Organization of FliN Subunits in the Flagellar Motor of *Escherichia coli*†

Koushik Paul and David F. Blair*

Department of Biology, University of Utah, Salt Lake City, Utah 84112

Received 21 December 2005/Accepted 10 January 2006

FliN is a major constituent of the C ring in the flagellar basal body of many bacteria. It is present in >100 copies per flagellum and together with FliM and FliG forms the switch complex that functions in flagellar assembly, rotation, and clockwise-counterclockwise switching. FliN is essential for flagellar assembly and switching, but its precise functions are unknown. The C-terminal part of the protein is best conserved and most important for function; a crystal structure of this C-terminal domain of FliN from *Thermotoga maritima* revealed a saddle-shaped dimer formed mainly from β strands (P. N. Brown, M. A. A. Mathews, L. A. Joss, C. P. Hill, and D. F. Blair, J. Bacteriol. 187:2890–2902, 2005). Equilibrium sedimentation studies showed that FliN can form stable tetramers and that a FliM₄FliN₄ complex is also stable. Here, we have examined the organization of FliN subunits by using targeted cross-linking. Cys residues were introduced at various positions in FliN, singly or in pairs, and disulfide cross-linking was induced by oxidation. Efficient cross-linking was observed for certain positions near the ends of the dimer and for some positions in the structurally uncharacterized N-terminal domain. Certain combinations of two Cys replacements gave a high yield of cross-linked tetramer. The results support a model in which FliN is organized in doughnut-shaped tetramers, stabilized in part by contacts involving the N-terminal domain. Electron microscopic reconstructions show a bulge at the bottom of the C-ring whose size and shape are a close match for the hypothesized FliN tetramer.

Many species of bacteria are propelled by rotating flagella (2) that obtain energy from the membrane ion gradient (16, 23; for reviews, see references 1 and 21) The flagella are built from about 25 different proteins, most of which have roles in forming the flagellar basal body, hook, and filament (22, 26). Only a few proteins function in rotation and switching. The membrane proteins MotA and MotB form ion-conducting complexes that function as the stator (3, 8–11, 33). The proteins FliN, FliM, and FliG form the rotor-mounted switch complex that functions in flagellar assembly, rotation, and clockwise-counterclockwise (CW/CCW) direction control (44, 45). The switch complex contains >100 copies of FliN (47), about 34 copies of FliM (47), and 25 copies of FliG (13, 18, 35, 36, 47). Together, these form the C ring, a 45-nm-diameter, drum-shaped structure at the bottom (the cytoplasm-proximal end) of the basal body (Fig. 1) (14, 19, 40).

FliG functions directly in rotation of the flagellum (17, 24). It interacts with the FliF protein that forms the MS ring (13, 15, 20, 30) and with the membrane-associated stator protein MotA (48) and must presumably be located in the upper (membrane-proximal) part of the C ring (Fig. 1). FliM and FliN have not been located precisely but are probably more distant from the membrane because they do not function as directly in rotation (24) and are not believed to interact directly with FliF or MotA. FliM is closely involved in direction switching (34) and contains a binding site for the CW-signaling protein CheY (28, 41, 43). FliN has been implicated in CW/CCW switching (17) and in the export process that occurs during flagellar assembly (42), but its exact functions are unknown.

The FliN protein of *Escherichia coli* or *Salmonella* contains 137 residues (27). The C-terminal part of the protein, from about residue 58 to the end, is best conserved and most important for function (37). A FliN protein lacking 57 N-terminal residues is sufficient for flagellar assembly and rotation, but the cells swarm at about one-third of the wild-type rate in soft agar (37) and show relatively slow, erratic motility in liquid (K. Paul and D. Blair, unpublished data). In the flagellum, FliN interacts with the C-terminal domain of FliM (FliM₄) (28, 41). Portions of FliM₄ show sequence homology to FliN (28) and may therefore show some similarity in folding. FliM might also bind to FliG, but the evidence for this is less strong (39).

Two crystal structures were recently solved for most of the FliN protein from *Thermotoga maritima* (Protein Data Bank [PDB] entries 1o6a and 1yab) (5). Both structures show a saddle-shaped dimer formed mainly from β strands, with short α helices at the ends (Fig. 2). The two structures differ somewhat in the position of α-helix 1, which packs near the body of the dimer in one crystal (1o6a) but extends to contact an adjacent dimer in the other (1yab). The FliN dimer exhibits a sizable surface hydrophobic patch, formed from the side chains of 5 residues (10 residues in the dimer) with a strongly conserved nonpolar character. A mutation in the hydrophobic patch (Val113→Asp) affects both flagellar assembly and switching (5).

Equilibrium sedimentation experiments showed that the FliN protein of *E. coli* forms a stable tetramer in solution and that the FliN and FliM proteins of *T. maritima* form a stable complex with the composition FliM₄FliN₄ (5). (Sedimentation experiments with FliM used the *T. maritima* proteins because *E. coli* FliM is prone to aggregation.) Accordingly, we proposed that FliM₄FliN₄ complexes might be structural units of the C ring (5). The subunit composition of the C ring is not yet firmly established, however; an estimate of subunit stoichiometry in purified basal bodies of *Salmonella enterica* serovar Typhimurium gave a FliM:FliN ratio closer to 1:3 (47). Be-
cause FlIMc shows sequence similarity to FlIN, it might be capable of replacing a FlIN subunit to form a FlIM-FlIN heterodimer, and such heterodimers in combination with FlIN dimers could account for a 1:3 subunit composition. The location and arrangement of the FlIN subunits in the C ring are also unknown. The related protein HrcOBC crystallized as an elongated tetramer in which the dimers are joined end to end (12). Patterns of residue conservation indicate that FlIN dimers might associate in a similar end-to-end fashion (5), but the subunit associations seen in FlIN crystals indicate that other arrangements are also possible, including some with the shape of a ring (Fig. 2C to E).

Here, we have used targeted cross-linking to test various models for the organization of FlIN. Cys residues were introduced in various positions on the surface of FlIN, singly or in pairs; disulfide cross-linking was induced; and the products were analyzed on immunoblots. The subunit replacement model was tested by making Cys replacements at predicted interfacial positions in FlIN and FlIMc. The results indicate that FlIN is organized in tetramers and does not form subunit-exchanged heterodimers with FlIMc. The FlIN tetramer is not organized like the tetramer seen in the HrcOBC crystal structure but has a doughnut shape similar to the subunit organization seen in one of the FlIN crystal structures. The FlIN doughnut seen in the crystal structure is puckered and as a result is predicted to be bistable (to exist in two equally stable conformations). In the flagellum, the two conformations would be nonequivalent and might be relevant for CW/CCW switching. Some Cys replacements were also made in the structurally uncharacterized N-terminal domain of FlIN. Occurrences of efficient cross-linking show that the N-terminal segments of two FlIN subunits are in proximity to each other and are near the hydrophobic patch of another dimer. These interactions appear to stabilize the tetramer, because a protein lacking the N-terminal domain formed mainly dimers. Electron microscopic reconstructions (40, 46) show a bulge at the bottom of the C ring of the appropriate size and shape to fit the FlIN tetramers.

**MATERIALS AND METHODS**

**Strains and media.** The *E. coli* strains and plasmids used are listed in Table 1. Procedures for transformation and plasmid isolation were described previously (38). TB contained 10 g tryptone and 5 g NaCl per liter. SB contained, per liter, 12-g tryptone, 24-g yeast extract, 5-mL glycerol, 3.8-g KH2PO4, and 12.5-g K2HPO4. Ampicillin (Amp) was used at 125 μg/mL in liquid medium and at 50 μg/mL in swarm plates. Chloramphenicol was used at 50 μg/mL in liquid and 12.5 μg/mL in swarm plates. IPTG (isopropyl-β-D-thiogalactopyranoside) was prepared as a 0.1 M stock in water.

**Site-directed mutagenesis and assays of swarming.** Single-stranded DNA preparation and site-directed mutagenesis were carried out according to the Altered Sites procedure (Promega), with flIN cloned in plasmid pLS4 (24) or with flIM cloned in pHT41 (38). The mutations were confirmed by DNA sequencing. The mutated flIN and flIM genes were transferred into plasmids (pHT39 and pDBF97, respectively) that allow IPTG-regulated expression from the tac promoter. Swarming in soft agar, swimming in liquid, and flagellation were assayed as described previously (38) by using the flIN deletion strain DFB223 transformed with wild-type or mutant plasmids. Swarm plates contained 0.2% agar, and an appropriate antibiotic(s).

**Cross-linking.** Initial cross-linking experiments used the catalyst Cu[1,10-phenanthroline]2 (Cu-phenanthroline) and were performed as described previously (4), with minor modifications. flIN deletion strain DFB223 or flIMDC deletion strain RP3098 was transformed with wild-type or mutant plasmids. RP3098 expresses no flagellar genes, except those expressed from plasmids, and thus does not assemble flagella. Cells were cultured overnight in TB and antibiotics at 32°C, then diluted 100-fold into TB containing appropriate antibiotics, and grown for 1 h at 32°C. The culture was then induced with 25 μM IPTG, and growth was continued for an additional 3.5 h. The absorbance of each culture was measured at 600 nm. The least-dense culture was transferred to a centrifuge tube in its entirety, and the other cultures were transferred in volumes adjusted (by using A600 readings) to give equal numbers of cells. The cells were pelleted (3,000 × g; 10 min) and resuspended in 200 μL lysis buffer (50 mM Tris [pH 8], 0.5 M sucrose, 10 mM EDTA, 0.2-mg/ml lysozyme). The cells were incubated on ice for 1 h and then rapidly diluted by the addition of 1.8 mL of ice-cold water. Samples were divided into two 1-mL fractions, and 110 μL of cross-linking reagent was added to one of the tubes and 110 μL of 50% ethanol was added to the other. The cross-linking reagent contained 4 mM CuSO4 and 16 mM 1,10-phenanthroline (pH 8). The samples were incubated at room temperature with gentle rotation for 5 min, sonicated for 15 s (50% duty cycle, power level 2; Branson model 450 sonifier), and incubated an additional 5 min. Reactions were quenched by the addition of N-ethylmaleimide (22 μL of a 1-mL stock in 95% ethanol) and EDTA (126 μL of a 0.5 M stock). A total of 100 μL of each sample was mixed with 100 μL of nonreducing gel-loading buffer, boiled, and used for electrophoresis.

For some cross-linking experiments using iodine, cells were cultured, collected as described above, and then treated with 0.2 mM I2, added from a 2-mM stock in 95% ethanol. N-Ethylmaleimide was added after 5 min, and then cells were mixed with gel loading buffer (lacking reducing agent), boiled, loaded on gels, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, as described below.

**SDS-PAGE and immunoblotting.** Protein samples were separated on 10% or 12% SDS-PAGE minigels (Bio-Rad MiniProtean system). Proteins were transferred to nitrocellulose using a semidry transfer apparatus (Bio-Rad). Rabbit polyclonal antibody against FlIN was prepared as described previously (37) and was used at an ∼1,000-fold dilution. Bands were visualized using the Super Signal West PicoLumol system (Pierce) and X-ray film. Bands were quantified by video densitometry using the public domain image-processing program NIH Image (developed at the U.S. National Institutes of Health and available online at http://rsb.info.nih.gov/nih-image/).

**Gel filtration chromatography.** The flIN deletion strain DFB223 was transformed with plasmids encoding either full-length FlIN or FlIN lacking 57 N-terminal residues. Cells were grown at 37°C in 500 mL SB-Amp broth to an absorbance of 0.7 at 600 nm, then IPTG was added to 400 μM, and growth was continued for an additional 3.5 h. The absorbance of each culture was measured at 600 nm. The least-dense culture was transferred to a centrifuge tube in its entirety, and the other cultures were transferred in volumes adjusted (by using A600 readings) to give equal numbers of cells. The cells were pelleted (3,000 × g; 10 min) and resuspended in 200 μL lysis buffer (50 mM Tris [pH 8], 0.5 M sucrose, 10 mM EDTA, 0.2-mg/ml lysozyme). The cells were incubated on ice for 1 h and then rapidly diluted by the addition of 1.8 mL of ice-cold water. Samples were divided into two 1-mL fractions, and 110 μL of cross-linking reagent was added to one of the tubes and 110 μL of 50% ethanol was added to the other. The cross-linking reagent contained 4 mM CuSO4 and 16 mM 1,10-phenanthroline in 50% ethanol and was freshly prepared from a 1 M stock of 1,10-phenanthroline in 95% ethanol and a 400-mM stock of CuSO4 in water. Samples were incubated at room temperature with gentle rotation for 5 min, sonicated for 15 s (50% duty cycle, power level 2; Branson model 450 sonifier), and incubated an additional 5 min. Reactions were quenched by the addition of N-ethylmaleimide (22 μL of a 1-mL stock in 95% ethanol) and EDTA (126 μL of a 0.5 M stock). A total of 100 μL of each sample was mixed with 100 μL of nonreducing gel-loading buffer, boiled, and used for electrophoresis.

**For some cross-linking experiments using iodine, cells were cultured, collected as described above, and then treated with 0.2 mM I2, added from a 2-mM stock in 95% ethanol. N-Ethylmaleimide was added after 5 min, and then cells were mixed with gel loading buffer (lacking reducing agent), boiled, loaded on gels, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, as described below.
continued for 3 h. Cells were pelleted and resuspended in 40 ml of buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Cells were lysed by passage through a French pressure cell, and the lysate was centrifuged (23,000 g; 45 min) to pellet the membranes. The supernatant was collected in a fresh tube. A 2-ml sample of the supernatant was filtered and loaded onto a Superdex-300 size exclusion column in a fast-performance liquid chromatography apparatus (ÅKTA; Amersham). The column was run in 50 mM Tris-HCl (pH 8.0)–200 mM NaCl, and fractions were collected and analyzed by SDS-PAGE and immunoblotting.

RESULTS

Shape of the FliN tetramer. The crystal structure is known for residues 68 to 154 of T. maritima FliN (5) (PDB accession numbers 1yab and 1o6a), corresponding to residues 51 to 137 of the E. coli protein. The cross-linking experiments used the protein from E. coli. The wild-type protein contains no Cys residues. In the part of FliN of known structure, 10 Cys replacements were made on the surface and 1 was made at a more interior position near the dimer twofold axis. Several Cys replacements were also made in the N-terminal domain, which has not yet been crystallized. The positions of the mutations are shown in Fig. 3, either on the crystal structure (for positions 54 and higher) or on a sequence alignment of the N-terminal segment (for the replacements in the N-terminal domain). A secondary-structure prediction for the N-terminal part of the protein is also shown. The Cys replacements in the

TABLE 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or property</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFB223</td>
<td>fliN-null strain</td>
<td>37</td>
</tr>
<tr>
<td>RP3098</td>
<td>flhDC deletion strain; expresses no chromosomal flagellar genes</td>
<td>J. S. Parkinson</td>
</tr>
<tr>
<td>pTBM30</td>
<td>P(<em>{\text{tac}}) expression vector(Ap(</em>{\text{p}})); parent of pH(_{\text{T39}})</td>
<td>29</td>
</tr>
<tr>
<td>pLS4</td>
<td>fliN in pAlter-1(Ap(_{\text{p}})); used for site-directed mutagenesis of fliN</td>
<td>37</td>
</tr>
<tr>
<td>pSB4</td>
<td>Expresses fliN codons 58–137 in pTBM30</td>
<td>37</td>
</tr>
<tr>
<td>pH(_{\text{T39}})</td>
<td>fliN in pTBM30; used to express FliN proteins with Cys replacements</td>
<td>24</td>
</tr>
<tr>
<td>pH(_{\text{T41}})</td>
<td>fliM in pAlter-1; used for site-directed mutagenesis and expression of fliM</td>
<td>24</td>
</tr>
<tr>
<td>pDB94</td>
<td>P(_{\text{tac}}) expression vector for fliM; Cm(^{\text{r}})</td>
<td>38</td>
</tr>
</tbody>
</table>
N-terminal domain were in a segment that is relatively well conserved and predicted to form an α helix.

First, we examined cross-linking of proteins with single Cys residues on the surface of the domain of known structure. One double mutant (77C/83C) was also studied, because these positions are predicted to be near each other in the HrcQBC-based model for the FliN tetramer (5, 12). The Cys-substituted FliN proteins were expressed in the fliN deletion strain DFB223, cells were lysed, and copper phenanthroline was added to catalyze oxidative cross-linking. The deletion in strain DFB223 is fully complemented by plasmid-encoded fliN (37), and so the cells in these experiments were flagellate. Parallel experiments were also conducted with an flhDC strain (RP3098) that does not express any chromosomal flagellar genes and that does not assemble flagella to determine whether similar subunit contacts occur when the protein is not assembled into motors. Proteins were resolved on SDS-PAGE gels, and the products of cross-linking were characterized on FliN immunoblots.

Wild-type FliN and most of the Cys-replacement proteins showed no cross-linking when treated with Cu-phenanthroline. Just four of the mutant proteins, with Cys replacements at positions 54, 57, 58, and 136, showed cross-linking upon oxidation (Fig. 4). (Numbers are for the E. coli protein; T. maritima numbers are higher by 17.) Each showed a single major cross-linked product with an apparent molecular weight of 42 kDa. Although this was larger than expected for a normally migrating FliN dimer (predicted mass, 31 kDa), cross-linked species often exhibit aberrant mobility, and other evidence described below supports its assignment as a FliN dimer. These four positions are near the ends of the FliN dimer (Fig. 3), in a region that is the major site of contact between dimers in the lyab FliN crystal structure (Fig. 2E) (5). This region did not contribute to the dimer-dimer interface in the HrcQBC-based model for the FliN tetramer (see Fig. S1A in the supplemental material). Cross-linking results were similar for the flagellum-forming strain and the flhDC strain (Fig. 4). Thus, the cross-linked product did not contain any flagellar proteins besides FliN, and the contacts through positions 54, 57, 58, and 136 did not require the protein to be assembled into flagella. Given the location of these residues at the ends of the (crystallographic) FliN dimer, the cross-linking was not between the two subunits of this dimer but must occur within a larger assembly—presumably, the FliN tetramer known from sedimentation equilibrium experiments (5).

Fluctuations in protein position or orientation might allow cross-linking between sites that are somewhat separated in the lowest-energy configuration (7). Previous studies found that such motional effects are minimized when iodine rather than copper phenanthroline is used for oxidation (4, 6); so to place tighter constraints on residue proximity, we examined cross-
replacement of a FliN subunit by FliM C. To test this subunit-exchanged heterodimer formation by FliM-FliN, we introduced a Cys replacement at position 58 near the twofold axis of the dimer and another at position 68 near the end(s) of the dimer. Cross-linking was induced with copper phenanthroline, and products were examined using immunoblots, as before. Representative results are shown in Fig. 6. In the flagellate strain, cross-linking of the 54C/68C double mutant gave a species with an apparent mass of about 70 kDa as the only major product. In the nonflagellate strain, the results were similar, except that a small amount of dimer was also observed. Because the 54C and 68C replacements individually showed very efficient cross-linking (Fig. 4 and 6), we assigned the 70-kDa band as a FliN tetramer cross-linked through both positions. The apparent mass was close to that expected (predicted tetramer mass, 62 kDa). Although the tetramer formed in very high yield, larger products were not observed in significant amounts.

**Organization of a segment in the N-terminal domain.** As discussed above, the N-terminal domain of FliN contributes to optimal swarming motility but is not essential for flagellar assembly. We hypothesized that the N-terminal segment might contribute to the stability of the FliN tetramer. To test this, we used gel filtration to examine the state of association of a FliN protein deleted for residues 1 to 57. The FliN(Δ1-57) protein ran with an apparent mass of about 20 kDa in the sizing column (Fig. 7), close to that expected for a dimer (predicted dimer mass, 16 kDa) and too small to be a tetramer. The full-length FliN protein, by contrast, exhibited a broad peak centered at around 100 kDa, consistent with previous sedimentation experiments indicating a tetramer with nonglobular shape (5). Thus, the N-terminal domain appears necessary for the formation of a stable FliN tetramer.

To examine further the organization of the N-terminal domain, we studied cross-linking of proteins with single Cys replacements in a relatively well-conserved, predicted α-helical segment (Fig. 3). An initial experiment used Cu-phenanthroline with Cys replacements positions 16, 19, 22, and 25. In each case, treatment with Cu-phenanthroline gave cross-linked dimer in high yield (Fig. 8A), indicating that the N-terminal segments of linking by iodine. Although yields were lower than those with copper phenanthroline, the proteins with Cys replacements at position 54, 57, or 136 showed a readily detectable cross-linked product upon treatment with iodine. Position 58, which showed a lower yield than the others with copper phenanthroline, did not show any cross-linking using iodine.

**FliN and FliMC do not form a subunit-exchanged heterodimer.** As discussed above, sequence similarities between FliN and FliMC raise the possibility that FliM-FliN heterodimers form by replacement of a FliN subunit by FliM C. To test this subunit-exchanged replacement model, we introduced a Cys replacement at a position that is near the twofold axis of the FliN dimer seen in the crystal structures (Leu 68 in *E. coli*, corresponding to Leu 85 in *T. maritima*). The L68C protein gave cross-linked FliN dimer in very high yield (Fig. 5), again with an apparent mass of approximately 42 kDa. We conclude that position 68 of each FliN subunit is close to position 68 of the other subunit in the dimer, as is seen in the crystal structures. Although the yield of cross-linking showed some variability between experiments, it was typically >80% and appeared to be quantitative (i.e., with no detectable monomer remaining) in some experiments. Such complete conversion into dimer would not be possible if a significant fraction of the FliN were present in FliM-FliN heterodimers formed by a subunit replacement. As a further test, we looked for cross-linking between FliN with Cys at position 68 and FliM with Cys at position 265, the residue that corresponds with FliN-Leu68 in a FliM-FliN sequence alignment. No FliM-FliN cross-linking was detected using either FliN or FliM immunoblots, and the major product was again FliN dimer (Fig. 5; the FliN blot is shown). We conclude that subunit-exchanged FliM-FliN heterodimers do not occur or are present as only a minor species. These data support a tetrameric FliN complex and argue against a complex with composition FliN(FliM)4.
different FliN subunits are in proximity to each other. The protein cross-linked through these positions migrated with an apparent mass of 35 kDa in gels, close to the mass expected for a FliN dimer.

To place tighter constraints on the arrangement of the N-terminal segment, we examined iodine-induced cross-linking of FliN proteins with Cys in 10 positions in the predicted helical segment (Fig. 3). Although yields were relatively low, the proteins with Cys at position 16, 19, or 20 produced cross-linked dimer at readily detectable levels (Fig. 3B). If this segment of FliN were helical, then these positions would lie on one face, and the pattern of cross-linking would suggest that the N-terminal segments of two FliN subunits approach most closely through this face.

Next, we examined cross-linking of FliN proteins with a Cys replacement at position 19 in the N-terminal domain and a second replacement at position 68 near the dimer twofold axis. Oxidation using copper phenanthroline gave two major products, both in the range expected for a tetramer. Two products in the range expected for a dimer and one in the range expected for a monomer.
Species larger than a tetramer did not occur in significant amounts. Results were similar in the flagellate strain and the nonflagellate \textit{flhDC} strain, and so the cross-linked products did not contain any flagellar proteins besides FliN. Because FliN in solution is known to form a stable tetramer and patterns of cross-linking were similar whether or not flagella were assembled, we concluded that the cross-linking occurred within rather than between FliN tetramers. Given the results with the individual Cys replacements at positions 19 and 68, one interpretation might be that FliN dimers joined by a 68-68 disulfide can cross-link into tetramers through position 19. Such tetramers could contain either three or four disulfide bonds (i.e., be either linear or closed), which could account for the occurrence of two major products. An alternative possibility is that the N-terminal segment is positioned near the hydrophobic patch, and cross-linking between positions 19 and 68 gives rise to tetramers and other products.

To distinguish between these possibilities, we first examined cross-linking of the 19C-68C double mutant by using iodine. The 19C replacement by itself showed only very weak cross-linking with iodine, and so iodine treatment should give little or no tetramer if tetramer formation requires the 19-19 disulfide. Iodine treatment of the 19C-68C double mutant gave both dimer and tetramer in high yield, as well as species at a higher molecular weight (Fig. 9B). Because iodine was not expected to induce efficient 19-19 cross-linking, this suggested that the tetramer and larger species were formed by cross-linking between positions 19 and 68. Some tetramer was also formed upon iodine treatment of the 16C-68C, 22C-68C, and 25C-68C double mutants (Fig. 9B). Positions 22 and 25, like 19, showed no iodine-induced cross-linking by themselves, and so the production of tetramer in the 22C-68C and 25C-68C mutants reinforces the conclusion that position 68 can cross-link to positions in the N-terminal segment.

Tetramer formation might still be due to 68-68 cross-linking in combination with 19-19 cross-linking, if we postulate that the Cys replacement at position 68 affects the position or mobility of the N-terminal segment to increase the efficiency of 19-19 cross-linking. Thus, as a further means of distinguishing the models, we examined cross-linking of FliN with one Cys replacements at position 19 and another at position 54. Copper phenanthroline was used for this experiment, because unlike iodine it allows efficient cross-linking through positions in the N-terminal segment, including position 19. One model predicts formation of tetramer upon treatment with copper phenanthroline, while the other predicts formation of only dimer (Fig. 9C). Some cross-linked dimer was present prior to treatment with the oxidation catalyst, as was also observed with the 54C single replacement. Treatment with Cu-phenanthroline gave an additional dimer form and a high yield of tetramer. This supports the model in which the N-terminal segments are in proximity to the hydrophobic patch of another dimer (Fig. 9C).
Function of the Cys mutant proteins. The FliN proteins that displayed efficient cross-linking were tested by swarming assays to determine whether their function was altered by the Cys replacements. The proteins with Cys replacements at positions 16, 19, 22, 25, 54, 57, and 136 allowed cells to swarm at approximately wild-type rates. The replacement at position 58 caused a roughly twofold reduction in swarming rate. The replacement at position 68, either alone or in combination with other Cys replacements, allowed flagellar assembly and vigorous swimming, but the cells swarmed at <1/10 the wild-type rate in soft agar and exhibited aberrantly smooth motility in liquid, indicating a CCW motor bias.

DISCUSSION

Based on patterns of sequence conservation, we suggested previously (5) that FliN might be organized similarly to the tetramer of HrcQBC (a FliN paralog) seen in a crystal structure (12). However, a subunit organization like that in the HrcQBC crystal cannot account for the pattern of disulfide cross-linking observed here with FliN (see Fig. S1 in the supplemental material). The positions of high-yield cross-linking instead indicate a doughnut-like arrangement, substantially similar to the organization seen in one of the FliN crystal structures (1yab) (Fig. 10A) (5). The subunits contacts in this arrangement are provided largely by α-helix 1, particularly positions 54 and 57. An alignment of FliN sequences (not shown) shows that hydrophobic character is very well conserved at these positions. Although helix 1 participates in close dimer-dimer contacts in one of the crystals (1yab) (5), it does not preserve these contacts in the other crystal form (1o6a), which might account for its different positioning in the two structures (Fig. 2B).

Our results show that little if any of the FliN in the cell occurs in the form of subunit-exchanged FliM<sub>C</sub>/FliN heterodimers. A Cys residue near the twofold axis of the FliN dimer gave disulfide-linked dimer in very high yield, and no FliM-FliN cross-linking was observed when a second Cys residue was introduced at the corresponding position in FliMC (Fig. 5). We therefore favor a model in which FliM<sub>FliN<sub>C</sub> units, rather than FliM<sub>FliN<sub>C</sub> units, are constituents of the C ring. Sedimentation equilibrium studies have established the stability of a FliM<sub>FliN<sub>C</sub> complex of the T. maritima proteins (5). The FliM protein of E. coli is less amenable to such studies, owing to its tendency to aggregate, but we are attempting to identify better-behaved fragments that might allow further tests of this model for the complex.

The shape and dimensions of the hypothesized FliN tetramer provide strong constraints on its location in the C ring. Electron microscopic reconstructions of the basal body at approximately 20-Å resolution (14, 40) show that a ring with these dimensions would not fit in the part of the C ring just under FliG but is a close match for the bulge at the bottom edge of the ring. The FliN tetramer appears a good fit for this location in both side and end views of the basal body (Fig. 10B). Given this location for FliN at the bottom and the previously inferred location for FliG at the top of the C ring (13, 15, 20, 25, 30, 48), we conclude that FliM must be situated in the middle of the C-ring wall (Fig. 10B). Although the crystal structure of FliM has not been reported, a crystal structure is
containing residues 15 to 25 is hypothesized to form an
the contribution of the segment to stability of the tetramer. The segment
lie near the top (15, 20, 25, 30, 48), it follows that FliM is located in the
view of the basal body is from reference 40 (with permission of the
FIG. 10. Model for FliN subunit arrangement and location. (A) Doughnut-like arrangement seen in the FliN crystal structure 1yab (5). Positions
that gave a high yield of cross-linked dimer are indicated. At each dimer
dimer interface, the structure shows interpretable density for residue 136
of only one subunit, extending only to residue 135 in the other subunit. Residue 136 occurs in an α helix; to obtain a rough estimate of its position,
this helix from the first subunit was overlaid on the (shorter) helix of the
second. (B) Proposed location of the FliN tetramers in the flagellar basal
body. The location and orientation of one FliN tetramer are indicated; the
flagellum would contain approximately 34 FliN tetramers in all. The side
view of the basal body is from reference 40 (with permission of the
publisher), and the end view is from reference 46 (with permission of the
publisher). Given this location for FliN and evidence that FliG must
lie near the top (15, 20, 25, 30, 48), it follows that FliM is located in the
middle of the C-ring wall, as indicated. (C, left) Arrangement of N-
terminal segments that can account for the cross-linking results and for
the contribution of the segment to stability of the tetramer. The segment
containing residues 15 to 25 is hypothesized to form an α helix. Two such
helices are in proximity to each other and to the hydrophobic patch of the
other FliN dimer, bringing residues 19 and 68 into proximity. Although
the arrangement pictured is stable enough to allow high-yield cross-link-
ing between positions 19 and 68, the results also indicate that the seg-
ments are dynamic (see Discussion). (Right) Detailed view of the hypo-
thesized interface between the N-terminal segments and the body of the
dimer. The view is along the segments, one pointing toward the viewer
and the other pointing away. Positions that cross-link most efficiently to
position 68 are shaded, and positions that allow some cross-linking be-
tween the two N-terminal segments are circled.

FIG. 10. Model for FliN subunit arrangement and location. (A) Dough-

nut-like arrangement seen in the FliN crystal structure 1yab (5). Positions
that gave a high yield of cross-linked dimer are indicated. At each dimer
dimer interface, the structure shows interpretable density for residue 136
of only one subunit, extending only to residue 135 in the other subunit. Residue 136 occurs in an α helix; to obtain a rough estimate of its position,
this helix from the first subunit was overlaid on the (shorter) helix of the
second. (B) Proposed location of the FliN tetramers in the flagellar basal
body. The location and orientation of one FliN tetramer are indicated; the
flagellum would contain approximately 34 FliN tetramers in all. The side
view of the basal body is from reference 40 (with permission of the
publisher), and the end view is from reference 46 (with permission of the
publisher). Given this location for FliN and evidence that FliG must
lie near the top (15, 20, 25, 30, 48), it follows that FliM is located in the
middle of the C-ring wall, as indicated. (C, left) Arrangement of N-
terminal segments that can account for the cross-linking results and for
the contribution of the segment to stability of the tetramer. The segment
containing residues 15 to 25 is hypothesized to form an α helix. Two such
helices are in proximity to each other and to the hydrophobic patch of the
other FliN dimer, bringing residues 19 and 68 into proximity. Although
the arrangement pictured is stable enough to allow high-yield cross-link-
ing between positions 19 and 68, the results also indicate that the seg-
ments are dynamic (see Discussion). (Right) Detailed view of the hypo-
thesized interface between the N-terminal segments and the body of the
dimer. The view is along the segments, one pointing toward the viewer
and the other pointing away. Positions that cross-link most efficiently to
position 68 are shaded, and positions that allow some cross-linking be-
tween the two N-terminal segments are circled.

FIG. 10. Model for FliN subunit arrangement and location. (A) Doughnut-like arrangement seen in the FliN crystal structure 1yab (5). Positions
that gave a high yield of cross-linked dimer are indicated. At each dimer
dimer interface, the structure shows interpretable density for residue 136
of only one subunit, extending only to residue 135 in the other subunit. Residue 136 occurs in an α helix; to obtain a rough estimate of its position,
this helix from the first subunit was overlaid on the (shorter) helix of the
second. (B) Proposed location of the FliN tetramers in the flagellar basal
body. The location and orientation of one FliN tetramer are indicated; the
flagellum would contain approximately 34 FliN tetramers in all. The side
view of the basal body is from reference 40 (with permission of the
publisher), and the end view is from reference 46 (with permission of the
publisher). Given this location for FliN and evidence that FliG must
lie near the top (15, 20, 25, 30, 48), it follows that FliM is located in the
middle of the C-ring wall, as indicated. (C, left) Arrangement of N-
terminal segments that can account for the cross-linking results and for
the contribution of the segment to stability of the tetramer. The segment
containing residues 15 to 25 is hypothesized to form an α helix. Two such
helices are in proximity to each other and to the hydrophobic patch of the
other FliN dimer, bringing residues 19 and 68 into proximity. Although
the arrangement pictured is stable enough to allow high-yield cross-link-
ing between positions 19 and 68, the results also indicate that the seg-
ments are dynamic (see Discussion). (Right) Detailed view of the hypo-
thesized interface between the N-terminal segments and the body of the
dimer. The view is along the segments, one pointing toward the viewer
and the other pointing away. Positions that cross-link most efficiently to
position 68 are shaded, and positions that allow some cross-linking be-
tween the two N-terminal segments are circled.

known for the related chemotaxis protein CheC (31). The
structure shows an ellipsoidal protein with dimensions (ap-
proximately 2.5 nm by 3.5 nm by 5 nm) that are a good fit for
this location.

Although we still lack detailed structural information on the
N-terminal part of FliN, the present results cast some light on
its function and organization. This domain contributes to the
stability of the tetramer, because a truncated FliN lacking
residues 1 to 57 formed primarily dimers. The cross-linking
results establish that the N-terminal segments of two FliN
subunits are in fairly close contact (Fig. 8) and that this assem-
bly of two N-terminal segments, most likely two α helices, is
positioned near the hydrophobic patch of another FliN dimer
(Fig. 9). If residues 15 to 25 form an α helix, as predicted, then
the observed pattern of cross-linking can be accounted for in a
simple model for the segment organization (Fig. 10C).

In E. coli, the N-terminal segment is joined to the rest of
FliN by a Gly-rich segment (GGG13-45) that should allow con-
siderable conformational flexibility. The cross-linking expe-
riments give two indications that the N-terminal segments are
not rigidly fixed but can undergo some movement relative to
each other and to the patch: yields of intersegment cross-
linking were much higher when Cu-phenanthroline was used
instead of iodine (Fig. 8A), and several different positions in
the N-terminal segment showed some ability to cross-link to
the same position (residue 68) in the main body of the protein
(Fig. 9B). Given this dynamic character, we predict that the
N-terminal segments could be displaced from the hydrophobic
patch readily, to allow interactions with other proteins, such as
FliH (K. Paul and D. F. Blair, unpublished data). Flexibility of
the N-terminal segment might also allow it to reach between
different tetramers; such bridging could account for the oc-
currence of products larger than tetramer in some of the cross-
linking experiments with double Cys proteins (Fig. 9B).

Studies of spontaneous mutants showed that FliN has some
role in CW/CCW switching (17). In this context, we note that
the FliN tetramer in the 1yab crystal structure is puckered, as
can be seen in side views (Fig. 10A and B; see Fig. S2 in the
supplemental material). If such puckering occurs in the FliN
tetramer in the flagellum, then the tetramer would be capable
of existing in two conformations (puckered in one direction or
the other). The two conformations would be equivalent in an
isolated tetramer and thus equally stable, but in the context of
the C-ring they would be nonequivalent and might correspond
to CW and CCW states of the switch (see Fig. S2 in the
supplemental material).

Finally, we note that the subunit arrangement we propose is
likely to be relevant for other species that contain FliN but
might be different in species that use the much larger protein
FliY. These include most gram-positive species and spiro-
chaeetes. The organization of FliY and the shape of the C ring
in those species remain to be examined.

ACKNOWLEDGMENTS

We thank Sandy Parkinson for strains; Jonathan McMurry, Bertha
González-Pedrajo, Dennis Thomas, Perry Brown, Bryan Lowder, and
Jacob Harmon for discussions and advice; Moisés Terrazas for assis-
tance with swarming-rate measurements; Stan Williams and Marian
Price-Carter for assistance with FPLC experiments; and Marian Price-
Carter for critical reading of the manuscript.