Rusty, Jammed, and Well-Oiled Hinges: Mutations Affecting the Interdomain Region of FliG, a Rotor Element of the *Escherichia coli* Flagellar Motor

Susan M. Van Way, Stephanos G. Millas, Aaron H. Lee, and Michael D. Manson*

Department of Biology, Texas A&M University, College Station, Texas 77843

Received 4 November 2003/Accepted 6 February 2004

The FliG protein is a central component of the bacterial flagellar motor. It is one of the first proteins added during assembly of the flagellar basal body, and there are 26 copies per motor. FliG interacts directly with the Mot protein complex of the stator to generate torque, and it is a crucial player in switching the direction of flagellar rotation from clockwise (CW) to counterclockwise and vice versa. A primarily helical linker joins the N-terminal assembly domain of FliG, which is firmly attached to the FliF protein of the MS ring of the basal body, to the motility domain that interacts with MotA/MotB. We report here the results of a mutagenic analysis focused on what has been called the hinge region of the linker. Residue substitutions in this region generate a diversity of phenotypes, including motors that are strongly CW biased, infrequent switchers, rapid switchers, and transiently or permanently paused. Isolation of these mutants was facilitated by a “sensitizing” mutation (E232G) outside of the hinge region that was accidentally introduced during cloning of the chromosomal fliG gene into our vector plasmid. This mutation partially interferes with flagellar assembly and accentuates the defects associated with mutations that by themselves have little phenotypic consequence. The effects of these mutations are analyzed in the context of a conformational-coupling model for motor switching and with respect to the structure of the C-terminal 70% of FliG from *Thermotoga maritima*.

*Corresponding author. Mailing address: Department of Biology, 3258 TAMU, Texas A&M University, College Station, TX 77843. Phone: (979) 845-5158. Fax: (979) 845-5158. E-mail: mike@mail.bio.tamu.edu.
tions can be compensated for by mutations in flIG. Three of these suppressors map to a part of flIG that encodes what is predicted to be a linker between the motility and assembly domains (17). Elements within this region, which encompasses residues 183 to 196, are predicted to function as a flexible hinge whose position determines how the motility domain of FlIG presents itself to MotA. To assess how residue changes in the linker affect flagellar performance, we performed random PCR mutagenesis of codons 183 to 196 of a plasmid-borne flIG gene. Based on the phenotypes of our mutants, we conclude that a simple two-state (CW/CCW) model is inadequate to describe the full behavior of the flagellar motor.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli strain DBF225 (26), which contains an in-frame deletion that removes the entire flIG coding region, was used as the host for flIG plasmids. The flIG gene was amplified from the chromosome of E. coli by using PCR. Unique HindIII and BamHI restriction site regions were engineered into the 5' and 3' ends of flIG, respectively. The amplified gene was subsequently cloned into the low-copy-number plasmid pHS576 (43) to form pSVW1, in which flIG is under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P<sub>lac</sub> promoter. The pSVW1 plasmid complements the flIG deletion in strain DBF225 to allow swarming in tryptone semisolid agar.

Localized random PCR mutagenesis. The DNA sequence that encodes the putative hinge of flIG was mutagenized using the two-primer method described by Perlak (35) and Deng and Nickoloff (12). Plasmid pSVW1 was used as a substrate for mutagenesis, and a doped oligonucleotide was utilized to introduce missense mutations into the putative hinge. The level of doping was such that each of the three alternate bases was present as a 1% contaminant. In a subsequent effort to increase the likelihood of obtaining transversions to expand the range of amino acid substitutions we found, the mutagenesis protocol was altered by doping positions occupied by pyrimidines with purines only and positions occupied by purines with pyrimidines only, at a level of 3%. A counterselection primer was employed during mutagenesis to insert a mismatch within a unique and nonessential SpeI restriction site in pSVW1.

Isolation of mutants. DBF225 cells were transformed with the mutantized flIG-encoding plasmids, and potential mutants were identified by screening for mutant motility phenotypes in tryptone swarm agar (10 g of Difco Bacto-Tryptone, 5 g of NaCl, and 3.25 g of Difco Bacto-Agar, all per liter, 1 mM IPTG, and 30 μg of chloramphenicol/ml). Mutants were restated in swarm agar to confirm their phenotypes and to measure swarm diameters. Plasmids for sequencing were isolated by using the Qiapgen plasmid purification protocol. The entire flIG gene was sequenced for each candidate mutant by using the ABI fluorescent dideoxy sequencing protocol.

Immunoblotting. DBF225 cells carrying pSVW1, pSVW2, or one of their mutant derivatives were grown with vigorous swirling at 32°C in tryptone broth containing 1 mM IPTG and 30 μg of chloramphenicol/ml. The next morning, the cultures were diluted 1:100 into the same medium and grown to an OD<sub>590 nm</sub> of 0.6 to 0.8. Cells were washed on nitrocellulose filters with 150 ml of dH<sub>2</sub>O and resuspended in dH<sub>2</sub>O to an OD<sub>590 nm</sub> of approximately 0.4. Cells were stained for 10 s with 1% ammonium molybdate.

RESULTS

Isolation and initial characterization of mutations affecting the FlIG hinge. The E. coli chromosomal flIG gene was amplified from the chromosome by PCR using Taq polymerase and cloned into a low-copy-number plasmid (pHS576) under control of the lac promoter. The resulting plasmid, pSVW1 (pflIG<sup>+</sup>), complemented the motility and chemotactic defects of the ΔflIG strain DBF225 and was used as a template for doped-oligonucleotide mutagenesis of flIG codons 183 to 196. Strain DBF225 was transformed with mutagenized, pooled pSVW1 DNA, and mutants were isolated by screening for altered swarm phenotypes on tryptone semisolid agar. After confirmation that the mutations were plasmid borne, the mutant flIG genes were sequenced to identify mutations.

Mutants were independently isolated from approximately 3,000 screened transformant colonies. The plasmids contained six unique missense mutations that cause the following amino acid substitutions: K189E (five times), G195A (twice), and G185S, R190P, and V196L (once each). One plasmid had a G→T transversion that introduces an A199S substitution. This information that the mutations were plasmid borne, the mutant flIG genes were sequenced to identify mutations.

All mutant plasmid-borne flIG genes, as well as the parental pSVW1 plasmid, contained a second mutation that causes an E232G substitution (Fig. 1). It was presumably introduced during PCR cloning, since the mutation is not in the chromosomal flIG gene of strain RP437. We therefore used site-directed mutagenesis to introduce Glu-232 into all plasmid-borne mutant flIG genes. The true flIG<sup>+</sup> plasmid was called pSVW2. DBF225/pSVW2 colonies swarmed appreciably

...
FIG. 1. Locations of residue substitutions in the connector between the assembly and motility domains of FliG. The residues within the expanded portion of the schematic of the protein primary sequence are within or immediately flank the region of FliG that corresponds to the long α helix that connects the assembly and motility domains (see the text). Residue substitutions in this region are indicated below the wild-type sequence. Residues Lys-192 to Gly-95 comprise the putative hinge. The positions of the Pro-168 through Ala-170 residues that are deleted in an extremely CW-biased mutant of S. enterica (46) and the position of the E232G substitution introduced during PCR cloning of fliG (see text) are also indicated.

Swimming behavior of single and double mutants. DFB225/pSVW1 swarms were 52% the diameter of DFB225/pSVW2 swarms (Tables 1 and 2). The G195A substitution in either plasmid eliminated swarming. The remaining pSVW2 mutants formed swarms with ≥45% the diameter of those made by DFB225/pSVW2. The A199S/E232G double mutant did not swarm, and the K189E/E232G, R190P/E232G, and V196L/E232G double mutants formed swarms that were 45% the diameter of those made by DFB225/pSVW2.

Swimming behaviors of single and double mutants. DFB225/pSVW1 swarms were 52% the diameter of DFB225/pSVW2 swarms (Tables 1 and 2). The G195A substitution in either plasmid eliminated swarming. The remaining pSVW2 mutants formed swarms with ≥45% the diameter of those made by DFB225/pSVW2. The A199S/E232G double mutant did not swarm, and the K189E/E232G, R190P/E232G, and V196L/E232G double mutants formed swarms that were 45% the diameter of those made by DFB225/pSVW2.

Swimming behaviors of single and double mutants. DFB225/pSVW1 swarms were 52% the diameter of DFB225/pSVW2 swarms (Tables 1 and 2). The G195A substitution in either plasmid eliminated swarming. The remaining pSVW2 mutants formed swarms with ≥45% the diameter of those made by DFB225/pSVW2. The A199S/E232G double mutant did not swarm, and the K189E/E232G, R190P/E232G, and V196L/E232G double mutants formed swarms that were 45% the diameter of those made by DFB225/pSVW2.

Swimming behaviors of single and double mutants. DFB225/pSVW1 swarms were 52% the diameter of DFB225/pSVW2 swarms (Tables 1 and 2). The G195A substitution in either plasmid eliminated swarming. The remaining pSVW2 mutants formed swarms with ≥45% the diameter of those made by DFB225/pSVW2. The A199S/E232G double mutant did not swarm, and the K189E/E232G, R190P/E232G, and V196L/E232G double mutants formed swarms that were 45% the diameter of those made by DFB225/pSVW2.

Swimming behaviors of single and double mutants. DFB225/pSVW1 swarms were 52% the diameter of DFB225/pSVW2 swarms (Tables 1 and 2). The G195A substitution in either plasmid eliminated swarming. The remaining pSVW2 mutants formed swarms with ≥45% the diameter of those made by DFB225/pSVW2. The A199S/E232G double mutant did not swarm, and the K189E/E232G, R190P/E232G, and V196L/E232G double mutants formed swarms that were 45% the diameter of those made by DFB225/pSVW2.
E232G mutant swarms had ≤13% the diameter of DFB225/pSVW2 swarms.

Behavior of free-swimming cells. Subjective analyses of the swimming behavior of DFB225 cells carrying pSVW2 or one of its mutant derivatives or of DFB225 cells carrying pSVW1 or one of its mutant derivatives are given in Tables 1 and 2, respectively. The swimming behavior of each mutant was generally consistent with its swarming properties and the rotational behavior of tethered cells of the mutant.

Effects of fliG hinge mutations on flagellar assembly. Mutations in FliG often affect flagellar assembly (25, 52). It therefore seemed possible that reduced motility might be a consequence of this effect. We therefore examined negatively stained preparations of whole cells to determine the average number of flagellar filaments on cells expressing various plasmid-encoded mutant FliG proteins. The mean number of filaments associated with cells of strain DFB225/pSVW2 was 3.2 (Table 1), slightly more than half the mean number (5.9) of flagella per cell of the wild-type strain, RP437. Except for the G195A, V196L, and A199S mutants, all of the single substitutions decreased the number of flagella to ≤1.5 per cell. The E232G substitution also had a severe effect, since DFB225/pSVW1 cells had an average of only 0.3 flagellum per cell (Table 2). Correspondingly low numbers of flagella, or none at all, were seen with the double mutants.

Analysis of tethered cells. Tethered DFB225 cells containing pSVW1, pSVW2, or one of their derivatives were analyzed to assess torque generation, rotational bias, and switching frequency of the mutant motors.

(i) Rotational velocity. The G185S, K189E, R190H, K192E, G194S, G195A, and V196L mutant cells rotated at speeds ≥20% those of DFB225/pSVW2 cells (Table 1) or cells of the wild-type parental strain, RP437 (data not shown). The mean speeds of A199S and R190P mutant cells were 70 and 30% of the wild-type speed, respectively. The E232G substitution slowed rotation significantly (Table 2), and very few K192E/E232G, G194S/E232G, or V196L/E232G cells rotated. No cells producing the G195A/E232G and A199S/E232G proteins spun. Surprisingly, R190P/E232G double mutant cells rotated faster than those of the R190P single mutant.

(ii) Directional bias of rotation. DFB225/pSVW2 cells had a mean 70% CCW bias (Table 1), somewhat higher than the 55% CCW bias of tethered cells of strain RP437 (data not shown). The K189E, R190P, and A199S mutants exhibited similar biases. The G194S and V196L substitutions increased the CCW bias to 85%, whereas the G185S and G195A substitutions introduced a very strong CW bias. The E232G substitution increased the CCW bias in otherwise wild-type FliG or in FliG with the K189E and K192E substitutions (Table 2), and it caused a dramatic increase in CCW rotation in combination with G185S (from 7 to 60% CCW bias). In contrast, E232G decreased CCW rotation modestly in combination with the G194S substitution and more strongly in combination with the R190P and V196L substitutions. Cells expressing the G195A/E232G and A199S/E232G FliG proteins did not rotate.

(iii) Switching frequency. The number of reversals per minute of single-mutant cells is shown in Table 1. DFB225/pSVW2 cells reversed a mean of 42 times per minute. The K189E, K192E, and V196L mutants had similar frequencies (33 to 40/min). The A199S mutant exhibited an increased mean reversal frequency of 77/min. The R190H and R190P mutants reversed relatively infrequently (15 and 10/min, respectively), although they had nearly wild-type CCW biases of 51 and 68%, respectively. The G194S mutant, although modestly CCW biased (84%), also reversed less often than expected (17/min) (Fig. 2). Cells of the strongly CW-biased G185S mutant made fewer reversals (22/min), and the G195A mutant was essentially locked in CW rotation.

The E232G substitution had little effect on reversal frequencies (Table 2) except in the K189E/E232G strain, which had a 97% CCW bias. R190H/E232G and R190P/E232G cells had reversal rates (22 and 34/min, respectively) that were higher than those of the corresponding single mutants. G185S/E232G cells exhibited an unusual behavior. Their mean reversal frequency (75/min) was high, and their mean CCW bias over the population of tethered cells examined was 60%, which is close

<table>
<thead>
<tr>
<th>Flig variant</th>
<th>Relative swarm diam*</th>
<th>Swimming behavior*</th>
<th>Flagella/cell</th>
<th>Rotational velocity*</th>
<th>Rotational bias</th>
<th>Switching frequency</th>
<th>Location of mutated residue*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E232G</td>
<td>52</td>
<td>~50% slow R/T</td>
<td>0.3 ± 0.5</td>
<td>4.9 ± 0.4</td>
<td>92 ± 2.2</td>
<td>22 ± 6.2</td>
<td>α-Helix H</td>
</tr>
<tr>
<td>G185S/E232G</td>
<td>43</td>
<td>&gt;90% slow R/T</td>
<td>1.0 ± 0.3</td>
<td>2.8 ± 0.5</td>
<td>60 ± 8.0</td>
<td>74 ± 18</td>
<td>See Table 1</td>
</tr>
<tr>
<td>K189E/E232G</td>
<td>13</td>
<td>~15% R/T</td>
<td>0.3 ± 0.1</td>
<td>3.8 ± 0.6</td>
<td>97 ± 2.5</td>
<td>2 ± 1.1</td>
<td>See Table 1</td>
</tr>
<tr>
<td>R190H/E232G</td>
<td>44</td>
<td>~15% slow R/T</td>
<td>0.7 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>64 ± 7.0</td>
<td>23 ± 3.3</td>
<td>See Table 1</td>
</tr>
<tr>
<td>R190P/E232G</td>
<td>13</td>
<td>~25% slow R</td>
<td>0.6 ± 0.2</td>
<td>4.1 ± 0.5</td>
<td>45 ± 8.3</td>
<td>34 ± 7.0</td>
<td>See Table 1</td>
</tr>
<tr>
<td>K192E/E232G</td>
<td>76</td>
<td>~5% slow R/T</td>
<td>0</td>
<td>2.2 ± 0.2</td>
<td>80 ± 4.6</td>
<td>34 ± 8.5</td>
<td>See Table 1</td>
</tr>
<tr>
<td>G194S/E232G</td>
<td>28</td>
<td>~2% slow R/T</td>
<td>0</td>
<td>3.7 ± 0.7</td>
<td>72 ± 4.7</td>
<td>38 ± 6.2</td>
<td>See Table 1</td>
</tr>
<tr>
<td>G195A/E232G</td>
<td>0</td>
<td>Nonmotile</td>
<td>NRC</td>
<td>46 ± 7.8</td>
<td>40 ± 5.9</td>
<td>See Table 1</td>
<td></td>
</tr>
<tr>
<td>V196L/E232G</td>
<td>5</td>
<td>Jiggle only</td>
<td>0.6 ± 0.3</td>
<td>3.5 ± 0.5</td>
<td>NRC</td>
<td>See Table 1</td>
<td></td>
</tr>
<tr>
<td>A199S/E232G</td>
<td>0</td>
<td>&lt;1% slow R/T</td>
<td>0.5 ± 0.1</td>
<td>NRC</td>
<td>NRC</td>
<td>See Table 1</td>
<td></td>
</tr>
</tbody>
</table>

* Mean diameters determined as for Table 1 and expressed as a percentage of the mean diameter of swarms made by the DFB225/pSVW2 strain.
* Swimming behavior recorded as in Table 1.
* The number of flagella per cell counted as for Table 1.
* Rotational velocities determined as for Table 1.
* Rotation biases recorded as in Table 1.
* Rotation biases scored as in Table 1.
* The residue corresponding to Gly-232 of the FliG Ec sequence is in α-helix H in the crystal structure of FliGp,v.
* Cells of the V196L/E232G double mutant did not exhibit significant translational movement, nor did they actively tumble. However, they moved more chaotically than would be expected from Brownian motion alone, based on the behavior of ΔfliG strain DFB225 (data not shown).
* NRC, no rotating cells in all microscopic fields observed.
to that of wild-type cells. However, some individual G185S/E232G cells were extremely CCW biased, others were very CW biased, and yet others spent nearly equal amounts of time in CW and CCW rotation. Some cells in each of these categories often stopped briefly without changing their direction of rotation. Although we scored these events as reversals, they may better be thought of as pauses in the sense used by Lapidus et al. (24) and Eisenbach et al. (14).

DISCUSSION

Based on genetic analyses of the *E. coli* and *Salmonella enterica* serovar Typhimurium fliG genes (17, 19, 25, 52) and a computer-generated prediction of secondary structure, Garza et al. (17) proposed that residues 183 to 196 of *E. coli* FliG constitute a flexible hinge between the N-terminal assembly and C-terminal motility domains of the protein. To study the role of this region in motor behavior, we mutagenized codons 183 through 196 of a plasmid-borne copy of fliG obtained by PCR cloning from the chromosome of wild-type *E. coli* strain RP437. The pSVW1 plasmid constructed in this way complemented strain DFB225 (H9004 fliG) for swarming in tryptone semi-solid agar. It was used as the template for random PCR mutagenesis. We subsequently found that pSVW1 contains a mutation that causes the E232G substitution in FliG. This adventitious change accentuated the effects of residue substitutions in the mutagenized region and facilitated detection of other mutations. The codon specifying the wild-type Glu-232 residue was introduced into pSVW1 to create plasmid pSVW2.

Identity of mutations affecting the FliG hinge. The six random missense mutations identified cause the residue substitutions G185S, K189E, R190P, G195A, V196L, and A199S. Mutations causing the R190H, K192E, and G194S substitutions, which were previously found as suppressors of a motB missense mutation (17), were also introduced into pSVW1 and pSVW2.

Effects of mutations on torque generation. Of the single substitutions, only R190P decreased the mean rotation speed by more than 50%—from 8.4 ± 0.6 revolutions per second (rps) to 2.7 ± 0.4 rps (Table 1). The A199S substitution caused a more modest reduction to 5.7 ± 0.3 rps. Since the rotational velocity of a tethered cell is directly proportional to the torque generated by the motor of the tethering flagellum (30), we conclude that most of these mutations by themselves have little influence on the force generated by the motor.

DFB225/pSVW1 cells rotated at 4.9 ± 0.4 rps (58% of the wild-type speed), and the mean rotational velocities of the double mutants were yet lower (Table 2). The most severe effect in mutants that produced rotating cells was seen with R190H/E232G (1.5 ± 0.2 rps). However, R190P/E232G cells spun faster than R190P cells (4.1 ± 0.5 versus 2.7 ± 0.4 rps).

The reason that the E232G substitution decreases torque in most cases is unknown, although defective interaction with FliM is apt to be at least partially responsible (see below).

Effects of mutations on directional bias. Some of the single mutations altered the CW/CCW ratio of rotation (Table 1). Tethered DFB225/pSVW2 cells had a 70% CCW bias. The flagella of two mutants spun primarily CW: G185S (7% CCW bias) and G195A (≤1% CCW bias). Two other mutants, G194S and V196L, had increased CCW biases (84 and 85%).

![Diagram](http://jb.asm.org/)

FIG. 2. Relationship between reversal frequency and directional bias for mutant flagellar motors. The absolute value of the mean deviation from 50% CCW rotation for a population of 20 cells of each mutant strain is shown on the ordinate. The mean number of reversals per minute for the same population of 20 cells of each mutant strain is plotted on the ordinate. (A) Strains containing plasmids derived from pSVW2 (FliG野生型). The ovals show the standard errors about the means for the values on the x and y axes. Deviations above 50% CCW rotation are indicated by open ovals; deviations below 50% are indicated by gray ovals. The FliG substitution in each mutant is indicated. WT is DFB225/pSVW2. The dotted line is a linear regression of the data points, and the dashed lines are the linear regression lines for the mean x and y values plus one standard error and minus one standard error. The data are from Table 1. (B) As in panel A, except that the strains contain plasmids derived from pSVW1 and the data are from Table 2.
The remaining single substitutions in the linker region had no significant effect on directional bias.

The mean CCW bias of DFB225/pSVW1 cells was 92% (Table 2). The E232G substitution had a mixed effect on directional bias in combination with second mutations. The CCW bias increased in four instances (G185S, K189E, R190H, and K192E) and decreased in three others (R190P, G194S, and V196L). No rotating cells were seen with A199S/E232G. Finally, G195A/E232G cells do not produce any flagella. Indeed, the E232G substitution uniformly and substantially decreased the number of flagellar filaments observed by electron microscopy (Table 2).

Effects of mutations on reversal frequency. To visualize the effects of the mutations on switching rates in a way that corrects for rotational bias, we plotted reversal frequency as a function of absolute directional bias (deviation from 50% CCW rotation) for each mutant (Fig. 2) and calculated a linear regression.

The data fit the linear regression reasonably well for most strains except at the extremes of CW and CCW bias, where reversal frequencies rapidly collapse to zero. The advantage of plotting the data this way is that the outliers stand out. For example, in Fig. 2A the R190H, R190P, and G194S mutants clearly reverse less often than would be expected based on their directional bias, whereas the A199S mutant reverses more often than expected. Of the double mutants with wild-type directional biases, G185S/E232G reversed more often than expected and fell significantly outside the predicted distribution (Fig. 2B). As expected, the 97% CCW-biased K189E/E232G mutant seldom reversed.

Effects on motor assembly, swimming, and swarming. The G185S, K189E, R190H, R190P, K192E, and G194S substitutions all decreased the mean number of flagella from 3.2 ± 0.6 per cell for DFB225/pSVW2 to ≈1.5 per cell. This decreased flagellation is not clearly reflected in the swarm diameters or swimming behavior of these mutants (Table 1). For example, the K189E, R190H, K192E, and G194S mutants form essentially wild-type swarms, and the first two produce >90% motile cells. We conclude that swarm formation is quite forgiving of moderate defects in flagellar assembly, perhaps because the best swimmers out of a genetically uniform population determine the rate at which the swarm rings advance.

The double mutants all had an average of ≈1.0 flagellum per cell (Table 2), and with three of them (K192E/E232G, G194S/E232G, and G195A/E232G) no flagella were found in 20 randomly chosen cells. This result is consistent with the very small number of motile cells in cultures of these three mutants (Table 2). In contrast, V196L/E232G and A199S/E232G cells were almost all nonmotile but had as many or more flagella than DFB225/pSVW1 cells. Thus, most of the flagellar motors of V196L/E232G and A199S/E232G cells must be nonfunctional.

A conformational-coupling model for directional switching. A theoretical treatment of directional control in the flagellar motor has been published recently (13). Based on the arguments advanced in that publication and the performance of the flagella of our hinge mutants, we have concluded that, at least under some conditions, motor switching behavior cannot be entirely accounted for by a simple two-state model. We have simplified our discussion by considering only conformational changes in the 26 FliG subunits (15, 20, 40) in the rotor. These events can be viewed as the output of the switching event. These changes in FliG conformation are strongly influenced by the fraction of the 34 FliM subunits per motor (45) that are bound to the tumble (CW rotation) regulator CheY-phosphate (10, 37).

We propose that the motor can exist in multiple states. At one extreme, all of the subunits are oriented to allow CW rotation. At the other extreme, all of the subunits are oriented to allow CCW rotation. Between these extremes are 25 hypothetical states in which 1 to 25 subunits can be in the CW orientation, with the remainder being in the CCW orientation. (This minimal estimate assumes that only the number of CW- and CCW-oriented subunits, rather than the distribution of CW- and CCW-oriented subunits around the ring, is relevant to overall motor behavior.) Reversals normally occur within a few milliseconds without a significant pause (1, 2, 23). This rapid switching is explained by Duke et al. (13) as the consequence of a high degree of allosteric coupling among the individual switch elements, a phenomenon they describe as conformational spread in a protein ring. The basic idea is that the conformation of one subunit strongly influences the conformation of one of its neighbors in an asymmetric fashion such that changes propagate in one direction or the other around the ring. Thus, even though each FliG subunit can occupy only two possible conformations, there are 27 possible states of the motor: all subunits in the CW orientation, all subunits in the CCW orientation, or 25 different intermediate ratios of CW and CCW subunits. The last condition would correspond to a transition state that is normally very brief but potentially becomes significant in mutant motors in which allosteric coupling among FliM or FliG subunits is impaired (see below).

Evaluation of the behavior of mutant motors with respect to the conformational-coupling model. The diverse phenotypes associated with different residue replacements in the hinge region of FliG can be evaluated in the context of the model shown in Fig. 3. Mutations that increase the stability (i.e., decrease the free energy) of either the CW or the CCW conformation of FliG are expected to generate motors that are CW or CCW biased relative to the wild-type motor. Note that this result could arise from making one extreme state more stable than in the wild type, one extreme state less stable, or a combination of these two effects.

Mutations that increase the total activation energy barrier between the two extreme states should decrease the reversal frequency, as is seen with the R190H, R190P, and G194S mutants (Fig. 2A). Mutations that decrease the activation energy should increase the reversal frequency, as is seen with the A199S mutant (Fig. 2A). One might expect that one could differentiate between a mutant motor in which one directional state becomes more stable than in the wild type as opposed to a mutant motor in which one directional state becomes less stable based on reversal frequencies. If the former is true, reversals of cells whose flagella are spinning in the dominant direction should be infrequent and reversals of cells whose flagella are spinning in the other direction should occur at the normal frequency. In the latter case, reversals of cells whose flagella are spinning in the dominant direction should occur at the normal frequency and reversals of cells whose flagella are
spinning in the other direction should occur more often than normal. We did not distinguish between CW-to-CCW and CCW-to-CW reversals in our tally of switches per minute. However, since tethered cells of the most strongly biased mutants reversed only rarely (Tables 1 and 2), we favor the interpretation that a directionally biased mutant motor is more stable than a wild-type motor in the conformation that corresponds to the predominant direction of rotation.

Two of the double-mutant motors (G185S/E232G and A199S/E232G) exhibited behaviors that we attribute to a disruption of normal conformational coupling among the FliG subunits. This result is in keeping with the generally deleterious effect on motor function that might be expected from random mutations.

A population of G185S/E232G cells showed no pronounced directional bias, but individual cells spun predominantly CW, predominantly CCW, or about equally in both directions (Table 2). Furthermore, many cells engage in frequent pauses, which we scored as reversals. Such longer pauses can be observed, even with wild-type cells, under some conditions (14, 24). We suggest that in the G185S/E232G motor these pauses are accentuated because the transition state, in which individual FliG subunits within a motor are in different conformations, sometimes persists long enough to be detected by eye.

The motors of the A199S/E232G double mutants may suffer a drastic loss of allosteric coupling among the FliG protomers (13). This effect might be particularly noticeable in tethered cells because generation of the high torque required to rotate a cell body may require greater synchrony among rotor subunits. The slower rotational speeds of tethered cells (Tables 1 and 2) of some of the mutants whose flagella are not paralyzed might reflect a more modest decrease in conformational coupling among FliG protomers, although there are clearly other equally plausible explanations. If the former explanation is correct, one might expect larger variations in rotational speed in these mutants. Since we did not specifically score variations in rotational velocity in our analysis of tethered cells, relatively subtle changes could easily have been missed.

The structural basis of mutant-motor phenotypes. The crystal structure of the C-terminal 70% (residues 104 to 335) of FliG from *T. maritima* (FliG<sub>Tm</sub>) has recently been determined (8, 27). FliG<sub>Tm</sub> shows significant sequence identity to *E. coli* FliG (FliGEc), with residues 104 to 335 of FliG<sub>Tm</sub> corresponding to residues 103 to 331 of FliG<sub>Ec</sub> (see Fig. 1). Residues 237 through 331 of FliG<sub>Ec</sub> (residues 239 to 335 of FliG<sub>Tm</sub>) form a stable domain that has been shown to interact directly with the large cytoplasmic loop of MotA (54, 55). The N-terminal 100+ residues of FliG comprise a domain that is attached to the MS ring, but whose structure is unknown. The intervening region contains two well-defined subdomains that interact with FliM (32).

In the FliG<sub>Tm</sub> crystal structure these two domains are connected by an extended α-helix (residues 172 to 193 in FliG<sub>Tm</sub>, corresponding to residues 171 to 191 in FliG<sub>Ec</sub>). This helix is flanked on its N-terminal end by a turn (residues 166 to 171 in FliG<sub>Tm</sub> and 165 to 171 in FliG<sub>Ec</sub>) that contains a Pro residue in both proteins. Residues 194 to 197 in FliG<sub>Tm</sub> (residues 192 to 195 in FliG<sub>Ec</sub>) comprise a turn. The conserved tandem Gly residues (Gly-196 and Gly-197 in FliG<sub>Tm</sub>, Gly-194 and Gly-195 in FliG<sub>Ec</sub>) may provide a flexion point for interdomain movement during switching events. The R190H and R190P substitutions, which are N terminal to the tandem Gly residues, and the A199S substitution, which is C terminal to them, decrease and increase the frequency of motor reversal, respectively. The significance of this correlation remains to be demonstrated.

Based on the FliG<sub>Tm</sub> structure, Glu-232 of FliG<sub>Ec</sub> is directly adjacent to a hydrophobic patch that is proposed to constitute a site of interaction with FliM (8). Also, a mutation in *E. coli*
flG that generates the E232V substitution decreases interaction of FliG with FliM (32). We therefore suspect that the defects in motor assembly and the changes in motor function associated with the E232G substitution are, at least in part, attributable to impaired interaction with FliM and might be reversed by overproduction of FliM and/or FliG (25). The possibility that the FliM interaction is weaker when FliG contains the E232G substitution could account for a longer duration of the transition state in some double-mutant motors.

A number of CW-biased flG mutations had previously been shown to change residues in the interdomain region of FliG from S. enterica serovar Typhimurium (19) and E. coli (25). The most potent of these is an in-frame deletion of the codons for Pro-169, Ala-170, and Ala-171 in S. enterica serovar Typhimurium. This mutation confers such an extreme bias that the defects in motor assembly and the changes in motor function with single-residue changes within the hinge region of FliG, summarized in Tables 1 and 2, should help elucidate the structural basis for control of the direction of flagellar rotation.

ACKNOWLEDGMENTS

We thank David Blair for supplying the ΔflG strain and FliG antibody and Christos Savva for help with electron microscopy. David Blair, Howard Berg, and Greg Reinhart provided useful suggestions about various aspects of our interpretation of the results. We are also grateful to the anonymous reviewers of earlier submissions of the manuscript for their candid comments and valuable suggestions, which we have tried to incorporate as they intended. The late-night heroic effort of Arjan Bormans to get the TIFF files of the figures up to snuff for electronic submission is especially appreciated.

This work was supported by grant DAAG55-97-1-0380 from the Army Research Office.

REFERENCES


