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The NMR Structure of FliK, the Trigger for the Switch of Substrate Specificity in the Flagellar Type III Secretion Apparatus

Shino Mizuno[1], Hirokazu Amida[2], Naohiro Kobayashi[3], Shin-Ichi Aizawa[1]* and Shin-ichi Tate[2]*

[1]*Department of Life Sciences, Prefectural University of Hiroshima, 526 Nanatsuka, Shobara, Hiroshima 727-0023, Japan*
[2]*Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8526, Japan*
[3]*Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

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The flagellar cytoplasmic protein FliK controls hook elongation by two successive events: by determining hook length and by stopping the supply of hook protein. These two distinct roles are assigned to different parts of FliK: the N-terminal half ($FliK_N$) determines length and the C-terminal half ($FliK_C$) switches secretion from the hook protein to the filament protein. The interaction of $FliK_C$ with FlhB, the switchable secretion gate, triggers the switch. By NMR spectroscopy, we demonstrated that FliK is largely unstructured and determined the structure of a compact domain in $FliK_C$. The compact domain, denoted the $FliK_C$ core domain, consists of two α-helices, a β-sheet with two parallel and two antiparallel strands, and several exposed loops. Based on the functional data obtained by a series of deletion mutants of the $FliK_C$ core domain, we constructed a model of the complex between the $FliK_C$ core domain and $FlhB_C$. The model suggested that one of the $FliK_C$ loops has a high probability of interacting with the C-terminal domain of FlhB ($FlhB_C$) as the FliK molecule enters the secretion gate. We suggest that the autocleaved NPTH sequence in FlhB contacts loop 2 of $FliK_C$ to trigger the switching event. This contact is sterically prevented when NPTH is not cleaved. Thus, the structure of FliK provides insight into the mechanism by which this bifunctional protein triggers a switch in the export of substrates.

Introduction

Many bacterial species can swim in liquid, using one flagellum or many flagella.[1] The flagellum self-assembles from a large number of different proteins; it is structurally divided into three substructures: the filament, the hook, and the basal body.[2,3] The assembly of the flagellum proceeds from the cell-proximal to the cell-distal substructures: the basal body formation is followed by the biogenesis of the hook and finally by growth of the filament.[4] The filament and the hook are external to the cytoplasmic

*Corresponding authors. E-mail addresses: aizawa@pu-hiroshima.ac.jp; tate@hiroshima-u.ac.jp.

Abbreviations used: NOE, nuclear Overhauser enhancement; HSQC, heteronuclear single quantum coherence; RDC, residual dipolar coupling; GST, glutathione *S*-transferase; hetNOE, heteronuclear nuclear Overhauser enhancement; EDTA, ethylenediaminetetraacetic acid; PDB, Protein Data Bank.

membrane; their components, the hook protein (FlgE) and the filament protein (FliC), are exported by a flagellar-specific secretion apparatus that belongs to the type III secretion system (T3SS).[1,4] In the Gram-negative bacterium *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), the filament has a variable length that depends upon the growth phase or the age of the filament. In contrast, the hook, a short tubular structure connecting the basal body and the filament, has a controlled length; the average length is 55 nm with a standard deviation of 6 nm.[5] Although its length is controlled, this rather large deviation, 10% of the average, suggests a loose mechanism of length control.

Two flagellar-specific proteins are known to be directly involved in hook length control: FliK and FlhB.[5,6] FlhB is an integral membrane protein that switches the substrate specificity of the T3SS.[7,8] FlhB has putative transmembrane domains in its N-terminal part and a hydrophilic globular domain in its C-terminal part,[9,10] which is cytoplasmic. The soluble C-terminal domain of FlhB ($FlhB_C$), undergoes autocleavage at the NPTH loop, where the N–P bond is cleaved; $FlhB_C$ is thereby divided into two subdomains: $FlhB_{CN}$ (residues 211–269) and $FlhB_{CC}$ (residues 270–383).[11,12] A site-directed mutant having APTH instead of NPTH prevents cleavage of the loop.[12,13] Mutants that slow down autocleavage of this loop cause delayed switching.[13] Autocleavage of the loop was suggested to cause a structural change in $FlhB_C$ and thereby to switch its substrate specificity.[12] However, recent X-ray studies that compared the structures of the non-cleaved and cleaved forms of the C-terminal domains of YscU and EscU (FlhB homologs of *Yersinia* and *Escherichia coli* injectisomes, respectively) have demonstrated that the cleavage of the NPTH loop does not alter the structure; the only structural changes are limited to the loop.[14–16] Thus, the substrate specificity switch does not seem to be mediated by the changes in the overall fold of $FlhB_C$.

FliK, a soluble cytoplasmic protein, is secreted during flagellar formation.[17] In *fliK* deletion mutants, flagellar filament formation is not initiated. Instead, the hook continues to grow, giving rise to the "polyhook" phenotype.[6,18] FliK appears to have two domains, the N-terminal domain ($FliK_N$) and the C-terminal domain ($FliK_C$), which are easily separated by limited proteolysis.[8,17,19,20] $FliK_N$ is less stable and is more easily degraded by prolonged proteolysis than $FliK_C$, which remains intact after prolonged proteolysis, suggesting that $FliK_C$ has a stable fold.[11] Conservation of the hydrophobic residues suggests that $FliK_C$ should have a structure in common with its homologs and orthologs in the T3S *s*pecificity *s*witching *s*ystem (T3S4) domain.[21] Mutant FliKs that have deletions in the T3S4 domain lose their ability to switch substrate specificity, suggesting that the T3S4 domain plays an essential role in the switch.[9,19] In summary, FliK is structurally and functionally divided into two parts: $FliK_N$ (residues 1–203) and $FliK_C$ (residues 204–405).[6,9] $FliK_N$ is directly involved in length control, while $FliK_C$ controls the hook length by switching the substrate specificity of the secretion apparatus. It should be noted that these two functions are not independent of one another. If $FliK_C$ is deleted, the hooks keep elongating into polyhooks even in the presence of intact $FliK_N$. In contrast, as long as $FliK_C$ is intact and even if $FliK_N$ is deleted, the hooks elongate to produce polyhooks with filaments attached, the so-called polyhook-filament phenotype.[5] Although there are several hypotheses for the role of FliK in determining hook length, none of them can fully explain all of the observatons.[19,22–24]

Biochemical experiments on FliK gave limited insight into the FliK structure, leading to inadequate hypotheses about its mechanism of action. We were motivated therefore to determine the FliK structure using NMR spectroscopy. In this study, we analyzed both the N- and C-terminal domains and compared their structural features with those of the full-length FliK. We confirmed earlier observations that $FliK_N$ is unstable and that $FliK_C$ has both an unstructured and a structured parts and a folded domain. We then solved the solution structure of the folded domain (core) of $FliK_C$. Based on results from a series of experiments using *fliK* mutants, we modeled the structure of a complex between the $FliK_C$ core domain and $FlhB_C$. We modeled the $FlhB_C$ structure based on the sequence homology with its homolog EscU.[16] In our model, the cleaved loop in $FlhB_C$ exposes the site of interaction with the $FliK_C$ core domain; this site of interaction is sterically blocked in the non-cleaved form of $FlhB_C$. Overall, the FliK structure obtained from the present NMR analyses sheds new light on the regulatory roles of FliK as a bifunctional player in the flagellar formation.

Results

Our strategy

In the alignment of amino acid sequences of FliK orthologs from various species, there are no conserved sequences in the N-terminal region ($FliK_N$), whereas conserved sequences are found in the C-terminal region ($FliK_C$) (Fig. 1a). Not surprisingly, genetic, biochemical, and molecular biological assays showed that $FliK_N$ is structurally disordered while $FliK_C$ has a compact globular domain.[6,9,24] $FliK_N$ contains an unusual sequence of Gly and Pro, which are structure-breaking residues (Fig. 1a). We employed PONDR† to predict disordered regions, that is, those that have a score greater than 0.5 (Fig. 1b).[26,27] Of the FliK structure, 61% is predicted to

† http://www.pondr.com/2daypage.html