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Interaction of FliS flagellar chaperone with flagellin

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Abstract Premature polymerization of flagellin (FliC), the main component of flagellar filaments, is prevented by the FliS chaperone in the cytosol. Interaction of FliS with flagellin was characterized by isothermal titration calorimetry producing an association constant of $1.9 \times 10^7\ M^{-1}$ and a binding stoichiometry of 1:1. Experiments with truncated FliC fragments demonstrated that the C-terminal disordered region of flagellin is essential for FliS binding. As revealed by thermal unfolding experiments, FliS does not function as an antifolding factor keeping flagellin in a secretion-competent conformation. Instead, FliS binding facilitates the formation of α-helical secondary structure in the chaperone binding region of flagellin.

Keywords: FliS; Flagellar chaperone; Flagellin; Flagellar export; Type-III export

1. Introduction

Bacteria swim by rotating flagellar filaments, each of which has a helical shape and works as a propeller [1]. External flagellar proteins, lying beyond the cytoplasmic membrane, are synthesized in the cell and exported by the flagellum-specific export apparatus from the cytoplasm to the site of assembly through the central channel of the flagellar filament. The flagellum-specific export system is a specialized type III export machinery [2].

Little is known about the export process of flagellar proteins through the hollow core of the flagellum to their site of polymerization. Owing to the small diameter of the export channel (25–30 Å) [3], proteins destined for incorporation into the growing flagellum are thought to be exported in a partially unfolded state, implying that premature folding and oligomerization in the cytosol must be prevented. It seems that cells prevent the cytosolic folding and interaction of these proteins applying flagellar chaperones [4–6] that specifically interact with individual axial proteins and may keep them in a secretion-competent conformation.

FliS acts as a substrate-specific chaperone facilitating the export of flagellin (FliC) [6,7], the main component of flagellar filaments. Both terminal regions of flagellin, comprising 66 N-terminal and 44 C-terminal residues, are disordered in the monomeric form whereas the central part of the molecule is composed of three well-folded domains [8,9]. The highly conserved disordered regions are essential for self-assembly of flagellin [10]. They become stabilized into α-helical bundles upon polymerization forming the core part of filaments [3].

FliS from *Salmonella typhimurium* consists of 135 amino acid residues [11] and predicted to be predominantly α-helical [7]. The first crystallographic structure of a flagellar chaperone, *Aquifex aeolicus* FliS [12], demonstrated that FliS adopts a novel fold, clearly distinct from those of the type III secretion chaperones. FliS effectively inhibits in vitro polymerization of flagellin [7]. Yeast two-hybrid assays indicated that the C-terminal disordered region of flagellin is essential for FliS binding [13]. Gel filtration chromatographic experiments suggested that *Salmonella* FliS forms stable homodimers which bind to FliC monomers [7]. Yet, the structure of *A. aeolicus* FliS in complex with a C-terminal fragment of flagellin shows a monomer to monomer interaction [12].

Here we addressed the mechanism of *Salmonella* FliS chaperone action. FliS–FliC complex formation was quantitatively characterized by isothermal titration calorimetry (ITC) to determine binding stoichiometry. Conformational rearrangements accompanying binding of FliS to FliC were also studied. We aimed at answering the question whether chaperone binding has only a local effect on the binding region of flagellin, or transmitted to other parts of the molecule keeping flagellin subunits in an unfolded and secretion-competent state.

2. Materials and methods

2.1. Protein expression and purification

Flagellin was prepared from *S. typhimurium* wilde-type strain, SJW1103, and purified as described [8]. Fragments of flagellin missing terminal segments of various sizes within the disordered terminal regions were prepared by limited proteolysis of monomeric flagellin

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Abbreviations: FliC, flagellin; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; CD, circular dichroism

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and purified by ion-exchange column chromatography [10]. The F(59-494) fragment was produced by Endoproteinase Lys-C (ELC), F(1-461) was prepared by Endoproteinase Glu-C (V8). ELC and V8 were obtained from Boehringer (Roche). Other chemicals were of analytical grade from commercial sources.

FliS was overproduced in *Escherichia coli* cells, strain BL21(DE3), carrying pET-3c-based plasmids. FliS producing bacteria were kindly provided by Hideyuki Matsunami (Dynamic NanoMachine Project, ICORP, JST, Japan). 20 ml overnight culture was inocculated into 1000 ml of LB broth, supplemented with Ampicillin (50 μg/ml) and Chloramphenicol (30 μg/ml), and incubated at 37 °C to reach an $OD_{600\ nm}$ of 0.6. After addition of IPTG to a final concentration of 1 mM, incubation was continued for another 4 h. The bacterial pellet was collected by centrifugation at 4500 rpm for 20 min and stored at −30 °C.

Purification of FliS protein was performed as follows: The bacterial pellet from 2 L culture was resuspended in 30 ml PBS, sonicated on ice to lyse the cells for 1 min, 3 times at a power of 14 W (CP-130, Cole-Palmer Vernon Hills, IL), and the suspension was centrifuged at 12 000 × *g* for 30 min at 4 °C. The crude inclusion body pellet was fully resuspended in 15 ml of 3% Triton X-100, 50 mM Tris–HCl, 1 mM EDTA (pH 8.0) and sonicated on ice. The solution was centrifuged at 8000 × *g* for 25 min at 4 °C to remove membrane fragments and cell debris. The supernatant was collected and the pellet was washed again. This step was repeated 2 times with Triton X-100, and then 2–3 times without Triton X-100. The collected supernatants were centrifuged at 38 000 rpm for 30 min at 4 °C. The inclusion body pellet was washed 2 times with 50 mM Tris, 1 mM EDTA, pH 8. Then, the pellet was dissolved in 18 ml of 6 M Guanidine–HCl, 50 mM Tris, pH 8, 10 mM EDTA containing 2 Complete mini protease inhibitor cocktail tablets (Roche) and incubated overnight at 4 °C on a shaker. The sample was centrifuged at 38 000 rpm for 30 min at 16 °C, and the supernatant was used for final purification by FPLC on a HiPrep Sephacryl 16/60 S-200 gel filtration column (Pharmacia Biotech) applying 20 mM Tris, 150 mM NaCl, pH 7.8, buffer.

The protein concentration of samples was determined from absorption measurements at 280 nm using extinction coefficients calculated from the known aromatic amino acid contents of the molecules [14]. Purity of protein samples was checked by SDS–PAGE using 12.5% and 20% polyacrylamide gels, stained with Coomassie blue R-250 (Merck).

2.2. Scanning microcalorimetry

Differential scanning calorimetric experiments were performed with a VP-DSC instrument (MicroCal, Northampton, MA) interfaced to an IBM PC for automatic data collection and instrument control. Calorimetric measurements were done in 20 mM Tris–HCl buffer solution (pH 7.8) at a scanning rate of 1 °C/min. Protein samples were extensively dialyzed against the buffer at 4 °C. In the calculations of molar thermodynamic quantities, the molecular masses used were 16.5 kDa and 51.4 kDa for FliS and FliC, respectively. Differential scanning calorimetry (DSC) data were analyzed with an Origin 5.0-based software package supplied by MicroCal.

2.3. Isothermal titration calorimetry

The experiments were carried out with a VP-ITC titration calorimeter (MicroCal, Northampton, MA). Measurements were done in 20 mM Tris–HCl, pH 7.8, at various temperatures. Protein samples were extensively dialyzed against the buffer at 4 °C. All solutions were thoroughly degassed before use by stirring under vacuum. FliS solution was loaded into the calorimetric cell and 10 μl portions of concentrated FliC solution were injected. To take into account heats of dilution, two blank titrations were performed; one injecting FliC solution into buffer and another injecting buffer into the FliS solution. The heat released by dilution of FliC was negligible. The averaged heats of dilution were subtracted from the main experiment. Calorimetric data were analyzed using MicroCal Origin software fitting them to a single binding site model.

2.4. Circular dichroism (CD) measurements

Far-UV CD spectra were recorded in the range of 200–260 nm at 0.5 nm intervals on a Jasco-720 spectropolarimeter. Samples were measured at room temperature in cylindrical fused quartz cells with a path length of 0.1 cm in 10 mM phosphate buffer (pH 7.0).

3. Results

3.1. Thermodynamic characterization of FliC–FliS association

Isothermal titration calorimetry was used to determine directly the energetics and stoichiometry of FliS–FliC interaction. FliS solution at a protein concentration of 0.2 mg/ml was loaded into the calorimetric cell and 10 μl portions of concentrated FliC solution (c = 5.5 mg/ml) were injected. The observed heat effects clearly indicated the interaction (Fig. 1, upper panel). The measured data were analyzed and nicely fitted using a one-site model (Fig. 1, lower panel). A large and favorable enthalpy of binding ($\Delta H = -12.9$ kcal/mol) was observed upon interaction at 25 °C, accompanied by an unfavorable binding entropy ($\Delta S = -9.9$ cal mol^{-1} deg^{-1}). The FliS–FliC association had an equilibrium association constant of 1.9×10^7 M^{-1}. Our calorimetric data clearly show that FliS interacts with FliC with a 1:1 stoichiometry (N = 1.00). It is worth noting that significant interaction persisted between FliS and FliC even at 55 °C, well-above the unfolding temperature of flagellin (data not shown).

To localize the binding regions terminally truncated fragments of flagellin were prepared by proteolytic digestion. F(59-494) was deprived of its disordered N-terminal region, while F(1-461) lacked a large portion of the C-terminal

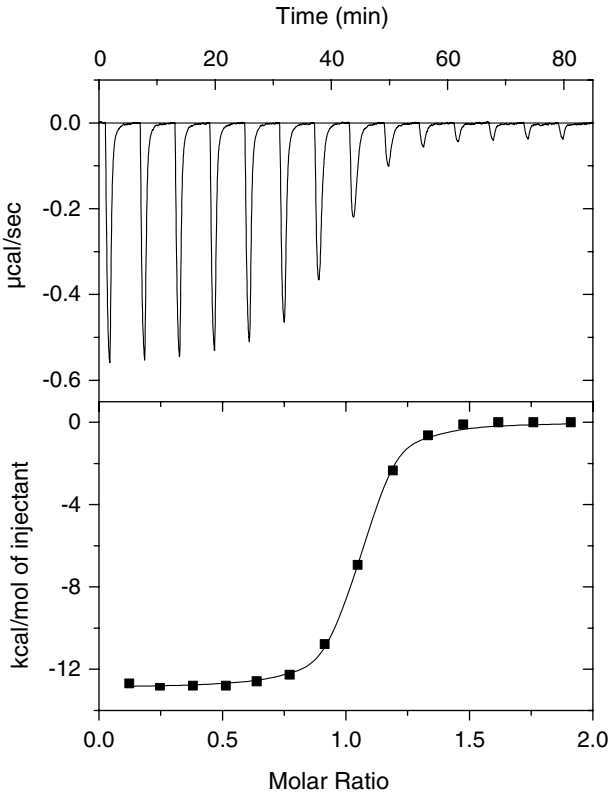

Fig. 1. Isothermal calorimetric titration of FliS with flagellin at 25 °C. Upper part: repetitive 10 μl injections of FliC (c = 5.5 mg/ml) into a FliS (c = 0.18 mg/ml) solution caused exothermic heat pulses. Lower part: changes in binding enthalpy (■) of the corresponding injections shown above as a function of the molar FliC to FliS ratio. The solid line is the least-squares fit of the data to a one binding site model resulting in the following parameters: stoichiometry N = 1.00, $K = 1.9 \times 10^7$ M^{-1}, $\Delta H = -12.9$ kcal/mol. Titrations were done in 20 mM Tris–HCl, pH 7.8, buffer.