Interaction of the disordered terminal regions of flagellin upon flagellar filament formation

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Abstract Helical filaments of bacterial flagella are built up by a self-assembly process from thousands of flagellin subunits. To clarify how the disordered terminal regions of flagellin interact upon filament formation, polymerization ability of various terminally truncated fragments was investigated. Fragments deprived of 19 N-terminal residues were able to bind to the end of filaments, however, only a single layer was formed. Removal of C-terminal segments or truncation at both ends resulted in the complete loss of binding ability. Our observations are consistent with the coiled-coil model of filament formation, which suggests that the α-helical N- and C-terminal regions of axially adjacent subunits form an interlocking pattern of helical bundles upon polymerization.

Key words: Bacterial flagellar filament; Flagellin; Disordered regions; Coiled-coil formation

1. Introduction

Bacteria swim by means of flagella. Helical filaments of bacterial flagella are constructed from subunits of a single protein, flagellin, by a self-assembly process [1]. Terminal regions of flagellin, about 65 N-terminal and 45 C-terminal residues, have no ordered tertiary structure in the monomeric form [2,3]. The disordered terminal regions become ordered into a highly α-helical conformation upon polymerization [4]. They belong to the most conserved parts of the molecule and are thought to be essential in assembly regulation [5,6]. There is a growing interest in understanding the role of unstructured protein segments in controlling macromolecular recognition and self-assembly [7,8].

The flagellar filament is composed of 11 strands of protofilaments, which are nearly longitudinal arrays of subunits. The central core of filament consists of a concentric double tubular structure, as revealed by X-ray fiber diffraction and electron microscopy (EM) studies at around 9 Å resolution [9–11]. The structures of flagellar filaments reconstructed from various terminally truncated flagellins under high precipitant concentrations have revealed that the disordered terminal regions are involved in the inner tube of the concentric tubular structure, and their direct interaction is responsible for the proper folding the filament core [12]. Recently, the crystal structure of the flagellin F41 fragment at 2.0 Å resolution has been determined [13], which directly revealed the protofilament structure except the truncated terminal regions, which contribute to the very core part of filament.

The N- and C-terminal regions of flagellin as well as other axial components of bacterial flagella, possess heptad repeats of hydrophobic amino acid residues [14], a characteristic of sequences that fold into α-helical coiled coils. It was suggested that the terminal regions of neighboring subunits interact to form a coiled-coil structure (Fig. 1). This interlocking organization has been postulated to be the common motif by which the axial proteins form a continuous structure [14].

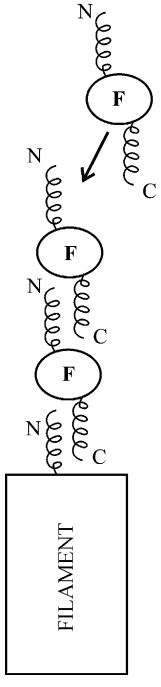

Fig. 1. Schematic diagram of the coiled-coil model of filament formation. The amphipatic helix-forming terminal regions of axially adjacent subunits are postulated to form an interlocking pattern of coiled-coil bundles upon polymerization.

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Abbreviations: ELC, endoproteinase Lys-C; V8, endoproteinase Glu-C from *Staphylococcus aureus* V8; FRET, fluorescence resonance energy transfer; CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; AF350, Alexa Fluor 350; AF488, Alexa Fluor 488

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This coiled-coil model of filament formation lacks direct experimental support. While X-ray fiber diffraction and EM studies located rod shaped densities corresponding to helical bundles aligned nearly parallel to the filament axis in the core region of the filament [9–11], it was not possible to trace the polypeptide chain and identify subunit boundaries. It is still an open question how the disordered terminal regions of neighboring subunits interact to form the inner core of flagellar filaments. A major motivation for this work was to obtain supporting evidence for the coiled-coil model of filament formation.

2. Materials and methods

2.1. Production of Cys-containing mutant flagellin

A cysteine residue was introduced into the central variable part of flagellin, which is expected not to be essential for self-assembly. At sequence position 356 of the wild-type flagellin of *Salmonella* SJW1103, a Ser residue was exchanged for Cys by site-directed mutagenesis. Mutagenesis was carried out on an M13mp18 single strand DNA containing the SJW1103 *fliC* gene in a similar way as described in [15]. The DNA fragment carrying mutated *fliC* was excised from the double-stranded M13mp18 RF DNA by *Kpn*I–*Bam*HI and inserted into a pKOT1 vector. A *fliC*$^-$ strain of *Salmonella typhimurium*, SJW2536, was transformed by this constructed plasmid. Wild-type and Cys-containing flagellins were isolated and purified as described in [2] with slight modifications. Treatment with 5 mM dithiothreitol was applied before the ion-exchange chromatographic purification of mutant flagellin to reduce intermolecular disulfide bonds.

2.2. Filament reconstruction and fragment preparation

Flagellar filaments were reconstituted from purified flagellin solutions by adding ammonium sulfate to a final concentration of 1.0 M. The filaments were washed two times by centrifugation and suspending them at a final concentration of 5 mg/ml in 10 mM phosphate buffer (pH 7.0) containing 150 mM NaCl. The suspension was sonicated for a few minutes to break the flagella into short pieces. This solution was used as a seed preparation in polymerization experiments.

Fragments of flagellin missing terminal segments of various sizes within the disordered terminal regions were prepared by limited proteolysis of monomeric flagellin and purified by ion-exchange column chromatography [5]. Endoproteinase Lys-C (ELC) and endoproteinase Glu-C (V8) from *Staphylococcus aureus* V8 were obtained from Boehringer (Roche), trypsin was purchased from Sigma. The F(20–494) fragment was obtained by proteolysis with ELC, F(1–461) and F(30–461) were produced by V8, while the F(67–450) fragment was prepared by tryptic digestion.

2.3. Fluorescence measurements

Various terminally truncated fragments of flagellin were labeled with fluorescent dyes, and their binding to the end of flagellar filaments was studied and compared by fluorescence resonance energy transfer (FRET) measurements. The thiol-reactive fluorescent dyes, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) or Alexa Fluor 350 C5 maleimide (AF350) were used as donor molecules, and Alexa Fluor 488 C5 maleimide (AF488) as an acceptor molecule in our FRET measurements [17]. Short flagellar filaments were prepared from purified (unlabeled) flagellin solution. Then, a thin layer of donor-labeled flagellin was polymerized onto the top of the short filaments at a monomer to filament ratio of 1:50 (w/w). Upon subsequent addition of AF488-conjugated subunits at a protein concentration of 0.1 mg/ml, binding was detected by FRET through the appearance of sensitized fluorescence of the acceptor at 512 nm. Fluorescent dyes were obtained from Molecular Probes.

Thiol-reactive fluorescent probes were covalently conjugated to Cys-containing mutant flagellin and its fragments using standard procedures [17]. Prior to the labeling reaction, intermolecular disulfide bonds were reduced by Tris-(2-carboxyethyl)phosphine. The unreacted labeling reagent was separated from the flagellin-dye conjugate by gel filtration on a Sephadex G-25 column. The degree of labeling was calculated using the following formula:

$$\frac{\text{moles of dye}}{\text{moles of protein}} = \frac{A_x}{\varepsilon} \times \frac{\text{MW of protein}}{\text{mg protein/ml}}$$

where A_x is the absorbance value and ε is the molar extinction coefficient of the dye at its absorption maximum wavelength. Extinction coefficients of 33 000, 19 000, and 65 000 (cm^{-1} M^{-1}) were used for CPM, AF350 and AF488 at their absorbance maximum of 384 nm, 346 nm and 495 nm, respectively [17]. The concentration of flagellin and its fragments was determined from absorption measurements at 280 nm [5] making correction for the absorbance of the dye at 280 nm [17]. The degree of labeling was usually in the range of 0.4–0.7 dye/protein molecule.

Polymerization ability of dye-conjugated flagellin molecules was checked by inducing filament formation with ammonium sulfate. Filaments were observed by fluorescence microscopy using an Olympus BX50 microscope with fluorescence attachment. FRET measurements between CPM- or AF350-labeled donor and AF488-labeled acceptor molecules were done on a Fluoromax-2 (Jobin-Yvon) computer controlled fluorescence spectrophotometer. All measurements were carried out in 10 mM phosphate buffer, pH 7.0, containing 150 mM NaCl.

3. Results

3.1. Preparation and labeling of terminally truncated fragments of flagellin

In order to get insight into the molecular mechanism of flagellar (proto)filament formation and explore how the disordered terminal regions construct the inner core of filaments, various terminally truncated fragments of flagellin were prepared and their polymerization ability was investigated. FRET was used to monitor binding of truncated flagellin fragments to the end of flagellar filaments. FRET is a highly distance-dependent interaction between two dye molecules without emission of a photon. Any process bringing the donor and acceptor into close proximity will result in energy transfer, therefore FRET is a very sensitive tool to detect macromolecular interactions.

The amino acid sequence of flagellin from *S. typhimurium* does not contain any Cys residues [16]. In order to achieve specific labeling with fluorescent dyes, a Cys residue was introduced into the central variable part of flagellin at sequence position 356 replacing a Ser residue by site-directed mutagenesis. This region of the molecule is known not to be essential for filament formation [1,15,18].

Cys-containing mutant flagellin was used for fragment preparation. Using appropriate proteases of high specificity, disordered terminal segments of flagellin can be removed in a predetermined way, and the resulting fragments can be easily purified by ion-exchange chromatography [5]. F(20–494) fragments, lacking 19 N-terminal residues of flagellin are easily produced by mild proteolytic treatment with ELC. The F(1–461) and F(30–461) fragments were obtained by digestion with V8 protease. F(1–461) has an intact N-terminal part but lacks 33 C-terminal residues, while F(30–461) contains truncations at both ends. Both disordered terminal regions were completely removed by tryptic digestion, resulting in the F(67–450) fragment.

Thiol-reactive fluorescent dyes were covalently conjugated to Cys-containing mutant flagellin and its terminally truncated fragments (F(20–494), F(1–461), F(30–461) and F(67–450)). CPM and AF488 [17] were chosen as donor and acceptor molecules, respectively, in energy transfer measurements (alternatively, in some experiments AF350 was used instead of CPM.) This donor–acceptor pair has a high quantum yield,

their absorption spectra are well-separated, and there is a large overlap between the emission spectrum of CPM and the absorption spectrum of AF488. CPM was excited at 394 nm and FRET was detected by the appearance of sensitized fluorescence of the acceptor at 512 nm. When AF350 was used the excitation wavelength was 347 nm.

To demonstrate that conjugation of fluorescent dyes to flagellin does not disturb filament formation, polymerization ability of CPM- or Alexa-labeled flagellin was tested by inducing filament formation with ammonium sulfate [19]. A dye-conjugated flagellin solution of 1.5 mg/ml was prepared in 20 mM Tris–HCl (pH 7.8) containing 150 mM NaCl, and 4.0 M ammonium sulfate was added to various final concentrations. After 1 day of incubation at room temperature, filament formation was observed by fluorescence microscopy. Our dye-labeled flagellin samples readily polymerized into helical filaments at ammonium sulfate concentrations higher than 0.2 M. At relatively low precipitant concentrations

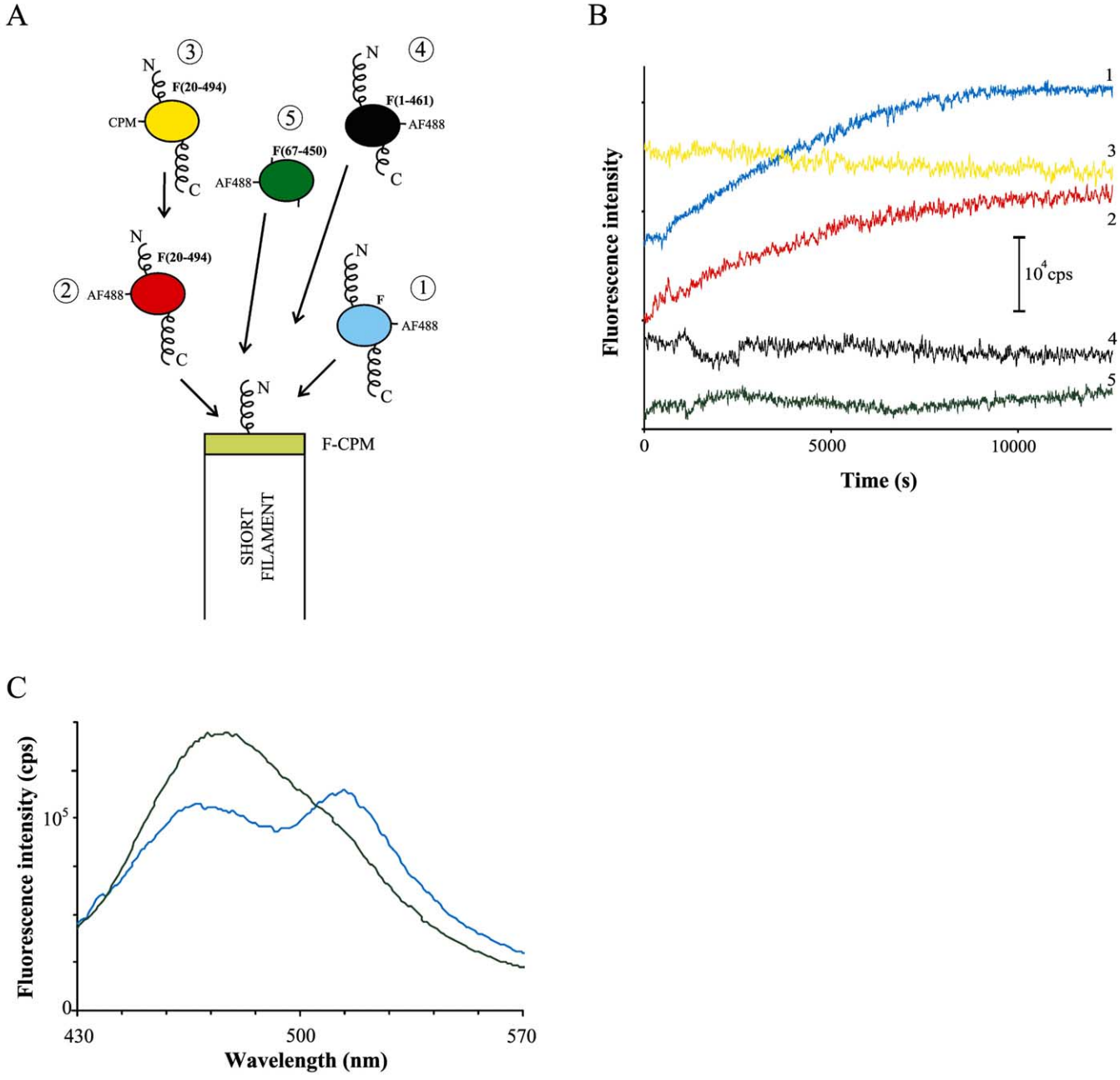

Fig. 2. Interaction of flagellin (blue), F(20–494) (red), F(1–461) (black) and F(67–450) (green) subunits with the end of flagellar filament. A: Short flagellar filaments covered by a thin layer of CPM-labeled flagellin (light green) were mixed with various AF488-conjugated subunits. B: Time course of interaction was monitored by FRET at 512 nm using an excitation wavelength of 394 nm. The increase of fluorescence signal indicated that flagellin or F(20–494) fragments were capable of binding to the end of filaments. On the contrary, F(1–461) and F(67–450) fragments lost the ability for polymerization. Although F(20–494) fragments could bind to the end of filaments, these F(20–494)-capped filaments were unable for further growth, as shown by the lack of energy transfer upon addition of CPM-conjugated F(20–494) subunits (yellow). (Binding curves are shifted along the *y*-axis for better arrangement.) C: Fluorescence emission spectra of donor and acceptor in the presence and absence of FRET. These spectra were recorded at the end of binding experiments for samples 1 and 5 of (B).

($\leq$0.4 M) long helical filaments appeared (not shown). These observations indicated that dye conjugation to flagellin did not interfere with the polymerization ability.

3.2. Binding of truncated fragments to the end of filaments

Flagellar filaments consist of tens of thousands of flagellin subunits. Polymerization of flagellin into flagella is a self-assembly process [1,20], which occurs only at one end of the filament corresponding to the distal one in native flagellum. We aimed at detecting binding of only a few molecules to the end of filaments. To increase sensitivity of our binding experiments, extremely short flagellar filaments were prepared. As a first step, short filaments were reconstituted from purified wild-type flagellin solution by adding ammonium sulfate at a high (1.0 M) concentration. Then, this filament sample was sonicated to decrease further the average length of filaments, and to increase the number of ends where subunit incorporation occurs. To minimize the fluorescence background, we started with unlabeled filaments and polymerized only a few CPM- or AF350-labeled flagellin subunits onto their ends to create a thin layer of donor molecules for energy transfer measurements. This filament preparation was used in subsequent binding studies (Fig. 2A).

As a control experiment, incorporation of intact flagellin subunits into the filament was monitored by FRET. Addition of AF488-conjugated flagellin to a solution of short filaments covered by CPM-labeled subunits resulted in a decrease of fluorescence intensity at the emission maximum of CPM (470 nm), while a new peak arose around 512 nm (Fig. 2C, blue). This increase of fluorescence signal at 512 nm was measured as a function of time (Fig. 2B, blue), and could be interpreted as a result of FRET between CPM-labeled and AF488-conjugated flagellin subunits bound to each other at the end of flagellar filaments. In control experiments, we could not observe any energy transfer between CPM- and AF488-labeled flagellin monomers in solution.

To explore the role of the N- and C-terminal disordered regions of flagellin in polymerization, interaction of various terminally truncated fragments with the end of flagellar filaments was investigated. Monitoring the polymerization of AF488-labeled fragments of flagellin by FRET revealed that F(20–494) fragments, deprived of 19 N-terminal residues, could effectively bind to the end of filaments (Fig. 2B, red). Upon addition of AF488-conjugated F(20–494), energy transfer clearly occurred between the CPM-labeled flagellin subunits and newly incorporated AF488-F(20–494) fragments, reflecting their close proximity.

On the contrary, removal of C-terminal segments resulted in the complete loss of binding ability. When AF488-conjugated F(1–461) or F(67–450) fragments were added to CPM–flagellin-capped filaments, we could not observe any increase in fluorescence intensity at 512 nm (Fig. 2B, black and green). Similarly, F(30–461) fragments were also unable to polymerize (not shown).

While F(20–494) fragments could bind to the end of filaments, they were unable to be used for continuous polymerization under physiological (precipitant-free) conditions. This was demonstrated by the following experiment: As a first step, AF488-labeled F(20–494) fragments were allowed to bind to the end of filaments as described above, resulting in filaments covered by acceptor-labeled (truncated) subunits. Upon subsequent addition of donor-labeled CPM-F(20–494) fragments, we could not observe any sign of energy transfer (Fig. 2B, yellow), indicating that F(20–494)-capped filaments are not capable of further filament growth. This experiment suggests that although F(20–494) fragments can attach to the end of native filaments, they can not bind to each other in the axial direction forming only a single layer of subunits. In control measurements, addition of CPM-conjugated flagellin to AF488-labeled seeds resulted in a binding curve very similar to that obtained for binding of AF488-conjugated flagellin to seeds covered by CPM-labeled subunits, indicating that the dipole geometry in both cases allows efficient energy transfer.

4. Discussion

The structure of the helical filaments of bacterial flagella has been determined at atomic resolution except the very inner core part [13], which is constructed from the disordered terminal regions of monomeric flagellin [12]. The disordered terminal regions exhibit intrinsically high propensity for amphipatic α-helix formation, which might be manifested in appropriate environments [21,22]. Indeed, polymerization of flagellin is accompanied by the stabilization of the unstructured parts into a highly α-helical conformation [1,4,9]. It has been hypothesized that the α-helical N- and C-terminal regions of axially neighboring subunits interact to form coiled-coil bundles upon polymerization [14].

Our observations can be well-explained based on the coiled-coil model of filament assembly (Fig. 1) assuming that subunits at the distal end of filaments have a free N-terminal end available for interaction with the C-terminal portion of flagellin molecules to be newly incorporated. F(1–461), F(30–461) and F(67–450) fragments of flagellin lost their polymerization ability because their truncated C-terminal region could not form helical bundles with the free N-terminal regions of flagellin subunits at the filament tip. Consistently, the F(20–494) fragment, having an intact C-terminal part, was able to bind to the end of filaments. Nevertheless, only a single F(20–494) layer could be formed since F(20–494) lacks 19 N-terminal residues, which prevented binding of F(20–494) subunits to each other in the axial direction. Thus, no energy transfer was observed upon addition of CPM-labeled F(20–494) to filaments covered by AF488-conjugated F(20–494) subunits. Thus, our results support the coiled-coil model of filament formation suggesting that the inner core of flagellar filament involves helical bundles formed by the interaction of α-helical N- and C-terminal regions of axially adjacent subunits.

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