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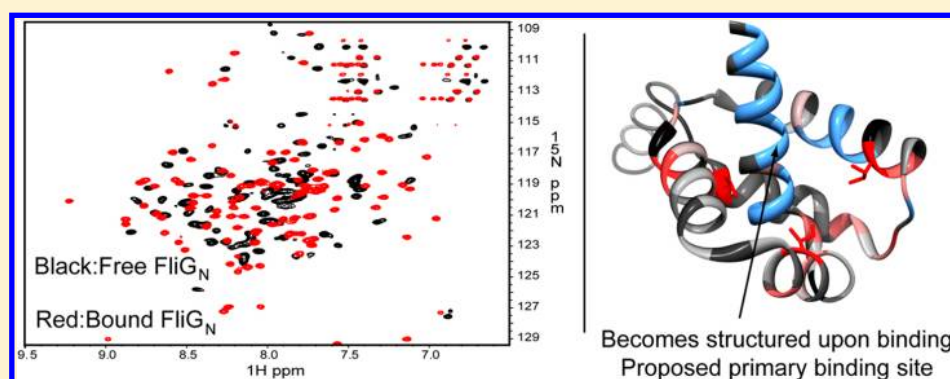
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Structural Insights into the Interaction between the Bacterial Flagellar Motor Proteins FliF and FliG

Robert Levenson, Hongjun Zhou, and Frederick W. Dahlquist*

Department of Chemistry and Biochemistry, University of California, Santa Barbara, California 93106-9510, United States

S Supporting Information



ABSTRACT: The binding of the soluble cytoplasmic protein FliG to the transmembrane protein FliF is one of the first interactions in the assembly of the bacterial flagellum. Once established, this interaction is integral in keeping the flagellar cytoplasmic ring, responsible for both transmission of torque and control of the rotational direction of the flagellum, anchored to the central transmembrane ring on which the flagellum is assembled. Here we isolate and characterize the interaction between the N-terminal domain of *Thermotoga maritima* FliG (FliG_N) and peptides corresponding to the conserved C-terminal portion of *T. maritima* FliF. Using nuclear magnetic resonance (NMR) and other techniques, we show that the last ~40 amino acids of FliF (FliF_C) interact strongly (upper bound K_d in the low nanomolar range) with FliG_N. The formation of this complex causes extensive conformational changes in FliG_N. We find that *T. maritima* FliG_N is homodimeric in the absence of the FliF_C peptide but forms a heterodimeric complex with the peptide, and we show that this same change in oligomeric state occurs in full-length *T. maritima* FliG, as well. We relate previously observed phenotypic effects of FliF_C mutations to our direct observation of binding. Lastly, on the basis of NMR data, we propose that the primary interaction site for FliF_C is located on a conserved hydrophobic patch centered along helix 1 of FliG_N. These results provide new detailed information about the bacterial flagellar motor and support efforts to understand the cytoplasmic ring's precise molecular structure and mechanism of rotational switching.

Directed bacterial motility is driven by the regulation of the rotational direction of the bacteria's flagella. Flagella alternate between clockwise and counterclockwise rotation, propelling the bacteria toward optimal environments through a "biased random walk" mechanism.^{1,2} The flagellum can be considered as being assembled upon an inner membrane ring (the MS-ring) composed of ~26 copies³ of the transmembrane protein FliF. Torque to rotate the flagellar motor is generated by peptidoglycan-anchored membrane protein complexes composed of two proteins, MotA and MotB, which encircle the rotating motor. Both transmission of torque from the Mot proteins and control of the rotational direction of the motor ("switching") rely on a ring of proteins at the cytoplasmic face of the flagellum called the C-ring. The C-ring is composed of three proteins with differing copy numbers: FliG (~26 copies per ring),⁴ FliM (~34 copies),^{5,6} and FliN (110–140 copies).^{7,8} FliG is an entirely α -helical protein composed of three domains connected by linkers. The first ~90 amino acids (in *Thermotoga maritima*) of FliG form the N-terminal domain, FliG_N, which

interacts with FliF and is discussed in greater detail below. The other two domains of FliG, FliG_M (residues ~110–190) and FliG_C (residues ~195–335), have been implicated in both assembly and switching of the motor.^{9,10} FliG_C is thought to form the direct torque-generating interactions with the Mot proteins through a series of charged residues located along a ridge in the domain.¹¹ FliM has been shown to be critical for switching¹² and has been shown both in vitro and in vivo to form interactions with both FliG_M and FliG_C.¹³ The exact roles of these interactions remain under intense investigation.^{14–17} FliN, which exists in an approximately 4:1 ratio to FliM, is thought to assemble in a pseudosymmetric tetrameric ring and interact with the C-terminal domain of FliM, and has been implicated in both assembly and switching of the flagellum.^{18,19}

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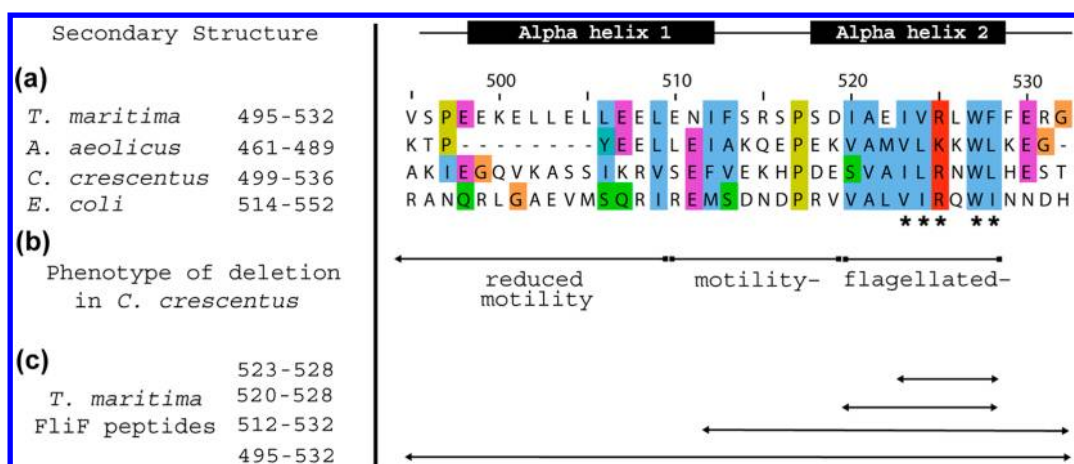


Figure 1. Sequence alignments, phenotypes of mutations, and FliF_C peptides used in this report. (a) Multiple-sequence alignment of the cytoplasmic C-terminal segment of FliF. The predicted secondary structure is shown above the sequences. The most conserved amino acids in α -helix 2, determined from analysis of a larger set of sequences, are marked with stars. Sequence numbers in the respective organisms are shown next to the organism's name. Alignment was performed using ClustalX.⁴¹ The multiple-sequence alignment image was made in Jalview⁴² and colored according to the default ClustalX scheme. (b) Phenotypes of *C. crescentus* mutants²⁷ with deleted segments in the indicated ranges. "Flagellated" mutants did not have visible flagellation. "Motility" mutants, while flagellated, did not show motility on soft agar plates. (c) Portions of FliF_C represented by the synthetic peptides that were tested in this paper.

The assembly of the flagellum is thought to begin with the organization of transmembrane protein FliF into the MS-ring.²⁰ Next, the C-ring is formed around the MS-ring.²¹ Because binding of FliG to FliF is essential for C-ring construction, and the C-ring is necessary for assembly of the remainder of the flagellum,^{22,23} the interaction between FliF and FliG represents one of the first identified steps in the assembly of the flagellum. Two-hybrid screening,²⁴ scanning deletions,⁹ and the generation of partially motile FliF–FliG fusion proteins^{25,26} have indicated that the first ~50 amino acids of FliG_N are essential for, and thus likely involved in, binding to FliF. An *in vivo* study using *Caulobacter crescentus* showed that deletions within the last 38 amino acids of FliF (excluding a few residues at the very C-terminus) affect flagellar assembly and bacterial motility, again presumably through their involvement in the interaction of FliF with FliG.²⁷ These 38 amino acids (FliF_C) are well-conserved and are predicted by secondary structure prediction algorithms to form two α -helices (Figure 1a). A highly conserved (residues 520–528 in *T. maritima*) portion of FliF_C, including a nearly absolutely conserved tryptophan (W527 in *T. maritima*), was found to be required for assembly (Figure 1b). An extended sequence, including the region just described and including an additional 10 amino acids (residues 510–528, in total, in *T. maritima*), was found to be essential for motility. Lastly, the full 38-amino acid FliF_C sequence (residues 491–528 in *T. maritima*) was identified as being necessary for full wild-type motility. In this study, we have reconstituted the interaction between FliF_C and FliG_N *in vitro* and have used a combination of NMR and other techniques to characterize their binding.

MATERIALS AND METHODS

Protein Purification. *T. maritima* FliG_N constructs were generated from a FliG_{NM} construct encoded on vector pJY5 provided by D. Blair (University of Utah, Salt Lake City, UT), with an N-terminal eight-His tag and a TEV protease site. The FliG_N constructs were created by introducing stop codons at appropriate sites. Constructs were confirmed by DNA sequencing.

All proteins were expressed in BL21-Rosetta cells grown at 37 °C. Cells grown in LB medium were induced with 1 mM IPTG at an OD₆₀₀ of ~0.5, followed by a 6 h expression after which cells were pelleted and frozen at –80 °C for later use. Isotopically enriched proteins were grown in M9 medium from starter cultures in LB using standard methods. Briefly, pelleted cells from overnight cultures of cells grown in LB were added to M9 minimal medium containing [¹⁵N]ammonium chloride, with [¹³C]glucose and/or D₂O when appropriate. After recovery had been allowed, cells were induced at an OD₆₀₀ of ~0.4 and allowed to express overnight before being harvested.

FliG_N protein, after cell lysis and a 10 min heat shock at ~70 °C, was purified by nickel-affinity chromatography. Column-bound FliG_N was washed with 130 mM imidazole, followed by elution from the column with 250 mM imidazole. FliG_N was further purified by size-exclusion fast performance liquid chromatography, providing pure protein as shown on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels. The purified protein was then concentrated and stored at 4 °C or frozen at –80 °C until it was used. The final buffer used for NMR and fluorescence experiments consisted of 50 mM sodium phosphate, 100 mM NaCl, and 0.5 mM EDTA (pH 6.5).

The DNA sequence encoding *T. maritima* full-length FliG_{NMC} was obtained from the Joint Center for Structural Genomics (JCSG). The FliG_{NMC} sequence, including an N-terminal six-His tag, was then subcloned into the pET-22b vector. Purification and buffer conditions were as described above, except FliG_{NMC} was washed with 30 mM instead of 130 mM imidazole.

When the reaction was performed, the N-terminal His tag on FliG_N was excised using AcTEV protease (Invitrogen). Digestion was performed at 30 °C until completion, using SDS–PAGE analysis for confirmation of cleavage.

Synthetic FliF_C Peptides. Synthetic FliF_{495–532} peptide (~90% pure) was obtained from EZ Biolab or NEO BioScience. Synthetic FliF_{512–532}, FliF_{520–528}, and FliF_{523–528} peptides (~80% pure) were obtained from Biomartik Corp.

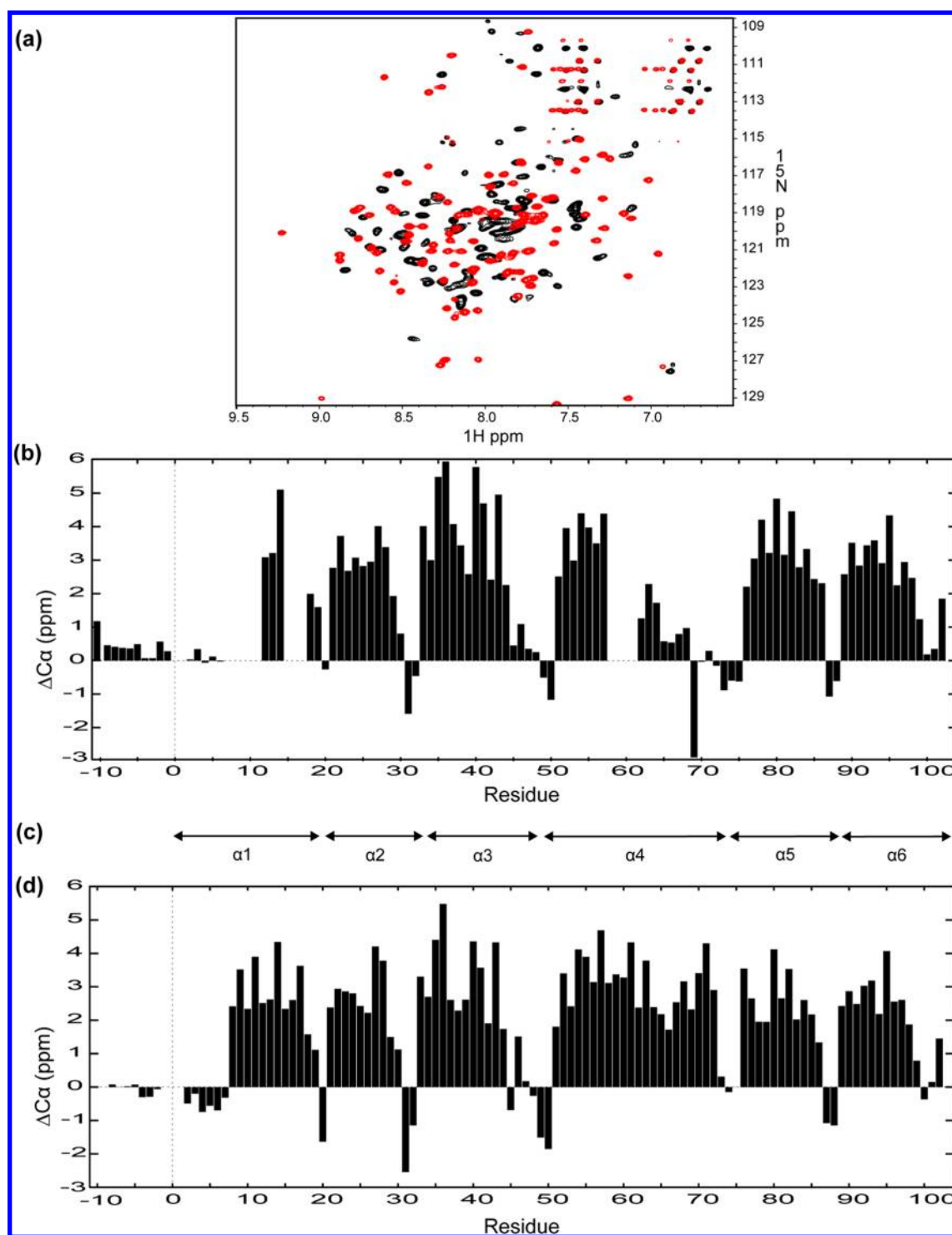


Figure 2. NMR data from FliG_N bound and not bound to FliF_{495–532}. (a) Overlay of ¹H–¹⁵N TROSY-HSQC spectra of [¹⁵N,²H]FliG_N (black) and [¹⁵N,²H]FliG_N with FliF_{495–532} (red). (b) C α chemical shift deviations from random coil values in free FliG_N. (c) Locations of α -helices in FliG_N, as observed in the *A. aeolicus* crystal structure and from computational predictions. (d) C α chemical shift deviations from random coil values in FliG_N bound to FliF_{495–532}.

For use in experiments, lyophilized peptide was dissolved in final buffer and then adjusted to the appropriate pH.

NMR Spectroscopy. NMR experiments were performed on either a Varian Inova 600 MHz or a Bruker Avance III Ultrashield Plus 800 MHz spectrometer. All experiments were performed at 40 °C with 8% (v/v) D₂O added to buffer. Initial assignments of FliG_N were made using HNCACB^{28,29} and CBCA(CO)NH^{29,30} pulse sequences with an ~0.5 mM ¹⁵N-

and ¹³C-labeled FliG_N sample. Later confirmation of FliG_N assignments and the assignments of ¹⁵N-, ¹³C-, and ²H-labeled FliG_N bound to unlabeled FliF_{495–532} was performed using HNCA, HNCACB, HN(CO)CA, and HN(CO)CACB sequences.^{29,31,32} Binding between labeled FliG_N and peptides was observed using ¹H–¹⁵N HSQC and ¹H–¹⁵N TROSY-HSQC pulse sequences. NMR data were processed using

nmrPipe,³³ and assignments were performed with a modified version of ANSIG.³⁴

Chemical shift assignments have been deposited in the BioMagResBank as entries 18309 (FliG_N) and 18310 (labeled FliG_N bound to unlabeled FliF_{495–532}).

Fluorescence Spectroscopy. FliG_N was purified to homogeneity before analysis. The synthetic peptide was prepared at appropriate concentrations as described above. The concentration of the peptide was determined using absorption of UV light at 280 nm ($\epsilon = 5500 \text{ M}^{-1} \text{ cm}^{-1}$). Each step in the shown titrations was measured 10 times and averaged. Titrations were repeated at least twice. Fluorescence analysis was performed using a Perkin-Elmer LS 55 fluorimeter and analyzed using FL WinLab.

Size-Exclusion Chromatography and Light Scattering. Size-exclusion chromatography with static light scattering analysis was performed using a system consisting of a Wyatt Technologies Minidawn TREOS multiangle light scattering detector, a Wyatt Technologies t-REX refractive index detector, a Waters UV detector, and an HPLC pump, with proteins separated on Wyatt Technologies HPLC size-exclusion columns. Purified individual proteins or protein complexes were run at $\sim 1 \text{ mg/mL}$. Wyatt Technologies ASTRA software was used for analysis and molecular weight determination. Size-exclusion chromatography–light scattering experiments were performed in 25 mM Tris, 200 mM NaCl, and 0.5 mM EDTA (pH 7.4).

RESULTS

All the experiments in this work were performed with proteins from *T. maritima*, a monotrichously flagellated thermophile originally harvested from thermal vents.³⁵ Proteins from *T. maritima* have been used in many structural studies of the flagellar C-ring, and crystal structures of FliG_{MC},¹⁰ the N-domain and middle domains of FliM,³⁶ and most of FliN⁸ now exist. More recently, the crystal structure of full-length FliG from *Aquifex aeolicus* was determined, providing the first structure of the FliG_N domain,¹⁵ although in the absence of FliF. There is good sequence conservation of FliG among *Escherichia coli*, *T. maritima*, *A. aeolicus*, and *C. crescentus*, so it likely that the general structure and mechanism of torque transmission of FliG is conserved among organisms. To facilitate NMR studies and to isolate the interaction of interest, we generated or obtained smaller protein constructs of FliG and FliF, respectively. On the FliG side, multiple FliG_N constructs were derived from a *T. maritima* FliG_{NM} construct generously provided by D. Blair. FliG_N protein constructs encoding residues 1–52, 1–73, and 1–89 of FliG were found to be unfolded, as judged by analysis of one-dimensional proton NMR and/or ^1H – ^{15}N correlated spectroscopy using ^{15}N -enriched purified protein (data not shown). A construct encoding residues 1–102, which includes the entire FliG_N domain and approximately one-half of the α -helix linker that connects FliG_N to FliG_M, was found to be stable and had moderately well-dispersed peaks (Figure 2a, black). This construct, which includes an N-terminal eight-His tag along with a TEV protease site, was used for the rest of this study and will be termed simply FliG_N. Removal of the affinity tag was found to have no significant effects on the NMR spectra of labeled FliG_N, so all data shown here were for FliG_N with the His tag attached.

NMR Characterization of FliG_N and FliF_C Peptide Binding. Using triply labeled (^{15}N , ^{13}C , and ^2H) protein,

FliG_N resonances were assigned using standard three-dimensional (3D) NMR techniques. Analysis of $\text{C}\alpha$ secondary shifts from random coil values indicates a six-helix protein (Figure 2b), in agreement with the published crystal structure of FliG_N from *A. aeolicus* FliG and computational secondary structure predictions (Figure 2c).^{37,38} We were able to assign $\sim 86\%$ (excluding affinity tag residues) of the protein backbone amide resonances. Missing resonances were grouped into two regions, composed of residues on helix 1 of FliG_N (amino acids 7–11 and 15–17) and a series of residues on helix 4 (amino acids 58–61). These resonances are likely broadened because of the dynamic properties of the residues, indicating a lack of structural rigidity in these areas. Helix 1 forms the central helix around which the rest of the domain is folded, and a lack of stable structure in helix 1 likely leads to increased conformational heterogeneity in the rest of the protein. Notably, the broadened resonances corresponding to amino acids 15–17 are located in the center of a highly conserved row of hydrophobic residues in the FliG_N sequence (Figure S1 of the Supporting Information). While examination of the crystal structure of FliG_N from *A. aeolicus* shows that some of these residues are buried in the core of the domain, others are completely or partially solvent exposed. Residues 58–61 of helix 4, also not detected because of resonance broadening, are located immediately next to helix 1 in the crystal structure of FliG_N, further indicating instability in the region surrounding the central helix. The lack of structure in this area of helix 4 propagates through to the helix 4–helix 5 turn.

As discussed above, an earlier in vivo study using *C. crescentus* showed that deletions of different portions of FliF_C caused varying phenotypic effects. To examine these effects, we purchased synthetic peptides from commercial sources corresponding to four selected portions of FliF_C, which we used to probe for an interaction with FliG_N (Figure 1c). These peptides correspond to the regions necessary in vivo for flagellar assembly (peptides FliF_{520–528} and FliF_{523–528}), the region necessary for motility (FliF_{512–532}, covering only helix 2 of FliF_C), and the region necessary for wild-type motility (FliF_{495–532}, including both helices 1 and 2 of FliF_C). We then tested for interactions between these peptides and [^{15}N , ^2H]-FliG_N using ^1H – ^{15}N TROSY-HSQC experiments. As the synthetic peptides were not enriched with NMR-visible isotopes, all the NMR analysis was performed from the perspective of the labeled FliG_N and does not provide direct information about the structure or conformational changes of the FliF_C peptides.

We observed no shifts in the spectrum of FliG_N when it was mixed with large excesses of peptides FliF_{520–528} and FliF_{523–528}, suggesting that these smaller peptides do not interact with FliG_N. Both larger peptides did bind FliG_N and caused widespread perturbations across the spectrum, indicating large conformational changes in FliG_N. Shifts in FliG_N upon peptide binding were notably different between FliF_{495–532} and FliF_{512–532}. Binding of FliF_{495–532} to FliG_N is shown in Figure 2a (red). Binding of FliF_{512–532} is compared with binding of FliF_{495–532} and the unbound FliG_N spectrum in Figure S2a of the Supporting Information. Although the overall FliG_N chemical shift perturbations from the two peptides are comparable in magnitude and direction, which shows that the general mode of binding is similar, few resonances in the bound spectra overlay. The spectrum of FliG_N bound to FliF_{512–532} is not intermediate between those of unbound FliG_N and FliG_N bound to FliF_{495–532}. Instead, the differences between the

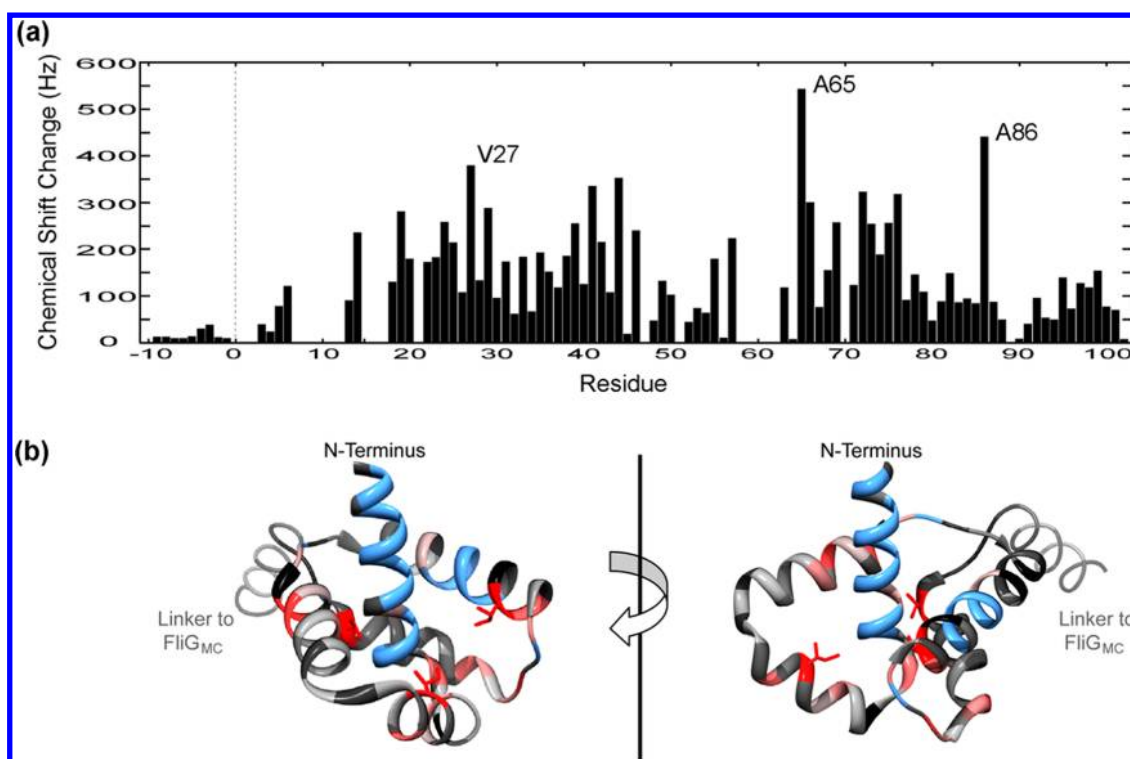


Figure 3. Chemical shift perturbations in FliG_N upon FliF_{495–532} binding. (a) Chemical shift perturbations to amide ¹⁵N–¹H resonances upon addition of FliF_{495–532} to ¹⁵N- and ²H-labeled FliG_N. The shifts are shown as absolute values. (b) Chemical shift perturbations from the NMR titration mapped onto the crystal structure of *A. aeolicus* FliG_N. The relative sizes of shifts increase in the following order: black < gray < red. Blue denotes residues for which a change cannot be mapped because of a lack of assignment in either the bound or unbound state, almost all due to missing residues in unbound FliG_N. The residues with the largest shifts are shown as sticks (corresponding in *T. maritima* to A86, A65, and V27, in clockwise order starting from the top left red residue on the left side). The protein image was created using UCSF Chimera.⁴³

spectra of FliG_N bound to the two peptides indicate that α -helix 1 of FliF_C plays an important structural role in FliG_N binding. This implies that the contact interface between FliF and FliG_N is widespread across both helices 1 and 2 of the FliF_C peptide. Because the FliF_{512–532} peptide could not reproduce the interaction of the larger FliF_{495–532} peptide (in addition to poor solubility), we chose to use FliF_{495–532} for further characterization.

Binding of FliF_{495–532} to FliG_N. Because binding to FliF_{495–532} (as well as FliF_{512–532}) occurred in the slow-exchange regime, 3D NMR experiments were used to assign the resonances of FliG_N when it was in complex with the peptide; ~99% of the FliF_{495–532}-bound FliG_N backbone amide resonances were assigned. Relative to that of unbound FliG_N, the spectrum of FliG_N bound to the FliF_{495–532} peptide was much sharper and had better dispersion of peaks. α secondary shifts show that the secondary structure of FliG_N bound to FliF_{495–532} is similar overall to that of unbound FliG_N (Figure 2d). However, the regions of FliG_N that were missing resonances and likely unstructured in the absence of FliF_{495–532} were found to be easily assignable in the peptide-bound state and to now possess significant secondary structure. This likely reflects a large reduction in the level of conformational exchange for these residues. Both helices 1 and 4, which had little structure in unbound FliG_N, have strong α -helical structure when bound to FliF_{495–532}. Changes in the chemical shifts of *T. maritima* FliG_N upon binding FliF_{495–532} (Figure 3a) are shown mapped onto the crystal structure of *A. aeolicus* FliG_N (Figure 3b). The three resonances showing the largest chemical shift perturbations upon peptide binding are

V27, A65, and A86 (all in *T. maritima* numbering), all partially buried hydrophobic amino acids oriented toward the central helix 1 of FliG_N. Large chemical shift perturbations are distributed across the first ~80 amino acids of FliG_N, covering helices 1–4. Helices 5 and 6 have relatively smaller perturbations, with the exception of A86, indicating that they are more distant from the interaction site. Because of the widespread changes in chemical shifts across the domain, it is not possible to determine a precise binding site for the peptide directly from chemical shift perturbation mapping. Altogether, the data indicate that the interaction between FliF_{495–532} and FliG_N is extensive and that the interaction between FliF_C and FliG_N likely causes large propagated conformational changes and provides significant stability to the N-terminal domain of FliG.

Characterization of FliG–FliF_C Complexes by Size-Exclusion Chromatography with Light Scattering. We noted while performing NMR experiments that the resonances of FliG_N were broader than expected for its predicted monomeric size (predicted $M_w \approx 13.9$ kDa), and that binding of FliG_N to the peptide led to an unexpectedly large increase in the sharpness of peaks. We thus used a size-exclusion chromatography–light scattering–refractive index system to characterize the oligomeric state of FliG_N alone and in complex with FliF_{495–532}. We found that *T. maritima* FliG_N forms a tight homodimer in the absence of peptide (calculated $M_w \approx 31$ kDa), while FliG_N bound to FliF_{495–532} forms a heterodimeric complex (calculated $M_w \approx 17$ kDa) (Figure 4a). Both complexes have very similar retention times on size-exclusion columns despite an ~10 kDa difference in molecular weight,

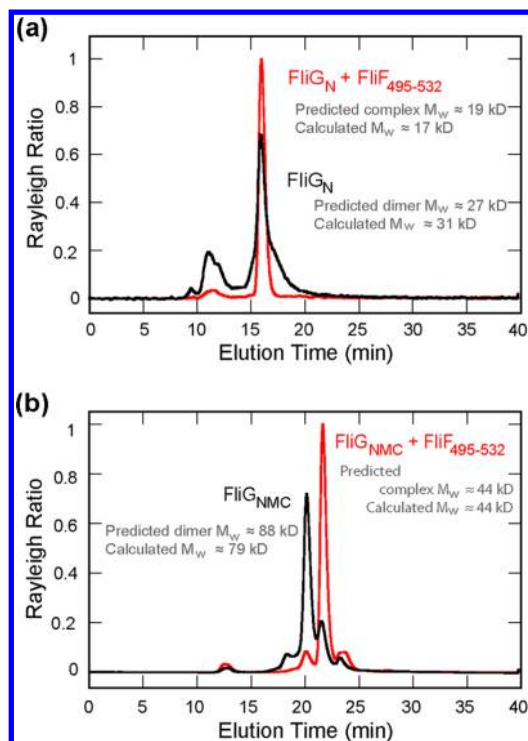


Figure 4. Size-exclusion chromatography–light scattering analysis of FliG constructs with and without FliF_{495–532}. (a) Chromatogram of the light scattering signal of *T. maritima* FliG_N with or without the FliF_{495–532} peptide. (b) Chromatogram of the light scattering signal of full-length *T. maritima* FliG_{NMC} with or without the FliF_{495–532} peptide.

possibly because of the cylindrical shape of the FliG_N construct used in these experiments. This indicates that the dimeric interface should occur along the longer cylindrical surface of the protein instead of on either of the cylinder's bases, which would be expected to cause a larger change in retention time. Despite the similar retention times, the oligomeric forms of the different complexes are clearly differentiated by the experimental setup because of the protein concentration calibration from the refractive index detector. To see if these results apply to the full FliG protein as well, we obtained a construct encoding full-length *T. maritima* FliG. Like FliG_N, purified full-length FliG forms a tight dimer in the absence of peptide (calculated $M_w \approx 79$ kDa) and forms a heterodimeric complex when bound to FliF_{495–532} (calculated $M_w \approx 44$ kDa) (Figure 4b). In this case, a difference in retention time is clearly observed. Because the middle and C-terminal domains of FliG do not oligomerize (data not shown), this dimerization of free FliG must be driven by the FliG_N domain.

Fluorescence Analysis of Peptide Binding. FliF_C contains a nearly absolutely conserved tryptophan residue (W527 in *T. maritima*). We therefore monitored the intrinsic fluorescence of W527 in FliF_{495–532}, the only tryptophan present in both *T. maritima* FliF_C and FliG_N, as a function of FliG_N to probe binding. Addition of FliG_N to the peptide led to an ~ 4 -fold increase in the apparent quantum yield as well as an ~ 10 nm blue shift in the emission maximum of the tryptophan (Figure 5a,b). Both effects indicate that W527 moves from a solvated to an unsolvated environment when interacting with FliG_N. The stoichiometry of binding was one FliF_C peptide to one subunit of FliG_N, in line with experiments indicating a 1:1 ratio of FliF to FliG in the flagellar motor.²⁶ The affinity of

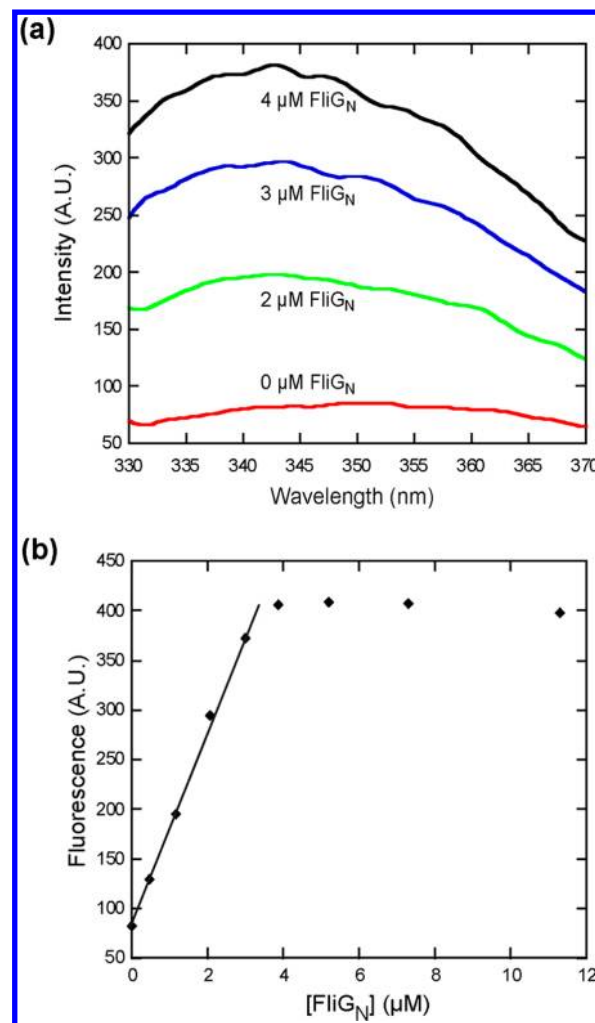


Figure 5. Changes in fluorescence from W527 in FliF_C upon FliG_N binding. (a) Fluorescence spectrum from 330 to 370 nm of a titration of ~ 4 μ M FliF_C peptide with FliG_N. (b) Fluorescence intensity ($\lambda_{\text{ex}} = 295$ nm, and $\lambda_{\text{em}} = 352$ nm) of another titration of ~ 4 μ M FliF_{495–532} peptide with FliG_N.

FliF_{495–532} was too strong to be measured by the fluorescence assay; binding was stoichiometric within the usable range of the fluorescence signal [lowest interpretable measurement using ~ 40 nM FliF_{495–532} (data not shown)]. We therefore estimate an upper bound for the dissociation constant in the low nanomolar range, though the affinity may indeed be significantly stronger than that. We also measured the affinity of the shorter FliF_{512–532} for FliG_N using the same fluorescence assay. From the binding curve, we measured a K_d of ~ 84 nM (Figure S2b of the Supporting Information), demonstrating that although there is strong binding between FliF_{512–532} and FliG_N, the shorter peptide interacts at least 1 order of magnitude more weakly with FliG_N than FliF_{495–532} does.

DISCUSSION

In this study, we have reconstituted and characterized in vitro the interaction between the C-terminal region of FliF and the N-terminal region of FliG (FliG_N). Previous in vivo work with *C. crescentus* has shown that a relatively small series of residues located at the C-terminus of FliF (FliF_C) are necessary for flagellar assembly and has noted distinct phenotypic effects for particular deletion mutations in this region. In particular,

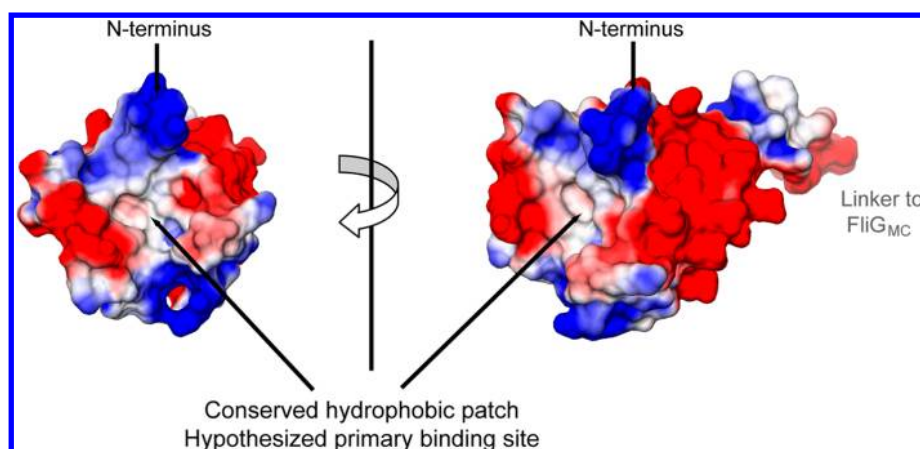


Figure 6. Poisson–Boltzmann electrostatic surface map of *A. aeolicus* FliG_N. Blue represents negative surface, red positive surface, and white neutral surface. Electrostatic field generated using the APBS⁴⁴ plugin for PyMOL. Image rendered using UCSF Chimera.

analysis has identified one region necessary for flagellar assembly, a larger region necessary for motility, and a yet larger area necessary for wild-type motility. We investigated the interaction between these proteins directly by obtaining synthetic peptides corresponding to these regions. Our data correlate the phenotypic effects of the *in vivo* mutants with differences in the binding between our FliF_C peptides and FliG_N *in vitro*. We observed that minimal peptides corresponding to the most highly conserved regions of FliF_C are not sufficient in themselves to ensure binding. However, the large increase in the apparent quantum yield of the highly conserved W527 in FliF_{495–532} upon FliG_N binding indicates that this region is certainly directly involved in binding when it is part of a larger peptide. One explanation for this disparity may be that the shorter FliF_{520–528} and FliF_{523–528} peptides are not sufficiently folded to form the necessary interactions with FliG_N. Observation of an upfield methyl ¹H peak in FliF_{512–532} and FliF_{495–532}, but not in the shorter peptides (data not shown), suggests that the longer peptides have some structure while the shorter ones do not. Differences in the chemical shift perturbations of FliG_N backbone resonances upon binding FliF_{512–532} or FliF_{495–532} indicate that both FliF_C helices 1 and 2 are involved in interactions with FliG_N. This conclusion correlates nicely with the *in vivo* observation that a FliF protein lacking helix 1 of FliF_C is sufficient for flagellar assembly and some motility but cannot support wild-type motility.

The reason for the importance of FliF_C helix 1 in ensuring wild-type motility is unclear. The interaction between FliF and FliG has been previously postulated to be purely structural, based on a lack of isolated switch-biasing mutants in the N-terminal region of FliG, despite attempts at their generation.³⁹ The second transmembrane segment of FliF is separated from the conserved helices 1 and 2 of FliF_C by ~35 unconserved amino acids. Though these residues might be expected to provide some flexibility in the positioning of FliF_C, it is possible that the observed phenotypic effects of the FliF_C helix 1 deletion mutants are due to either a forced translocation or reorientation of the FliG_N domain, therefore preventing the connected middle and C-terminal domains from assuming their normal arrangements. A FliF–FliG fusion mutant, with some reduced motility,²⁵ was shown by EM analysis to have altered C-rings, possibly explaining its motility defects.²⁶ Alternatively, the weakened affinity of a shortened FliF_C for FliG_N might lead to a greater degree of dissociation of FliG from FliF *in vivo*,

which could have effects on motility because this intermolecular interaction is necessary for transmitting the torque from the stators to the body of the flagellum. Photobleaching experiments using a wild-type *E. coli* FliG–GFP fusion showed no fluorescence recovery, indicating that there is normally little or no turnover of FliG in the C-ring.⁴⁰ Lastly, it is possible that the correlation of an *in vivo* motility effect with the observed difference in binding reflects an unidentified role of the FliG_N–FliF_C complex in torque conduction or switching, though what that role might be is unknown.

Using fluorescence spectroscopy, we were able to probe binding of FliF_{495–532} to FliG_N. As expected, we measured a 1:1 binding stoichiometry between FliF_C and FliG_N. We were able to determine an upper bound for the dissociation constant between FliF_{495–532} and FliG_N in the low nanomolar range. The interaction between FliF and FliG has previously shown itself to be very hardy: pH 2.5 conditions are necessary to dissociate FliG from FliF in flagellar basal body extracts.²⁷ Given that FliG is responsible for transmitting the torque from the Mot proteins to the basal body of the flagellum, it would be expected that this intermolecular interaction is very strong, as keeping these domains together is crucial for normal flagellar function.

We observed that *T. maritima* FliG tightly dimerizes through the FliG_N domain in the absence of FliF_C. A ¹⁵N- and ¹³C-edited NOESY⁴⁵ of a mixture of ¹⁵N- and ¹³C-labeled FliG_N domains shows that there is a broad interface between subunits on the face of FliG_N formed by helices 2, 3, and 5 (Figure S3a,b of the Supporting Information). The dimer we observe in our experiments is symmetrical, because only one set of peaks is seen in our NMR spectra. Because this dimer disappears upon FliF_C binding, it is likely that peptide binding causes propagated conformational changes that eliminate the dimerization interface between subunits. It is unknown what the biological role, if any, of this dimerization may be. It may play a role in preventing assembly of FliG with FliM–FliN complexes until FliG is associated with FliF, or it may simply stabilize the FliG_N domain until it can bind FliF. FliG from *A. aeolicus* has been reported to be a monomer in solution, and we are not aware of a conclusive determination of the oligomeric state of *E. coli* FliG, though there are implications it may be monomeric as well.²⁴

Binding of FliF_{495–532} to FliG_N leads to widespread conformational changes in the FliG_N domain and causes the stabilization of FliG_N helices 1 and 4. These widespread

changes in FliG_N upon FliF_C binding can be explained by a primary binding site located on the highly conserved hydrophobic patch centered along FliG_N helix 1 (Figure 6). In this model, helix 2 of FliF_C directly interacts with central helix 1 of FliG_N, propagating conformational changes through and stabilizing the tertiary structure of the FliG_N domain. The stabilization of helix 1 is reflected in the large perturbations of the chemical shifts of FliG_N hydrophobic residues oriented toward the central helix. Deletion mutations have previously shown that the first ~50 amino acids of FliG, encompassing helices 1–3 of FliG_N, are necessary for flagellar assembly. Because the interactions between FliF_C and FliG_N are widespread, FliG_N helices 2 and 3 (as well as additional portions of the protein, such as the helix 4–helix 5 loop) may also be involved in secondary interactions with FliF_C. Helices 5 and 6 of FliG_N, predicted to be most distal from our proposed binding site, are the smallest shifters upon FliF_C binding. This postulated binding site on FliG_N would also provide a very favorable orientation for assembly of the C-ring: the FliF binding site would be directly opposite the linker helix that connects FliG_N to the FliG_{MC} domains. Work to determine an atomic-resolution structure of the FliG_N–FliF_C complex is ongoing.

■ ASSOCIATED CONTENT

■ Supporting Information

Multiple-sequence alignment of FliG_N from *T. maritima*, *A. aeolicus*, *C. crescentus*, and *E. coli* (Figure S1); an overlay of ¹⁵N–¹H TROSY-HSQC spectra of FliG_N, FliG_N bound to FliF_{495–532}, and FliG_N bound to FliF_{512–532} (Figure S2a) and a fluorescence experiment showing the titration of FliF_{512–532} with FliG_N (Figure S2b); and an overlay of the ¹⁵N–¹H HSQC spectrum of FliG_N with NOEs observed between a mixture of ¹⁵N- and ¹³C-labeled FliG_N (Figure S3a) and mapping of interfacial residues with NOEs onto an *A. aeolicus* structure of FliG_N (Figure S3b). This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: (805) 893-5326. E-mail: dahlquist@chem.ucsb.edu.

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■ ABBREVIATIONS

NMR, nuclear magnetic resonance; HSQC, heteronuclear single-quantum coherence; TROSY, transverse relaxation-optimized spectroscopy; HPLC, high-performance liquid chromatography.

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