Targeted Disulfide Cross-Linking of the MotB Protein of *Escherichia coli*: Evidence for Two H^+ Channels in the Stator Complex†

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ABSTRACT: Bacterial flagella are turned by rotary motors that obtain energy from the membrane gradient of protons or sodium ions. The stator of the flagellar motor is formed from the membrane proteins MotA and MotB, which associate in complexes that contain multiple copies of each protein. The complexes conduct ions across the membrane, and couple ion flow to motor rotation by a mechanism that appears to involve conformational changes [Kojima, S., and Blair, D. F. (2001) *Biochemistry 40*, 13041−13050]. Structural information on the MotA/MotB complex is very limited. MotA has four membrane-spanning segments, and MotB has one. We have begun a targeted disulfide-cross-linking study to probe the arrangement of membrane segments in the MotA/MotB complex, beginning with the single membrane segment of MotB. Cys residues were introduced in 21 consecutive positions in the segment, and disulfide cross-linking was studied in MotA/MotB complexes either in membranes or detergent solution. Most of the Cys-substituted MotB proteins formed disulfide-linked dimers in significant yield upon oxidation. The yield of dimer varied regularly with the position of the Cys substitution, following the pattern expected for a parallel, symmetric dimer of α-helices. In a structural model based on the cross-linking experiments, critical Asp32 residues that are believed to facilitate proton movement are positioned on separate surfaces of the MotB dimer and so probably function within two distinct proton channels. Regions accessible to solvent were mapped by measuring the reactivity of introduced Cys residues toward *N*-ethyl maleimide and a charged methanethiosulfonate reagent. Positions near the middle of the segment were inaccessible to sulhydryl reagents. Positions within 6−8 residues of either end, which includes residues around Asp32, were accessible.

Many species of bacteria swim by means of semirigid helical filaments that are turned by rotary motors in the cell membrane. The filament/motor organelle is called a flagellum. The rotary motors obtain energy from the membrane gradient of protons or, in some marine or alkalophilic species, sodium ions. In most species, the motors can rotate either CW[1] or CCW, and controlled switching between CW and CCW directions of rotation is the basis for directed movement such as chemotaxis (see refs *1*−*3* for reviews).

The molecular mechanism of the flagellar motor is not understood. Although more than 40 proteins are needed for assembly and operation of the bacterial flagellum, only five—MotA, MotB, FliG, FliM, and FliN—appear to be involved in torque generation (*4*, *5*). FliG, FliM, and FliN function in a complex that is attached to the base of the flagellum (*6*) and that probably rotates with the filament (but see also ref *7*). Of these three proteins on the rotor, FliG is the one most involved in the generation of torque (*8*, *9*). FliM is most important in CW−CCW switching (*10*), and FliN may have mainly a structural role (*9*, *11*). The stator is formed from the membrane proteins MotA and MotB, which function together to conduct ions across the membrane and to couple ion flow to motor rotation (*12*, *13*). MotA and MotB bind to each other and can be co-isolated via an affinity tag on MotB (*14*). A recent study using gel-filtration chromatography showed that the *V. alginolyticus* homologues of MotA and MotB, called PomA and PomB, form a complex with an apparent mass consistent with four copies of PomA and two copies of PomB (*15*).

MotA has four membrane-crossing segments, two short segments in the periplasm, and two long segments in the cytoplasm (Figure 1). The cytoplasmic domain of MotA contains two charged residues, Arg90 and Glu98, that are important for motor rotation and that interact with charged residues of the rotor protein FliG (*20*). Two proline residues (Pro173 and Pro222 in the *Escherichia coli* protein) are conserved near the cytoplasmic ends of the third and fourth membrane segments of MotA. These Pro residues are important for motor rotation, possibly functioning to regulate conformational changes that couple proton movement to rotor movement (*21*, *22*). MotB has a small N-terminal domain in the cytoplasm, a single transmembrane segment, and a

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; β-ME, β-mercaptoethanol; CW, clockwise; CCW, counterclockwise; EDTA, ethylenediamine-tetraacetic acid, MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; NEM, *N*-ethyl maleimide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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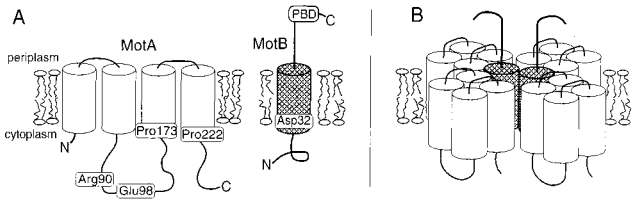

FIGURE 1: (A) Membrane topologies of the MotA and MotB proteins, and approximate locations of Asp32 and other functionally important residues identified by mutational studies. The topology of MotA is based on the hydrophobicity profile reported by Dean et al. (*16*) and cysteine-accessibility experiments of Zhou et al. (*17*). MotB topology is based on the sequence from Stader et al. (*18*) and PhoA-fusion experiments of Chun and Parkinson (*19*). "PBD" signifies a sequence motif believed to function as a peptidoglycan binding domain. The most hydrophobic segment of MotB contains residues ca. 29−49, the positions mutated in this study. (B) Probable stoichiometry of MotA subunits (light gray, four copies) and MotB subunits (dark gray, two copies) in the stator complex, based on sizing chromatography experiments of Sato and Homma (*15*).

large domain in the periplasm that includes a motif believed to bind peptidoglycan and so provide an anchor to the cell wall (*19*, *23*). An aspartate residue (Asp32 in *E. coli* MotB) is conserved near the cytoplasmic end of the MotB membrane segment and is the only conserved, titratable residue of the motor essential for rotation. Mutations in Asp32 prevent motor rotation (except the D32E mutation, which allows slow rotation), and also prevent proton flow, indicating a role in proton transfer (*24*). In the study detailed in the previous paper in this issue, we obtained evidence that proton association/dissociation at Asp32 would cause conformational changes in the MotA/MotB complex, which might work on the rotor to drive rotation.

Structural studies of MotA and MotB have been hampered by the fact that they are membrane proteins and have so far resisted crystallization. Useful information on the arrangement of membrane segments in integral membrane proteins can be obtained by using targeted disulfide cross-linking, a method pioneered by Falke and Koshland (*25*) and since used by several groups to investigate the membrane segments of bacterial chemoreceptors (*26*−*28*) and other integral membrane proteins (e.g., refs *29* and *30*). In this approach, cysteine residues are introduced into various positions in one or more membrane segments, and Cys pairs that can form a disulfide bond upon oxidation are identified. Because the distance between Cys residues is a major factor affecting rates of disulfide formation, the method provides information on residue proximities. This information can be used to develop a model for the arrangement of the membrane segments, both within a single molecule and between different molecules in an oligomeric complex. An advantage of the method is that it can use the protein(s) in membranes and so does not require detergents or purification steps that might disrupt fragile complexes.

Here we present the results of a targeted cross-linking study of the membrane segment of MotB. Cysteine residues were introduced, one at a time, at positions 29−49 of MotB. All of the mutations except D32C permitted motor rotation, indicating that the introduced Cys residues did not perturb the conformation of the MotA/MotB complexes. The Cys-substituted proteins were oxidized by using either iodine or the catalyst $Cu[1\text{-}10\text{-phenanthroline}]_3$, and cross-link formation was monitored by immunoblots. The observed pattern of cross-linking shows that the membrane segments of two MotB molecules associate in a symmetric dimer of α-helices. The Asp32 residues are displayed on separate surfaces of the MotB dimer, where they are likely to function within two distinct proton channels.

EXPERIMENTAL PROCEDURES

Strains and Plasmids. Strain BL21(De3) was used as host for overexpression of proteins using the *T7* promoter (*31*). Strain RP6894 (deleted for *motA* and *motB*) was a gift from J. S. Parkinson (University of Utah) and was used in assays of function of plasmid-encoded mutant MotB proteins. Mutations in *motB* were made by the Altered Sites (Promega) procedure in plasmid pRF5, a derivative of pAlter-1 that encodes both *motA* and *motB* behind the *T7* promoter. The *motA* gene in plasmid pRF5 was first mutated to change the native Cys240 residue to Ser. In a complementation assay using RP6894 as host, this replacement caused only a small (20%) reduction in swarming rate on soft-agar tryptone plates. Plasmid pRF5 gives high-level expression of MotA and MotB in T7-polymerase containing hosts and levels comparable to wild-type in hosts that do not encode T7-polymerase. Experiments with partially purified MotA/MotB complexes used plasmid pTB1, which encodes MotA and MotB with a hexahistidine tag attached at the C-terminus, and which also uses the *T7* promoter (*14*). Four Cys replacements in MotB (positions 31−34) were moved from plasmid pRF5 into pTB1 by transferring an *Mlu*I−*Nsi*I fragment that contains the mutated segment. Experiments to study cross-linking of MotB in the absence of MotA used plasmid pGM1, which expresses MotB from the *tac* promoter, at levels 3−5-fold wild-type when induced with 100 μM IPTG. Selected Cys replacements in MotB (single replacements at positions 38 and 40, and the triple replacement 36/42/49) were transferred to pGM1 on an *Mlu*I−*Nsi*I fragment. All mutations and ligation steps were confirmed by DNA sequencing. Sequencing and oligonucleotide synthesis were performed by core facilities at the University of Utah. Plasmids were isolated using the QIAprep miniprep system (Qiagen).

Swarming Rates. Rates of swarming in soft agar were measured as described before (*32*). The cells were strain RP6894 (Δ*motA*Δ*motB*) transformed with plasmid pRF5 encoding MotA and either wild-type or mutant MotB. Two *motB* mutants and the wild-type control were spotted onto tryptone plates containing 0.28% agar, and swarm diameters were measured at regular intervals to determine the rate. Reported rates are relative to the wild-type control present on the same plate.

SDS−PAGE and Immunoblotting. Protein samples were separated on SDS−PAGE mini-gels (Bio-Rad MiniProtean II system). Protein was transferred to nitrocellulose using a semi-dry transfer apparatus (Bio-Rad Transblot SD). Rabbit polyclonal antibody against MotB was prepared as described previously (*24*) and was used at a dilution of 1/1200. HRP-conjugated secondary antibody (Pierce) was used at 1/10000. Bands were visualized using the Super Signal West Pico luminol system (Pierce) and X-ray film. Bands were quantified by using a video densitometer and the software package NIH image.

Isolation of Membranes. A freezer stock of BL21(De3) cells expressing the Cys-mutant MotB proteins from plasmid

pRF5 was used to inoculate 35 mL of LB-broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl/L) containing 100 μg/mL ampicillin, and cultures were grown overnight (approximately 16 h) with shaking at 37 °C. Cells were harvested by centrifugation (3000*g*, 10 min), and the cell pellet was resuspended in 1 mL of 150 mM Tris, pH 8.0, 50 mM EDTA, and 0.6 mg/mL lysozyme (hen egg-white, Sigma) in a 1.7 mL microfuge tube. Samples were incubated at 0 °C for 30 min, frozen (at −70 °C) and thawed once, then lysed by two rounds of sonication (Branson model 450 sonifier, power level 3, 50% duty cycle, ice temperature) for 90 and 60 s. Unlysed cells were pelleted by centrifugation (2000*g*, 10 min) and the supernatant was transferred to a fresh tube. Membranes were collected by centrifugation (16000*g*, 45 min), and the supernatant was removed and replaced with 450 μL of PBS, pH 8.0 (containing per liter 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4). The membranes were resuspended by sonication (power level 1, 50% duty, room temperature) for 10−15 s, and stored at −20 °C.

Protein Solubilization and Partial Purification of MotA/MotB Complexes. Membranes were solubilized using CHAPS (Sigma), by combining equal volumes of thawed membranes and 5% aqueous CHAPS. This solution was immediately diluted 10-fold into 50 mM Tris, pH 8.0, 5 mM EDTA, 200 mM NaCl, and an additional 1/10 volume of 5% CHAPS was added to bring the final CHAPS concentration to 0.68%. Following 10 min of incubation at room temperature, the solution was centrifuged (16000*g*, 10 min) to pellet insoluble material, and the supernatant was transferred to a new tube. The brief exposure to 2.5% CHAPS was found to increase the efficiency of solubilization. Partially purified MotA and MotB in CHAPS were obtained by batchwise affinity purification using His-Bind resin (Novagen), as described previously (*14*).

Cross-Linking with Iodine. Membrane samples for cross-linking were prepared by mixing 10 μL of thawed membranes with 90 μL of PBS (pH 8.0). CHAPS-solubilized membrane proteins were either used directly in cross-linking experiments or, to gauge dilution effects, were diluted 10- or 50-fold in PBS supplemented with CHAPS (0.5%). MotB proteins with Cys residues near either end of the membrane segment (positions 29, 30, 48, and 49) showed significant levels of preexisting cross-link in nonoxidized membrane samples prepared by the protocol above. For these positions, cross-linking experiments used whole cells from midlog cultures. Saturated overnight cultures in 2.0 mL of LB plus 100 μg/mL ampicillin were diluted 200-fold into fresh broth and grown at 37 °C with aeration to OD_{600nm} ca. 0.5. Cells were pelleted by centrifugation and resuspended in 200 μL of 50 mM Tris, pH 8, 200 mM NaCl, and 5 mM EDTA. The sample was then split into two 100 μL aliquots, one for the nonoxidized control and the other for oxidation by iodine.

Crystalline iodine (Sigma) was dissolved in 95% ethanol to make a 500 mM stock solution which was stored at room temperature. Experiments used a 10 mM working solution freshly prepared in 50% ethanol. All cross-linking experiments were done at room temperature (ca. 22 °C). Iodine-oxidized samples were exposed to 200 μM iodine for 2−3 min, quenched with 20 mM NEM (Sigma) for at least 2−3 min, then denatured by addition of SDS to 5% followed by heating to 95 °C for 3 min. NEM-only control samples were exposed to 20 mM NEM for 2−3 min, then denatured in SDS as above. Samples were either frozen for later use or were prepared for SDS−PAGE by adding $^1/_4$ vol 4× loading buffer (8% SDS, 8% glycerol, 50 mM Tris pH 6.8, and 0.13% bromphenol blue).

Cross-Linking with Cu [1,10-phenanthroline]$_3$. Cells from saturated overnight or midlog cultures were collected by centrifugation and resuspended in 200 μL of buffer containing 50 mM Tris, pH 8.0, 0.5 M sucrose, 10 mM EDTA, and 0.2 mg/mL lysozyme and put on ice for 1 h to produce spheroplasts. Cells were lysed by rapid 10-fold dilution into ice-cold water, and 0.11 volume of a Cu[1,10-phenanthroline]$_3$ working stock was added to experimental samples, while non-oxidized controls received NEM to 20 mM. The Cu[1,10-phenanthroline]$_3$ working stock contained 4 mM Cu(II) and 16 mM 1,10-phenanthroline, prepared by diluting a 1 M solution of 1,10-phenanthroline (Sigma) in 95% ethanol, and a 400 mM solution of $CuSO_4$ (Sigma) in water, into 50% ethanol. Samples were incubated at room temperature for 5 min followed by sonication (power 3, 50% duty, 15 s), then incubated for an additional 4 min at room temperature. The reaction was stopped by adding NEM (to 20 mM) and EDTA (to 50 mM) (non-oxidized controls received only the EDTA), followed by denaturation with SDS−PAGE buffer and heating.

Blocking of Disulfide Formation by Sulfhydryl Reagents. For most NEM-protection experiments, samples were treated with 20 mM NEM for 1 min, 200 μM iodine for 2−3 min, and then denatured. At some positions (indicated in the figure legend), Cu[1,10-phenanthroline]$_3$ gave a better yield of dimer and was used as oxidant in the NEM-block experiments. Spheroplasts (prepared as above) were treated with 20 mM NEM for 1 min at room temperature, then lysed by 10-fold dilution into water that already contained 400 μM Cu(II) and 1.6 mM 1,10-phenanthroline, to ensure that the reagent gained immediate access to both sides of the membrane. Cross-linking was allowed to proceed at room temperature, and stopped by adding EDTA to 50 mM and additional NEM sufficient to give a final concentration of 20 mM.

Blocking experiments with MTSET (Anatrace) also used whichever oxidation protocol gave the better yield of dimer at a given position. For iodine experiments, overnight cultures (3 mL of LB with 100 μg/mL ampicillin) were grown from freezer stocks at 37 °C with shaking. The cultures were pelleted by centrifugation (1100*g*, 15 min) at room temperature in a Beckman CS-6 clinical centrifuge. Cells were resuspended in 300 μL of 50 mM Tris, pH 7.0, and 20 mM EDTA. The EDTA and Tris were found necessary in whole-cell experiments with MTSET to increase access of the reagent to the inner membrane. After incubating on ice for 15 min, the samples were separated into three 100 μL aliquots. One aliquot was exposed to only NEM (20 mM), another to iodine (5 mM, 2−3 min) followed by NEM (20 mM), and the third to 1 mM MTSET from a 100 mM stock in water (kept at 0 °C) for 2 min at room temperature, and then iodine (5 mM, 2−3 min, room temperature), then NEM (20 mM, room temperature). For Cu[1,10-phenanthroline]$_3$ experiments, 200 μL of spheroplasts (prepared as described above) were treated with 1 mM MTSET for 2 min at room temperature, diluted to 1.5 mL with additional spheroplast buffer (minus lysozyme), then centrifuged (ca. 4000*g*, 8 min)

Table 1: Swarming Motility of MotB Mutants[a]

mutation	relative swarming rate	mutation	relative swarming rate
A29C	0.82 ± 0.10	F40C	0.40 ± 0.03
Y30C	0.70 ± 0.02	F41C	0.81 ± 0.06
A31C	0.76 ± 0.07	L42C	0.99 ± 0.15
D32C	0.00	V43C	1.01 ± 0.03
F33C	0.45 ± 0.03	M44C	1.02 ± 0.09
M34C	0.89 ± 0.06	W45C	0.21 ± 0.09
T35C	1.05 ± 0.09	L46C	0.92 ± 0.11
A36C	0.76 ± 0.09	I47C	0.94 ± 0.11
M37C	0.99 ± 0.09	S48C	0.95 ± 0.08
M38C	0.85 ± 0.12	I49C	0.92 ± 0.07
A39C	0.99 ± 0.08		

[a] Swarming rates are means ± SD for three determinations, and are relative to wild-type controls present on the same plates.

to remove residual MTSET or derivatives that were found to interfere with Cu[1,10-phenanthroline]$_3$-mediated cross-linking. Spheroplasts were resuspended in 100 μL of spheroplast buffer (minus lysozyme) and then lysed and oxidized by dilution into water containing Cu[1,10-phenanthroline]$_3$, as described above.

RESULTS

Cysteine Substitutions and Disulfide Cross-Linking of MotB. The wild-type MotB protein of *E. coli* contains no Cys residues. Using site-directed mutagenesis, cysteine replacements were made in 21 consecutive positions (residues 29−49) of MotB. This is the most hydrophobic segment of the protein and should thus correspond at least approximately to the segment inserted into the bilayer. The Cys-containing MotB proteins were expressed from plasmid pRF5, which also expresses a Cys-less form of MotA (containing the mutation C240S). To test function of the mutant MotB proteins, the mutant plasmids were introduced into a *motA-motB* deletion strain, and rates of swarming in soft (0.28%) agar were measured. Most of the mutant MotB proteins supported swarming at better than 70% of wild-type (Table 1). Three replacements that involved a large reduction in side-chain volume (F33C, F40C, and W45C) functioned at between 20 and 50% of wild-type, and the D32C mutant was nonfunctional, as expected for a required site of protonation.

To study disulfide cross-linking, Cys-containing MotB and Cys-less MotA were expressed in cells, and membranes (200−300 μg of total membrane protein) were isolated and treated with iodine (200 μM) for 2−3 min at room temperature. The membrane-permeant sulfhydryl reagent NEM was then added to prevent further cross-linking upon denaturation, and samples were electrophoresed and analyzed by immunoblots with anti-MotB antibody. Membranes exposed only to NEM were used as nonoxidized controls. Following iodine treatment, a band was observed at 65−70 kDa in all of the Cys-replacement mutants (Figures 2 and 3). This is about twice the mass of MotB (34 kDa, and with an apparent mass of 35−39 kDa in SDS−PAGE). The band was not detected on anti-MotA immunoblots, and its appearance required a Cys residue in MotB but not in MotA, showing that it does not contain MotA (Figure 2, panels A and B). The 65−70 kDa band was also observed when cross-linking was carried out on MotA/MotB complexes solubilized in CHAPS, partially purified by means of a hexahistidine tag on MotB, and was diminished in intensity upon addition of β-mercaptoethanol (Figure 2C). Coomassie staining of the solubilized, affinity-purified samples showed that MotA and MotB were the only abundant proteins in the mass range that could give rise to a 65−70 kDa cross-linked species containing MotB. Together, these results demonstrate that the 65−70 kDa band is a disulfide-linked dimer of MotB. The yield of the 65−70 kDa cross-linked species was not greatly decreased when the detergent-solubilized proteins were diluted 50-fold, showing that the cross-linking occurred within preexisting

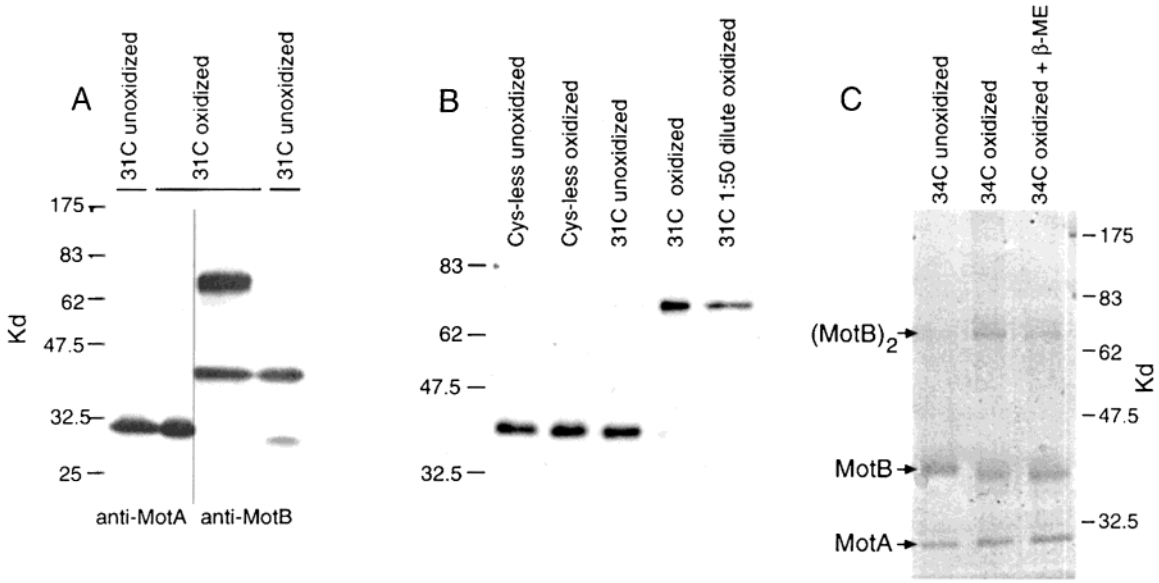

FIGURE 2: Identification of the disulfide-cross-linked MotB dimer. (A) Anti-MotB and anti-MotA immunoblots showing that the cross-linked product contains MotB but not MotA. This experiment used iodine for oxidation, in whole cells. (B) Cross-linking of membrane proteins solubilized in CHAPS and effect of dilution. MotB was either Cys-less or with Cys at position 21, as indicated. Oxidation was by Cu[1,10-phenanthroline]$_3$. An anti-MotB immunoblot is shown. The sample in the rightmost lane was diluted 50-fold prior to cross-linking. (C) Cross-linking in partially purified MotA/MotB complexes, with a Cys residue in MotB position 34 but none in MotA. Shown is a Coomassie-stained gel of membrane proteins solubilized in CHAPS and partially purified via a hexahistidine tag on MotB. Cross-linking was induced by iodine.

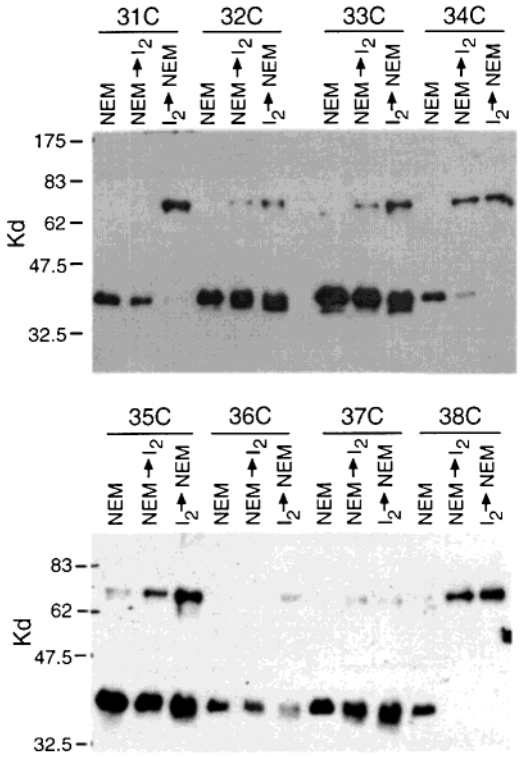

FIGURE 3: MotB immunoblots showing disulfide cross-linking of MotB proteins with Cys in 8 positions. For each position, three experiments are shown. "NEM" is a control in which samples were treated only with NEM (20 mM). "NEM → I_2" is an experiment to test accessibility of the position to NEM, by treating samples first with NEM (20 mM) for 1 min, then cross-linking with I_2 (200 μM). "I_2 → NEM" is an experiment in which samples were treated first with iodine (200 μM) for 2−3 min, then with NEM (20 mM). The dimer yields in iodine-treated samples in this experiment were as follows: 31C, 100%; 32C, 14%; 33C, 27%; 34C, 100%; 35C, 24%; 36C, 17%; 37C, 2%; 38C, 100%.

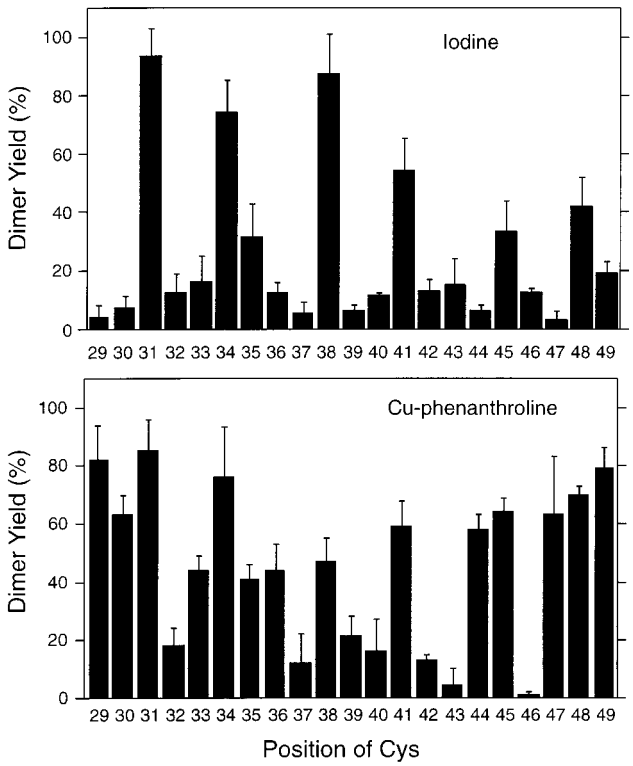

FIGURE 4: (A) Summary of yield of disulfide-cross-linked MotB dimer, vs position of the introduced Cys residue, with iodine (200 μM) as oxidant. Data are averages ± SD for at least three determinations. The yield at position 48 makes allowance for some (on average 15% and at most 25%) preexisting cross-linked dimer. (B) Summary of yield of disulfide-cross-linked MotB dimer, using Cu[1,10-phenanthroline]$_3$ to induce cross-linking. Data are averages of three determinations ± SD.

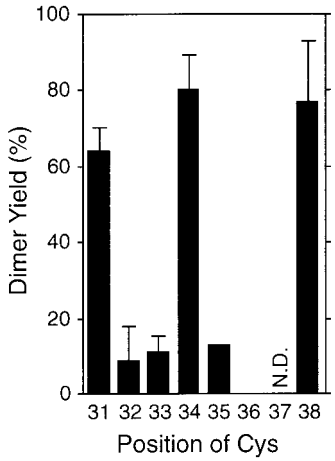

FIGURE 5: Iodine (200 μM) mediated disulfide cross-linking of Cys-containing MotB proteins solubilized and diluted 10-fold in a CHAPS buffer. At positions showing error bars, data are averages ± SD for at least three determinations. The value for position 35 is the average of two determinations, and for position 36, a single experiment that gave no cross-linked dimer. N.D., not determined.

complexes and not by a collisional mechanism (Figure 2B).

The efficiency of disulfide formation varied with the position of the Cys substitution (Figures 3 and 4). Most notably, the positions giving a high yield showed a clear periodicity of 3−4 residues, with maxima at positions 31, 34, 38, 41, 45, and 48. Positions 31, 34, and 38 showed essentially complete conversion to dimer in some experiments and showed better than 70% conversion in the majority of experiments. On an α-helix, the high-yield positions cluster on one face. The critical residue Asp32 is removed from this face by one residue, or about 100° (cf. Figure 7).

We also studied iodine-induced cross-linking of five multiple-Cys replacements, obtained by accident during the mutagenesis (32/38; 36/47; 39/49; 36/42; and 36/42/49). The Cys residues in most of these multiple mutants would be displayed on different faces of an α-helix and so should provide additional opportunity for cross-linking into species larger than dimers, if such species are present. All of the multiple-Cys mutants produced cross-linked MotB dimer, but none produced species larger than 65−70 kDa in significant yield (data not shown).

For a subset of positions (*31*−*38*), iodine-mediated cross-linking was also studied in membrane samples solubilized in 0.68% CHAPS, a detergent shown previously to allow co-isolation of MotA with His-tagged MotB (*14*). Cross-linking experiments in CHAPS also used 200−300 μg of total membrane protein and 200 μM iodine. Anti-MotB immunoblots showed the same dimer band, and the pattern of dimer yield vs position was similar to that seen with proteins in membranes (Figure 5).

The MotB dimer can form in the absence of MotA, because it was also seen when selected Cys-containing MotB proteins (Cys at position 38 or 40) were expressed from plasmid pGM1, which does not express MotA. As in the presence of MotA, yield was high with Cys at position 38

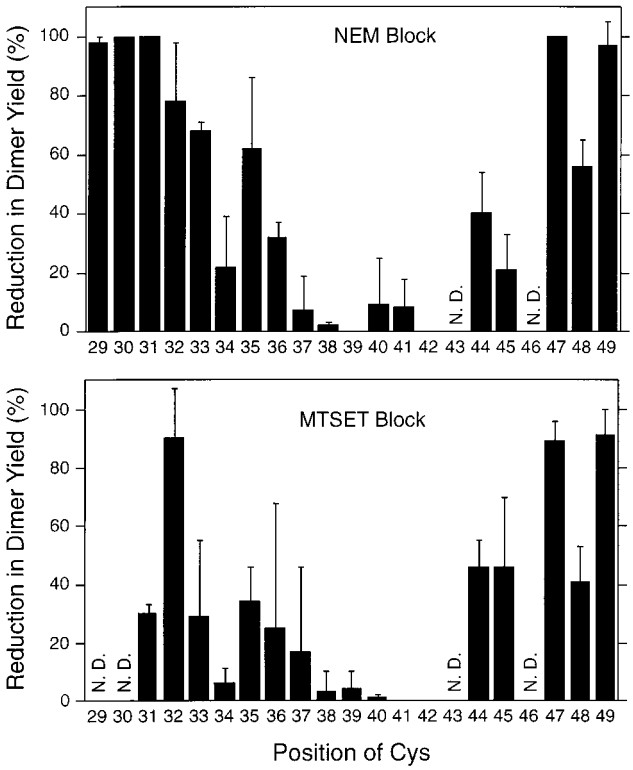

FIGURE 6: Reaction of the introduced Cys residues with sulfhydryl reagents, assayed by the resulting block of disulfide cross-linking. (A) Reduction in yield of MotB dimer caused by pretreatment with NEM (20 mM) for 1 min, as a function of position of the Cys residue. Oxidation was by iodine at most positions, but Cu(II)[1,-10-phenanthroline]$_3$ was used at positions 29, 30, 36, 44, 45, 47, 48, and 49, where it gives better dimer yield than iodine. N.D., not determined. (B) Reduction in yield of MotB dimer caused by pretreatment with MTSET (1 mM) for 2 min. Cu[1,10-phenanthroline]$_3$ was used to induce cross-linking at positions 44, 45, 47, 48, and 49; iodine was used at other positions. N.D., not determined.

(74 ± 23%; mean ± SD for four determinations) and lower at position 40 (21 ± 5%, four determinations). The MotB−MotB association is less stable in the absence of MotA, however. When the triple-Cys mutant MotB protein (36/42/49) was expressed from pGM1, it accumulated in membranes to a level similar to the single mutants, but in contrast to the experiment with MotA present, it did not yield any MotB dimer upon oxidation.

Cross-Linking Catalyzed by Cu[1,10-phenanthroline]$_3$. In the presence of oxygen, Cu(II)[1,10-phenanthroline]$_3$ is an efficient catalyst of disulfide bond formation and has been used in several targeted-cross-linking studies (e.g., refs *26* and *28*). Whereas iodine causes rapid (within seconds) oxidation of cysteine sulfhydryl groups either to disulfides or to other oxidized species that can no longer form disulfides, Cu[1,10-phenanthroline]$_3$ produces oxidizing species that appear gentler in the sense that cross-linking can continue for several minutes and time courses can be measured. To see if a different method of oxidation gave the same pattern of cross-linking yields, we used Cu[1,10-phenanthroline]$_3$ to catalyze cross-linking of Cys-substituted MotB proteins in membranes. Results are summarized in Figure 4B.

The overall pattern of cross-linking with Cu-phenanthroline was similar to that seen with iodine. Some differences were evident, however. Yield was lower at position 38, near the middle of the membrane segment, whereas yields were higher at positions near the ends. Certain positions near the ends that gave low yields with iodine showed moderate yields with Cu-phenanthroline, making the helical periodicity less clear there. As discussed below, this probably reflects the longer lifetime of reactive intermediates produced by Cu-phenanthroline and a resultant increase in the distance over which cross-linking can occur.

Accessibility of Introduced Cys Residues to Sulfhydryl Reagents. The extent of chemical modification of cysteine sulfhydryl groups can be monitored by the resulting reduction in yield of disulfide-linked dimer. While a number of factors can affect rates of sulfhydryl modification, accessibility to solvent should be a major one, and overall patterns of reactivity should, therefore, reflect patterns of solvent exposure. We measured the relative reactivity of introduced Cys residues to NEM, by comparing dimer yield in membranes treated first with iodine and then blocked with NEM versus membranes treated with NEM for 1 min prior to oxidation by iodine. Examples of NEM-block experiments are shown in Figure 3 (middle lane for each Cys mutant). The efficiency of protection by NEM was computed as the fractional reduction in dimer yield. Positions near the ends of the membrane segment showed efficient protection by NEM, whereas positions 37−42, in the middle of the segment, showed little protection (Figure 6A). Protection appeared to be less efficient at positions near the putative MotB−MotB interface; the block by NEM was less complete at positions 34 and 48 than at adjacent positions (cf. model in Figure 7).

NEM is membrane-permeant and not very large and so might gain access to parts of a membrane protein exposed to lipid or to other membrane segments. As a stricter probe of accessibility to solvent water, we examined reactions of the introduced Cys residues with a positively charged methanethiosulfonate reagent, MTSET (*33*−*35*). MTSET reacts with the thiolate form of a Cys side chain to form a mixed disulfide. The pattern of cross-linking inhibition was basically like that seen with NEM; positions near either end of the segment were readily blocked by the reagent, whereas positions 38−42 in the middle of the segment were not blocked (Figure 6B). Protection was again less efficient at interfacial positions. The block by MTSET was less complete at positions 31, 34, and 48 than at adjacent positions.

DISCUSSION

The side chain of cysteine is nonpolar and relatively small, and so Cys substitutions in a membrane segment are not expected to cause major perturbations to structure. Only 4 of 21 Cys substitutions in the membrane segment of MotB had significant effects on function. Three of these (the replacements of Phe33, Phe40, and Trp45) involve large decreases in side-chain volume, and the fourth is the replacement of the critical residue Asp32. Protein levels for all the mutants were comparable, and so even those mutations that impair function do not appear to destabilize the protein. It is likely, therefore, that the Cys-substituted proteins retain a native fold and normal subunit associations within the MotA/MotB complex.

Dimer Model of MotB. A straightforward interpretation of the cross-linking data is that the membrane segment of

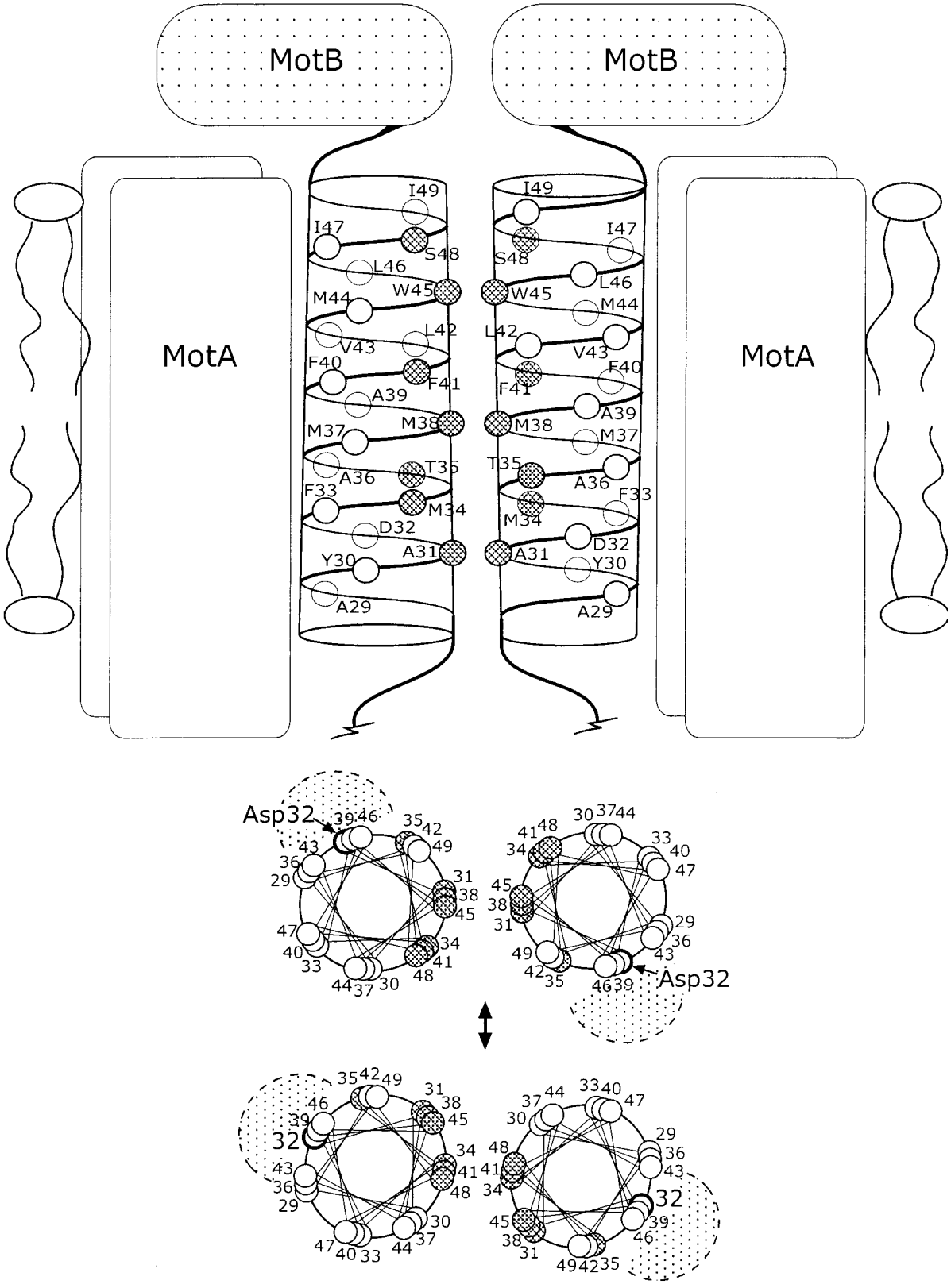

FIGURE 7: Arrangement of MotB membrane segments deduced from the disulfide cross-linking results. (Top) Side-view of the hypothetical arrangement of the membrane segments of two MotB molecules, in a symmetric dimer of α-helices. Multiple (probably four) copies of MotA are also present, shown surrounding the MotB dimer. The MotA subunits are not drawn to scale. Here and in the bottom panel, shaded circles denote positions that gave 30% or better yield of disulfide-linked dimer in the cross-linking experiments using iodine as oxidant. *Bottom panel:* End-view of the approximate arrangement of MotB membrane segments, looking from the cytoplasmic side of the membrane. Because positions 31, 34, 38, 41, 45, and 48 all give a high yield of cross-linking, the segments must have some motional freedom about their long axes. This motional freedom is represented by the two arrangements shown, which differ by 40° rotations of each segment. Because the two critical Asp32 residues are not at the interface, they might function in two distinct proton channels, indicated by the stippled semicircles.

MotB is an α-helix and that two such segments form a symmetric dimer (Figure 7). The association of MotB molecules is direct and does not require MotA, because cross-linking was efficient even in the absence of MotA. The very efficient cross-linking of Cys residues at some putative interfacial positions (31, 34, and 38; Figure 4) shows that the two MotB segments in the dimer are in close contact and also points to a symmetric association in which the dimer interface is formed from equivalent residues of the two molecules.

The cross-linking results provide good evidence that the native MotA/MotB complex contains two copies of MotB. Cross-linking of MotB into a dimer occurs within a preexisting complex rather than by a collisional mechanism, because the yield of cross-linked dimer was not much reduced when the membranes were solubilized with detergent and diluted by 50-fold. The complex does not contain three copies of MotB, because in that case the dimer yield should not exceed 67%, whereas actual yields were 100% in some experiments and greater than 70% in many experiments. The complex is unlikely to contain four or more copies of MotB, because cross-linked species larger than the dimer were not seen in significant yield with Cys in any single position or in two or three positions on different surfaces of the MotB helix. A MotB dimer is in accord with the recent sizing-chromatography study of the *Vibrio* proteins PomA and PomB (*15*), which gave evidence of a stoichiometry $(PomA)_4(PomB)_2$.

The yield of cross-linked dimer showed clear α-helical periodicity, particularly in the experiments using iodine (Figure 4A). Nevertheless, positions predicted by the model (Figure 7) to be on opposite faces of the complex gave a measurable yield of cross-linked dimer. Such cross-linking over a long distance has been noted in other targeted-disulfide studies of membrane proteins (e.g., refs *27* and *28*) and is due to flexibility of the protein coupled with the ability of a disulfide bond to trap rare conformations. In a targeted-disulfide study of the galactose binding protein, Careaga and Falke (*36*) showed that disulfide cross-linking can trap conformations in which helices are moved by at least 15 Å, or rotated by at least 35°, from their most stable position and orientation.

Effects of Oxidation Strategy. We found appreciable differences between iodine and copper-phenanthroline as oxidants. Some of the differences may be due to a more restricted access of oxidizing species produced by copper-phenanthroline to the interior of membrane segments. The major factor is probably a longer lifetime of the intermediates produced by copper-phenanthroline, which would allow trapping of more-distorted conformations. In a cross-linking study of an especially flexible segment of the chemoreceptor Tar, 58 consecutive Cys substitutions gave uniformly high yields of disulfide cross-linking by copper-phenanthroline (*37*), with no periodicity apparent. Helical periodicity in cross-linking rates was seen, however, when the copper-phenanthroline was buffered at a low concentration by adding EDTA (*37*, *38*). Although both strategies have been used with good success in targeted-disulfide studies and copper-phenanthroline might be better in some instances, our results show that, in the present case, iodine gives a more interpretable pattern of disulfide yields.

Solvent-Accessible Regions in the MotA/MotB Complex. The blocking experiments with sulfhydryl reagents, particularly the charged reagent MTSET, show that much of the MotB segment is accessible from the solvent. MTSET is positively charged and sizable (fitting in a cylinder ~1 nm in length and 0.6 nm in diameter), yet reacted with Cys residues about one-third of the way in from either end of the segment. This is consistent with a channel function of the MotA/MotB complexes. We propose that part of the ion pathway may consist of aqueous cavities that extend some distance into the MotA/MotB complex from either end. The MotA/MotB complex is not open to significant transmembrane ion flow when it is not associated with motors, however, as overexpression of the MotA and MotB proteins does not cause a proton leak (*13*). The complex functions in the motor to couple ion flow to rotation, and so it must contain elements that regulate proton movement at some point(s) along the pathway. The present results indicate that the elements regulating ion flow are likely to be in the middle one-third of the channel, the region inaccessible to the sulfhydryl reagents. Access appears unrestricted from the cytoplasm to Asp32, and to even the deeper residue 35, a finding that has significance for the motor mechanism given the critical role of Asp32 (see the previous paper in this issue).

Two Proton Channels. In a cross-linking-based model for the MotB dimer (Figure 7), the two Asp32 residues are not at the interface between MotB molecules but are displayed on separate faces. Given this arrangement, it seems likely that the two Asp32 residues function in two distinct channels rather than a single channel. Sizing chromatography experiments gave evidence of four copies of MotA (PomA) in the stator complex (*15*), and each MotA has four membrane segments. Sixteen MotA membrane segments would seem sufficient to form a framework for two proton channels, one on either side of the MotB dimer. Physiological data are also more consistent with a two-channel complex than a single-channel complex. The motor contains several (ca. 8) independent torque generators (*39*), and careful measurements show that each produces a torque of about 300 pN nm (*40*). FliG, the protein of the rotor that interacts with the stator, is present in some 25−35 copies/motor (*41*−*43*). If a single proton flowed through the MotA/MotB complex each time it moved past a FliG subunit on the rotor, then the proton stoichiometry would be about 35 protons/stator complex/revolution. The maximum torque possible, given a typical value of the protonmotive force (~160 mV), would then be about 140 pN nm per stator complex. If, instead, two protons flow through the stator complex each time it passes a rotor subunit, then the maximum torque would be about 280 pN nm per complex, close to the measured value.

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