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A Molecular Mechanism of Bacterial Flagellar Motor Switching

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Summary

The high-resolution structures of nearly all the proteins that comprise the bacterial flagellar motor switch complex have been solved; yet a clear picture of the switching mechanism has not emerged. Here we use NMR to characterize the interaction modes and solution properties of a number of these proteins, including several soluble fragments of the flagellar motor proteins FliM and FliG and the response-regulator CheY. We find that the switch signal, activated CheY, binds to a previously unidentified region of FliM, adjacent to the FliM-FliM interface. We also find that activated CheY and FliG bind with mutual exclusivity to this site on FliM, because their respective binding surfaces partially overlap. These data support a model of CheY-driven motor switching wherein the binding of activated CheY to FliM displaces the carboxy-terminal domain of FliG (FliG_C) from FliM, modulating the FliG_C-MotA interaction, and causing the motor to switch rotational sense as required for chemotaxis.

Introduction

A remarkable feature of the flagellar rotary motor is that it is bi-directional; using the same proton motive force it can produce torque in either the counter-clockwise (CCW) or clockwise (CW) rotational senses and can switch rapidly between the two. Flagellar motor switching is necessary for chemotaxis; *Escherichia coli* mutants that are locked in either sense of rotation are non-chemotactic (for a review see 1). This study focuses on the molecular events that initiate switching of the flagellar motor.

The small protein CheY binds to the flagellar motor and affects its rotational sense. A sharp transition from a CCW to CW motor bias is observed as the intracellular concentration of phospho-CheY (CheY~P) rises 2. CheY~P is generated at the chemoreceptor complexes and diffuses to the flagellar motors 3. When CheY~P concentrations are low, the default CCW sense of flagellar rotation is retained and the flagella bundle, causing the bacterium to swim in a relatively straight line. When a threshold level of CheY~P is reached, the motor switches to the CW sense and the flagellar bundle comes apart briefly, resulting in a random reorientation of the cell. This alternation between 'runs' and 'tumbles' enables the cell to travel up a gradient of attractant molecules or down a gradient of repellent molecules 4. At a molecular level, temporal increases in environmental quality cause chemoreceptors to inactivate the CheY kinase CheA, resulting in low concentrations of CheY~P, thereby suppressing the tumble response and producing longer runs in the favorable direction.

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Conversely, decreases in environmental quality cause activation of the CheY-kinase CheA, resulting in high CheY~P levels and a tumble event that reorients the cell in a new direction. This trial-and-error approach to directed motility results in the characteristic bacterial “random-walk.”

The aim of this study is to determine how CheY~P interacts with the flagellar motor to initiate motor reversal. A group of proteins known as the flagellar motor switch complex forms the binding site for CheY on the motor. These complexes assemble into a radial array with a barrel-like shape, identified as the C-ring of the motor. Each complex can bind one CheY~P and can subsequently act in concert with other CheY~P-bound complexes to cause motor switching. Each complex contains one FliM, four FliNs, and usually contains FliG. In *Escherichia coli* and *Salmonella*, a typical flagellar motor C-ring contains approximately 34 copies of FliM and 26 copies of FliG 5. FliM has been shown to contain an N-terminal region that binds CheY~P, and is therefore called the flagellar motor switch protein 6. FliG is known to contain charged surface residues that are directly involved in torque-generation, is located in physical proximity to the Mot complexes, and is thought to interact with MotA to affect the sense of the motor 7; 8. FliM and FliG have been shown to interact 8, and it is thought that FliG is responsible for communicating the bound-state of FliM to the Mot complexes 9; 10. FliN appears central to rotor assembly and may provide a scaffold upon which the functional interactions of FliG and FliM are assembled.

FliM and FliG have modular architectures, containing N-terminal (FliM_N, FliG_N), middle (FliM_M, FliG_M), and C-terminal (FliM_C, FliG_C) domains, and are known both to interact and self associate 10. Figure 1 shows a schematic representation of the switch components used in this study. A number of high-resolution structures of regions of these proteins from *T. maritima* have been solved. These include FliG_C 7, FliG_{MC} 8, FliM_M 11, FliN 12, and a peptide version *E. coli* FliM_N 13; 14; 15. Additionally high-resolution structures of *T. maritima* CheY are available 16, as are the structures of the highly similar *E. coli* CheY with and without phosphoanalog BeF₃⁻ 17; 18 as well as in complex with *E. coli* FliM_N 13; 14; 15. A number of insights have emerged from these studies, including identification of (i) conformational changes upon phosphorylation of CheY that enable it to bind tightly to FliM_N in a pocket far from the active site 13, (ii) a FliG_C surface that contains charged residues and interacts with MotA to generate torque 7; 19, (iii) a structural feature resembling a hinged-bar that connects the two domains of FliG_{MC} 8, and (iv) a structural model of the FliM_M 34-mer as it is arranged in the C-ring 11. Recent EM reconstructions of intact motors have also allowed researchers to accurately determine stoichiometries of, and place spatial boundaries on, the motor components 5. These reconstructions offer a stunning picture of the flagellar motor, however they are not sufficiently resolved to facilitate unambiguous docking of the component crystallographic structures. Finally, a number of studies have used cross-linking or site-directed mutagenesis to identify putative interaction surfaces amongst various switch complex proteins 11; 20; 21; 22. Despite these discoveries, the mechanism by which CheY~P switches the motor remains poorly understood. We have used NMR to characterize the interaction of a soluble fragment of *T. maritima* FliM (FliM_{NM}) with *T. maritima* BeF₃⁻-CheY (a stable CheY~P analog) and several soluble fragments of *T. maritima* FliG. We show that, in addition to binding the FliM_N region of FliM_{NM} at the expected target-binding pocket (anticipated from the *E. coli* CheY-FliM_N structure), a surface of CheY containing the phosphorylation site associates with FliM_M. The CheY~P binding surface on FliM_M is adjacent to a surface of FliM used to self-associate. Since switching is thought to proceed by a relative reorientation of associated FliM subunits (by formation of a new FliM_M-FliM_M interface 11), any effect of CheY binding at the FliM interface would be expected to affect motor bias. This second interface site is likely to be the binding site for CheY~P in certain CheY-responsive motors that lack

FliM_N 23 and may contain the catalytic residues that enable the FliM homolog FliY to act as a CheY phosphatase in *Bacillus subtilis* and other bacterial species 24.

We have also used NMR to characterize the FliM_{NM}-FliG_C and FliM_{NM}-FliG_M complexes in the presence and absence of BeF₃⁻-CheY. The FliM_M domain of FliM_{NM} binds to FliG_C near the surface that contains charged residues that are indispensable for torque-generation. These FliG_C residues form functionally important interactions with the torque generating stator component MotA 7; 19. In addition to defining the relevant binding surfaces, the NMR data indicate that when CheY binds to FliM_{NM}, FliG_C is displaced from its binding site on FliM_M. Conversely, FliG_M binds to FliM_M without regard to CheY occupancy. Consistent with these observations, we find that the CheY-FliM_M interface overlaps the FliG_C-FliM_M interface, whereas the surface of FliM_M that interacts with FliG_M apparently does not overlap the FliG_C/CheY site. We speculate that in the context of the motor, the displacement of FliG_C by CheY modulates the proximal FliG_C-MotA interface, and thereby the switch state of the motor. In total, these data suggest a molecular route by which CheY-binding could switch the rotational sense of the flagellar motor, allowing us to propose a model of the flagellar switch mechanism.

Results

Sequential Assignment and Secondary Structure Analysis

The 2D ¹H-¹⁵N NMR HSQC spectra of FliM_{NM} and FliG_{MC} are well dispersed and exhibit mostly sharp resonances at 45°C (Supplemental Material Figures 2 and 3, respectively). Backbone resonance assignments of both proteins were determined for ²H/¹⁵N/¹³C-labelled samples using a set of 3D experiments. Resonance peak positions of selectively labeled ¹⁵N-Tyr, ¹⁵N-Phe, ¹⁵N-Ala, and ¹⁵N-Leu FliM_{NM} samples were also considered during assignment of FliM_{NM}. Very few reliable assignments could be made in the linker connecting the two domains of FliM_{NM}, perhaps due to conformational heterogeneity and/or solvent exchange effects in this region. For FliG_{MC}, most of the residues in the linker region were detected and could be assigned.

The amide resonance peaks of wild-type *T. maritima* CheY have been assigned 25. The binding of BeF₃⁻ to *T. maritima* CheY results in resonance peak perturbations in similar regions of the protein as those observed in a previous study when *E. coli* CheY binds BeF₃⁻18. In *E. coli*, this complex has been shown to act as stable analog of the short-lived phosphorylated form 26. Backbone resonances of *T. maritima* CheY, BeF₃⁻-CheY, and BeF₃⁻-CheY in complex with unlabeled FliM-19 (a synthetic peptide consisting of the 19 N-terminal residues of *T. maritima* FliM) were assigned as described in Supplemental Experimental Procedures and are shown in Figure S1.

FliM_N is Flexibly Linked to FliM_M and Retains Structure

The CheY target region (FliM_N) is short, typically comprising fewer than twenty residues at the FliM N-terminus. This region is linked to FliM_M by a stretch of approximately 35 residues that are not well conserved (except for positions 39 and 40 which are almost invariably Asp/Tyr) and are typically rich in hydrophilic sidechains. The amide resonance peaks assigned to residues in the N-terminal domain of FliM_{NM} are significantly more intense than those in the middle domain, precluding the possibility that the two domains undergo rotational diffusion as a single rigid entity in solution.

Resonance peaks associated with FliM_N are moderately well dispersed (precluding a fully random-coil conformation). Additionally, secondary structure derived from alpha carbon chemical shift values 27; 28 for residues in the FliM_N agree well with the crystallographically determined secondary structure (Supplemental Material Figure 4),

indicating that FliM_N is at least partially structured in solution, even in the absence of CheY. Previous hydrodynamic measurements showed that a similar FliM construct, comprising the N-terminal region, linker, and middle domains, has a radius of gyration much larger than would be expected for a globular protein of its molecular weight 11. In contrast, a construct containing only FliM_M gave values typical for a globular domain of its size 11. By measuring backbone ¹⁵N T₁ and T₂ relaxation times for FliM_{NM}, the current study confirms that FliM_N has significant conformational freedom relative to the middle domain in FliM_{NM} (Supplemental Material Figure 4). While the FliM_M domain tumbles in solution at a rate typical for a globular protein of its molecular weight ($\tau_C \approx 13$ ns at 45°C), the FliM_N region tumbles significantly faster ($\tau_C \approx 5$ ns, treating the motion of each amide as independent of the others). In light of the mobility of FliM_N, the linker region connecting FliM_N and FliM_M clearly functions as a flexible tether. The previous hydrodynamic study did not attempt to ascribe the observed flexibility to the FliM_N or the linker regions, instead the authors suggest that the entire N-terminal segment of FliM_{NM} (including the FliM_N and linker region) is conformationally disordered 11. The results of our current study indicate that, perhaps paradoxically, the FliM_N region is significantly structured although the flexible “tether-like” linker allows it significant conformational freedom.

The correlation time of the FliM_N region of FliM_{NM} is longer than would be expected for an isolated 19-mer peptide. This likely reflects some rigidity in a partial structure and the spatial constraints imposed by the flexible linker connecting the two regions, a feature that has been shown to be important for signaling in a similar chemotaxis proteins²⁹, (see Supplementary Materials discussion).

The NMR data also show that FliM_N remains mobile relative to FliM_M when FliM_{NM} binds to FliG_{MC}. In these experiments, amide resonance peaks associated with the FliM_M domain broaden beyond detection when FliM_{NM} forms a complex with FliG_{MC}. This suggests that FliG_{MC} may be able to bind and bridge to FliM monomers. More experiments are needed to investigate this possibility. By contrast, the resonance peaks associated with the FliM_N region of FliM_{NM} do not lose dispersion and remain sharp under the same conditions.

CheY Binds to Both FliM_N and FliM_M

In *E. Coli*, FliM_N binds to the $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface of CheY. Several high-resolution structures of this complex have been determined 13; 14; 15. Our NMR data show that when BeF₃⁻-activated *T. maritima* CheY is saturated with FliM-19, resonances from a cluster of residues on this same surface shift, suggesting a common binding mode for the CheY-FliM_N interaction from both sources. The primary sequences of both of these protein regions are highly similar between the two organisms. Therefore, although there is currently no high-resolution structure of *T. maritima* CheY-FliM_N complex available, the proteins appear to associate in much the same manner as in *E. coli*.

When BeF₃⁻-activated CheY is titrated into a sample of FliM_{NM}, resonance peaks in both the N-terminal and middle domains of FliM_{NM} shift substantially (Figure 2A). Such shifts are not seen in the presence of BeF₃⁻ alone. When *T. maritima* CheY alone is added to FliM_{NM}, resonances corresponding to FliM_N show chemical shifts while most of the FliM_M resonances do not shift, suggesting that activation of CheY generates a distinctly different complex than does CheY alone.

Several lines of evidence argue that BeF₃⁻-activated CheY binds to both domains of FliM_{NM}. To discriminate a *bona fide* binding event from effects such as propagated conformational change, an amide-based contact point strategy was employed. This amide based strategy is similar to the methyl contact point strategy we used previously to characterize large protein complexes 30. The essential feature of this method is that only

resonances at the protein-protein interface are broadened when a deuterated binding partner is substituted with a protonated binding partner. Comparing the amide resonances of ^2H , ^{15}N -FliM_{NM} in complex with either ^2H -CheY or ^1H -CheY, several residues (100, 198, 212 and 213) that shift substantially when FliM binds ^2H -CheY are also broadened when bound to ^1H -CheY suggesting that the middle domain of FliM is in physical contact with CheY.

The middle domain of FliM is sufficient to form the observed CheY-FliM_M complex, since CheY binds the same surface of a FliM construct containing only the middle domain, although the magnitude of the observed resonance peak shifts are significantly smaller and the affinity is dramatically reduced.

The resonance peak intensities of FliM_{NM} also become more uniform when CheY binds, indicating that the FliM_N domain loses mobility when CheY binds. This result is consistent with previous hydrodynamic data showing that the extended conformation of FliM_{NM} collapses to a globular structure when CheY binds 11.

BeF₃⁻-CheY-induced perturbations in the FliM_M portion of FliM_{NM} map to a discrete region on the FliM_M surface, with the largest perturbations observed for Ser-95 and Val-96 that cap the C-terminus of FliM_M α -helix two (Figure 2B). This region also contains two other affected residues (Met-97 and Ile-98) that form a solvent-exposed hydrophobic patch, as might be expected for a protein interaction surface (Figure 2B).

FliM_M is in Direct Contact with the Active Site Surface of CheY

In an attempt to identify the BeF₃⁻-CheY surface used to interact with the middle domain of FliM, we compared the resonance peaks of the BeF₃⁻-CheY-FliM-19 complex with those of the CheY-FliM_{NM} complex (Figure 3A). Resonances in the FliM_N-binding region of CheY are in similar positions for the two complexes, yet both shift substantially from the corresponding BeF₃⁻-CheY peaks. Therefore, the CheY-FliM_N interaction does not appear to be perturbed by the presence of the linker or middle domain of FliM. Conversely, resonance peaks corresponding to a cluster of surface residues that includes the CheY phosphorylation site are in different positions when bound to either FliM_{NM} or FliM-19. This suggests that FliM_M binds to this region of BeF₃⁻-CheY. (Figure 3B). A comparison of the interaction with deuterated versus protonated FliM_{NM}, as described above, confirms that this region of BeF₃⁻-CheY binds to FliM_M.

FliM_{NM} Binds to FliG_{MC}, FliG_C, and FliG_M

Samples of ^{15}N labeled FliG_{MC}, FliG_C and FliG_M were titrated with unlabeled FliM_{NM} and their interactions were monitored until saturated with FliM_{NM}. Labeled FliM_{NM} samples were also observed when titrated with the FliG constructs. When labeled FliG_{MC} is titrated with FliM_{NM} the NMR peaks broaden beyond detection presumably due to the increased size of the resulting complex. When observing labeled FliM_{NM} in the presence of unlabeled FliG_{MC}, the peaks also broaden beyond detection except for the FliM_N resonances that stay sharp. To circumvent the limitations caused by the size of this complex, the FliG_{MC} construct was divided into its two domains creating the FliG_C and FliG_M constructs. Both constructs were found to bind FliM_{NM} as judged by chemical shift perturbation. Labeled FliG_C was then titrated with FliM_{NM} and resonance peak shifts were observed to exchange between free and bound in the intermediate to slow exchange regime on an NMR timescale (Figure 4). This made an exact determination of the binding affinity of FliG_C for FliM_{NM} difficult to determine. We estimate a dissociation constant of approximately 200 micromolar. The resonances of FliG_C that shifted upon interaction with FliM_{NM} were identified by assigning the backbone resonances of FliG_C. When mapped on the crystal

structure of FliG_C, these residues form a patch near the residues required for torque generation (Figure 4). When labeled FliM_{NM} is titrated with FliG_C, the resonances that shift map to a surface on the FliM_{NM} crystal structure (Figure 5) and overlapped with the region where CheY binds to FliM_{NM}.

CheY Modulates Formation of the FliM_{NM}-FliG_C Complex

A three-way protein titration was performed with BeF₃⁻-CheY and observed from the perspectives of either labeled FliM_{NM} or FliG_C. First, labeled FliG_C was saturated with unlabeled FliM_{NM} to form the FliG_C-FliM_{NM} complex. Next this complex was saturated with BeF₃⁻-CheY. Resonance peaks were monitored throughout the titration. When BeF₃⁻-CheY is added to the FliG_C-FliM_{NM} complex, the FliG_C resonances generally return to the positions observed for FliG_C alone (Figure 4). The addition of CheY to a FliM_{NM}-FliG_C complex disrupts the association FliG_C and FliM_{NM}. Similarly when the CheY-FliM_{NM} complex is saturated with FliG_C, resonance peaks of CheY generally return to the position observed when CheY is bound to FliM_N only. Therefore, we conclude that the interaction of FliG_C and CheY with FliM_M is mutually exclusive. This competition has the form of an allosteric modulation of activated CheY affinity by FliG_C. In the absence of FliG_C, activated CheY has higher affinity for FliM_{NM} by virtue of its interactions with both FliM_N and FliM_M. When FliG_C is bound to FliM_M, CheY has a lower affinity since it only interacts with FliM_N.

Formation of the FliM_{NM}-FliG_M Complex Is Not Modulated by CheY

The same multiple complex titrations were performed using the middle domain of FliG with FliM_{NM} and CheY. When FliG_M-FliM_{NM} complex is titrated with CheY the FliG_M resonance peaks do not shift significantly, retaining their positions observed for the two-way complex despite some broadening due to increased molecular size. Therefore the binding of CheY does not perturb the FliG_M and FliM_{NM} interaction.

Discussion

CheY Interacts with the Middle Domain of FliM

The NMR data presented indicate that a surface of FliM_M including a hydrophobic patch at the C-terminus of α -helix two interacts with a surface of CheY that includes the active site and α -helix one. This previously unreported association takes place in addition to the known association of CheY with the target region at the N-terminus of FliM. A single CheY is able to associate with both regions of FliM simultaneously, by virtue of the flexible linker connecting FliM_N to FliM_M.

The presence and location of the CheY-FliM_M interface is consistent with the results from a number of previous studies. In co-precipitation assays, the CheY-FliM interaction was detected even for truncated FliM mutants that lacked FliM_N 9. Several FliM mutations in the middle domain have been shown to rescue non-chemotactic CheY mutants 31. These second-site revertants were generally found in a region of FliM_M near the surface found here to bind CheY. CheY has also been shown to bind full-length FliM more tightly than FliM_N, implicating a second interaction site 32; 33.

In some species, flagellar motors contain FliM proteins that lack FliM_N altogether, yet remain CheY-responsive 23. Therefore, it is clear that the CheY-FliM_N interaction is not required for CheY to modulate the state of the motor. Rather, a different CheY interaction - perhaps with the site on FliM_M identified in this study - has primacy in the switching process, at least in the abovementioned species. In support of this contention, there are no reports of CheY responsive motors that lack both FliM_N and FliM_M (or their homologues).

Our data indicate that the CheY phosphorylation site is at the CheY-FliM_M interface. Since a number of CheY phosphatases (FliY, CheC, CheX, known as the CYK protein family 34) share the FliM_M fold 11; 35, the CheY-FliM_M interaction we identify may enable the FliM homolog FliY to catalyze CheY~P dephosphorylation. Proteins in the CYK family are thought to function using the same strategy as the unrelated CheY-phosphatase, CheZ, which inserts catalytic residues directly into the CheY active site 34; 36. Indeed, the catalytic residues in CheC/CheX shown to be responsible for CheY-phosphatase activity are conserved in FliY, but not in FliM 11; 34. Our data suggest that activated CheY binds near alpha helix 2 and that would appear to place it far from the active sites inferred from CheC/CheX in alpha helix 1. Experiments are in progress to clarify this point.

Implications for Flagellar Motor Cooperativity and Sensitivity Enhancement

Flagellar motor switching is extremely sensitive to CheY~P concentration (Hill coefficient $\approx 10^2$). Crane, Blair and coworkers proposed that switching involves a relative reorientation of the FliM subunits, and that this happens by modification of the FliM_M-FliM_M interface 11. CheY could convert the two-state motor switch in an all-or-none manner by interacting more favorably with switched subunits than with unswitched subunits 37. According to this Monod, Wyman and Changeux (MWC) formalism, CheY~P discriminates the state of individual FliM proteins as switched (CW) or unswitched (CCW), and interacts more favorably with the former to promote switching.

The NMR data presented in this study indicate that CheY~P binds to a surface of FliM_M adjacent to the FliM_M-FliM_M interface 11. In fact, one of the residues of *T. maritima* FliM (Arg-94) shown to be at the CheY~P-FliM_M interface in the present study was also able to crosslink to neighboring FliM_M subunits 11. The proximity of the CheY-binding site to the FliM_M homo-oligomer interface illustrates the potential for interplay between CheY-binding and remodeling of the FliM_M-FliM_M interface. Our data suggest that CheY (or more specifically, the tethered CheY~P-FliM_N complex) could promote switching by interacting more favorably with a CW FliM_M-FliM_M interface than a CCW FliM_M-FliM_M interface. In the context of the Crane model of the FliM_M 34-mer 11, our data indicate that the CheY binding site on FliM_M is on the inside of the C-ring barrel.

Under the previous assumption that CheY interacts with a single site on FliM, it is difficult to reconcile the low cooperativity of CheY-binding (Hill coefficient ≈ 1 38) with the high cooperativity of switching (Hill coefficient $\approx 10^2$). Previous authors have speculated that the signal amplification happens within the switch, after CheY~P binds, although the mechanism of this amplification was unknown 38. It is also unclear how the observed sensitivity-enhancement could be generated if CheY interacts only with the flexibly tethered FliM_N region. It may be that the dual-binding site architecture of FliM that we have described can lend clarity to these issues. This architecture would allow the observed low cooperativity of binding and high cooperativity of switching. This is because CheY~P binding to the motor is driven primarily by the strong interaction with FliM_N, yet we imagine that motor switching is driven by the subsequent weak interaction with FliM_M. Our data obviates the need to explain the sensitivity-enhancement with the formation of the CheY-FliM_N complex alone, since interactions with FliM_M must clearly now be considered as well. In the MWC formalism, the large number of FliM subunits in the motor can generate high cooperativity in switching with a relatively small change in CheY affinity between the CW and CCW states thus producing low cooperativity in CheY binding. We imagine that activated CheY binding to FliM_N alone is not cooperative and does not, in itself, cause cooperative switching. In this view, cooperative interactions are likely caused by FliM_M-FliM_M interactions as suggested by the Blair and Crane laboratories 11. These interactions would then modulate the affinity of activated CheY for FliM_M.

FliG Changes Conformation when CheY and FliM Associate

An unanticipated finding of this study is that FliG_C and CheY cannot bind simultaneously to the FliM_M region of FliM_{NM}. NMR binding experiments indicate that titrating BeF₃⁻-CheY into a stoichiometric complex of FliG_C and FliM_{NM} displaces FliG_C (Figure 4). Similarly, titration of FliG_C into a stoichiometric complex of BeF₃⁻-CheY and FliM_{NM} displaces CheY from FliM_M (although CheY remains bound to the FliM_N region). By using NMR chemical shift perturbation mapping to define the FliM_M-FliG_C and FliM_M-CheY interfaces, we find that there is a significant overlap between the binding surfaces of FliG_C and CheY on FliM_M, primarily at the C-terminal cap of FliM_M alpha helix 2 (Figure 5). In the context of the fully assembled motor, these data prompt us to speculate that (i) under default conditions where no CheY is bound (CCW rotation), FliG_C and FliM_M associate as in solution, and (ii) the binding of CheY to FliM_M displaces FliG_C from its binding site on FliM_M.

In contrast, FliG_M remains complexed to FliM_M both in the presence and absence of CheY, and in the presence or absence of FliG_C. The NMR data clearly indicate that three-way FliG_M-FliG_C-FliM_{NM} and FliG_M-CheY-FliM_{NM} complexes are possible and form readily in solution. This result indicates that the surface used by FliG_M to bind the FliM_M domain does not overlap the CheY- or FliG_C-binding surfaces on FliM_M. The assignments of FliG_M and characterization of the relevant binding interfaces on its surface will be described elsewhere.

FliG_C interacts directly with MotA to generate torque, and a surface of FliG_C containing several charged residues that are critical for generating torque in *E. coli* has been identified 7: 19. These residues are highly conserved across different species, and are similar in *E. coli* and *T. maritima*. The surface used by FliG_C to bind FliM_{NM} and MotA are in close proximity, although they do not overlap (Figure 6).

The NMR data presented indicate that when CheY binds to FliM_{NM}, FliG_C is displaced from FliM_M but FliG_M is not. Because the two domains of FliG are clearly covalently attached in the physiological motor, such a scenario would require a drastic reorientation of FliG_C relative to FliG_M. Analysis of the FliG_{MC} structure indicates that such a drastic relative reorientation of domains may be possible, by virtue of the hinged-bar characteristic of the linker connecting FliG_M to FliG_C 8. The two domains of FliG_{MC} are spatially separated by a rod-like helix. A pair of glycine residues at the C-terminal end of this helix makes what has been described as a “hinge” 8. This hinge is conserved as a glycine pair, is likely to confer relative conformational freedom to the FliG_M and FliG_C, and appears to be important for motor switching 39.

A Model of CheY-Based Motor Switching

For CheY to initiate motor switching, it must affect the interaction between rotor and stator components that are responsible for generating torque. FliM, the binding site for CheY in the motor, does not directly interact with the torque-generating Mot complexes. However, FliM binds to FliG, and FliG interacts directly with the Mot complexes 40. Mutational data indicate that a particular group of charged residues in the C-terminal domain of FliG are responsible for torque generation, and that this surface contacts the torque generating MotA protein. The surface of FliG_C indicated in the present study to interact with FliM_{NM} is directly adjacent to this region of FliG_C that contains the conserved charge residues that generate torque by interacting with MotA (Figure 6). The present NMR data indicate that FliG_C is displaced from FliM_M when CheY and FliM associate. By disrupting the FliM-FliG_C interaction, CheY-binding to FliM is expected to affect the orientation and/or conformation of the proximal Mot binding surface of FliG_C. These results indicate a molecular route by which CheY could affect motor bias (Figure 7).

There are eight fewer FliG than FliM proteins in a typical *E. coli* or *S. typhimurium* flagellar motor. This means that CheY~P can interact with the FliM_M in the motor in two ways: With and without needing to displace FliG_C. If each FliG_C in the motor forms a one-to-one complex with FliM_M, as in solution, that would leave eight “naked” FliM_Ms, with the remaining 24 being “clothed” by FliG_C. Displacing FliG_C requires energy, so CheY will bind these “naked” FliM_M sites before binding to the “clothed” FliM_Ms. Because these events will happen sequentially as CheY~P concentrations rise, we refer to them as Phase 1 and Phase 2. In Phase 1, low CheY~P concentrations allow the CheY~P-FliM_N complexes to bind to corresponding FliM_M subunits lacking a bound FliG_C (which number at minimum 8 for the typical motor). We imagine that Phase 1 is unproductive with respect to switching, since it does not affect the rotor/stator interaction. In Phase 2, the CheY~P concentration and motor-occupancy reach a threshold, and the CheY~P-FliM_N complexes can only bind to the remaining FliM_M subunits that have a bound FliG_C (at most 24 for the typical motor), causing that FliG_C domain to be displaced. We anticipate that Phase 2 is responsible for motor switching, since modulating the nature of the default FliG_C-MotA interaction is tantamount to modulating the rotor/stator interaction.

The Tethered-Bait Binding Strategy

If CheY can initiate switching by binding only to FliM_M in certain motors 23, what is the purpose of FliM_N? CheY interacts with full-length FliM with only moderately higher affinity than FliM_N, indicating that CheY binds to FliM_M with low affinity. Consistent with this notion, the association of CheY and FliM_M was not detected in previous calorimetric studies 11. While the reported CheY- FliM_M interface was formed between a construct containing only the FliM_M domain and the BeF₃⁻-CheY-FliM-19 complex *in trans* by NMR, the affinity was quite low ($K_d \approx 1$ mM).

A similar binding strategy that involves a mobile high-affinity recognition element, or bait, tethered to a second low-affinity binding site is prevalent among chemotaxis signal transduction complexes; in addition to the FliM/FliY and CheY~P complex, the CheA-CheY 41:42, CheZ-CheY 32, Tar/Tsr-CheR 43, and Tar/Tsr-CheB 44 complexes all associate using a similar tethered bait strategy. In these complexes, which represent every regulated protein-protein interaction in the simplest and best understood (*E. coli*) chemotaxis signal cascade, the tethered bait is typically required for the target to associate effectively with the second, often catalytic, site, as seen for FliM. Computational models suggest that a tethered bait can present the second site with local concentrations of bound target as high as several millimolar 45. Several DNA-binding proteins take advantage of this phenomenon by connecting a high-affinity site to a low-affinity site with a flexible linker 46. This strategy also increases the substrate-target encounter frequency, thereby enhancing the potential enzymatic rate at the second site 29. In the case of the CheR-Tar chemotaxis signaling complex, simulations show this effect to be indispensable for effective catalysis, and therefore necessary for chemotaxis, consistent with the impaired phenotypes of mutants lacking the bait region 29.

Because the tethered baits typically exhibits a strong preference for substrate over product (or signal [CheY~P] over non-signal [CheY], in the case of FliM), this strategy may also act as a selectivity filter for the second site. Enhanced presentation of the signal/substrate is especially advantageous to the low affinity site when high background concentrations of non-signal/product molecules might otherwise effectively compete. For enzymes, this separation of functions would allow the catalytic site to optimize catalytic rate without regard for ligand selectivity (performed in this case by the bait), and is likely to enable the uniquely rapid requirements of chemotaxis signal processing. For FliM, this strategy appears to also allow the motor its high sensitivity to CheY~P with relative insensitivity to inactive CheY.

Experimental Procedures

Protein Preparation

FliM_{NM} and FliG_{MC} were expressed using plasmids pKP43 and pJY5, respectively. Both plasmids were kind gifts from David Blair (University of Utah). The pKP43 plasmid encodes amino acids 1 to 229 of *T. maritima* FliM with an octa-histidine tag and TEV protease site at the N-terminus 11. In some experiments with FliM_{NM}, the histidine tag was removed by treatment with TEV protease. This produced no changes in the NMR resonances of FliM_M but some FliM_N resonances either shifted into highly overlapped regions or were lost due to dynamics or hydrogen exchange. The pJY5 plasmid, a derivative of pKM260, encodes amino acids 104 to 334 of *T. maritima* FliG and also contains a TEV-cleavable histidine tag at the N-terminus. FliG_M (104-193) and FliG_C (193-334) plasmids were constructed from the pJY5 plasmid and sub-cloned into pet22b vector for expression. The resulting FliG_C contains a C-terminal histidine tag.

E. coli BL21 (λDE3) cells were used to produce all proteins. Following growth to an OD₆₀₀ of 0.6 at 37°C, cells were induced using 1 mM IPTG. Ampicillin was used in the growth media at 100 µg/ml. Cells producing CheY or FliG constructs were harvested after 4 hours at 37°. Cells producing FliM_{NM} were harvested after 6 hours at 30°C.

FliM_{NM} was purified as described previously 11 with the following modifications: an initial heat-shock at 80°C for 15 minutes was added, and AcTEV protease (Invitrogen) was substituted for thrombin. Full length *T. maritima* CheY was expressed and purified as described previously 25. FliG constructs were purified as follows: cells were resuspended in French Press Buffer A (25mM Tris pH 7.5, 300mM NaCl, and 5mM Imidazole). The cells were ruptured using a 40k French Press cell. The solution was then placed in an 80°C water bath for 15 minutes, followed by a 16k centrifugation for 45 minutes. The supernatant was then loaded onto a nickel-chelating column, washed with 25mM imidazole and eluted with 200mM imidazole. The eluate was dialyzed into NMR buffer A (25 mM Tris 7.5, 200 mM NaCl, 0.5 mM EDTA). After dialysis the eluate was concentrated to 2ml and then subjected to gel filtration in the same buffer. The fractions containing the desired protein (as detected by SDS-PAGE gel) were selected and then concentrated to 0.5 to 2 mM. For samples that required removal of the N-terminal affinity tag, AcTEV protease (Invitrogen) was used following the manufacturer's instructions. The purification tag on the N-terminus of FliM_{NM}, containing the histidine and TEV tags, was left intact for all experiments except for those involving CheY binding, where the tag was removed with TEV protease. Production of isotopically enriched protein samples is described in the Supplemental Experimental Procedures.

NMR spectroscopy

All spectra were recorded at 45°C on a Varian Inova 600 MHz spectrometer equipped with a cryoprobe, except for the FliM_{NM} backbone assignment spectra which were recorded at 40°C to facilitate the long-term stability of the protein. All samples included 25 mM Tris pH 7.5, 200 mM NaCl, 0.5 mM EDTA, and 0.02% sodium azide, and 8% (v/v) D₂O. All FliM NMR samples also contained 10% (v/v) d-methanol and 5 mM DTT, which were found to increase the long-term stability of the protein. Backbone assignments were determined using sequential connections in the following pairs of experiments: HNCACB, and CBCA(CO)NH 47; 48; 49. In the case of FliG_{MC} TROSY-optimized versions of these experiments were used to assign the backbone.

Protein titration experiments were performed using 100 to 200 micromolar ¹⁵N or ²H, ¹⁵N labeled protein and various levels of either unlabeled or ²H labeled interaction partners. All samples included 25 mM Tris pH 7.5, 200 mM NaCl, 0.5 mM EDTA, and 0.02% sodium

azide, and 8% (v/v) D₂O. For experiments involving BeF₃⁻ activated CheY the above buffer was used and in addition it contained 10 mM MgCl₂, 8 mM NaF and 2 mM BeSO₄. The ¹⁵N labeled interaction partner was observed using either conventional ¹H-¹⁵N HSQC spectra or TROSY HSQC spectra.

Determinations of ¹⁵N T₁, T₂ and NOE relaxation properties of FliM_{NM} were made at 45°C using standard pulse sequences 50; 51; 52.

The data were processed using nmrPipe 53 and resonance assignments were performed on a Linux operating system using a modified version of ANSIG 54; 55. Resonance assignments are described in the Supplementary Materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A schematic representation of the components of the flagellar switch. CheY is a diffusible protein that binds to the 19 residue recognition sequence at the N-terminus of FliM (shown as M_N in the figure). FliM also interacts with FliG. The two C-terminal and middle domains of FliG, FliG_C (shown as G_C) and FliG_M, are joined by an apparently flexible glycine-glycine linker. The FliG_C also interacts with the stator components (shown as MotAB) to generate torque.

Figure 2.

Activated CheY interacts with the FliM middle domain in FliM_{NM}.

A. ^1H - ^{15}N TROSY HSQC NMR spectra of ^2H , ^{15}N labeled FliM_{NM} (treated with TEV protease to remove the poly-histidine tag) alone (black) and in the presence of ^2H labeled $\text{BF}_3\text{-CheY}$ (red). FliM_{NM} is present at 150 μM and CheY is present in excess (200 μM). Resonance peaks corresponding to the FliM_M domain of FliM_{NM} that are perturbed most with activated CheY are labeled. Arrows indicate the shift from bound to free. The residue with the largest shift, 96, and other significantly perturbed residues (e.g. 95, 97, 98) map to the C-terminus of FliM α -helix two. The remaining labeled resonance peaks form a surface surrounding FliM α -helix two, putatively located on an exposed region of FliM_M on the inside face of the C-ring barrel, adjacent to a FliM_M-FliM_M homo-oligomer contact.

B. Activated CheY binds to a discrete surface of FliM_M, centered on α -helix two. The residues most affected when $\text{BF}_3\text{-CheY}$ binds are shown in red. Residue 96, which is perturbed more than any other FliM_{NM} residue, is shown in magenta. The interfaces that are thought to link neighboring FliM_M monomers in the assembled motor are shown in green.

The point of origin for the linker that connects FliM_M to FliM_N is colored cyan. Blue shows the N-terminus of FliM_M. The drawings were made with PyMol and the solid representation is a Connolly surface⁵⁶ as calculated by PyMol⁵⁷.

Figure 3.

The FliM middle domain in FliM_{NM} interacts with a region containing the phosphorylation site of activated CheY.

(A) ^1H - ^{15}N TROSY HSQC NMR spectra at 45°C of the BF_3 -CheY-FliM_{N19} complex (black) and the BF_3 -CheY-FliM_{NM} complex (red). CheY is ^2H , ^{15}N labeled and present at 200 μM in both cases. Excess FliM_{N19} (800 μM) is present in one sample and excess ^2H labeled FliM_{NM} (230 μM) is present in the other. The buffer for both contains 50 mM TRIS, 50 mM NaCl, 0.5 mM EDTA, 1 mM BeSO_4 , 3mM NaF and 10 mM MgCl_2 . Resonance peaks corresponding to residues in activated CheY that differ in the two spectra are perturbed specifically by the presence of FliM_M and are numbered. The peaks with the largest shifts correspond to residues 14 and 107.

(B) Perturbed resonance peaks map to a region of CheY (magenta) that includes the phosphorylation site (yellow) and α -helix one (top of the structure) and is not near the

recognition peptide binding pocket (position 106 labeled in cyan, bound FliM peptide is shown on the left image only). The drawing is a Connolly surface⁵⁶ as calculated by PyMol⁵⁷.

Figure 4.

When activated CheY binds to FliM_{NM}, it disrupts the FliM-FliG_C association. ¹H-¹⁵N HSQC NMR spectra of ¹⁵N labeled FliG_C alone (black), the FliG_C-FliM_{NM} complex (blue) and the FliG_C-FliM_{NM} complex after the addition of BeF₃⁻-CheY (red) are shown. FliG_C was present at 200 μM in 25 mM Tris pH 7.5, 200 mM NaCl, 0.5 mM EDTA, and 8% (v/v) D₂O (black). Unlabeled FliM_{NM} was added to 450 μM in the same buffer (blue). Then MgCl₂, BeSO₄ and NaF were added to final concentrations of 10 mM, 2 mM, and 8 mM respectively. No changes were seen in the spectra (not shown). Then unlabeled CheY was added to a final concentration of 600 μM (red). The resonance peaks that shift form a surface of FliG_C responding to the formation of the FliM_{NM} complex (Figure 5). The resonance peaks corresponding to these residues of FliG_C shift back to their free (black) positions when FliM_M is saturated with CheY. In the insets, the path of peak shifts for two residues of FliG_C are shown. Because CheY and FliG do not interact (data not shown) this result indicates that the release of FliG_C from FliM_{NM} is a consequence of the interaction between activated CheY and FliM_{NM}.

Figure 5.

A shared surface of FliM_M is affected when activated CheY and FliG_C bind. Residues perturbed when activated CheY binds are shown as red spheres. Residues perturbed by FliG_C are shown as yellow spheres. Residues perturbed by both FliG_C and activated CheY are shown as cyan spheres. The two views of FliM_M are related by a 90° rotation. The drawing was generated with PyMol57.

Figure 6.

Residues of FliG_C that are perturbed when FliM_{NM} binds (blue spheres) are seen in proximity to FliG_C residues that are important for torque generation (red). These FliG residues are conserved and form a charged ridge on the FliG_C surface that is thought to interact with the cytoplasmic domain of MotA to generate rotational torque. The surface formed by these residues is directly adjacent to the FliM_M interaction sites. The two views of FliG_C are related by a 90° rotation. The drawing was generated with PyMol.

Figure 7.

Proposed molecular mechanism of CheY promoted flagellar motor switching. In the absence of CheY~P binding, some stochastic switching of the motor occurs, however, it is predominantly in the default CCW (*E. coli*) or CW (*T. maritima*) sense of rotation that produces smooth swimming as shown on the left of the figure. At low concentrations, CheY~P does not interact with the motor, and it retains its strong default bias, (CCW for *E. coli*, upper red arrow). As CheY~P concentration increases, CheY~P interacts with FliM_N and FliM_M sequentially: The affinity of CheY~P for FliM_M alone is weak. CheY binding is driven by tight binding to the tethered FliM_N target. The interaction of CheY~P with FliM_M displaces the FliG_C domain from its binding site on FliM_M. This requires a large-scale reorientation of the FliG_C domain, modifying the proximal surface of FliG_C relative to the stator components that generate torque and are anchored to the membrane and

peptidoglycan. We imagine that this event reverses the rotational sense of the motor. Theoretical work has established that FliG reorientation relative to MotA could switch the sense of rotation of the motor. Recent experimental work has reported that FliG can act as a molecular “clutch” and that perturbing the FliG_C-MotA interaction directly affects the rotational output of the motor 58.