Enzymatic Characterization of FliI
AN ATPase INVOLVED IN FLAGELLAR ASSEMBLY IN SALMONELLA TYPHIMURIUM*

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FliI is a protein needed for flagellar assembly in Salmonella typhimurium. It shows sequence similarity to the catalytic β subunit of the FₙFᵥ-ATPase and is even more closely related to putative ATPases in Type III bacterial secretory pathways. A His-tagged version of FliI, which was fully functional in complementation tests, was purified to homogeneity. It had an ATPase activity of 0.16 s⁻¹ at 25 °C and pH 7, and a Kₘ for ATP of 0.3 mM; Mg²⁺ was required. The activity was not affected by inhibitors of the Fₙ-, V-, or P-type ATPases, or inhibitors of the Type I or Type II bacterial secretory pathways. Mutations K188I and Y363S decreased the ATPase activity about 100-fold, increased the Kₘ about 10-fold, blocked flagellar assembly, and were dominant. Other FliI mutations that disrupted flagellar protein export were found near the N terminus; they permitted essentially wild-type ATPase activity, were not dominant, and showed a dosage-dependent phenotype. We propose that FliI has a C-terminal ATPase domain and an N-terminal domain that interacts with other components in the flagellum-specific export apparatus.

The flagellum of Salmonella typhimurium and many other bacteria consists of a long helical filament, a short hook, and a basal body with a central rod and several rings (see Ref. 1 for a review). Only two of the structural components are exported by the general secretory pathway, which involves processing of an N-terminal signal peptide (2, 3); these two proteins, FlgI and FlgH, are the subunits of the outer-membrane and periplasmic rings of the basal body. All of the other external proteins are exported by a flagellum-specific pathway. These proteins (the subunits of the rod, hook, hook cap, hook-filament junction, filament, and filament cap) are thought to travel through a hollow channel in the nascent structure (4, 5) and assemble at its distal end (6, 7). The flagellar export apparatus is presumed to be at the base of the flagellum and involves at least three protein components, one of them being FliI (8).

Three classes of bacterial protein secretory pathways have been described (9). The Type I pathway (10), exemplified by the hemolysin secretion pathway of Escherichia coli, is signal peptide-independent; one of its components contains an ATP-binding cassette (ABC)³ and is called an ABC transporter or traffic ATPase. The ATPase activities of two such transporters, HlyB from E. coli and PrsD from Erwinia, have been described (11, 12). The Type II or general secretory pathway is signal sequence-dependent and proceeds in two stages: from cytoplasm to periplasm, and from periplasm to the external medium (13). The first step employs SecA as the ATPase that catalyzes pre-protein insertion and translocation. The most extensively studied Type II secretion system is that of pullulanase in Klebsiella oxytoca. PulE and its homologs contain a consensus ATP-binding site and have been termed traffic ATPases (14).

A third type of secretory pathway, Type III, has recently been identified in a variety of animal and plant pathogenic bacteria (1, 9, 15). This pathway is signal peptide-independent and involves many protein components. Type III secretory systems have several components homologous to proteins implicated in flagellar protein export (1, 15). Thus, although virulence protein secretion and flagellar protein export have different end results (host targeting versus organelle assembly), they are clearly related.

The flagellar protein export system and the Type III secretion systems all contain a putative ATPase component, which in the case of the flagellar system is FliI. Surprisingly, FliI and its homologs show similarity to the catalytic β subunit of the proton-translocating FₙFᵥ-ATPase (8).

Dreyfus et al. (16) demonstrated ATP binding by S. typhimurium FliI but failed to detect ATP hydrolysis. Since then, ATPase activity has been demonstrated for glutathione S-transferase fusions of the S. typhimurium Type III secretion component InvC (17) and FliI (18). In this study, we have purified a His-tagged version of FliI and characterized its ATPase activity in detail. We have also examined the effects of selected mutations on ATPase activity and on flagellation and motility.

EXPERIMENTAL PROCEDURES

Bacterial Strains—S. typhimurium SJW1103 is wild-type for motility and chemotaxis (19); MY644 is a temperature-sensitive (ts) fliI mutant (20); SJW2702 is a nonflagellate fliI null mutant (21). E. coli XL1-Blue (Stratagene, La Jolla, CA) was used as the recipient in cloning experiments, and BL21(DE3)pLysS (22) was used for overproduction of FliI.

Chemicals and Enzymes—Primers for PCR amplification and sequencing were synthesized using a model 392 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). Chemicals and enzymes were obtained from the following sources: Ampli-Taq DNA polymerase (Perkin-Elmer); Ni-NTA-agarose resin (Qiagen, Chatsworth, CA); ATP, ADP, GTP, ITP, CTP, phosphoenolpyruvate, malachite green, and ammonium molybdate (Sigma); NADH, pyruvate kinase, and lactate dehydrogenase (Boehringer Mannheim); bafilomycin A1, aurovertin B, and AMP-PNP (Calbiochem). All other chemicals were of at least reagent grade. Molar extinction coefficients (ε) for the common nucleotides were taken from Ref. 23. Other ε values used were: ITP, ε₁₉ₒ = 12,200; bafilomycin A1, ε₄₅₀ = 25,000 (in methanol); aurovertin B, ε₇₉₂ =

MES, 2-(N-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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³ The abbreviations used are: ABC, ATP-binding cassette; Ni-NTA, Ni-nitrilotriacetic acid; AMP-PNP, 5′-adenosyl imidodiphosphate; AMPSO, 3-(cyclohexylamino)-1-propanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DCCD, dicyclohexylcarbodiimide; IPTG, isopropyl-1-thio-β-D-galactopyranoside;
dark-field light microscopy (32). Overexpression and Purification of FliI—E. coli BL21(DE3)pLysS was transformed with pET-FLAG-19b-based plasmids containing fliI alleles. Cells were grown overnight at 30 °C in 10 ml of Luria medium containing 50 μg/ml ampicillin, placed in 1 liter of the same medium, and grown at 25 °C to an optical density at 600 nm of 0.5. IPTG was added to 0.2 mM, and growth was continued for 10 h. The cells were harvested (10,000 × g; 15 min) and the pellets resuspended in 50 mM Tris-Cl, pH 8.0, 500 mM NaCl, 5 mM EDTA, 1 mM β-mercaptoethanol. The cells were disrupted by passage three times through a French press (SLM Instruments, Urbana, IL) at 4000 p.s.i. The lysate was centrifuged (17,000 × g, 40 min) to pellet unlysed cells and inclusion bodies. The supernatant was centrifuged at 150,000 × g for 2.5 h and the pellet discarded. The soluble fraction was loaded on a column containing 5–10 ml of Ni-NTA-agarose pre-equilibrated with binding buffer at a flow rate of 10–15 ml/h. The column was washed with 50 ml of binding buffer and then with 50 ml of binding buffer plus 25 mM imidazole. FliI was eluted with a gradient of 25–250 mM imidazole in binding buffer. Fractions containing FliI were identified by SDS-PAGE, pooled, and concentrated to 5 ml (YM-30 membrane, Stirred Cell 8050, Amicon, Beverly, MA). After overnight dialysis (Spectra/Per 1 membrane) against 4 liters of 20 mM Tris-Cl pH 8.0, 250 mM EDTA, 1 mM β-mercaptoethanol (with 1 change), FliI was further concentrated to about 0.5 ml (Centriprep-30 and Centricon-30 concentrators, Amicon). Protein concentration was measured spectrophotometrically by intrinsic absorbance at 280 nm or by the Bradford dye binding assay using bovine serum albumin as a standard (Bio-Rad). All stages of the purification were performed at 4 °C.

**PLASMIDS USED IN THIS STUDY**

| Plasmid | Vector† | FliI product | Reference |
|---------|---------|--------------|-----------|
| pET-FLAG-19b | pET19b | Not applicable | This work |
| pIK2101 | pET11a | Wild-type | 16 |
| pIFF600 | pET22b | Wild-type | This work |
| pIFF200/pIFF700 | pET/Pbr | His-tagged wild-type | This work |
| pIFF201 | pET | His-tagged C52T | This work |
| pIFF203/pIFF703 | pET/Pbr | His-tagged L12P* | This work |
| pIFF204 | pET | His-tagged L12P | This work |
| pIFF205/pIFF705 | pET/Pbr | His-tagged K87/C12P | This work |
| pIFF206/pIFF706 | pET/Pbr | His-tagged K87/C12P | This work |
| pIFF207/pIFF707 | pET/Pbr | His-tagged K188E | This work |
| pIFF209/pIFF709 | pET/Pbr | His-tagged Y363S | This work |

† pET, pET-FLAG-19b (see “Experimental Procedures”); pTrc, pTrc99A.

The extent of flagellation and motility were examined by high intensity

## DNA Techniques and Plasmid Constructions—Recombinant DNA techniques were carried out by standard protocols (24). DNA sequencing by the dideoxynucleotide method (25) used Sequenase enzyme (U. S. Biochemical Corp.). Direct sequencing of PCR products was performed by a modified snap-cooling procedure (26).

Plasmids used are listed in Table 1. The expression vector pET19b (Novagen Inc., Madison, WI) has a T7lac promoter-operator region and an optimal ribosome binding sequence (27), followed by DNA encoding the fliI sequence and the 3′ end of fliI (29), and the mutant allele where the mutation L12P was introduced into the wild-type gene (see “Experimental Procedures”).

34,400; vanadate, [γ-33]P–3,630.

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**RESULTS**

Overproduction and Properties of the FliI Protein—pIFF200 (encoding His-tagged wild-type FliI) or pIFF600 (encoding untagged wild-type FliI) was used to transform SJW2702, a nonflagellated fliI null mutant. Both transformants swarmed equally well (Fig. 1) and were comparable to untransformed wild-type strain SJW1103, establishing that the His-tagged FliI protein was fully functional.

His-tagged FliI was overproduced under the control of the T7 promoter using pIFF200 and E. coli BL21(DE3)pLysS. Following induction by IPTG, the amount of His-tagged FliI increased greatly, comprising more than half of the total cellular protein (Fig. 2, cf. lanes 1 and 2). Most of it formed inclusion bodies, which could be removed by medium speed centrifugation (lane 3). Following high speed centrifugation of the supernatant to remove membrane fragments, about 5% remained in the soluble fraction (lane 4), which should then be pre-equilibrated to pre-equilibrity on a nickel column (lane 6) and concentrated, yielding up to 10 mg from 1 liter of culture. Immunoblotting using anti-FLAG antibody or anti-FliI antibody (16) confirmed the iden-
Fig. 1. Complementation analysis of wild-type and mutant fli alleles. SJW2702 (fli null) was transformed with various PET-based plasmids. pIFF600 (top row, left) contained an intact wild-type fli allele, whereas the other plasmids had fli alleles with an N-terminal His tag: wild-type (plasmid pIFF200, top row, right); C52T (plasmid pIFF201), L12P (plasmid pIFF203), and R7C/L12P (pIFF205). Cells were spotted on soft tryptone agar plates containing ampicillin (pIFF209), vector alone (pET-FLAG-19b) (top row, left to right); K188I (pIFF206), K188E (pIFF207), Y363S (pIFF209), vector alone (pET-FLAG-19b) (bottom row, left to right). Cells were spotted on soft tryptone agar plates containing ampicillin and incubated at 30 °C for 7 h.

Fig. 2. SDS-PAGE (12.5%) of samples during purification of His-tagged FliI. Lane 1, total protein of uninduced cells transformed with pIFF200. Lane 2, total cell protein following induction with IPTG. Lane 3, pellet of French press cell lysate, containing the FliI inclusion bodies. Lane 4, soluble fraction following high speed spin (load to Ni-NTA column). Lane 5, flow-through from Ni-NTA column. Lane 6, final pool of purified FliI eluted from Ni-NTA column. The arrow indicates the position of the His-tagged FliI. Molecular masses (in kilodaltons) of standards are shown on the left.

The FliI protein was essentially pure, as judged by Coomassie staining. To exclude the possibility that there might be small amounts of highly reactive ATPase contaminants, we assayed the activity of purified FliI in situ in native gels. The only major band identified by the ATPase assay co-migrated with the only major band identified by Coomassie staining (Fig. 4). Mutant versions of FliI involving a loss of positive charge (FliI R7C/L12P and FliI K188I) ran slightly faster than wild-type FliI, confirming the presence of the mutation.

ATP was the best substrate for the reaction (Fig. 5a). GTP and ITP produced much lower reaction rates, and CTP essentially did not serve as a substrate at all. The reaction followed Michaelis-Menten kinetics with a V_max at 25 °C of 0.2 μmol of ATP hydrolyzed/min/mg of protein (corresponding to a k_cat of 0.16 s⁻¹) and a K_m for ATP of about 0.3 mM (Fig. 5b). ATP hydrolysis required a divalent cation (Fig. 5c). When Mg²⁺ was varied from 0.06 to 10 mM, the data fit well to the Michaelis-Menten equation and yielded a K_m for Mg²⁺ of about 1.1 mM. Mn²⁺ could substitute, but reaction rates were reduced by about 70%. Zn²⁺, Co²⁺, and Ni²⁺ functioned almost as well as Mg²⁺ at lower concentrations but were inhibitory above 2.5 mM. No ATP hydrolysis was detected when Ca²⁺ was used. Maximal activity of FliI was observed around pH 8 (Fig. 5d), where it was about 2-fold higher than at the standard assay pH of 7. Activity decreased abruptly at acidic pH and above pH 9.5. At acidic pH, denaturation and aggregation occurred, but protonation or deprotonation of critical residues may also have affected activity.

ADP was a competitive inhibitor, with a K_i of 0.26 mM (Fig. 5e). We tested inhibitors of the P-, V-, and F-type ion-translocating ATPases, of the SecA component of the general secretory pathway, and of the ATPase component of the Type I secretory pathway (Table II); none of these compounds had an inhibitory effect on FliI.

Characterization of fli Mutant Strains and FliI Mutant Proteins—MY644 is a temperature-sensitive fli mutant (8, 20). At the permissive temperature of 30 °C, MY644 cells swarmed, although not as well as wild-type cells (Fig. 6a). In liquid medium, they were significantly less motile than wild-type cells; few cells had more than one flagellar filament long enough to be visible by high intensity dark-field microscopy (32), and even these failed to form a flagellar bundle. At the restrictive temperature of 42 °C, MY644 cells had almost no swarming ability (Fig. 6b), whereas wild-type cells swarmed even better than at 30 °C, presumably because of a combination of higher torque generation, faster growth, and softer agar. The fli allele in MY644 was found to cause the mutation L12P near the N terminus. When SJW2702 (fli null) was transformed with the PET-based plasmid pIFF203 bearing the mutant allele from MY644, swarming was similar to that of MY644 and showed the same temperature-sensitive phenotype (Fig. 6). We also made the mutation L12P in the wild-type fli allele; SJW2702 transformed with the resulting plasmid (pIFF204) behaved identically to cells transformed with pIFF203, establishing that the L12P mutation alone was responsible. Placing the L12P allele in the pTrc99A vector to give plasmid pIFF703 improved swarming at both temperatures (Fig. 6), indicating a dosage effect (see below). In liquid medium, cells transformed with pIFF703 and grown at the per-
missive temperature were seen to be well flagellated (5–8 flagella/cell) and highly motile. A double mutation at the N terminus, R7C/L12P, had more severe effect on motility than the L12P mutational one (Fig. 1). Flagellation, motility, and swarming were improved when the allele was expressed from the pTrc99A-based plasmid pIFF705. The temperature sensitivity associated with the L12P mutation persisted in the double mutant.

His-tagged versions of these mutant proteins were purified. FliIL12P was found to have an ATPase activity comparable to that of wild-type FliI, and FliIR7C/L12P had an activity higher than that of wild-type FliI (Table III). Thus, these residues at the N terminus of the protein are not needed for catalytic activity. This region of the protein is predicted to form an α helix. CD spectra of the mutant proteins showed small but reproducible decreases (of 5% and 8%, respectively) in the ratio of ellipticity at 222 nm versus 208 nm and a slight blue shift of the minimum at 208 nm, both of these changes indicating that the mutant proteins have reduced helical contents. However, they showed the same subdomain melting temperature (51°C) as the wild-type protein, indicating that this is not a property of the N terminus.

Site-directed mutations (K188I, K188E, D272N, and Y363S) had been made previously at positions that were predicted to be catalytically important based on homology with the β subunit of the F1-ATPase. These mutant alleles had shown very poor complementation of an E. coli fliI mutant (16). We created His-tagged versions of three of these alleles (K188I, K188E, and Y363S). The corresponding plasmids (pIFF206, 207, and 209) failed to complement SJW2702 (Fig. 1). When the alleles were overexpressed from pTrc99A-based plasmids (pIFF706, 707, and 709) the K188I and K188E mutations still gave no complementation, and in liquid medium less than 1% of the cells gave any indication of motility; even on these, there were no flagellar filaments long enough to be seen in the microscope. With the Y363S mutation, a small percentage of the cells were slightly motile, but again no filaments could be seen.

The activities of FliI K188I and Y363S were about 100-fold lower than that of wild-type FliI, and the K_m values for ATP were about 10-fold higher (Table III). The mutant proteins had essentially the same CD spectra as wild-type FliI, but the melting temperature was reduced by 5°C, indicating that these residues probably lay within the subdomain that was undergoing denaturation. FliI K188E gave at least a 100-fold lower yield, and we were unable to characterize its enzymatic activity.

Dosage and Dominance Effects—The wild-type and mutant fliI alleles in pTrc99A or pET-FLAG-19b were used to test for dosage or dominance effects over the wild-type chromosomal allele of SJW1103 (Fig. 7). Immunoblotting (using either anti-FliI or anti-FLAG antibody) established that, for growth in Luria medium, the amount of FliI with the pTrc99A-based plasmids was about 50,000 molecules/cell, whereas with the pET-based plasmids in host lacking the T7 polymerase, it was below the detection limit of approximately 500 molecules/cell.

Expression of His-tagged wild-type FliI from a pTrc99A-based plasmid neither enhanced nor inhibited swarming of wild-type SJW1103 cells, and neither FliI L12P nor R7C/L12P had any dominant effect over the wild-type protein (Fig. 7). In contrast, all three catalytic mutant alleles were dominant, the effect being more pronounced with FliIK188I and K188E than with FliI Y363S. In liquid medium, FliI K188I and K188E were seen to greatly reduce the motility of SJW1103 (about 10% of the cells were poorly motile, and their filaments were too short to be seen). Cells making FliI Y363S were fairly motile, and in the dark-field microscope, a few filaments, which sometimes formed a bundle, could be seen on some cells. None of the mutant alleles showed dominance when they were being expressed at low levels from the pET vector, indicating that the effect was dosage-dependent.

Analysis was also performed using MY644 (FliI L12P) as host. With both the pTrc99A-based and the pET-based plasmids, the wild-type fliI allele was dominant. Synthesis of FliI L12P from the pTrc99A-based plasmid pIFF703 (and to a lesser degree from the pET-based plasmid pIFF203) improved the swarming of MY644, consistent with the phenotype of SJW2702 transformed with these plasmids (Fig. 6c). Thus, higher levels of FliI L12P improved function. The same was true of the R7C/L12P double mutant allele, although the effect...
was not as pronounced. Among the catalytic mutant alleles, both K188I and K188E showed dominance with both vector systems, whereas the Y363S allele showed it only with the pTrc99A-based vector.

DISCUSSION

The flagellar morphogenetic pathway of *S. typhimurium* has been studied extensively (1, 37). The process by which many flagellar proteins are translocated from the cytosol to the distal end of the growing structure, however, is not understood. Several proteins are believed to be implicated (8). Among these is FliI, which is especially interesting because of its similarity to the catalytic β subunit of the F₁-ATPase and to homologous proteins in Type III bacterial secretion systems; FliI has been identified in a variety of diverse bacterial species in addition to *S. typhimurium* (8) and *E. coli*² including the Gram-positive bacterium *Bacillus subtilis* (38), the spirochete *Borrelia burgdorferi* (39), and the photosynthetic bacterium *Rhodobacter sphaeroides* (40, 41).

A previous study, using FliI purified from inclusion bodies, failed to detect any ATPase activity (16), perhaps because the

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² M. Kihara and R. M. Macnab, unpublished data.
TABLE II
Effect of ATPase inhibitors on FliI ATPase activity
ATPase activity was measured using the malachite green assay (see "Experimental Procedures"). Stock solutions of bafilomycin A1, aurovertin B, and DCCD were prepared in dimethylsulfoxide, methanol, and ethanol, respectively. The final solvent concentration in the sample assay mixture (and in controls without the compound) was less than 1%. FliI was preincubated with the compounds for 10 min before addition of ATP to initiate the reaction. The effect of DCCD was also measured at pH 6.5, following the procedure of Satre et al. (64). In control experiments, vanadate and DCCD were shown to inhibit the F-type plasma membrane ATPase of N. crassa by more than 80%.

| Compound       | FliI ATPase activity | Specific for* | Reference |
|----------------|----------------------|---------------|-----------|
| None           | 100                  | P, I          |          |
| Vanadate (0.4 mM) | 130                  | P, I          | 11, 12, 65 |
| Bafilomycin A1 (16 μM) | 105                  | V             | 66        |
| KNO3 (10 mM)  | 93                   | V             | 67        |
| NaN3 (10 mM)  | 98                   | F, A          | 68, 69    |
| Aurovertin B (60 μM) | 96                   | F             | 57        |
| AMP-PNP (1 mM) | 94                   | F, V          | 58, 67    |
| DCCD (10 μM to 50 mM) | 120–200              | P, V, F       | 54, 64    |

* P, phosphorylation type ATPase (formerly known as E1, E2 type); V, vacuolar type ATPase; F, F1-F0 type ATPase; A, SecA component of the general (Type II) secretory pathway of bacteria; I, ATPase of the Type I bacterial secretory pathway.

TABLE III
Kinetic constants of wild-type and mutant FliI
ATPase activity was measured using the malachite green assay as described under "Experimental Procedures." ATP concentrations were varied from 0.17 to 5 mM. Data were fit to the Michaelis-Menten equation to obtain $K_m$ and $V$, using the HYPER program (70). Standard errors were less than 10% except for those of K188I and Y363S FliI, which were 20–30%.

| FliI         | $K_m$ (mM) | $V$ (μmol/min/mg) |
|--------------|------------|-------------------|
| Wild-type    | 0.26       | 0.20              |
| L12P         | 0.25       | 0.1               |
| R7C/L12P     | 0.24       | 0.48              |
| K188I        | 1.0        | 0.002             |
| Y363S        | 4.0        | 0.004             |

Fig. 7. Swarm plates illustrating dosage and dominance effects associated with wild-type and mutant versions of FliI. SJW1103 (wild-type) was transformed with plasmids derived from the pTrc99A vector. The plasmids were (from left to right) as follows: Top row, pIFF700 (wild-type fliI), pTrc99A vector; middle row, pIFF703 (FliI L12P), pIFF705 (FliI R7C/L12P); bottom row, pIFF706 (FliI K188I), pIFF707 (FliI K188E), pIFF709 (FliI Y363S). Photograph was taken after 6 h at 30 °C.

Fig. 6. Temperature sensitivity and dosage effect associated with the L12P mutation in FliI. Cells were spotted on soft tryptone agar plates and incubated at 30°C (a) or 42°C (b) for 6 h. Top left, strain SJW1103 (wild-type). Top right, MY644 (FliI L12P). Bottom left, SJW2702 (fliI null) transformed with the pET-based plasmid pIFF203 (FliI L12P). Bottom right, SJW2702 transformed with the pTrc99A-based plasmid pIFF703 (FliI L12P).

The His-tagged FliI we have studied is fully functional in complementation tests (Fig. 1), giving confidence that its enzymatic and other properties reflect those of the untagged protein. It was active as the monomer and had a simple non-cooperative ATPase activity of 0.2 μmol/min/mg (0.16 s$^{-1}$) in an assay mixture containing 5 mM ATP and 5 mM Mg$^{2+}$ at pH 7 (Fig. 4), conditions that are close to those in vivo (pH around 7.6 and ATP around 3 mM; Refs. 42 and 43). This ATPase activity is comparable to those seen for ATPases of other protein export and secretory systems (11, 12, 44, 45), including InvC, the FliI homolog in the S. typhimurium virulence secretion system (17).

It is much lower than the multisite (steady-state) activity of F$_1$-ATPase ($\approx 50$ s$^{-1}$), but is 100-fold higher than the unisite activity ($\approx 10^{-3}$ s$^{-1}$) (46).

We have identified several residues that are important catalytically (Table III). Lys-188 of FliI is within the motif A (Gly-$X_X$-Gly-Lys), where $X$ represents any amino acid), which is found in many ATPases (47) and lies within the nucleotide binding site (36). The mutation K188I decreased ATPase activity by 100-fold, establishing that Lys-188 is an essential residue for catalysis, as is the equivalent residue in other ATPases (12, 48–50). Tyr-331 in the E. coli F$_1$ β subunit is a conserved amino acid residue among the known β subunits (51), and

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3 E. Silva-Herzog and G. Dreyfus, personal communication.
modification by 2-azido-ATP or other affinity labeling reagents results in inhibition of catalysis (46, 52). In mitochondrial β, the corresponding residue has been shown to be in the nucleotide binding site and to have a hydrophobic interaction with the adenine ring of ATP (36). The substitution Y331S (but not Y331F) in E. coli β increases the K_m value by 30-fold and decreases the activity by 10-fold, suggesting that this residue is important for ATP binding but is not directly involved in catalysis (53). We found similar effects with the substitution Y363S at the equivalent position in FliI, further indicating that FliI and the F1, β subunit are structurally related.

FliI has sequence similarity to the catalytic subunits of the F1, vacuolar, and archaeobacterial ATPases (8). To see whether this might be reflected at the mechanistic level, we tested various known inhibitors of ATPases (see Ref. 54 for a review) (Table II). Inhibitors of P( phosphorylation)-type ATPases had no effect on FliI activity, which is not surprising since these ATPases employ a very different reaction mechanism. Inhibitors of F-type or V-type ATPases also had no effect on FliI. Interestingly, although sodium azide strongly inhibits the multisite ATPase activity of F1, it does not affect its unitive activity (46, 55); FliI is active as monomer and so might be expected to more closely resemble the unitive activity. Aurovertin B inhibits F1-ATPase by binding to Arg-398 in the β subunit (56, 57); there is no corresponding Arg in FliI. AMP-PNP is a nonhydrolyzable ATP analog, which impairs the normal cooperativity of F1 (58); since no cooperativity was associated with FliI, AMP-PNP should at most act as a competitive inhibitor. We did not observe inhibition by AMP-PNP to at least 1 mM, indicating that its binding affinity must be quite low. DCCD also did not inhibit FliI. In fact, it caused a stimulation of activity, suggesting it may be inhibiting a feature of the protein that itself regulates ATPase activity.

The divalent cation specificity of FliI also showed significant differences from that of the F1-ATPase. At 5 mM ATP, Mg2+ was stimulatory up to at least 10 mM, whereas for the F1-ATPase, it is inhibitory above 2.5 mM; and Ca2+ could not support the activity of FliI, whereas it is almost as effective as Mg2+ for the F1-ATPase (46, 59).

We conclude from the inhibition studies and the metal ion dependence that, in spite of the similarities between FliI and F1, β at the sequence level, their reaction mechanisms are probably rather different. At least part of the difference may derive from the fact that FliI is reactive as monomer (although its state in vivo is not known) whereas β is part of a large, highly cooperative complex.

We imagine that, in the living cell, ATP hydrolysis by FliI is coupled to some other process, which entails physical interactions with one or more other flagellar proteins. We do not yet know what that process is. One possibility is the energization of the protein transport process; another is chaperone function. We favor the former possibility, since known chaperones with ATPase activity, such as DnaK, differ from FliI in several regards. They have extremely high affinity for ATP (typically in the submicromolar range, cf. 0.3 mM with FliI) (60, 61) and low reactivity (turnover numbers in the 10−9 s−1 range, cf. 0.16 s−1 for FliI). Also, they are considerably larger (~70 kDa, cf. 49 kDa for FliI).

The filament is by far the major substructure of the flagellum, consisting of about 20,000 flagellin subunits. There are typically about 5 flagella on a wild-type cell, and so about 100,000 flagellin subunits have to be assembled within a generation time (~75 min in Luria medium at 25 °C, the temperature at which ATPase activity was assayed). This corresponds to an assembly rate of about 22 s−1. If FliI is implicated in the process, and there are about 1000 FliI molecules/cell (8), this would require a k_cat of at least 0.02 s−1, if one ATP has to be hydrolyzed per flagellin subunit exported and assembled. The observed k_cat of 0.16 s−1 for purified FliI indicates that it is capable of performing the task.

An important next step will be to identify the components that interact with FliI and the part of FliI that is responsible for the interaction. In the latter regard, the differences between the properties of the catalytic mutations and the N-terminal mutations R7C and L12P are highly suggestive. The catalytic mutations cause not only the expected severe decrease in enzymatic activity but also dominance over wild-type FliI. This is what would be expected if the mutant proteins were still competent to interact with other flagellar components, because they would sequester these components but would be unable to perform the ATPase-linked function. Conversely, if the N-terminal mutations impair the interaction, they would be recessive and wild-type FliI could function unimpeded with the other flagellar components. Thus there is a strong prediction that at least some part of the N-terminal region of FliI is responsible for the interaction, a prediction that is reinforced by the fact that the corresponding sequence is lacking in F1, β. FliI has a substantial N-terminal overhang with respect to β, and credible similarity between the two sequences does not commence until around residue 110 of FliI (8). Mutations such as R7C or L12P at the N terminus might perturb the interaction between FliI and other flagellar proteins. It is interesting that the R7C/L12P double mutation reproducibly stimulated ATPase activity, suggesting that the N terminus may exert a regulatory effect on the catalytic mechanism (cf. the stimulatory effect of DCCD).

The fact that the L12P mutation has an adverse effect on filament assembly immediately upon shift to the restrictive temperature (rather than after times comparable to the generation time) suggests that the interaction may be a transient one and that FliI is probably not tightly and permanently locked into the flagellar structure (cf. discussion in Refs. 8 and 16). The dosage dependence effect of the various mutant FliI proteins further supports the idea that the interaction may be a transient one in which the free concentration is important.

We suggest that FliI functions as a two-domain protein, with an N-terminal domain engaged in some flagellum-specific process and a C-terminal domain, which is responsible for the ATPase activity that drives the process. Deletion analysis should allow definition of the boundary between these two predicted domains.

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