

Conformational Change in the Stator of the Bacterial Flagellar Motor[†]

Seiji Kojima and David F. Blair*

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

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ABSTRACT: MotA and MotB are integral membrane proteins of *Escherichia coli* that form the stator of the proton-fueled flagellar rotary motor. The motor contains several MotA/MotB complexes, which function independently to conduct protons across the cytoplasmic membrane and couple proton flow to rotation. MotB contains a conserved aspartic acid residue, Asp32, that is critical for rotation. We have proposed that the protons energizing the motor interact with Asp32 of MotB to induce conformational changes in the stator that drive movement of the rotor. To test for conformational changes, we examined the protease susceptibility of MotA in membrane-bound complexes with either wild-type MotB or MotB mutated at residue 32. Small, uncharged replacements of Asp32 in MotB (D32N, D32A, D32G, D32S, or D32C) caused a significant change in the conformation of MotA, as evidenced by a change in the pattern of proteolytic fragments. The conformational change does not require any flagellar proteins besides MotA and MotB, as it was still seen in a strain that expresses no other flagellar genes. It affects a cytoplasmic domain of MotA that contains residues known to interact with the rotor, consistent with a role in the generation of torque. Influences of key residues of MotA on conformation were also examined. Pro173 of MotA, known to be important for rotation, is a significant determinant of conformation: Dominant Pro173 mutations, but not recessive ones, altered the proteolysis pattern of MotA and also prevented the conformational change induced by Asp32 replacements. Arg90 and Glu98, residues of MotA that engage in electrostatic interactions with the rotor, appear not to be strong determinants of conformation of the MotA/MotB complex in membranes. We note sequence similarity between MotA and ExbB, a cytoplasmic-membrane protein that energizes outer-membrane transport in Gram-negative bacteria. ExbB and associated proteins might also employ a mechanism involving proton-driven conformational change.

Motile bacteria swim by rotating flagella, filamentous organelles that extend from the cell body and function as propellers. Each flagellum is driven at its base by a rotary motor fueled by the transmembrane gradient of protons or (in some species) Na⁺ ions (1–3; for reviews, see refs 4–6). The flagellar motor is reversible, and controlled switching between CW¹ and CCW rotation is the basis for directed movements such as chemotaxis. While physiological and mutational studies have provided important insights into the workings of the motor, the molecular mechanism of torque generation is not yet understood.

Genetic studies in *Escherichia coli* and *Salmonella typhimurium* have shown that only a handful of proteins function in the generation of torque. Three proteins of the rotor, FliG, FliM, and FliN, have been implicated in motor rotation on the grounds that they can be mutated to give a flagellated

but nonmotile phenotype (e.g., refs 7 and 8). FliG, FliM, and FliN form a complex (9–12) mounted on the inner (cytoplasmic) face of the membrane-embedded MS-ring (13, 14). The FliG/M/N assembly has been termed the “switch complex” because certain mutations in these proteins affect the switching between CW and CCW rotation. The chemotactic signaling protein phospho-CheY binds to FliM to increase the probability of CW rotation, presumably by triggering a conformational change in the complex (15–17). Each motor contains some 25–40 copies of FliG, which attach directly to the MS-ring (13, 18–20). About 35 copies of FliM and more than 100 copies of FliN form a drum-shaped structure termed the C-ring that attaches to FliG and extends ~15 nm into the cytoplasm (13, 21, 22).

FliG is closely involved in the generation of torque, whereas FliM and FliN appear less involved (23–25). A ca. 100-residue C-terminal domain of FliG (FliG-C) functions specifically in the generation of torque, being essential for rotation but dispensable for flagellar assembly. This domain contains a set of charged residues that are especially important for rotation (Lys264, Arg281, Asp288, Asp289, and Arg297 in the protein of *E. coli*). These residues function redundantly (i.e., no single one is critical) and charge appears to be their important property (26). The crystal structure of FliG-C shows the charged residues clustered along a prominent ridge on the domain (27).

The stator is formed from the integral membrane proteins MotA and MotB, both essential for rotation but dispensable

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* To whom correspondence should be addressed. Phone: (801) 585-3709. Fax: (801) 581-4668. E-mail: blair@bioscience.utah.edu.

¹ Abbreviations: 4-APMSF, *p*-amidinophenylmethanesulfonyl fluoride; ATP, adenosine 5'-triphosphate; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CW, clockwise; CCW, counterclockwise; DTT, dithiothreitol; IAA, 3-β-indoleacrylic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

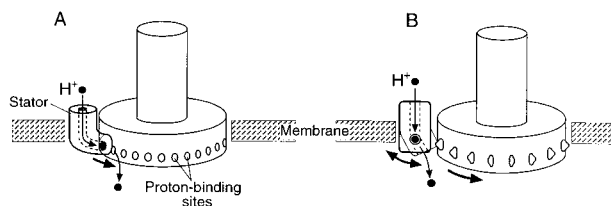


FIGURE 1: Contrasting models for torque generation by the proton-fueled flagellar motor. (A) At some step(s) along their path through the motor, the protons must bind to the rotor and be carried by it. (B) Protons follow a path within just the stator, driving cyclical conformational changes in the stator that apply force to the rotor.

for assembly of normal-looking flagella. Each flagellar motor contains several MotA/MotB complexes that surround the MS-ring (28) and that function independently to generate torque (29, 30). MotA has four membrane-crossing segments, and MotB has one (31–34). MotA and MotB bind to each other (10) in a complex with probable stoichiometry (MotA)₄(MotB)₂ (35). This complex functions to conduct protons across the membrane and to couple proton movement to rotation of the motor (36, 37). MotB has a large domain in the periplasm that includes a peptidoglycan-binding motif presumed to anchor the stator complexes to the cell wall (34, 38).

Residues of MotA that are important for function include two charged residues in the cytoplasmic domain, Arg90 and Glu98, that interact with the functionally important charged residues of FliG (39, 40). Like the charged residues of FliG, Arg90 and Glu98 of MotA function redundantly, and charge appears to be their key property. Two Pro residues of MotA located at the cytoplasmic ends of membrane segments, Pro173 and Pro222, are also important for rotation and might function to regulate conformational changes occurring during the torque-generating cycle (41). In MotB, an aspartic acid residue near the cytoplasmic end of the membrane segment, Asp32, is conserved and critical for motor rotation. A survey of conserved residues in MotA, MotB, FliG, FliM, and FliN found that no other titratable residue is critical for motor rotation (42). Asp32 is likely to have a direct role in the conduction of protons.

In the years since the discovery of flagellar rotation, many hypotheses for the mechanism have been proposed (reviewed in ref 43). The models are diverse, but can be classified according to whether the proton pathway includes elements of both the rotor and stator or is confined to just the stator (Figure 1). Because the mutational studies found no critical titratable residues on the rotor, we currently favor models in which protons remain within the stator. In this case, proton flow must be coupled to rotation by some means other than direct proton-rotor contact. Our hypothesis is that protonation of Asp32 in MotB drives conformational changes in the stator, which work on the rotor to drive rotation.

Limited proteolysis has been well established as a probe of conformational changes in proteins, including some membrane proteins (44, 45). A successful recent example is the Ca²⁺ pump of sarcoplasmic reticulum (see ref 46 for a review of this enzyme). The Ca²⁺ pump was probed by limited protease treatment of various conformations induced by the substrates (Ca²⁺, ATP, P_i) (refs 47–49), and sites of conformation-dependent protease susceptibility were mapped to particular segments in the cytoplasmic domain. The structure of the transporter in its Ca²⁺-bound form was

recently determined at 2.2 Å resolution by X-ray crystallography (50). A comparison of this Ca²⁺-bound structure to an 8-Å-resolution electron-crystallographic map of the Ca²⁺-free form (51) showed that large domain movements must occur upon binding of Ca²⁺, and identified the segments likely to move the most. These correlate well with the sites that show Ca²⁺-dependent protease susceptibility.

Here, we test for conformational changes in the MotA/MotB complex by using limited proteolysis. Patterns of proteolysis of MotA were compared in wild-type MotA/MotB complexes and complexes with mutations in key residues of one or both of the proteins. The results support a mechanism in which the stator undergoes significant changes in conformation.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. The wild-type strain RP437, *motA* deletion strain RP6894, and *flhDC* deletion strain RP3098 were gifts from J. S. Parkinson (University of Utah). Experiments in which MotA and MotB were overexpressed from the *T7* promoter used strain BL21(De3) (52). Plasmid purification and DNA manipulations used standard methods (53). Mutations in *motA* and *motB* were made in plasmid pRF4 (42) using the Altered Sites procedure (Promega) and then transferred into plasmid pDFB45 (32), which has *motA* and *motB* under control of the *trp* promoter. For co-isolation assays, mutations in *motA* and/or *motB* were transferred to plasmid pTB1 (10), which expresses MotA and C-terminally His-tagged MotB from the *T7* promoter. Assays of dominant-negative effects used plasmid pJZ18, which has *motB* under control of the *tac* promoter (42).

Isolation of Membranes. Right-side out membranes were isolated by using the method of Konings and Kaback (54). Inside-out membranes were isolated by the method of Rosen (55) with slight modifications. RP6894 or RP3098 cells transformed with plasmid pDFB45 were cultured overnight at 37 °C in LB-amp (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 100 µg/mL ampicillin), diluted 100-fold into 250 mL of LB-amp, and recultured to an OD_{600nm} of 0.1. The *trp*-operon inducer IAA was added to a final concentration of 25 µg/mL, from a 10 mg/mL stock solution in ethanol. Cells were grown at 37 °C to an OD_{600nm} of 0.8, then harvested by centrifugation and re-suspended in 20 mL of ice-cold membrane buffer (50 mM Tris-Cl pH 7.5, 50 mM NaCl, 250 mM sucrose, 0.5 mM DTT, 10% glycerol, and 2 mM CaCl₂). The cell suspension was passed through a French pressure cell two times at relatively low pressure (4000 psi). DNase I (10 µg/mL) and MgCl₂ (5 mM) were added and the solution was incubated for 10 min at 22 °C. Unbroken cells were removed by centrifugation at low speed (5000g, 10 min), and membranes were collected by ultracentrifugation (100000g, 60 min, SW27 rotor). Membranes were resuspended in TNC buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, and 2 mM CaCl₂), frozen by immersion in an ethanol/dry ice bath, and stored at –80 °C.

Protease Treatment. Membranes (2.5 mg/mL protein) in TNC buffer were digested with 0.25 mg/mL trypsin (Sigma) at room temperature (ca. 22 °C) for the times indicated in the figures. Digestions with clostripain (Sigma) used 1.0 mg/mL membrane protein and 0.2 mg/mL clostripain in TNC

buffer, at 42 °C for the times indicated. Clostripain-digestion experiments at varied pH used the same conditions, except the buffer was 50 mM MES-Tris, 50 mM NaCl, and 2 mM CaCl₂, adjusted to pH values ranging between 4.5 and 7.5. Reactions were stopped by 1 mM 4-APMSF (trypsin experiments) or 2 mM iodoacetamide (clostripain experiments). Immunoblots with a range of sample loadings were used to construct standard curves to allow estimation of relative MotA concentrations in undigested membrane samples, and these concentration estimates were used to equalize sample loadings on the SDS-PAGE gels shown. Gels were blotted onto nitrocellulose membranes and MotA fragments were detected using polyclonal primary antibody raised against the large cytoplasmic domain (residues 48–174) of MotA (41), and secondary antibody and other reagents from the SuperSignal West Pico chemiluminescent procedure (Pierce). Band intensities on immunoblots were measured by video densitometry using the image-analysis program NIH-Image.

Cell Fractionation. Cell fractionation experiments used strain RP3098 transformed with plasmid pDFB45. Inside-out membranes were treated with trypsin (2.5 mg/mL membrane protein and 0.25 mg/mL protease) for 30 min at 22 °C or with clostripain (1.0 mg/mL membrane protein and 0.2 mg/mL protease) for 20 min at 42 °C. An equal volume of 8 M urea/1 M NaCl solution was added, and the samples were incubated for 30 min at 22 °C. Samples were then centrifuged at 16000g for 15 min, the supernatant was transferred to another tube, and membrane pellets were resuspended in a volume equal to that of the unfractionated starting sample. Equal volumes of all samples (unfractionated, supernatant, and pellet) were loaded on gels for analysis by immunoblotting.

Co-Isolation Assay. Detergent solubilization of MotA and MotB, and affinity purification of His-tagged MotB, were done basically as described before (10). Briefly, MotA and MotB proteins were expressed from plasmid pTB1 in strain BL21(De3), cells from a 20 mL saturated culture in LB were lysed by sonication, and membranes were collected by centrifugation. Membranes (4–8 mg/mL protein) were solubilized using CHAPS (1% w/v), and the solution was applied to His-bind resin (Novagen). The resin was washed two times with detergent-containing buffer (20 mM Tris-Cl pH 7.9, 0.5 M NaCl, and 0.3% CHAPS) containing 5 mM imidazole then three times with the same buffer containing 30 mM imidazole. His-tagged MotB and associated MotA were eluted in the same buffer except containing 300 mM imidazole. Material eluted from the His-bind resin was analyzed by immunoblotting.

Measurement of Swarming Rates in Soft Agar. Plasmid pJZ18 carrying wild-type or mutant variants of the *motB* gene was transformed into the wild-type strain RP437. Plasmid pJZ18 has *motB* under control of the IPTG-inducible *tac* promoter. Cells were cultured overnight at 32 °C in T-broth (1% bacto-tryptone, 0.5% NaCl) containing 100 µg/mL ampicillin. One microliter of each saturated culture was spotted onto plates containing T-broth, 0.28% bacto-agar, 100 µg/mL ampicillin, and various concentrations of IPTG. Plates were incubated at 32 °C, and swarm sizes were measured at regular intervals (typically 45 min). Plots of swarm diameter versus time were fitted to a line, and the slope was used to compute the swarming rate.

RESULTS

Conformational Change in MotA Induced by Asp32 Replacements in MotB. Because Asp32 of MotB is the only conserved titratable residue found to be critical for rotation, we hypothesized that proton binding/dissociation reactions at Asp32 might drive conformational changes in the stator that apply force to the rotor (41, 42; cf. Figure 1). Conformational changes are important in the action of other motor proteins (for review, see ref 56). If protonation or mutation of Asp32 were shown to affect the conformation of the MotA/MotB complexes, that would support a conformation-based mechanism for the generation of torque.

To test for the hypothesized conformational changes, we used limited-trypsin digestion of MotA in membrane-bound complexes with either wild-type or mutant MotB. Physiological (37), genetic (57), and biochemical studies (10, 35) show that MotA and MotB form a complex. Using a plasmid that has both genes under control of the *trp* promoter, MotA was expressed in cells together with either wild-type MotB or MotB with the mutation Asp32→Asn. The asparagine replacement was examined first because its effects (to neutralize the side chain and introduce a hydrogen-bond donor) are most like protonation. Proteolysis experiments used inside-out membrane vesicles because the cytoplasmic domain of MotA contains all but one of the sites for trypsin digestion (Figure 2) and also contains the residues Arg90 and Glu98, which form a site of interaction with the rotor (40). Inside-out vesicles were prepared by a French-press protocol and the orientation of membranes was confirmed by comparing tryptic digests of these vesicles with digests of right-side-out vesicles produced by enzymatic cell lysis. As expected, MotA was more resistant to trypsin digestion in right-side-out vesicles than in inside-out vesicles, whereas MotB (a mostly periplasmic protein) was more resistant to digestion in inside-out vesicles (Figure 2).

Inside-out membrane vesicles containing MotA and MotB were treated with trypsin (0.25 mg/mL) for 1–30 min at 22 °C, and MotA fragments were detected on immunoblots using polyclonal antibody raised against the large cytoplasmic domain. A representative immunoblot (Figure 3) shows that at digestion times between 5 and 20 min, an 8-kDa MotA fragment was produced in much higher yield when MotB was wild-type than when it had the D32N mutation. The altered protease susceptibility of the MotA coexpressed with D32N-mutant MotB is consistent with a conformational change in the MotA/MotB complex but could also be caused by mutation-induced dissociation of MotA from MotB. To address this possibility, we did the same experiment with membranes that contained MotA but no MotB. The pattern of tryptic fragments was similar to that seen in the presence of wild-type MotB and different from that seen with D32N-mutant MotB (Figure 3C). We also used a co-isolation assay with His-tagged MotB to show that the D32N mutation did not weaken the binding of MotA to MotB (data not shown). The D32N mutation in MotB must therefore alter the protease susceptibility of MotA by altering its conformation or accessibility within the MotA/MotB complex and not by causing it to dissociate from MotB.

Requirements for the Conformational Change. We carried out limited-proteolysis experiments with several different replacements of MotB Asp32 to determine what features of

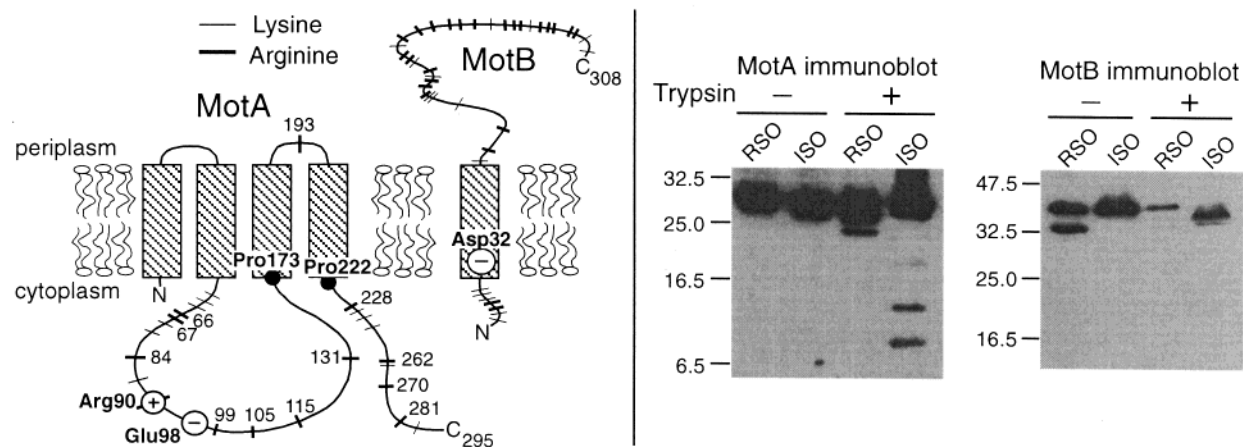


FIGURE 2: (Left) Schematic of the MotA and MotB proteins showing the membrane topologies, residues important for function that were identified by mutational studies, and sites cleavable by clostripain (Arg, thick lines) or by trypsin (Lys or Arg, thin and thick lines). Numbers are given for Arg residues of MotA. (Right) Experiments to confirm the orientation of membranes isolated by French press (inside-out, ISO) or by enzymatic cell lysis (rightside-out, RSO). Membranes were treated with trypsin for 20 min, and fragments were detected on immunoblots using antibodies against MotA or MotB, as indicated. Samples contained equal amounts of MotA or MotB prior to protease treatment. The lower band seen on the MotB immunoblot in the absence of trypsin (RSO membranes) is a MotB breakdown product produced during membrane isolation, whose intensity varied from experiment to experiment.

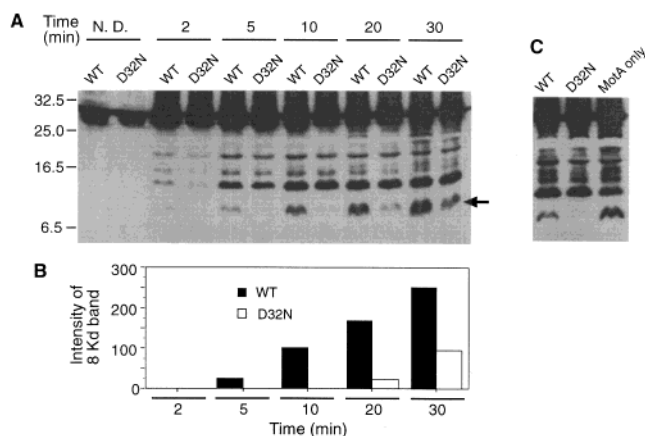


FIGURE 3: Effect of the Asp32→Asn mutation in MotB on protease susceptibility of MotA in the MotA/MotB complex. (A) Representative anti-MotA immunoblot showing MotA fragments produced by trypsin treatment of MotA/MotB complexes in ISO membranes. MotA was wild-type, and MotB was either wild-type or with the mutation D32N, as indicated. N.D., not digested. (B) Quantification of the 8-kDa bands in the experiment of panel A. (C) Comparison of MotA fragments produced after 20 min trypsin digestion of wild-type MotA/MotB complexes, D32N-mutant complexes, and MotA in membranes without any MotB.

the side chain are important in regulating conformation of the MotA/MotB complex. Results are summarized in Figure 4 (top). The Ala, Ser, Cys, and Gly replacements of Asp32 were like the Asn replacement, causing a ca. 5-fold decrease in the yield of 8-kDa fragment after 20 min of trypsin digestion. All of these replacements are small and uncharged, in these ways resembling a protonated Asp residue. Medium-size replacements, irrespective of charge, did not cause the conformational change seen with the small replacements; Leu, Glu, or Arg replacements of Asp32 did not significantly alter the yield of 8-kDa fragment. The D32W mutation had an effect opposite that of the small replacements, causing a small but reproducible increase in the level of 8-kDa fragment after 20 min of digestion.

MotA and MotB bind fairly well to each other but do not bind strongly to other flagellar proteins (10), and so the

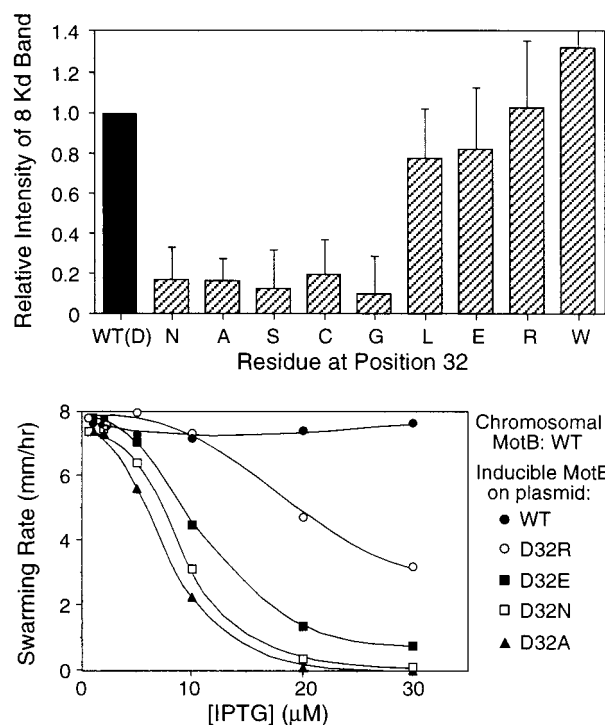


FIGURE 4: (Top) Effect of 9 different Asp32-replacements on the yield of 8-kDa fragment after 20 min digestion with trypsin. Yields for the mutants are relative to wild-type, and are the mean (\pm SD) of three independent experiments. (Bottom) Dominant-negative effects of four different Asp32-mutant MotB proteins. Mutant MotB proteins were expressed from an IPTG-regulated plasmid in wild-type cells, and rates of swarming in semisolid (0.28%) agar were measured. Swarming rates are the mean of two determinations, which differed by an average of 0.34 mm/h.

observed conformational change probably occurs in a complex that contains only MotA and MotB. To see whether any other flagellar proteins are required for the conformational change, we carried out limited trypsin digestion of MotA/MotB complexes in membranes that contained no other flagellar proteins. These experiments used membranes isolated from a mutant strain deleted for the genes *flhD* and *flhC*, which encode regulatory factors necessary for the

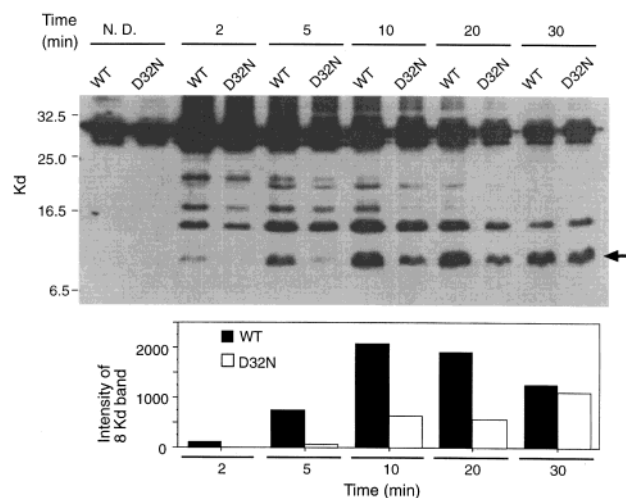


FIGURE 5: Proteolysis of the MotA/MotB complex in membranes that contain no other flagellar proteins. MotA and MotB were expressed from a plasmid in the *flhDC* strain RP3098, which does not express any chromosomal flagellar genes. Inside-out membranes were treated with trypsin and MotA fragments were detected by immunoblot. The 8-kDa bands (arrow) are quantified in the lower panel. N.D., not digested.

expression of all chromosomal flagellar genes (58). When the membranes contained no flagellar proteins besides MotA and MotB, limited-digestion experiments gave the same result: at intermediate times of digestion, an 8-kDa MotA fragment was produced in higher yield when MotB was wild-type than when it harbored the D32N mutation (Figure 5). Taken together with the fact that MotB binds to MotA (10), this shows that Asp32 mutations in MotB directly affect the conformation and/or exposure of MotA molecules in the MotA/MotB complex.

Correlation of Conformational Changes with Dominance of Asp32 Mutations. We reported previously that Asp32-mutations in MotB are very dominant, meaning that the mutant proteins impair motility strongly when expressed in wild-type cells (42). We also noted strong dominance in mutations of the conserved residues Pro173 and Pro222 in MotA, and proposed that such mutations might put the stator in an aberrant conformation that impedes rotation driven by functional (wild-type) complexes present in a motor. Since the initial experiments showed that mutations of Asp32 do cause conformational changes, we asked whether these conformational changes are correlated with the dominance of the mutations. To allow better quantification of dominant-negative effects, a set of *motB* mutations (D32N, D32A, D32E, and D32R) was transferred onto a plasmid that allows variable, IPTG-regulated expression of MotB. The mutant plasmids were introduced into wild-type cells, and rates of swarming were measured in soft-agar plates containing various concentrations of IPTG. Results are shown in Figure 4 (bottom). The magnitude of dominant-negative effects varied with the mutation, following the sequence D32A \approx D32N > D32E > D32R. Thus, the most strongly dominant mutations are the same as the ones that produced the largest effects on conformation in limited-proteolysis experiments (compare top and bottom panels of Figure 4).

Sites Affected by the Conformational Change. MotA contains many sites for cutting by trypsin (Lys or Arg), many pairs of which could yield an 8-kDa fragment. To localize the digestion sites that produce the 8-kDa fragment, we

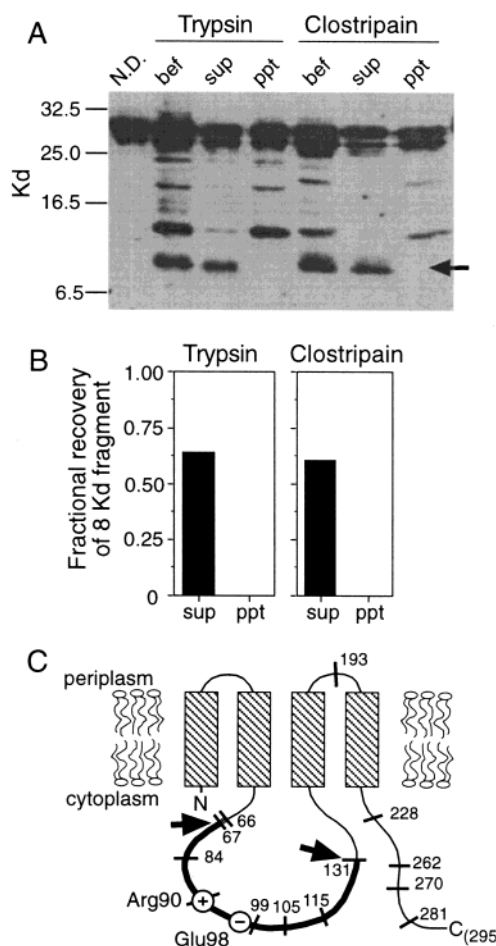


FIGURE 6: (A) Separation of soluble and membrane-associated MotA fragments. The experiment used strain RP3098 ($\Delta flhD flhC$) transformed with a plasmid that expresses MotA and MotB. Soluble and membrane fractions were separated by centrifugation at 16000g for 15 min. The arrow indicates the 8-kDa fragment. "bef", unfractionated sample; "sup", supernatant; "ppt", pellet; N.D., not digested. (B) Relative amounts of 8-kDa fragment recovered in the supernatant and pellet fractions in the experiment of panel A. (C) Sketch of MotA showing sites of possible cleavage by clostripain (Arg residues), and the segment of the protein (thick line) assigned to the 8-kDa fragment. The fragment is produced by cutting at residue 66 or 67 at the N-terminus, and residue 131 at the C-terminus (thick arrows). Residues that interact with the rotor, Arg90 and Glu98, are also indicated.

fractionated trypsin-treated samples to separate membrane-bound MotA fragments from soluble fragments. Most of the tryptic fragments of MotA sedimented with the membranes, but the 8-kDa fragment was found only in the supernatant of samples spun at 16000g for 15 min (Figure 6), indicating that the fragment does not contain any membrane-inserted segments. Note that since Arg and Lys residues are absent from the membrane segments of MotA (Figure 2), tryptic fragments that contain any part of a membrane segment will contain at least one entire segment. Because the antibody used for the immunoblots was raised against the first cytoplasmic domain of MotA, the 8-kDa fragment must be contained within this domain.

Whereas trypsin cuts at both Arg and Lys residues, the protease clostripain cuts only at Arg. Treatment with clostripain also yielded an 8-kDa fragment that was soluble (Figure 6) and present in higher yield with wild-type MotB than with D32N-mutant MotB (Figure 8). This implies that

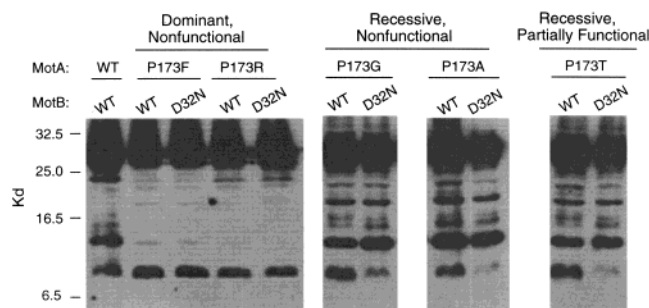


FIGURE 7: Effects of Pro173 mutations on proteolysis of MotA in MotA/MotB complexes. Experiments used inside-out membranes containing MotA (either wild-type or with the Pro173 mutations indicated) and MotB (either wild-type or with the D32N mutation). Samples were treated with trypsin at 22 °C for 20 min, and MotA fragments were detected by immunoblot.

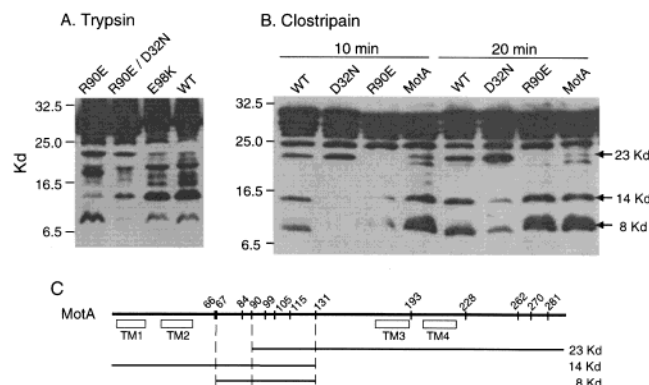


FIGURE 8: Proteolysis of MotA/MotB complexes with mutations in residues Arg90 or Glu98 of MotA. (A) Digestion with trypsin. Membranes containing MotA (wild-type, R90E, or E98K, as indicated) and MotB (either wild-type or D32N) were digested with trypsin at 22 °C for 20 min, and MotA fragments were detected by immunoblot. (B) Digestion with clostripain. Membranes containing MotA and MotB, either wild-type or with the indicated mutations, were treated with clostripain at 42 °C for 10 or 20 min, and MotA fragments were detected by immunoblot. Lanes marked "MotA" are membranes that contained only wild-type MotA. (C) Map of Arg residues in MotA, and suggested assignments for 8-, 14-, and 23-kDa fragments discussed in the text.

the 8-kDa fragment is produced by cutting at Arg residues, at both ends. The size of the fragment and the locations of Arg residues in the cytoplasmic domain of MotA (Figure 2) then indicate that it is produced by cutting at residue 66 or 67 at the N-terminus, and residue 131 at the C-terminus (Figure 6, bottom). The 8-kDa fragment thus contains the residues of MotA (Arg 90 and Glu 98) that interact with the rotor (40).

Effects of Low Solution pH. The results with various Asp32 mutants (Figure 4) imply that protonation of Asp32, to give a small uncharged side chain, should cause a conformational change in the MotA/MotB complexes. To test more directly the effects of protonation, we carried out proteolysis experiments at lower pH, using clostripain (which unlike trypsin remains active at low pH). Several bands changed in intensity when pH was lowered to 5.5, and the yield of 8-kDa fragment was significantly reduced in the wild-type at pH 4.5. The intensity changes were seen in both the wild-type and D32N mutant, however, and the reduction in 8-kDa band intensity was also seen in a sample that contained only MotA (data not shown). Thus, we did not observe any effects that can be solely attributed to protonation of Asp32.

Effects of Mutating Key Residues of MotA. Residue Pro173 of MotA is conserved across species and is important for motor rotation. It is probably an important determinant of conformation, and on the basis of mutant phenotypes, we proposed that it might control conformational changes in the stator that couple proton movement to rotor movement (41). To probe the effects of Pro173 replacements on conformation, we examined patterns of proteolysis of MotA with 5 different mutations in this position (P173G, P173A, P173F, P173R, or P173T), in complexes with either wild-type MotB or D32N-mutant MotB.

The MotA mutations P173F and P173R are nonfunctional and strongly dominant (41). These mutations caused a substantial change in the proteolysis pattern that was different from the effects of MotB Asp32 mutations (Figure 7). The 8-kDa fragment was produced in somewhat higher yield than in the wild-type, and a number of larger fragments were produced in much lower yield. Further, the P173F and P173R mutations prevented the conformational change ordinarily induced by the D32N mutation (Figure 7). A co-isolation assay using His-tagged MotB showed that MotA and MotB remained associated with each other in the P173F/D32N double mutant (data not shown). These results show that the P173F and P173R replacements produce an altered and possibly locked conformation in the MotA/MotB complexes.

The P173G and P173A mutations of MotA are also nonfunctional, but unlike the P173F and P173R mutations, they are recessive (41). These mutations did not affect MotA conformation as judged by patterns of limited proteolysis and also did not hinder the conformational change induced by the D32N mutation in MotB. The mutation P173T is partially functional and recessive. Like the P173G and P173A mutations, it did not alter the MotA-proteolysis pattern and did not prevent the conformational change induced by the D32N mutation (Figure 7).

Arg90 and Glu98 are in a cytoplasmic domain of MotA, probably in a site distinct from Pro173 and MotB Asp32. They participate in electrostatic interactions with charged residues of the rotor protein FliG (40). These stator-rotor interactions are essential for rotation, but their precise role is not known. In a recent proposal for the rotation mechanism, we suggested that such interactions might synchronize proton gating in the stator with the movement of the rotor, ensuring that protons can enter and bind to Asp32 only when the rotor is properly positioned to be pushed by the resulting conformational change. It is of interest to know whether Arg90 or Glu98 are directly important for determining the conformation of MotA/MotB complexes in membranes. To address this, we carried out limited-proteolysis experiments on MotA with mutations in Arg90 or Glu98, expressed along with wild-type MotB or, in some cases, D32N-mutant MotB.

The experiments used the charge-reversing mutations R90E and E98K in MotA, previously shown to prevent motor rotation (39). The E98K mutation did not alter the pattern of tryptic fragments (Figure 8A). The R90E mutation did alter the pattern of tryptic fragments, but the changes could be due mainly to the loss of a trypsin-digestion site at residue 90 (cf., experiments below using clostripain). The R90E mutation clearly did not interfere with the conformational change caused by the D32N mutation; the yield of 8-kDa fragment was decreased in the R90E/D32N double mutant relative to the R90E-only mutant. Thus, Arg90 and Glu98

do not appear to be strong determinants of conformation of MotA/MotB complexes in membranes.

We also digested the R90E-mutant protein with clostripain, which gives simpler fragment patterns that are easier to interpret (Figure 8B). As in the experiment above, the 8-kDa fragment was produced in higher yield when MotB was wild-type than when it had the D32N mutation, and in this experiment, a ~14-kDa band also showed higher yield with wild-type MotB than with D32N-mutant MotB. When the R90E mutation was present in MotA, a band at ~23 kDa disappeared and the 8 and 14-kDa bands increased in intensity at the 20-min time point. These results can be accounted for by the loss of the digestion site at residue 90. The ~23-kDa fragment is the only band to disappear in the R90E mutant and is very likely the larger fragment produced by cleavage at Arg90 (residues 91–295; Figure 8C). The 8-kDa fragment contains residues ~67–131, and so removal of the clostripain-digestion site at residue 90 is expected to increase its yield. The 14-kDa fragment is probably the smaller fragment (residues 1–131) produced by cutting at residue 131, in which case it is also expected to show increased yield when Arg90 is mutated.

Above, we found that MotA present in membranes without any MotB gave a trypsin-digestion pattern similar to that of MotA in MotA/MotB complexes. With clostripain, the fragment pattern with MotA alone was again basically like that with MotA/MotB complexes, but some differences were noted. In the MotA-only samples the ~23-kDa fragment was somewhat decreased in intensity, 14- and 8-kDa bands were somewhat increased, and a set of weak bands appeared in the 18–21 kDa range (Figure 8). This indicates that the presence of MotB alters slightly the clostripain susceptibility of some Arg residues in the cytoplasmic domain of MotA.

DISCUSSION

A mutational survey of titratable residues in the flagellar motor (42) led us to propose that protonation/deprotonation of Asp32 in MotB might cause cyclical conformational changes in the stator that drive movement of the rotor. Here, we probed conformational changes in the stator by examining the protease susceptibility of MotA/MotB complexes in membranes. The pattern of fragments produced during digestion with trypsin proved useful as a probe of conformational change: various small, uncharged replacements of MotB Asp32 caused a ca. 5-fold reduction in yield of an 8-kDa tryptic fragment of MotA. The mutations did not cause MotA to dissociate from MotB, and the change in proteolysis pattern did not require any flagellar proteins besides MotA and MotB. Together, these observations show that the mutations in residue 32 of MotB alter the conformation and/or accessibility of MotA within the MotA/MotB complex.

Protonation of the native aspartic acid residue would likewise give a small, uncharged side chain, and in terms of the functional groups present, a protonated Asp is something between Asn and Ser, both of which cause the conformational change. Thus, protonation of Asp32 should cause the same or a very similar conformational change in the MotA/MotB complexes. Joining this to the evidence that implicates Asp32 in proton conduction (42), we conclude that proton flow through the motor will drive cyclical conformational changes in the MotA/MotB complexes. Judging from the magnitude of the effect on protease susceptibility, the conformational changes could be substantial. We note that the MotA/MotB

complex might have access to two (or more) conformations in both its protonated and deprotonated forms; our results would then indicate that the equilibrium between conformations is biased strongly by protonation.

Clostripain-digestion experiments at pH 4.5 did not reveal any effects solely attributable to protonation of MotB Asp32 (data not shown). In an effort to protonate Asp32 specifically, we also examined the effects of energizing everted membranes, using NADH as electron donor. Energizing the membranes did not affect the pattern of fragments produced by trypsin. In another study of the MotA/MotB complexes, we probed the accessibility of introduced Cys residues to sulfhydryl reagents and found that Asp32 and neighboring residues are readily accessible from the cytoplasmic side of the membrane (see following paper in this issue). If Asp32 can be protonated from the cytoplasmic side but not from the periplasmic side, in MotA/MotB complexes in membranes, then energizing the membranes will decrease rather than increase the proton concentration at Asp32, and so it is not expected to induce the conformational change.

Does the conformational change in the stator act upon the rotor, as it should if it has a role in rotation? As described in the Results, the evidence combines to show that the 8-kDa fragment is produced by cleavage at Arg66 (or 67) and Arg131, in the larger cytoplasmic domain of MotA (Figure 6). The conformational change thus alters protease susceptibility at sites in MotA that flank the rotor-interacting segment (Arg90–Glu98). This conformational change could, therefore, move parts of MotA at the rotor–stator interface. Also relevant is the finding that mutations in Asp32 or Pro173 that cause the largest conformational changes are also the most dominant, and that dominant Pro173 mutations, but not recessive ones, lock the conformation of the stator so that it is unresponsive to mutation of residue 32 (Figures 4 and 7). The very strong dominance of certain MotA and MotB mutations prompted us to suggest before that those mutant proteins might interfere with (i.e., jam) rotation of the rotor. In support of the jamming hypothesis, we found that some especially dominant mutations of MotA (the ones studied were in Pro222) were less dominant when the cells contained a FliG mutation that increases rotor–stator clearance (41). The correlation with dominance is thus additional reason to think that the conformational changes observed here affect the rotor–stator interface.

The Glu replacement of Asp32 has little effect on the proteolysis pattern of MotA, implying that conformation is not much affected. Moreover, D32E mutants retain some ability to rotate their motors, implying that both the protonated and deprotonated conformations of the stator are at least basically normal. The D32E mutant swims quite slowly, however, implying that some step during the torque-generating cycle is slowed. A slow step can account for the significant dominance of the D32E mutation (Figure 4). If both conformations of the stator (protonated and deprotonated) are normal in imposing constraints on rotor movement, but the conformations are slow to interconvert, then D32E-mutant complexes will retard the rotation driven by faster, wild-type complexes. The D32R mutation prevents motor rotation and can be presumed to prevent proton-driven conformational changes in the stator, yet is less dominant than the other mutations. If strong dominance is due to rotor–stator jamming, as now seems likely, then the lesser dominance of the D32R replacement would indicate a

conformation that is pressed less tightly against the rotor or is more flexible.

The conformational change observed here may provide means for investigating some motor processes *in vitro*, because it involves only MotA and MotB and so might also be observed in purified MotA/MotB complexes. We have investigated several detergents and have found a few that are effective in extracting the MotA/MotB complexes from membranes while preserving the D32N-induced conformational change (unpublished observations). This shows that the conformational change can occur outside of membranes and can serve as a probe of the integrity of MotA/MotB complexes during purification.

The occurrence of significant conformational change in the stator has implications not only for the present-day mechanism but also for the evolution of the flagellar motor. A membrane complex that undergoes proton-driven conformational changes could perform useful work in contexts other than (and simpler than) the flagellar motor, and ancestral forms of the MotA/MotB complex might have arisen independently of any part of the rotor. We queried the sequence database using the sequence of the best-conserved part of MotA (the segment containing membrane segments 3 and 4) from *Aquifex aeolicus*, a species whose lineage is deeply branched from other bacteria. In addition to the expected MotA homologues, the search returned a protein sequence from the archaeal species *Methanobacterium thermoautotrophicum* (protein MTH1022) that shows significant sequence similarity not only to MotA but also to the protein ExbB (Figure 9). ExbB is a cytoplasmic-membrane protein that functions in conjunction with ExbD, TonB, and outer-membrane receptors to drive active transport of certain essential nutrients across the outer membrane of Gram-negative bacteria. The energy for this transport comes from the proton gradient across the inner membrane. Thus, MotA and ExbB are both components of systems that tap the proton gradient to do work some distance away (at either the rotor-stator interface or the outer membrane; Figure 9).

Other features also point to a connection between the Mot and Exb systems. MotA functions in a complex with MotB, which as noted contains the critical residue Asp32 near the cytoplasmic end of its single membrane segment. ExbB functions in a complex with ExbD, which likewise has a single membrane segment with a critical Asp residue near its cytoplasmic end (Asp25 in ExbD of *E. coli*; ref 59). Although ExbB has only three membrane segments in contrast to the four in MotA, the membrane segments that show sequence similarity have the same topology. The protein TonB is also present in the complex with ExbB and ExbD (59, 60) and would provide an additional membrane segment to round out the topological correspondence (Figure 9). ExbB contains a well-conserved Pro residue (Pro141 in *E. coli* ExbB) that is the counterpart of Pro173 of MotA. Although MotB and ExbD do not share close sequence similarity apart from the critical Asp residue, in certain positions in the membrane segment the residues most common in MotB proteins are also common in ExbD proteins. Finally, like the MotA/MotB complex the ExbB/ExbD complex contains multiple copies of each protein (61). Together, these facts make a reasonable case for an evolutionary connection between the Mot proteins of the flagellar motor and the Exb proteins of outer-membrane transport (and by extension the TolQ/TolR proteins, which are related to ExbB/ExbD but whose functions are less understood).

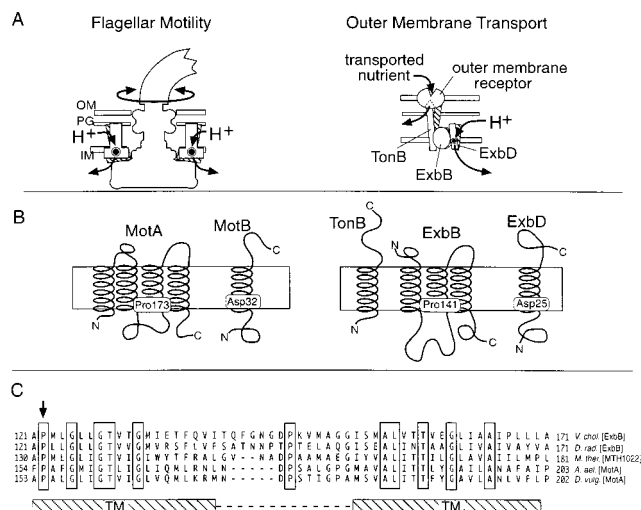


FIGURE 9: Correspondences between the MotA/MotB and ExbB/ExbD complexes. (A) Functions of the complexes. Both use the membrane proton gradient as a source of energy to drive processes occurring outside of the membrane (either the application of force to the flagellar rotor, or active transport of nutrients across the outer membrane). As shown here very diagrammatically, the complexes are hypothesized to do work by undergoing conformational changes as protons move on and off critical Asp residues in MotB and ExbD, respectively. (B) Membrane topologies. The ExbB/ExbD complex also contains the protein TonB, which has a single membrane segment with topology (N-terminus in) like that of segment 1 of MotA. Pro and Asp residues important for function and conserved in both systems are shown. (C) Alignment of a ca. 50-residue segment from selected MotA and ExbB sequences. Shown below are approximate positions of membrane segments (segments 3 and 4 of MotA, or segments 2 and 3 of ExbB). The arrow indicates a Pro residue that is conserved in both systems and known to be important for function of MotA (Pro173 in MotA of *E. coli*). *V. chol.*, *Vibrio cholerae*; *D. rad.*, *Deinococcus radiodurans*; *M. ther.*, *Methanobacterium thermoautotrophicum*; *A. ael.*, *Aquifex aeolicus*; *D. vulg.*, *Desulfovibrio vulgaris*. Using the *M. thermoautotrophicum* MTH1022 sequence fragment to query the database of finished and unfinished bacterial genomes (National Center for Biotechnology Information, 5/21/01), pairwise BLAST E-values for these alignments are for *V. cholerae* [ExbB], 1E-6; for *D. radiodurans* [ExbB], 9E-5; for *A. aeolicus* [MotA], 0.004; and for *D. vulgaris* [MotA], 0.002.

Our current view of conformational changes and their role in torque generation is summarized in Figure 10. Salient points are as follows. In wild-type MotA/MotB complexes apart from motors, Asp32 is most often deprotonated and is not accessible to protons from the periplasm (panel A). Protonation of Asp32, or mutation to a small neutral residue such as Asn, changes the conformation of the stator so that it presents an altered surface to the rotor (panel B). Dominant mutations of Pro173 (e.g., P173F) give a third conformation (panel C), which is locked in the sense of being unresponsive to mutation of Asp32 (panel D). In the flagellar motor, an appropriate alignment of the rotor and stator triggers the opening of a gate that controls access from the periplasm, so that a proton can enter and bind to Asp32. Protonation causes a conformational change in the stator that pushes the rotor through a small angle (panels E and F). The proton then dissociates from Asp32 into the cytoplasm. This reverses the conformational change, driving further rotation as the protruding parts of the stator engage the next "tooth" of the rotor (panel G).

Recent physiological studies conducted over a wide range of rotation speeds provide evidence that the flagellar motor

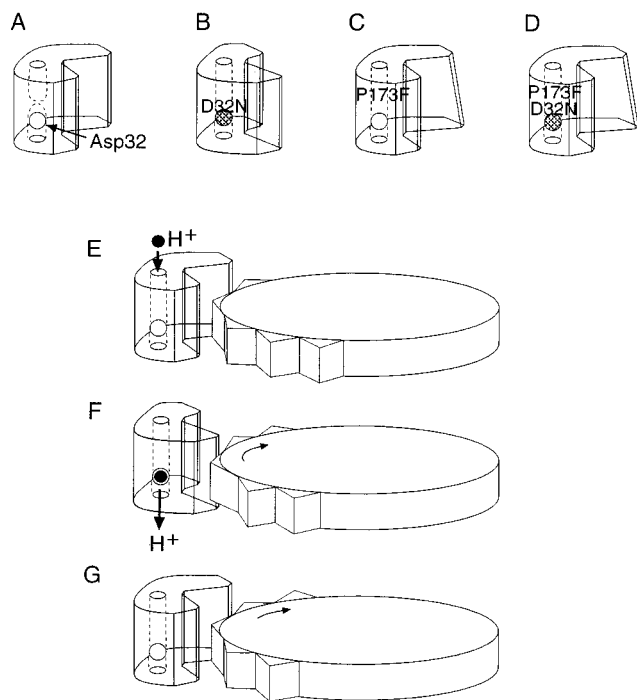


FIGURE 10: Hypothesized role of stator conformational changes in the generation of torque by the flagellar motor. Each panel shows a very simplified cartoon of one stator complex (of the ca. 8 complexes present in a flagellar motor). (A) In wild-type complexes in the membrane, Asp32 is most often deprotonated and is not accessible to protons from the periplasm. (B) Mutation of Asp32 to Asn, which is presumed to mimic protonation, puts the stator complex in a different conformation in which rotor-interacting parts (the right-hand side) of the stator move. (C) The mutation P173F in MotA (or another dominant mutation of Pro173) puts the stator in a third conformation that resembles the deprotonated more than the protonated state, but which is distinct from both. (D) The P173F-mutant complex is locked in the sense that adding the Asp32 → Asn mutation has no effect on its conformation. (E) In the flagellum, an appropriate alignment of the stator with the rotor triggers opening of a gate in the stator, admitting a proton which binds to Asp32. (F) Binding of the proton to Asp32 forces a conformational change, which works on the rotor to drive rotation through a small angle. (G) Following the conformational change, the proton dissociates to the cytoplasm, reversing the conformational change and causing further rotation.

uses a power-stroke mechanism in which rotor movement occurs synchronously with dissipation of the available chemical energy (62, 63). A power-stroke mechanism contrasts with "Brownian ratchet" models in which forward movement of the rotor occurs thermally, and chemical energy is used to engage a latch at the appropriate time(s) to prevent backward slippage. The mechanism pictured in Figure 10 is in accordance with the recent physiological findings in utilizing power strokes to drive rotation, one following protonation of Asp32 and another following deprotonation, both obtaining energy from exothermic conformational changes.

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