*Articles*

# Solubilization and Purification of the MotA/MotB Complex of *Escherichia coli*†

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ABSTRACT: Bacterial flagella are driven at their base by a rotary motor fueled by the membrane gradient of protons or sodium ions. The stator of the flagellar motor is formed from the membrane proteins MotA and MotB, which function together to conduct ions across the membrane and couple ion flow to rotation. An invariant aspartate residue in MotB (Asp32 in the protein of *E. coli*) is essential for rotation and appears to have a direct role in proton conduction. A recent study showed that changes at Asp32 in MotB cause a conformational change in the complex, as evidenced by altered patterns of protease susceptibility of MotA [Kojima, S., and Blair, D. F. (2001) *Biochemistry 40* (43), 13041−13050]. It was proposed that protonation/deprotonation of Asp32 might regulate a conformational change in the stator that acts as the powerstroke to drive rotation of the rotor. Biochemical studies of the MotA/MotB complex have been hampered by the absence of a suitable assay for its integrity in detergent solution. Here, we have studied the behavior of the MotA/MotB complex in a variety of detergents, making use of the protease-susceptibility assay to monitor its integrity. Among about 25 detergents tested, a few were found to solubilize the proteins effectively while preserving certain conformational properties characteristic of an intact complex. The detergent dodecylphosphocholine, or DPC, proved especially effective. MotA/MotB complexes purified in DPC migrate with an apparent size of ∼300 kDa in gel-filtration columns, and retain the Asp32-modulated conformational differences seen in membranes. $^{35}$S-radiolabeling showed that MotA and MotB are present in a 2:1 ratio in the complex. Purified MotA/MotB complexes should enable in vitro study of the proton-induced conformational change and other aspects of stator function.

Bacterial flagella consist of helical propellers turned by rotary motors in the cell membrane (*1*, *2*). The flagellar motors are fueled by the membrane gradient of protons (*3*) or sodium ions (*4*). Bacteria direct their swimming toward nutrients, optimal temperatures, or other factors that favor survival. In many species, the pattern of swimming is controlled by reversals between CW[1] and CCW motor rotation, in response to various sensory inputs. In *E. coli* or *Salmonella*, for example, CCW rotation causes a cell to swim smoothly whereas CW rotation causes the cell to tumble (*2*, *5*). When the concentration of a chemoattractant is increasing with time, as it does when a cell is swimming up a gradient of the attractant, CCW intervals are prolonged and the cell progresses up the gradient (*6*). Some other species steer by other means, periodically stopping their motor or modulating the motor speed (*7*, *8*). For reviews of the flagellar motor see refs *9*−*12*.

The rotor of the flagellar motor contains a protein assembly termed the "switch complex" that functions in rotation and CW/CCW switching (*13*, *14*). The switch complex is formed from the proteins FliG, FliM, and FliN, each present in many copies (*14*−*16*). FliG is most important for rotation (*17*, *18*), and contains charged residues that interact with the stator

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[1] Abbreviations: 4-APMSF, *p*-amidinophenylmethanesulfonyl fluoride; BCA, bicinchoninic acid; BME, β-mercaptoethanol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; $C_{12}E_8$, dodecyloctaethylene glycol ether; cmc, critical micellar concentration; CCW, counterclockwise; CW, clockwise; CyFos-5, 5-cyclohexyl-1-pentylphosphocholine; CyFos-6, 6-cyclohexyl-1-hexylphosphocholine; CyFos-7, 7-cyclohexyl-1-heptylphosphocholine; DDAO, *N*-dodecyl-*N*,*N*-dimethylamine *N*-oxide; DDAPS, *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate; DG, *n*-dodecyl β-D-glucopyranoside; DM, *n*-dodecyl β-D-maltopyranoside; DPC, *n*-dodecylphosphocholine; EDTA, ethylenediaminetetraacetic acid; Fos-Choline-8, *n*-octylphosphocholine; Fos-Choline-9, *n*-nonylphosphocholine; Fos-Choline-10, *n*-decylphosphocholine; Fos-Choline-11, *n*-undecylphosphocholine; Fos-Choline-12, *n*-dodecylphosphocholine; Fos-Choline-13, *n*-tridecylphosphocholine; Fos-Choline-14, *n*-tetradecylphosphocholine; Fos-Choline-15, *n*-pentadecylphosphocholine; Fos-Choline-16, *n*-hexadecylphosphocholine; Fos-MEA-8, octylphospho-*N*-methylethanolamine; Fos-MEA-10, decylphospho-*N*-methylethanolamine; Fos-MEA-12, dodecylphospho-*N*-methylethanolamine; IPE, isotridecylpoly(ethylene glycol ether); IAA, 3-β-indoleacrylic acid; IPTG, isopropyl β-D-thiogalactopyranoside; MEGA-10, decanoyl-*N*-methylglucamide; NEM, *N*-ethylmaleimide; OG, *n*-octyl β-D-glucopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Thesit, dodecylpoly(ethylene glycol ether); Tris, tris(hydroxymethyl)aminomethane.

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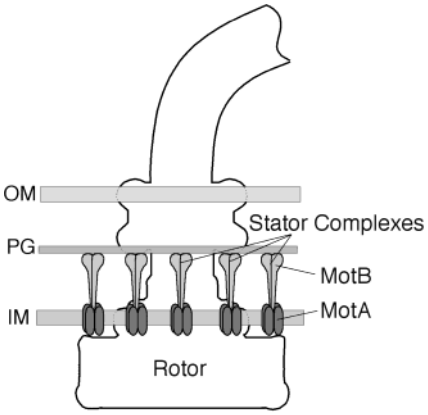


FIGURE 1: Structure at the base of the flagellum in a Gram-negative bacterium. The rotating part is shown in white, and the stator in shades of gray. The stator consists of multiple complexes of the membrane proteins MotA and MotB, which are believed to attach to peptidoglycan via the periplasmic domain of MotB. Results of the present study and of Sato and Homma (*36*) are consistent with the subunit composition $MotA_4MotB_2$, as pictured.

(*19*, *20*). FliM is most important for CW/CCW direction switching (*21*) and is the target for binding of phospho-CheY, a signaling molecule that is regulated by the chemosensory pathway and promotes CW rotation (*22*). The role of FliN is not known, but may be primarily structural (*16*, *17*). The stator is formed from the membrane proteins MotA and MotB (*23*−*25*). Each motor contains several MotA/MotB complexes, which function independently to conduct ions across the membrane and produce the torque for rotation (*26*−*29*) (Figure 1). In freeze-fracture electron micrographs, the stator complexes appear as circular arrays of particles in the cytoplasmic membrane (*25*, *30*). These particles surround a central "stud" whose dimension is consistent with the rod structure. In the micrographs of *E. coli* membranes, each array contains from 10 to 12 particles. Torque-restoration experiments, on the other hand, suggest that each motor in *E. coli* has eight independent torque-generating units (*27*).

The mechanism of torque generation is not understood in detail, but recent results suggest that much of the action occurs within the stator complexes. The protons that energize the motor appear to follow a path that is contained within the stator and that includes an invariant Asp residue in MotB, Asp32. This residue in the stator is essential for motor rotation, whereas no titratable residue of the rotor is singly critical (*31*). Charge-neutralizing mutations in Asp32 of MotB cause a conformational change in the stator, which affects a domain of MotA known to interact with the rotor (*32*). Our working hypothesis is that protonation of Asp32 causes a conformational change in the MotA/MotB complex that works on the rotor to drive rotation.

Physiological (*29*) and genetic (*33*, *34*) studies gave the first indications that MotA and MotB function together in a complex. Subsequent biochemical studies showed that, when either MotA or MotB is isolated by means of an affinity tag, the other protein is copurified (*35*, *36*). In a study using gel filtration and quantification of Coomassie-stained bands on gels, Sato and Homma (*36*) presented evidence for a subunit composition of $PomA_4PomB_2$ in the stator complex of the sodium-driven motor of *Vibrio*. (The $Na^+$-using motor of *Vibrio* is located at the cell pole, and the stator proteins are called Pom for polar motility.) PomA/PomB complexes were purified and reconstituted into liposomes, and were shown to catalyze $Na^+$ translocation, albeit at a relatively slow rate. The size of the membrane-inserted complex was not determined, and the subunit composition of the functional unit remains uncertain. Biochemical studies of the MotA and MotB proteins are less advanced. Some initial efforts to purify the MotA/MotB complex have been reported (*35*), but procedures have not yet been established for solubilizing the complex in intact form.

A limitation in our previous studies of the Mot protein complex has been the lack of a suitable assay for its integrity in detergent solution. The Asp32-regulated conformational change appears to be important for function, and it requires MotA and MotB to be present together in the complex (*32*). It thus provides a means for monitoring the intactness of the complex, which we have used here to screen a variety of detergents. We find that a few detergents can solubilize the MotA/MotB complex efficiently while preserving its conformational properties as assayed by limited proteolysis. In an appropriate detergent, the complex can be isolated by affinity chromatography and gel filtration, and exhibits Asp32-regulated conformational responses similar to those observed in membranes. Thus, the conformational change requires no proteins besides MotA and MotB, and can be studied in vitro using purified material. The size of the purified complex, together with the MotA:MotB ratio determined by radiolabeling, indicates the subunit composition $MotA_4MotB_2$.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains and Plasmids. E. coli* strains and plasmids used are listed in Table 1. Plasmid purification and other DNA manipulations used standard procedures. The mutation D32N in MotB was made in plasmid pRF4 using the altered sites procedure (Promega) and then transferred into plasmid pDFB45, pSK11, pSK15, pSK16, or pSK17 by transfer of restriction fragments.

*Construction of Plasmids That Overexpress MotA*−*His and MotB.* Plasmids for overexpression of His-tagged MotA together with MotB were constructed by PCR. Sequences encoding an oligo-His tag were attached to the 3′ end of the *motA* gene by amplification of the gene on plasmid pDFB45, using an upstream primer that anneals to internal sequences of *motA* and a downstream primer (5′-CCG CCG *CTC GAG* TCA ATG GTG ATG GTG ATG GTG ATG GTG TGC TTC CTC GGT TGT CGT-3′) that encodes an *Xho*I/*Ava*I site (italics) and eight His residues. The amplified fragment was digested with *Bst*BI and *Xho*I and cloned into similarly digested pTB1, to make pSK9. The *motB* gene was amplified, again using pDFB45 as template, using the upstream primer 5′-CCG CCG CTC **GAG G**AA ***AT***A TGA AGA ATC AAG CG-3′ and the downstream primer 5′-CCG CCG CTC GAG TCA CCT CGG TTC GGC TGA-3′. Boldface type indicates the Shine−Dalgarno sequence, and boldface italic type indicates two nucleotides that were altered to disrupt the hairpin structure in the mRNA (*37*). The amplified *motB* fragment was digested with *Ava*I and cloned into the *Xho*I/*Ava*I site in pSK9 to make pSK11, which encodes *motA*−*his*$_8$ and *motB* under the control of the T7 promoter. To put *motA*−*his*$_8$ and *motB* behind the *trp* promoter, a *Bst*BI−*Nsi*I fragment of pSK11 that encodes C-terminal parts of MotA

Table 1

| strain or plasmid | relevant genotype or properties | source or ref |
|---|---|---|
| *E. coli* | | |
| RP437 | Wild type for motility and chemotaxis | J. S. Parkinson |
| RP6894 | Δ*motA motB* | J. S. Parkinson |
| BL21(De3) | host for overexpression from the T7 promoter | *38* |
| Plasmids | | |
| pDFB45 | $P_{trp}$−*motA motB* Ap$^r$, parent of pSK15,16 | *29* |
| pRF4 | $P_{T7-}$*motA motB* Ap$^r$, used in making *motB* mutation D32N | *31* |
| pTB1 | $P_{T7}$−*motA motB*−*His*$_6$ Ap$^r$, parent of pSK9,11,17 | *35* |
| pSK9 | $P_{T7}$−*motA*−*his*$_8$ Ap$^r$ | this study |
| pSK11 | $P_{T7}$−*motA*−*his*$_8$ *motB* Ap$^r$ | this study |
| pSK15 | $P_{trp}$−*motA*−*his*$_8$ *motB* Ap$^r$ | this study |
| pSK16 | $P_{trp}$−*motA*−*his*$_8$ *motB* Ap$^r$, optimized SD sequence | this study |
| pSK17 | $P_{T7}$−*motA*−*his*$_8$ *motB* Ap$^r$, optimized SD sequence | this study |

and N-terminal parts of MotB was transferred into the corresponding sites in pDFB45. To improve *motB* expression from pSK11, additional nucleotide changes were made according to VanWay et al. (*37*), to optimize the Shine−Dalgarno sequence and further disrupt the hairpin structure in the mRNA. Changes were introduced by PCR using the same downstream primer as before and the upstream primer 5′-CCG CTC G**AG GAG GT**T TAA TAT GAA GAA ***CAC*** AGC GCA TCC GAT TAT TGT C-3′. The optimized S−D sequence is shown in boldface. Three nucleotides that were changed to disrupt secondary structure in the transcript are in boldface italics; these cause the amino acid replacement Gln4 → Thr in MotB, which was shown not to affect function (*37*). The amplified *motB* fragment was digested with *Ava*I and *Nsi*I and cloned into pSK15, making pSK16. To place the new *motA*−*his*$_8$/*motB* construct behind the T7 promoter, a *Bst*BI−*Nsi*I fragment was transferred into pSK11, giving pSK17. Plasmids encoding the D32N mutation in MotB were made in the same way as described above for the wild type, except using mutant pDFB45 encoding the D32N replacement as template. Sequences of the constructs in the region of the MotA−MotB junction are shown in Figure 3A.

*Detergent Solubilization of MotA/MotB Complexes.* Membranes containing MotA/MotB complexes with either wild-type or D32N-mutant MotB were isolated as described previously (*32*). Membranes were resuspended in TNCG buffer (50 mM Tris−Cl, pH 7.5, 50 mM NaCl, 2 mM $CaCl_2$, 20% (v/v) glycerol) to a final concentration of 2.5 mg/mL protein and then solubilized by adding 1/10 volume of 10×-concentrated detergent solution, typically for 1 h at 4 °C. With some detergents, a 15 min incubation was found to be as effective in solubilizing the proteins and was used in later experiments. Unsolubilized material was removed by centrifugation (16000*g*, 15 min) at 4 °C. Pellets were resuspended in a volume equal to that of the starting sample. Equal volumes of unfractionated, supernatant, and pellet samples were loaded on SDS−PAGE gels for analysis by immunoblotting, using polyclonal antibody raised against the cytoplasmic domain of MotA and procedures described previously (*32*).

*Protease-Digestion Assay for Screening Detergents.* Solubilized stator complexes containing either wild-type MotB or D32N-mutant MotB were digested with Trypsin (Sigma), using protocols similar to those reported previously (*32*). The optimal protease concentration and temperature of digestion were found to vary among the detergents, and were as follows: OG, DM, DDAPS, IPE, and the Fos-Choline detergents (including DPC), 0.1 mg/mL Trypsin on ice for 5 or 10 min; CHAPS and CHAPSO, 0.2 mg/mL Trypsin at room temperature for 5 or 10 min; DDAO, 0.05 mg/mL Trypsin on ice for 5 or 10 min; DG, Thesit, and C12E8, 0.2 mg/mL Trypsin on ice for 5 or 10 min; MEGA-10, 0.05 mg/mL Trypsin at room temperature for 5 or 10 min. The procedure with DPC was later refined to use just 0.05 mg/mL Trypsin on ice for 5 min; this was the procedure used to obtain the data in Figure 2. Trypsin reactions were stopped by addition of 1 mM 4-APMSF. Products of proteolysis were analyzed by SDS−PAGE and immunoblotting with MotA antibody, as described previously (*32*). The yield of an 8 kDa MotA fragment was shown previously to be diagnostic of a conformational change regulated by mutations at Asp 32 (*32*). The intensities of this band from wild-type or D32N-mutant complexes were compared by using video densitometry and the program NIH-Image.

Some proteolysis assays were done on the proteins in membranes (Figure 3D). These used procedures described previously (*32*).

*Swarming Assays.* Cells of the *motAB*-deletion strain RP6894 were transformed with plasmids expressing the Mot proteins from the *trp* promoter (pDFB45 or pSK16). Fresh transformants were cultured overnight at 32 °C in TB-Ap (1% bacto-tryptone, 0.5% NaCl, 100 μg/mL ampicillin), and then 1 μL was spotted onto TB-Ap plates containing 0.28% bacto-agar. Plates were incubated at 32 °C until sizable swarm rings developed (typically about 9 h) and then photographed.

*Preparation of Membrane Proteins for Tests of Expression Level.* To examine the relative levels of chromosomally expressed MotA and MotB in a wild-type strain, RP437 cells were cultured overnight at 32 °C in 5 mL of TB, and the membrane was isolated as described previously (*30*). To examine relative levels of MotA−His and MotB expressed from plasmids pSK11 and pSK17, the plasmids were transformed into strain BL21(De3) (*38*), and then the membranes were isolated as above and diluted to give Mot protein levels comparable to those in samples of wild-type membranes. Samples were analyzed by SDS−PAGE and immunoblotting using the MotA and MotB antibodies in a 2:1 mixture (A/B).

*Overproduction and Purification of MotA−His/MotB Complexes.* BL21(De3) cells were transformed with plasmid pSK17, and fresh transformants were inoculated into 1 L of SB (1.2% bacto-tryptone, 2.4% yeast extract, 1.25% $K_2HPO_4$, 0.38% $KH_2PO_4$, 0.5% (v/v) glycerol) containing 100 μg/mL

ampicillin. Cultures were incubated at 24 °C with shaking to an $OD_{600}$ of ~0.2, then induced with 0.4 mM IPTG, and cultured overnight at 24 °C. Cells were collected by centrifugation and resuspended in 20 mL of membrane buffer (50 mM Tris−Cl, pH 7.5, 50 mM NaCl, 250 mM sucrose, 0.5 mM DTT, 10% glycerol, 2 mM $CaCl_2$), then sonicated, passed through a French pressure cell, and sonicated again. Samples were centrifuged at 8000*g* for 10 min at 4 °C to remove undisrupted cells, and then membranes were collected by centrifugation (100000*g*, 60 min, SW27 rotor). Membranes were resuspended in 1 mL of buffer A (20 mM Tris−Cl, pH 8.0, 300 mM NaCl, 5 mM imidazole) containing 20% glycerol, frozen by immersion in an ethanol/dry ice bath, and stored for later use. For solubilization, membrane samples (typical protein concentration 20−30 mg/mL) were thawed and diluted into buffer A containing 20% (w/v) glucose and 0.05% (w/v) DPC (Anatrace) to a final concentration of 1.0 mg of protein/mL. Following 15 min of gentle rotation at 4 °C, samples were diluted 4-fold into buffer A containing 20% glycerol and 0.03% DPC and then rotated for 10 min more. Samples were spun (23000*g*, 15 min, 4 °C, SS-34 rotor) to remove unsolubilized material. The supernatant was mixed with His-Bind resin (Novagen) (typically a 1 mL bed volume) prewashed with the same buffer, incubated at room temperature for 30 min with gentle mixing, and then packed into a column. The column was washed with 15 mL of buffer A containing 20% glycerol and 0.03% DPC and then 15 mL of the same buffer except containing 60 mM imidazole. The MotA−His/MotB complexes were eluted in ~5 mL of the same buffer except containing 300 mM imidazole. The affinity-purified samples were concentrated, when necessary, by using Macrosep centrifugal devices (Pall Life Sciences), and applied to a Superdex 200 size-exclusion column connected to an FPLC system (Amersham Biosciences). The column was equilibrated and run in buffer A containing 20% glycerol and 0.03% DPC at a flow rate of 0.35 mL/min. The peak fractions containing both MotA and MotB were collected and stored at −70 °C. Protein concentrations were assayed by the BCA method using a commercial reagent kit (Pierce).

*Proteolysis Assay of Purified MotA/MotB Complexes.* Purified MotA/MotB at a concentration of ~25 $\mu$g/mL was digested with 25 $\mu$g/mL Trypsin on ice for the times indicated in TNCG buffer containing 0.01% DPC and 250 $\mu$g/mL BSA to slow digestion to a rate comparable to that with unpurified samples. The lower DPC concentration was used to ensure that the complex did not dissociate. The reaction was stopped by adding 1 mM 4-APMSF, and MotA fragments were analyzed on immunoblots as described above.

*Partial Purification of Radiolabeled MotA/MotB Complexes.* Proteins in growing cells were radiolabeled with [$^{35}$S]-methionine by the method of Tabor and Richardson (*39*) with minor modifications. Cells of BL21(De3) freshly transformed with plasmid pSK17 were inoculated into 20 mL of M9 medium supplemented with thiamine (1 $\mu$g/mL) and 18 amino acids (30 $\mu$g/mL, minus cysteine and methionine) and ampicillin (100 $\mu$g/mL), and grown overnight at 24 °C, giving $OD_{600}$ values in the range 0.15−0.4. A 1.5 mL aliquot of this culture was transferred into a test tube, and IPTG was added to give a final concentration of 0.4 mM. A 1 $\mu$L aliquot of L-[$^{35}$S]Met (10 mCi/mL; Amersham Biosciences) was added, and incubation was continued for 4 h at 24 °C. Cells were pelleted, resuspended in 1 mL of spheroplast buffer (50 mM Tris−Cl, pH 8, 10 mM EDTA, 0.5 M sucrose, 200 $\mu$g/mL lysozyme), and kept on ice for 30 min and at room temperature for 15 min. Spheroplasts were pelleted (16000*g*, 5 min), resuspended in 1 mL of 20 mM Tris−Cl, pH 7.5, 5 mM $MgCl_2$, then mixed by gentle rotation for 15 min at room temperature. Unbroken cells were removed by low-speed centrifugation (735*g*, 3 min), and the membranes were collected (16000*g* for 15 min). The pellet was suspended in 200 $\mu$L of buffer A containing 20% glucose and 0.05% DPC and put on ice for 15 min. The sample was diluted 5-fold into buffer A containing 20% glycerol and no DPC, and put on ice for another 15 min. Unsolubilized material was removed by centrifugation (16000*g*, 15 min), and the supernatant was mixed with 100 $\mu$L (bed volume) of His-Bind resin prewashed with buffer A containing 20% glycerol and 0.01% DPC. After 30 min of gentle rotation at room temperature, unbound material was removed by a brief (~5 s) spin. The resin was washed twice with the same buffer (1 mL/wash) and then three times with the same buffer except containing 60 mM imidazole (1 mL/wash). Complexes were eluted in 100 $\mu$L of the same buffer except containing 300 mM imidazole for 10 min on ice. The eluted material was mixed with SDS loading buffer and boiled, and serially diluted samples were loaded onto an SDS−PAGE gel. Radioactivities of MotA and MotB on the dried gel were measured using a PhosphorImager (Molecular Dynamics). The MotA:MotB ratio was calculated from plots of radioactivity vs protein loaded, taking into account the numbers of Met and Cys in each protein (14 Met + 1 Cys in MotA−His, 12 Met in MotB).

## RESULTS

*Solubilization of MotA/MotB Complexes.* In experiments to probe conformational changes in the stator, we found that mutations in the putative proton-transferring residue of MotB (Asp32) alter the protease susceptibility of sites in a cytoplasmic domain of MotA (*32*). A comparison of various mutations in MotA and MotB gave evidence that the conformational change is relevant for function, and preliminary experiments with the detergent CHAPS showed that the protease susceptibility of MotA remained responsive to MotB mutations in solubilized complexes. These results suggested that patterns of protease susceptibility might provide a means to monitor the integrity of the complex in detergent solution. We have used the protease-susceptibility assay here to identify detergents that can solubilize the complexes efficiently while preserving the conformational responses to mutation seen in membranes.

To determine which detergents are effective in solubilizing MotA/MotB complexes, membranes containing the Mot proteins were treated with a variety of detergents, at concentrations equal to or above the cmc, and the soluble and insoluble fractions were separated by centrifugation. Anti-MotA immunoblots were used to estimate the relative amounts of protein in the supernatant and pellet fractions. Examples of solubization data are shown in Figure 2A, and results for 26 detergents are summarized in Table 2. Certain detergents were only marginally effective in solubilizing the complex, as exemplified by dodecyl glucoside and CHAPS. Others, such as DPC, were very effective in solubilizing the protein from membranes.

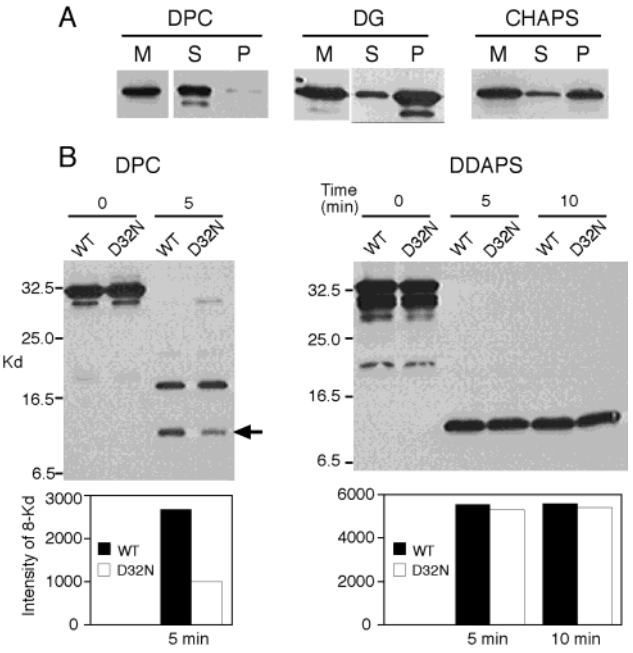


FIGURE 2: Representative results of detergent-solubilization experiments. (A) Anti-MotA immunoblots showing solubilization of MotA from membranes by three detergents of varying efficiency. Detergents were used at the concentrations given in Table 2. Samples were fractionated by centrifugation. M = membranes, S = soluble fraction, and P = pellet. (B) Integrity of the MotA/MotB complexes, as monitored by limited proteolysis. Complexes contained either wild-type MotB or D32N-mutant MotB, as indicated. Following solubilization, samples were treated with Trypsin for the times shown, and MotA fragments were examined on immunoblots. Conformational differences seen in the membranes are also seen in DPC, but not in DDAPS. Solubilization and proteolysis were carried out in solutions containing 20% glycerol. Experiments were done at least three times, and typical results are shown.

The integrity of the detergent-solubilized MotA/MotB complexes was assessed by the limited-proteolysis assay. Experiments with the membrane-bound complex showed that mutations in Asp32 of MotB cause a conformational change in MotA, as evidenced by the increased yield of an 8 kDa fragment derived from the MotA cytoplasmic domain (*32*). A similar result with a detergent-solubilized complex would indicate that this conformational response has not been disrupted by detergent. Examples of limited-proteolysis results are shown in Figure 2B, and results for 22 detergents are summarized in Table 2. A subset of the detergents that were effective in solubilizing the complex also preserved the Asp32-modulated conformational properties as assayed by limited proteolysis. These included DPC and a number of related Fos-Choline detergents with slightly longer or shorter hydrocarbon chains, and two detergents of the CyFos family. DPC was selected for further study, as it was among the most efficient in solubilizing the complex (Figure 2A) and consistently showed Asp32-modulated conformational differences in the limited-proteolysis experiment (Figure 2B). Solubilization in DPC was found to be especially efficient when carried out in the presence of 20% glucose, followed by dilution into a buffer containing 20% glycerol.

Certain detergents, exemplified by DDAPS (Figure 2B), solubilized the complex efficiently but eliminated the Asp32-regulated conformational differences. This might reflect dissociation of the complex or other factors such as reduced flexibility. Whatever the exact cause, it indicates that DDAPS and like-behaving detergents are not suitable for isolating the complex in functional form.

Table 2: Solubilization of MotA/MotB by Detergents[a]

| detergent | cmc (%, w/v) | tested concn (mg/mL) | efficiency of solubilization[b] | wt vs D32N conformation difference |
|---|---|---|---|---|
| CHAPS | 0.46 | 0.5 | +[c] | Y |
| CHAPSO | 0.50 | 0.5 | + | N |
| DDAO | 0.05 | 0.05 | +++[d] | Y |
| DDAPS | 0.12 | 0.2 | +++ | N |
| OG | 0.58 | 1.0 | +++ | Y[e] |
| DM | 0.009 | 0.02 | ++[c] | Y |
| DG | 0.005 | 0.01 | + | N |
| Thesit | 0.005 | 0.02 | ++[c] | Y |
| C12E8 | 0.005 | 0.02 | ++[c] | Y |
| IPE | 0.007 | 0.05 | ++ | N |
| MEGA-10 | 0.22 | 0.3 | +++ | N |
| Fos-Choline-8 | 3.4 | 3.3 | + | np[f] |
| Fos-Choline-9 | 1.2 | 1.2 | ++ | np |
| Fos-Choline-10 | 0.35 | 0.5 | +++ | Y |
| Fos-Choline-11 | 0.062 | 0.5 | +++ | Y |
| Fos-Choline-12(DPC) | 0.047 | 0.05 | +++ | Y |
| Fos-Choline-13 | 0.027 | 0.05 | +++ | Y |
| Fos-Choline-14 | 0.0046 | 0.05 | +++ | Y[g] |
| Fos-Choline-15 | 0.0027 | 0.05 | +++ | Y[h] |
| Fos-Choline-16 | 0.0005 | 0.05 | +++ | Y[h] |
| Fos-MEA-8 | 0.59 | 0.5 | + | np |
| Fos-MEA-10 | 0.15 | 0.5 | ++ | Y |
| Fos-MEA-12 | 0.014 | 0.5 | ++ | np |
| CyFos-5 | 0.15 | 0.5 | ++ | Y |
| CyFos-6 | 0.094 | 0.5 | +++ | Y |
| CyFos-7 | 0.022 | 0.2 | +++ | Y |

[a] Experiments were in the presence of 20% glycerol. Results were similar in the absence of glycerol, except the complex showed a greater tendency to dissociate. [b] Solubilization efficiencies were scored as follows: +++, more than 50% in supernatant; ++, approximately 50% in supernatant; +, less than 50% in supernatant. [c] Ineffective in solubilizing the proteins when they were overproduced. [d] Promoted proteolytic breakdown of MotA. [e] The MotA/MotB complex dissociated during purification. [f] Not performed. [g] Differences in the proteolysis patterns were relatively weak. [h] Differences in the proteolysis patterns involved fragments other than 8 kDa.

*Purification of the Stator Complex.* To facilitate purification of the MotA/MotB complex, His tags were attached to either the N-terminus or C-terminus of MotA. The N-terminal tag eliminated motility (not shown), but a C-terminal tag was tolerated (Figure 3C). The *motA* and *motB* genes are translationally coupled in the native operon, and insertion of the His tag sequences is expected to disrupt this coupling (Figure 3A). Cells expressing MotA−His from the initial construct (pSK11) gave a MotA:MotB ratio lower than that in wild-type cells (Figure 3B). To obtain a more normal MotA:MotB ratio, we introduced mutations to improve the efficiency of the Shine−Dalgarno sequence at the start of *motB* and to prevent formation of the hairpin structure in the mRNA, following the procedure of VanWay et al. (*37*) (Figure 3A). With these modifications the MotA:MotB ratio was closer to that of the wild type (Figure 3B), and cells swarmed at closer to wild-type speeds in soft agar (Figure 3C).

To see whether the His-tagged MotA retains conformational properties like the untagged protein, the limited-proteolysis experiment was done on complexes containing His-tagged MotA (and either wild-type or D32N-mutant MotB, as usual). Both in membranes and in DPC-solubilized samples, an 8 kDa MotA fragment was produced in greater yield when MotB was the wild type than when it had the

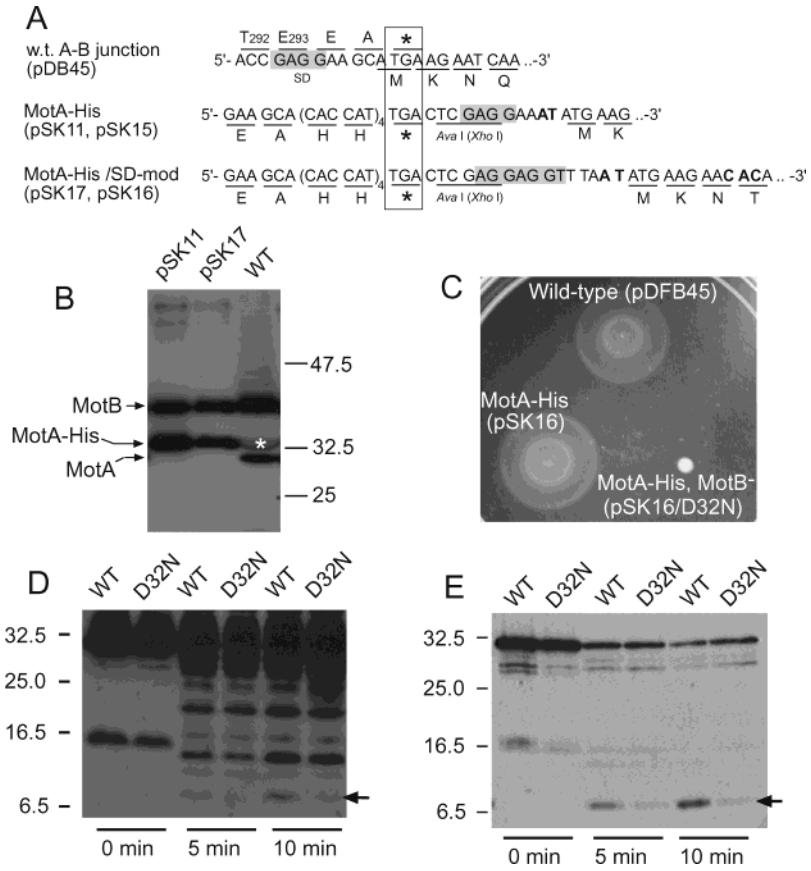


FIGURE 3: Properties of His-tagged MotA constructs. (A) Sequences of the *motA*−*motB* junction region in the wild-type operon (top), in a variant with a His tag at the C-terminus of MotA (middle), and in the variant with the His tag on MotA plus additional modifications to increase the efficiency of translation of *motB* (bottom). (B) Relative levels of MotB and MotA (or His−MotA) in wild-type cells (WT), in a construct that overexpresses MotA−His and MotB (pSK11), and in the construct with further modifications to enhance translation of *motB* (pSK17). The wild-type lane was loaded with undiluted cell membranes; the asterisk indicates a cross-reacting band seen at high concentrations of membrane protein. The pSK11 and pSK17 lanes were loaded with membranes diluted approximately 1000-fold. The Mot proteins were visualized by immunoblotting with anti-MotA and anti-MotB antibodies in a 2:1 mixture. (C) Swarming in soft-agar plates of cells expressing wild-type MotA and MotB, His-tagged MotA and wild-type MotB, or wild-type MotA and the nonfunctioning MotB mutant D32N. The cells contained a chromosomal *motAB* deletion. (D) Asp32-regulated conformational differences in membrane-bound complexes containing His-tagged MotA, as detected by limited proteolysis. Complexes contained wild-type MotB or D32N-mutant MotB, as indicated. The proteins were expressed from plasmid pSK16, in the *motAB*-deletion strain RP6894. (E) Limited-proteolysis experiment on the His-tagged construct solubilized in DPC (0.05%). Proteins were expressed from plasmid pSK16, in strain RP6894.

D32N mutation (Figure 3D,E), the same effect seen with the untagged complexes in membranes (Figure 2). Thus, the complexes containing His-tagged MotA appear intact by the criterion of protease susceptibility.

Steps in the purification of the complex are summarized in Figure 4. To increase expression levels of the proteins, the construct was moved into a vector that uses the T7 promoter. In a T7-polymerase-containing host, the MotA−His and MotB proteins accumulated to high levels and remained with the membrane fraction upon centrifugation. The overexpressed proteins were solubilized effectively by DPC, and purification on a Ni-IDA histidine-affinity column gave material that contained MotA−His and MotB as the major components. As judged from quantification of Coomassie-stained bands, the MotA:MotB ratio was about 2:1 in the affinity-purified material.

Gel-filtration chromatography was used to purify the complexes further and obtain a rough estimate of their size. Columns were run in a range of detergent concentrations to identify conditions for obtaining intact, well-dispersed complexes. When the DPC concentration was below the cmc (0.01%), the proteins remained together but eluted in the void volume, indicating aggregation. At a concentration above the cmc (0.05%), the MotA−His and MotB eluted from the column separately (data not shown). At an intermediate concentration (0.03%), most of the MotA−His eluted together with MotB in a peak at 300 kDa (Figure 5A). Immunoblots showed that the 300 kDa peak contained both MotA and MotB (Figure 5C), and Coommassie staining (not shown) indicated a MotA:MotB ratio close to 2:1. The purified complexes in 0.03% DPC retained the same mobility in gel filtration after storage for several days at −70 °C (Figure 5A).

As noted, the plasmid used for overexpression of the MotA/MotB complexes (pSK17) contained modifications to enhance the translation of *motB*. When the proteins were expressed from a plasmid that lacks these modifications (pSK11), the MotA:MotB ratio was increased, and the gel-filtration profile shifted to ~150 kDa, with a shoulder near the 300 kDa position (Figure 5B). Immunoblots showed that the 150 kDa peak consisted mainly of MotA, and only a small amount of MotB (Figure 5C).

To see whether the Asp32-regulated conformational differences were retained in purified stator complexes, a limited-

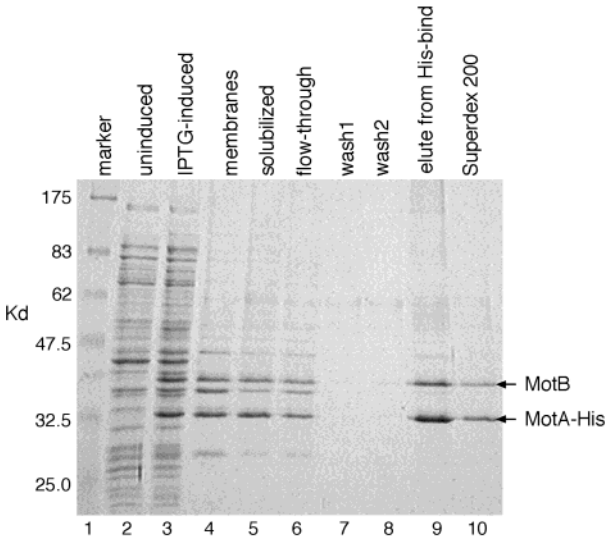


FIGURE 4: Steps in purification of the MotA/MotB complex. Proteins are resolved on a Coommassie-stained 10% SDS−PAGE gel. Key: lanes 2 and 3, whole-cell lysates before and after induction by IPTG; lane 4, isolated membranes; lane 5, soluble fraction following solubilization with DPC; lane 6, flow-through from the His-Bind column; lanes 7 and 8, His-Bind washes; lane 9, complexes eluted by 300 mM imidazole; lane 10, complexes further purified by gel filtration (Superdex 200).

proteolysis experiment was carried out on the material purified by His-Bind and gel-filtration columns. The purified complexes exhibited a conformational difference similar to that observed in membranes and detergent-solubilized samples (Figure 6). Thus, the effect requires only MotA and MotB and no other proteins.

The efficiency of Coommassie staining can vary between proteins, so an apparent A:B ratio of 2:1 might correspond to an actual ratio as great as 4:1 or as small as 1:1. To obtain a more reliable estimate of the A:B ratio, we radiolabeled proteins by growing cells on [$^{35}$S]methionine, purified the MotA−His/MotB complexes on a His-Bind column, and quantified the proteins on gels using a phosphorimager. Results for one such experiment are shown in Figure 7. The MotA:MotB ratios estimated in three independent experiments were 1.8:1, 2.0:1, and 2.3:1.

## DISCUSSION

The limited-proteolysis assay proved useful for classifying detergents, by rapidly identifying ones that perturb the conformational properties of the MotA/MotB complex. Of the detergents studied here, those in the Fos-Choline or CyFos families were most effective. DPC, also called dodecylphosphocholine or Fos-Choline 12, released most of the MotA/MotB proteins from the membrane, particularly when used in the presence of 20% glucose. As importantly, complexes purified in this detergent retained Asp32-regulated conformational differences similar to those seen in membranes. The Fos-Choline and CyFos detergents contain a phosphocholine headgroup, and the resemblance to phospholipid might be a factor in their performance. We have not examined the effects of added phospholipids upon the stability or conformation of the complex.

The other detergents failed, in one way or another, to yield solubilized MotA/MotB complexes suitable for purification. Certain detergents (e.g., DDAO) solubilized the proteins

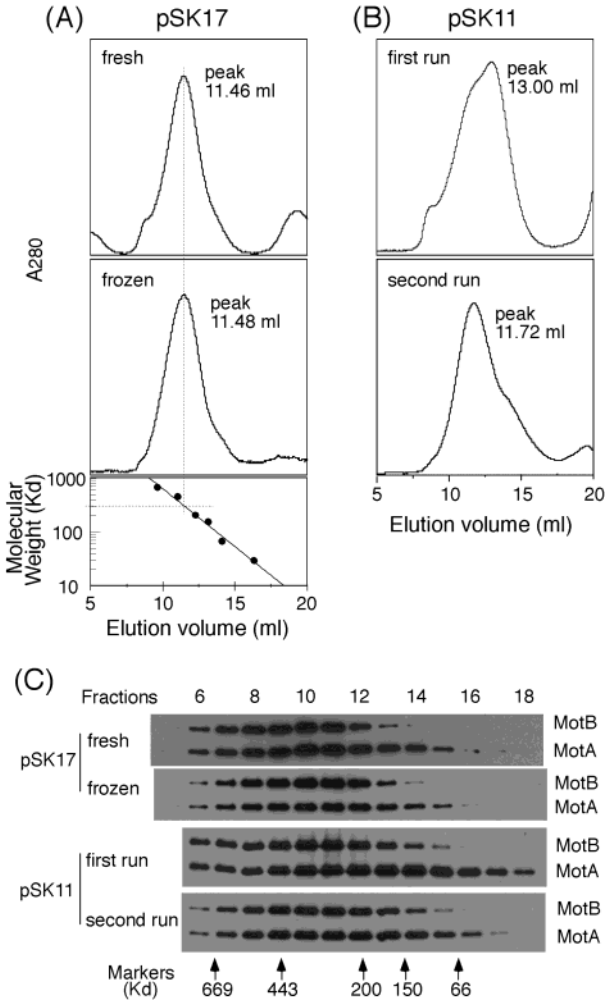


FIGURE 5: Gel-filtration chromatography of MotA−His/MotB complexes in 0.03% DPC. (A) Results using the overexpression plasmid pSK17, which encodes MotA−His and is also modified to enhance translation of *motB* (see the text). The upper panel shows the chromatogram obtained with freshly prepared complexes, and the lower panel shows results with complexes frozen at −70 °C for 3 days. A calibration curve using globular-protein standards is shown at the bottom. The mass standards were thyroglobulin (669 kDa), apoferritin (443 kDa), $\beta$-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), and carbonic anhydrase (29 kDa). (B) Results using the overexpression plasmid pSK11, which encodes MotA−His but has no modifications to enhance translation of *motB*. The top panel shows the chromatogram obtained with samples freshly purified on a His-Bind column, and the bottom shows a chromatogram obtained by rerunning the early-eluting fractions from the first run (fractions 10−12). (C) Immunoblots of fractions from the gel-filtration columns, using anti-MotA and anti-MotB antibodies in a 2:1 mixture.

efficiently but made them subject to proteolysis, as evidenced by the appearance of MotA breakdown products in the absence of any added protease. Some detergents were effective in solubilizing the MotA/MotB proteins when they were expressed at only moderate levels, but less effective when the proteins were overproduced (e.g., DM or CHAPS). Some detergents were effective in solubilizing the complex and also preserved nativelike conformational properties immediately following solubilization, but allowed the complex to dissociate during the column steps used in purification (e.g., OG). When used at an appropriate concentration (0.03%), DPC did not exhibit these problems (Figures 5 and

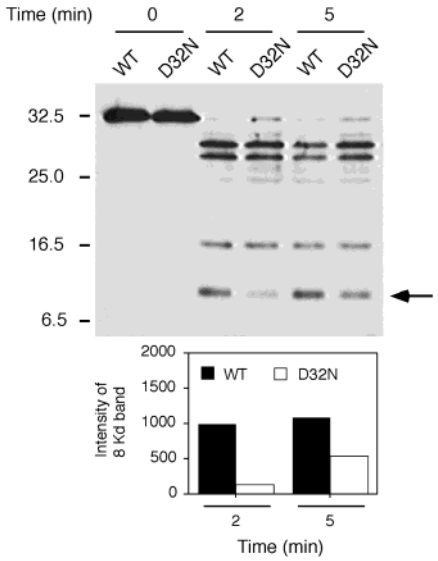


FIGURE 6: Limited-proteolysis experiment on MotA−His/MotB complexes purified on a His-Bind column and by gel filtration. Proteolysis was carried out in a buffer containing 20% glycerol and 0.01% DPC.

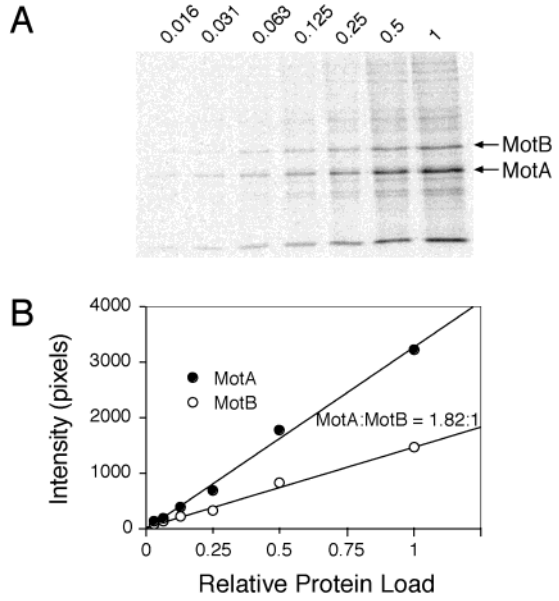


FIGURE 7: Radiolabeling of Mot proteins with [$^{35}$S]methionine. (A) Phosphorimager scan of radiolabeled MotA−His/MotB proteins purified by affinity chromatography. The gel was loaded with varying amounts of total protein, as indicated at the top. (B) Intensity of radioactivity in the MotA and MotB bands, vs relative protein load. Linear fits to the data give a MotA:MotB ratio of 1.82:1 for the data shown; the average MotA:MotB ratio from three independent determinations was 2.04:1.

6). A higher concentration of DPC (0.05%) caused some dissociation of the complex during gel filtration.

In 0.03% DPC, most of the MotA and MotB migrate together in gel-filtration columns, with an apparent mass of 300 kDa. The MotA:MotB ratio in the complex is 2:1, as determined by $^{35}$S-radiolabeling (Figure 7). Given the masses of MotA−His and MotB (33.1 and 34.2 kDa, respectively), a complex with stoichiometry $MotA_6MotB_3$ would have a mass just over 300 kDa. The complex must also contain bound detergent, however, and current models for detergent binding indicate that DPC molecules bound to the complex should add 20 kDa or more to its mass (*40*). Considerations of shape also argue against a 6:3 subunit stoichiometry. The MotA/MotB complex has large domains on both sides of the membrane and membrane-embedded parts that contain only about 20% of the total protein mass. Given this mass distribution, the complex must have a shape that is significantly nonspherical.

Because both the bound detergent and the nonspherical shape of the complex should increase its apparent mass in gel filtration, we conclude that the subunit stoichiometry cannot be $MotA_6MotB_3$, and is more likely $MotA_4MotB_2$. If the complex is very highly elongated, then even a complex with composition $MotA_2MotB_1$ might fit the data. A targeted disulfide-cross-linking study of MotB showed, however, that the complex contains at least two copies of MotB, and probably exactly two (*41*). Thus, taking all the constraints together, we conclude that the subunit composition is $MotA_4MotB_2$. Cross-linking experiments presented in the following paper in this issue provide additional, independent evidence for the $MotA_4MotB_2$ subunit stoichiometry. The same subunit composition has been inferred for the $Na^+$-conducting PomA/PomB stator complex of *Vibrio alginolyticus* (*36*).

The relationship between the $MotA_4MotB_2$ complex and the particles seen in freeze-fracture electron micrographs (*25*) is not clear. The freeze-fracture views were from the periplasmic side of the membrane, and the particles might correspond to the periplasmic domains of individual MotB subunits. If so, then the presence of two MotB subunits in each torque-generating complex might help to reconcile the eight torque generators inferred from torque-restoration experiments (*27*) with the 10−12 particles per ring seen in freeze-fracture micrographs (*25*). If each particle corresponds to a MotB periplasmic domain, then a motor could have as many as 16 particles. Sixteen particles have been seen in rings associated with the flagella of *Aquaspirillum serpens* (*30*) and a motile *Streptococcus* (*25*). The *E. coli* rings have fewer particles than this, but most motors in wild-type *E. coli* cells also appear to have fewer than the full complement of torque generators, as judged by torque measurements (*27*). Another possibility is that some complexes are lost from the rings during sample preparation and are not included in the count.

We previously used an *flhDC* mutant that does not express any chromosomal flagellar genes to show that the Asp32-modulated conformational differences do not require any flagellar proteins besides MotA and MotB (*32*). The present results with purified complexes show that, with the possible exception of bound lipids, no cellular molecules besides MotA and MotB are required to observe the conformational differences. The complex appears at least moderately stable as judged by the limited-proteolysis experiment; in 0.03% DPC (or lower) the Asp32-regulated conformational differences were retained through purification steps lasting as long as 3 h at 4 °C, and the complexes remained intact for at least several days when frozen at −70 °C.

A limitation in studies of the flagellar motor has been the absence of suitable in vitro assays for its function. Full in vitro reconstitution of motor function poses a difficult challenge, as it would require purified rotor as well as stator assemblies, and means for holding them in their proper relative position while applying a proton motive force. Because the conformational change persists in purified complexes that contain only two proteins, it might provide

a way to study some functionally relevant processes of the motor in vitro.

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