_M molecules are arranged in an antiparallel fashion involving interactions of the long helices, $\alpha 1$ and $\alpha 1'$ 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 a 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 and 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 would point toward the cytoplasm. This antiparallel 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 $\alpha 1 - \alpha 1'$ side of FliM, with the opposite side of the adjacent subunit to generate a back-to-front repeating chain and not the antiparallel front-to-front and back-to-back arrangements 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.



Fig. 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} (aqua) 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} : FliG_{M}).

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 derived from the crystal structure described above. The second model is based on the $FliM_M$: $FliG_{MC}$ co-crystal structure (PDB code 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 185–196) is not apparent in the structure. The third model is based on the FliG_{MC} structure (PDB code 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 [30,50]. This model explains the symmetry mismatch between the MS-

ring and the 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. 3a).

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 the antiparallel (model I) arrangements of the FliM:FliG heterotetramer in solution. Unmodified FliM did not show any crosslinked 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-linked [19] (Fig. 4a). 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



Fig. 3. (a) Possible arrangements of FliM and FliG in the C-ring. Model I is an antiparallel dimer of the heterodimer of FliM:FliG as observed in the structure mentioned above (PDB code 4QRM). Model II is a parallel back-to-front arrangement of the heterodimer of FliM_M:FliG_{MC} (PDB code 4FHR). Model III is based on the FliG_{MC} crystal structure (PDB code 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 [30]. (b) 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 code 2HP7). The stacked middle and C-terminal domains of FliG_{MC} (PDB code 4FHR) shown with the EHPQR motif that mediates contacts with FliM (side chain denoted as sticks) and residue substitutions used to test interfaces (stars).

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 higherorder species, as expected by the formation of symmetric disulfides (Fig. 4b). The observed crosslinking 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 Cys185 variant did not crosslink at all, as predicted from the $C^{\beta}-C^{\beta}$ 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 Cys57 (Fig. 4c). Overall, the cross-linking results not only provide strong evidence for a parallel arrangement of FliM subunits in the presence of FliG but also reveal contributions from antiparallel dimers. Clearly, the antiparallel configuration must form in solution to some extent because it crystallizes. Most important is that the cross-linking data also find 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



Parallel Antiparallel on FliM Â Å 64 32.1 11.9 64/185 12 31.8 57 32.4 33.7 57/185 20 41.2 164 34.2 10.7 185 33.9 54.9

Fig. 4. Cross-linking studies on FliM in the 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 (lanes 1 and 2), 64/185 (lanes 3 and 4), 57 (lanes 5 and 6), 57/185 (lanes 7 and 8), 164 (lanes 9 and 10), and 185 (lanes 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 h of incubation with the initiator. Multimers are generated only for a parallel arrangement (indicated by asterisk). (c) The distances in angstroms (Å) separating C^{β} atoms expected for a parallel or antiparallel arrangement of FliM in the presence of FliG.

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 leads to altered interactions between FliM_M and FliG_C [63]. To probe interactions between $FliG_C$ and $FliM_M$ in T. maritima, we performed pull-down assays between $FliM_{NM}$ and two $FliG_C$ variants: $FliG_{204-335}$ 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 (Fig. S1). 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, we conducted PDS on spinlabeled 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 [64–68]. 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 [64–69]. 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-tetramethylpyrolinyl-3-methyl)-methane sulfonate] to produce the nitroxide side

chain known as R1 (Fig. 3b). FliM has one native, buried cysteine (Cys214), 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 (Fig. S2a).

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 produce 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 and Fig. S3). 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, along the helices α 1 and α 1', and the fourth site, D121, resides on α 3, closer to the center of the molecule (Fig. 3b).

	Antiparallel Model I distances (Å)	Parallel			PDS
		Model II distances (Å)	Model III distances (Å)	Model IV distances (Å)	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

Table 2. Model-predicted C^{β} – C^{β} distances and PDS separations (see Fig. S3 and Fig. 8)

In cases where the observed PDS signal consists of several related peaks, multiple R_{max} values are provided and the dominant peak is marked with an asterisk (*). 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).

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 the C-terminal domains is 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 antiparallel 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



Fig. 5. FliG-dependent oligomerization of FliM. (a) Time domain data of FliM 60-R1 showing increase in FliM dimerization in the presence of both domains of FliG, but not $FliG_M$ or $FliG_C$. (b) Two possible arrangements of FliM and FliG based on the observations from the time domain signal.

distributions for FliM 60-R1, 64-R1, and 167-R1 are broad and somewhat bimodal, with peaks at 20-32 Å and 45 Å (Table 2). Thus, these distributions suggest contributions from multiple species, which could include both parallel and antiparallel 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 and Fig. S3). 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 (Fig. S2a).

Interprotein 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 (*q*-*m*', from different heterodimers within a tetramer). The FliM 60-R1 and FliG 160-R1 spin pairs produce a wide distribution that can be assigned to overlapping spin separations from m-m(31 Å), g-g(43 Å), and m-g(35 Å). FliM 167-R1 and FliG 160-R1 produced a wide bimodal distribution, with a peak at ~31 Å and another one at ~44 Å. For the FliM 60-R1 and FliG 174-R1 spin pairs, 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 ~31 Å corresponds to overlapping separations from m-m', g-g', and m-g spin sites (Table 2). In summary, the interprotein distance restraints not only reflect the m-q 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, these data are more consistent with a parallel arrangement, where the spin separations are broadened by flexibility within the oligomer.

Interprotein interactions from spin labels on FliM_M and FliG_C

Based on previous cross-linking data [50], spinlabeled 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 (Fig. S1).

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, and 227) on the PDS distributions (Figs. 3b, 6, and 7). As expected, substitution of FliG Q129 to W in the EHPQR motif caused loss of the *m*-*q* heterodimer distances (and broadening of the distribution) reported both by the FliG 160-R1 and FliM 121-R1 spin pair (Fig. 6a) and by the FliG 160-R1 and FliM 60-R1 spin pair (Fig. 6b). Thus, the Q129W substitution weakens the m-ginteraction. In contrast, the dipolar signals corresponding to q-q' 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 weaken 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 they 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 (ΔPEV) (PDB code 3AJC) variant from *T. maritima* [44], as well as in crystal structures of $FliG_{MC}$ (PDB) code 1LKV), Helicobacter pylori FliG_{MC} (PDB code 3USW), and Aquifex aeolicus FliG_{FL} (PDB code 3HJL), although there is some ambiguity as to whether this interaction is intermolecular or intramolecular. These mutagenesis results indicate an intermolecular contact between FliG_C and FliG_M (model III) that mediates formation of higher-order FliG:FliM complexes.



Fig. 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 wild type 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.



Fig. 7. Residue substitutions in the $FliG_{C}$ hydrophobic patch leave the FliM:FliG heterodimer intact but disrupt higher-order assembly. Time domain data (left) and corresponding distance distributions (right) for spin labels at FliG 160-R1:FliM 60-R1 spin pair (orange) and (a) FliG L227W 160-R1:FliM 60-R1 (red) and (b) FliG I204W 160-R1:FliM 60-R1 (blue) spin pair. A loss in a peak at distance 47 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 and 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.

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 (Fig. S2b). 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_{C} : $\text{FliG}_{M + 1}$ interaction a fliG_C: $\text{FliG}_{M + 1}$ interaction fliG_C: $\text{FliG}_{M + 1}$ 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 spinspin separations upon further oligomerization, which we take to be primarily tetramerization based on MALS experiments (Fig. 8a and Fig. S2a). Spin labels on FliG alone in the presence of FliM assign the FliG:FliG homodimer distance (q-q'). 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 Eq. (1) in Materials and Methods]. We also allow for asymmetry in the assembly by fitting two distinct intermolecular distances m-q' and q-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-R1 and FliM 121-R1), 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 (Fig. S4) that all 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. (Fig. S4). 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. 8b-f) matched well with the experimental distance distribution data with the adjusted R^2 parameter for each distance distribution ranging from 0.89 to 0.97. Nevertheless, the four models were clearly distinguished, with the more structurally similar ones giving closer agreement (Fig. S4). Starting parameters that varied by several angstroms in Gaussian mean or width generally converged to the same solution. Similar analysis for the antiparallel arrangement produced considerably worse fits (Fig. S5). 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 (Fig. S6).

18 distances extracted from the fits 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 and Table S1). 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 that 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 ~47 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 ~47-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



Fig. 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. Gray 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' (beige-filled), m-m' (green-filled), m-g = m'-g' (blue-filled), m-g' (pink-filled), and m'-g (violet-filled) 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), beige-filled area represents the FliG 305-R1:FliG 305-R1 distance, green-filled area represents the FliG 174-R1:FliG 174-R1 distance, blue-filled area represents the FliG 305-R1:FliG 305-R1:FliG 174-R1 intramolecular distance, and pink-filled area 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.



Fig. 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.

FliG_C:FliG_M (PDB code 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 µs and samples prepared in D₂O to increase spin dephasing times (Fig. S3).

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 CheY [72]) 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 CheYpP 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 state [73] produced similar effects on the 60-R1 distribution as CheY-pP (Fig. S7). 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 $\alpha 1'$ (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 the presence of CheY-pP (Fig. 10d). The short FliM:FliM distances (~20 Å) from spin labels on the $\alpha 1 - \alpha 1'$ 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



Fig. 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.

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 not only 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 arrange-

ment, the relevance to C-ring assembly is guestionable. 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 pulldown 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 (Fig. S2b and 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} (Δ PEV) (PDB codes 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 [57-59]. 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, as well as 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, and 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 *H. pylori* switch proteins shows 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 suggests 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 S. 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 [30,50]. Recent photobleaching experiments confirm these subunit ratios, but they interestingly find that the number of subunits is quite variable and appears to change with the functional state of the rotor [75-77]. From a spatial perspective, the smaller circumference of the MS-ring compared to the C-ring would necessarily accommodate fewer copies of FliG (26 subunits) than FliM (34 subunits). The stoichiometry mismatch could be explained if the C-ring is "gapped" 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 (Fig. S8). 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 approximately 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 the 34-fold symmetric outer Cring [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 torgue is generated by interactions involving the stator and FliG_C alone.

Activated CheY interactions with FliM

The chemotaxis signal is highly amplified within the switch [80]. 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, fluorescence resonance energy transfer results show that binding of CheY-P to FliM is about 5-fold less cooperative than 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 the antiparallel 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 /and 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 switch [26]. 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 switching [20]. 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 baseline 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 (Agilent Technologies) 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, and 5 mM imidazole]. Cells were sonicated and centrifuged at 22,000 rpm for 1 h 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 Hise-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. We added 5–10 mM MTSSL nitroxide spin label (Toronto Research, Toronto, ON) to the column and incubated it at room temperature for 4 h 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) and 150 mM NaCl].

Crystallization

Crystals of the FliM_M:FliG_M complex were obtained by vapor diffusion at room temperature from a 2 μ L drop containing 1 μ L of well solution [0.1 M imidazole (pH 6.5) and 1.0 M sodium acetate trihydrate; Hampton Research] and 1 μ L of a mixture of two proteins FliM_M, and FliG_M (~20 mg/mL in GF buffer). Crystals were observed in 5 days. Crystals were optimized by varying precipitant concentration and pH of buffer to obtain better diffraction-quality crystals, with the optimized condition as follows: 0.1 M imidazole (pH 6.5) and 1.2 M sodium acetate trihydrate.

Data collection, structure determination, and refinement

Data were collected at Cornell High Energy Synchrotron Source station A1 using the ADSC Quantum 210 CCD. Crystals were briefly soaked in 30% glycerol before mounting for X-ray exposure. Data were scaled by HKL2000 [82] and the structure of the complex was obtained by PHENIX AutoMR [83] with PDB code 3SOH (FliM_M:FliG_M structure) as a search model. The asymmetric unit was composed 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 [83].

Disulfide cross-linking studies

Cross-linking studies on FliM and FliG were performed according to Bass *et al.* with minor modifications. Copperphenanthroline (Cu-phen) was used as the initiator. Concentrated proteins in GF buffer were diluted with disulfide reaction buffer, as described previously [84]. 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. We quenched 8 μ L of the reaction with equal volume of 2×SDS with 10 mM imidazole after 1 h of incubation. We ran 15 μ L of this mixture on the SDS-PAGE gel after heat treatment at 90 °C for 2 min. 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 50 mM imidazole]. Proteins were incubated in 40 µL Ni-NTA with the binding buffer for 30 min 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. We added 2× SDS loading dye to the resin and boiled it for 5 min at 90 °C and centrifuged it at 13,000 rpm for 5 min. The supernatant was used for SDS-PAGE analysis. To demonstrate the binding of various constructs of FliG (75 µM) to FliM (100 µM) in the absence or presence of CheY/CheY-P (100 µM), we performed pull-down assays with His-tagged proteins as described previously [85] with minor modifications. For samples that 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 phosphonomethyl trifluoromethanesulfonate and 125 mM CaCl₂ in 125 mM 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid/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. Reverse-phased 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 and 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 min on ice in GF buffer with 40% glycerol before flash freezing them in liquid N₂. For PDS measurements in the presence of CheY-pP, 50 µM of CheY-pP was added to the mixture and incubated as described above. For experiments in D₂O proteins were exchanged into buffers made up in D₂O (50 mM Tris pH 7.5; 150 mM NaCl) and incubated on ice for 30 min before flash freezing.

PDS measurements

Four pulse DEER experiments were conducted at 60 K on a 17.3-GHz FT ESR spectrometer, which is modified to perform PDS experiments [54,86,87]. 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 method [88] and refined by the maximum entropy regularization method [89].

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:

$$P(r) = S\left\{ \left(\frac{f1^2}{\sigma 1}\right)G1 + \left(\frac{f2^2}{\sigma 2}\right)G2 + \left(\frac{2f1f2}{\sigma 3}\right)G3 \qquad (1) + \left(\frac{f1f2}{\sigma 4}\right)G4 + \left(\frac{f1f2}{\sigma 5}\right)G5 \right\}$$

where S is a normalization factor; G1, G2, G3, G4, and G5 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 f1 and f2 are spin-labeling occupancies for each site. Non-linear curve fitting was used to optimize the free parameters in Eq. (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, widths were constrained to 6 Å or lower except for one spin (FliG 174-R1) that had a very wide distribution. For distance distributions from structured residues, a 6-Å width serves as a practical upper limit [67]. A modified Eq. (1) was used [see Eq. (2)] to model the antiparallel arrangement (model I), which generates four unique distances (m-m', g-g', m-g, and m-g') on hetero-tetramer formation.

$$P(r) = S\left\{\left(\frac{f1^2}{\sigma 1}\right)G1 + \left(\frac{f2^2}{\sigma 2}\right)G2 + \left(\frac{2f1f2}{\sigma 3}\right)G3 + \left(\frac{2f1f2}{\sigma 4}\right)G4\right\}$$
(2)

Gaussian fitting of distance distributions followed the procedure of Georgieva et al., using OriginLab software [69].

Assessment of spin-label occupancy

The modulation depth for N-coupled spins represents the fraction of A spins effected by B spin pumping and is given by $\Delta(p) = (1 - (1 - p))^{N-1}$. For a pair of spins, modulation depth becomes *p* provided that there is 100% spin labeling and that the spins are in range of measurement. For other cases, the modulation depth is a function of spin-label occupancy (f), $\Delta(p,f) = (1 - (1 - fp))^{N-1}$. The pulse sequence used to calculate the spin-labeling occupancy had pulse widths of 16 ns, 32 ns, 32 ns and a pump pulse of 32 ns. For the 17.3-GHz spectrometer, *p* is approximately 0.23 for the abovementioned pulse sequence. The spin-label occupancy was hence calculated from the modulation depth of the control two-label experiments.

Multi-angle light scattering

Size-exclusion chromatography coupled with MALS was used to study the molar mass of the various protein fragments. Proteins (1.5 mg/mL) were run at room temperature on an size-exclusion chromatography column (WTC050N5; Wyatt) pre-equilibrated with GF buffer. The column is coupled to an 18-angle scattering detector (DAWN HELIOS II, Wyatt Technologies). For the FliM:FliG complex, individual components were mixed and incubated for 30 min. Analysis and molecular weight determination were carried out with Wyatt technologies ASTRA. Bovine serum albumin (Sigma) was used as a control for data quality.

Accession numbers

The atomic coordinates of the structure have been deposited to the Protein Data Bank with the accession code 4QRM.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2014.12.009.

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Abbreviations used:

PDS, pulsed dipolar ESR spectroscopy; CW, clockwise; CCW, counterclockwise; EM, electron microscopy; MALS, multi-angle light scattering; DEER, double electron electron resonance.

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