Role of the N-terminal domain of FliI ATPase in bacterial flagellar protein export

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Abstract

FliI, the ATPase involved in bacterial flagellar protein export, forms a complex with its regulator FliH in the cytoplasm and hexamerizes upon docking to the export gate composed of integral membrane proteins. The extreme N-terminal region of FliI is involved not only in its interaction with FliH but also in its oligomerization, but the regulatory mechanism of oligomerization remains unclear. Using in-frame 10-residue deletions within the 100 residues of the N-terminal domain, we demonstrate that the first 20 residues are required for FliH binding and that the conformation of the N-terminal domain is sensitive to the export function, even though the oligomerization and FliH-binding ability are retained and the ATPase activity is maintained in most of the deletion variants.

1. Introduction

For construction of the bacterial flagellum responsible for motility, most of flagellar components are exported by the flagellum-specific protein export apparatus, which consists of six integral membrane proteins (FliA, FliB, FliO, FliP, FliQ, FliR) and three soluble proteins (FliH, FliI, FliJ)\textsuperscript{[1,2]}. The integral membrane components, which are postulated to be located in the putative central pore of the basal body MS ring, acts as an export gate of the export apparatus\textsuperscript{[2]}. FliI is the ATPase\textsuperscript{[3]}. FliI forms a complex with FliH that acts as a negative regulator to prevent FliI from wasting ATP in the cytoplasm\textsuperscript{[4]}. The FliH-FliI complex delivers export substrates to the export gate and helps initial entry of the substrates into the cytoplasm\textsuperscript{[4]}. The FliH-FliI complex delivers export substrates that acts as a negative regulator to prevent FliI from wasting ATP in the cytoplasm and hexamerizes upon docking to the export gate composed of integral membrane proteins. The extreme N-terminal region of FliI is involved not only in its interaction with FliH but also in its oligomerization, but the regulatory mechanism of oligomerization remains unclear. Using in-frame 10-residue deletions within the 100 residues of the N-terminal domain, we demonstrate that the first 20 residues are required for FliH binding and that the conformation of the N-terminal domain is sensitive to the export function, even though the oligomerization and FliH-binding ability are retained and the ATPase activity is maintained in most of the deletion variants.

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2. Materials and methods

2.1. Bacterial strains, plasmid and media

Bacterial strains and plasmids used in this study are listed in Table 1. Lauria broth (LB) and soft tryptone agar plates were prepared as described previously [15].

2.2. Swarming motility assays

Fresh colonies were inoculated on soft tryptone agar plates and incubated at 30 °C to evaluate the swarming size.

2.3. Purification of FliI and measurements of the ATPase activity

His-FliI and its 10-residue deletion variants were purified by nickel affinity chromatography as described before [15] with a minor modification. Fractions containing His-FliI or its deletion variants were pooled and dialyzed overnight against 20 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 1 mM DTT. The malachite green ATPase activity assay [16] was performed as described [4]. The ATPase activity reported here are the averages of at least two independent assays.

2.4. Pull-down assays

Soluble fractions from the SJW1368 (ΔcheW-flhD) cells carrying the pTrc99A-based plasmids co-overproducing both FliH and His-FliI or its deletion variants were loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) agarose column. After washing the column with binding buffer (20 mM Tris–HCl, 500 mM NaCl), proteins were eluted with binding buffer containing imidazole by a stepwise increase in the imidazole concentration of 60, 100, 200, 300 and 600 mM.

Fig. 1. The primary structure of FliI with three functional domains and scanning deletion variants mapped in the 3D structure. (A) Three functional domains of FliI and in-frame deletion variants of FliI. wt is wild-type, and Δ1, Δ2, etc. are referred to as FliIΔ1, FliIΔ2, etc. in the text. All the deletion variants contain a 10-amino-acid deletion of residues 1–10, 11–20, and so on, respectively. Various properties associated with these variants are indicated as: Compl, complementation; Domi, Dominance; ΔfliHI inhib, inhibitory effect on the motility of the fliH-fliI double null mutant; FliH p.d., pull-down of untagged FliH with His-tagged FliI by Ni-NTA chromatography; Solu, solubility. Open squares represent positive results, closed square negative results, and gray squares weakly positive results. ND, not done. The export inhibition of the ΔfliHI-fliI strain by Fli deletion variants implies their binding to the export gate. (B) Cα backbone trace of the N-terminal domains in the hexamer model of FliI based on the structure of FliI (PDB ID code 2DPY) [9]. Two neighboring protomers are colored to indicate the region of scanning deletion: Δ3, blue; Δ4, cyan; Δ5, blue–green; Δ6, green; Δ7, lime; Δ8, yellow; Δ9, orange; Δ10, red. Arrowheads point to residues 35–38, which is located at the inter-subunit interface.
2.5. Structural comparison between FliI and F1 ATPase

The α and β subunits of F1 ATPase from bovine mitochondria (PDB ID code: 1BMF) [10] and thermophilic Bacillus PS3 (PDB ID code: 1SKY) [11] were superimposed onto FliI (PDB ID code: 2DPY) [9] as described before [9].

3. Results

3.1. Complementation and negative dominance of FliI deletions

To investigate the role of the N-terminal domain of FliI in its oligomerization control, we used a series of in-frame deletions of FliI (FliIΔ1 through FliIΔ10), each having a 10-amino-acid deletion (Fig. 1A). These deletions did not affect protein stability (data not shown). Then, we transformed a flil null mutant with the plasmids encoding these FliI deletion variants, and analyzed the motility of the resulting transformants (Fig. 2). FliIΔ4 through FliIΔ10 recovered the motility of the flil mutant to some degree while the rest did not even after prolonged incubation (Fig. 2B), indicating that the first 30 residues are essential for protein export. Interestingly, the N-terminal 26 residues have a flexible conformation in solution although it is predicted to form α-helix [14,17].

To analyze their negative dominance effect on the motility of wild-type cells, we transformed a Salmonella wild-type strain, SJW1103, with the plasmids encoding these FliI deletion variants and analyzed the motility of the resulting transformants on soft agar plates (Fig. 2C). FliIΔ2 through FliIΔ4 inhibited wild-type motility (Fig. 2C), indicating that they retain the ability to interact with proteins involved in the flagellar protein export process. In contrast, the others did not exert any dominant negative effects (Fig. 2C), suggesting that they cannot be incorporated into the export apparatus.

3.2. FliH binding ability of deletion variants of FliI

To clarify the FliH binding site in FliI, we carried out pull-down assays by Ni-NTA affinity chromatography (Fig. 3). Unlike wild-type FliI (Fig. 3A), His-FliIΔ1 and His-FliIΔ2 did not interact with FliH (Fig. 3B and C). In contrast, the other deletion variants bound to FliH at wild-type levels (Fig. 3D for His-FliIΔ3; data not shown for the rest). Therefore, we conclude that the N-terminal 20 residues of FliI are necessary for its interaction with FliH. In agreement with this, it has been shown that residues 1–18 of Helicobacter pylori FliI are essential for the interaction with FliH [14].

The flil null mutant is non-motile (vector control in Fig. 2A and B), while a flil null mutant and a flil-flil double null mutant are weakly motile (vector control in Fig. 2D), suggesting that FliH inhibits flagellar protein export in the absence of FliI presumably due to a non-functional docking of FliH onto the export gate [6]. Albeit rather weak, the significant motility recovery of the flil null mutants by FliIΔ4 through FliIΔ10 (Fig. 2B) may be caused by relieving the FliH-inhibitory effect by these FliI deletion variants sequestering FliH in the cytoplasm and facilitating the release of FliH from the export gate. Although this hypothesis cannot explain why FliIΔ3 has no recovery effect because FliIΔ3 binds FliH as well as FliIΔ4 through FliIΔ10. We then examined the effect of the FliI deletion variants on the motility of the flil-flil double null mutant. The swarming motility assay in Fig. 2D shows that many of the FliI deletion variants on the motility of the fliI-flil double null mutant can still dock to the export gate in some improper ways to interfere with the gating/export process by the gate complex alone.

![Fig. 2](image-url)

**Table 1**

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<td>[13]</td>
</tr>
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<td>pTRC99A/His-FLAG-FliI</td>
<td>[5]</td>
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<td>pTF700H</td>
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<tr>
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Fig. 2. Swarming motility assay for characterization of in-frame 10-residue deletions of FliI. (A), (B) Complementation of the motility of MKM30 (Δflil) transformed with pTrc99A-based plasmids encoding various deletion variants of FliI. vector, pTrc99A; wt, wild-type FliI; Δ1 through Δ10, FliIΔ1 through FliIΔ10, respectively. (C) Dominant negative effect on the motility of SJW1103 (wild-type) transformed with the same plasmids. (D) Motility of a flil-flil null mutant transformed with the same plasmids. Plates were incubated at 30°C for 6 h (A and C) and 24 h (B and D).
activity, suggesting that the extreme N-terminal region of FliI is responsible for regulation of the catalytic mechanism through its oligomerization control [5,13]. Therefore, we measured the ATPase activity of purified His-FLAG-tagged FliI deletion variants at a constant protein concentration of 0.2 mg/ml (Table 2). The ATPase activity of FliIΔ1 was approximately a half of the wild-type level (Table 2), and this variant did not form the hexamer ring even in the presence of phospholipids and an ATP analog, Mg-ADP-AlF4 (Supplementary Fig. 1). This is in agreement with the previous data shown by Minamino et al. [13]. In contrast, the ATPase activity of FliIΔ2 was significantly higher than that of wild-type FliI, and the ring structure was observed by electron microscopy (Supplementary Fig. 1). These results suggest that residues 2–10 of FliI are responsible for the hexamer ring formation.

Most of the other deletion variants showed reduced ATPase activities probably due to structural changes in the N-terminal domain. Interestingly, the ATPase activity of FliIΔ4 was about six-fold higher than that of wild-type FliI (Table 2). Although FliI bound to FliIΔ4 and suppressed its ATPase activity, the enzymatic activity of FliIΔ4 in the FliH-FliIΔ4 complex was still higher than that of the FliH-FliI complex (data not shown). These results suggest that residues 31–40 of FliI suppress the ATPase activity whether FliH is bound or not. Residues 31–40 form a hairpin loop structure with residues 32–35 forming a β-sheet and residue 35–38 forming a loop that appears to interfere with the hexamerization of the N-terminal domains. We therefore made another deletion variant of FliI missing residues 35–38, and carried out the same characterization as for other FliI deletion variants. FliIΔ3 (35–38) showed a moderate level of complementation and negative dominance effect (data not shown), and its ATPase activity was about two-fold higher than that of wild-type FliI (Table 2).

4. Discussion

In spite of the striking structural similarity with the α/β subunits of F1-ATPase, FliI tends to be monomeric in solution even in the presence of an ATP analog [13]. This is consistent with the fact that the ATPase activity of FliI is much lower than that of F1-ATPase, because ATP hydrolysis requires the conserved arginine residue in the neighboring subunit and therefore oligomerization is essential for the catalytic activity. The αββα hetero-hexamer of F1-ATPase is stabilized by the extensive inter-subunit interaction between the N-terminal domains. Detailed comparison of the N-terminal domain of FliI with those of the α/β subunits of F1-ATPase indicates that the main-chain conformation involved in the inter-subunit interaction is rather different although the core barrel structure can be superimposed relatively well onto each other (Fig. 4). The FliI hexamer model, which is constructed by fitting the N-terminal domain of FliI onto those of the α/β subunits of the F1-ATPase, shows steric-hindrances in the subunit interface, suggesting that a conformational change is required to form the hexamer formation. The N-terminal 26 residues of monomeric FliI in solution are not compactly folded and therefore accessible to the protease [17]. Interestingly, deletion of the first seven residues causes a conformational change in the remaining 19 residues of the unstable N-terminal region FliI, suppressing both its oligomerization and ATPase activity [13]. Deletion of 9 residues from 2 to 10 reduced the ATPase activity compared to the wild-type level. Deletion of 9 residues from 2 to 10 resulted in the wild-type level (Δ1 in Table 2) and abolished the FliI ring formation (Supplementary Fig. 1), suggesting that residues 2–10 are required for FliI oligomerization to fully exert the enzyme activity. In contrast, deletion of 10 residues from 11 to 20 resulted in an elevated ATPase activity (Δ2 in Table 2) and did not affect the ability of FliI to form the ring structure (Supplementary Fig. 1), suggesting that residues 11–20 may inter-

Fig. 3. Retardation of untagged FliI by N-His-FLAG-tagged deletion variants of FliI in the Ni-NTA pull-down assay. The crude soluble fractions from SJW1368 (ΔcheW-flhD) cells overproducing both proteins were applied to a Ni-NTA column and eluted at the imidazole concentrations indicated. (A) wild-type FliI (B) FliIΔ1, (C) FliIΔ2, and (D) FliIΔ3. Each fraction was analyzed by SDS–PAGE and stained with Coomassie brilliant blue. Positions of the N–His–FLAG-tagged FliI forms and of untagged FliH are indicated on the right.

Table 2

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<td>A(35–38)</td>
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* The ATPase activity was measured using the malachite green assay [16]. Relative ATPase levels are normalized to the ATPase activity of wild-type FliI.
correlation was found between their recovery level and the ATPase activity (Table 2). The recovered motility was more or less comparable to that of the fliH-fliD double null mutant (Fig. 2B and D), suggesting that the recovery is at least partly due to the relief of the FliH-inhibitory effect by deletion variants sequestering FliH, which is independent of ATP hydrolysis. However, many of the deletion variants further suppressed the slow export process by the export gate alone probably by docking to the gate, and some of the deletion variants appear to retain, albeit at an extremely low level, the proper function of Fli by docking to the gate with FliH. In any case, the levels of such recovery are extremely low (Fig. 2A) and show no correlation with the levels of ATPase activity of the deletion variants in comparison to the wild-type level, indicating that the interactions of Fli or the FliH-Fli complex with the export gate must be significantly reduced by deformed conformations of the N-terminal domain by the 10-residue deletions (Fig. 2A). As discussed above, the N-terminal 26 residues of monomeric Fli in solution are not compactly folded [17] and deletion of the first seven residues make the remaining 19 residues of the unstable N-terminal region more stably folded [13]. The deletion interferes not only with FliH binding but also with Fli oligomerization [13]. Interestingly, deletion of the first seven residues also significantly abolishes the bypass effect of Fli on the FliH defect, suggesting that the deletion reduces the ability of Fli to dock to the platform of the export gate made of six integral membrane proteins [5,13]. These results in turn strongly indicate that a correct conformation of the N-terminal domain is essential for proper interactions of Fli with other components of the export apparatus, such as the gate complex and FliH, to exert an efficient export function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.01.026.

References


Fig. 4. Structural comparison of the N-terminal domains of the αβ subunits of FliI ATPase and FliI. Arrows indicate the regions that interact with the neighboring subunits in the hexamer. (A) The α subunits from bovine mitochondria (cyan) (PDB ID code: 1BMF) [10] and the thermophilic Bacillus PS3 (green) (PDB ID code: 1SKY) [11] are superimposed onto FliI (blue) (PDB ID code: 2DPY) [9]. (B) The β subunits from bovine mitochondria (orange) and the thermophilic Bacillus PS3 (red) are superimposed onto FliI (blue).
similarity of FliI to F0F1, vacuolar, and archaeabacterial ATPase subunits. J. Bacteriol. 173, 3564–3572.


