Stabilization of flagellar filaments by HAP2 capping

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Received 5 March 2004; revised 20 April 2004; accepted 11 May 2004

Available online 31 May 2004

Edited by Amy McGough

Abstract In vivo growth of bacterial flagellar filaments by self-assembly of flagellin is promoted by a capping structure composed of a pentameric assembly of hook associated protein 2 (HAP2). Isolated native filaments with intact HAP2 cap exhibited higher melting temperature ($\Delta T_m = 4$ °C) and significantly increased resistance against heat-induced depolymerization than non-capped ones. Reconstituted filaments were also stabilized by HAP2 binding, but the obtained filament–HAP2 complexes were less stable than native assemblies. Their fast depolymerization at elevated temperatures and sensitivity to proteolysis indicated that native-like filament–HAP2 complexes are rarely obtained by in vitro reconstitution. A procedure was developed to isolate perfectly capped native filaments to facilitate high-resolution structural analysis.

Keywords: Bacterial flagellum; Self-assembly; Flagellar cap; Capping protein; HAP2; FliD

1. Introduction

Bacteria swim by rotating helical flagellar filaments [1] which are constructed from a single protein, flagellin. The filament is a tubular structure composed of 11 protofilaments ([2], and references therein). The atomic model of the flagellar filament has recently been determined by X-ray diffraction [3] and electron cryomicroscopy [4]. In vitro polymerization of flagellin subunits into filaments occurs spontaneously under appropriate conditions upon addition of short filaments as seeds [5] or a precipitant such as ammonium sulfate (AS) [6].

In vivo filament growth requires a cap structure tightly attached to the distal end of filament [7]. The cap is a pentameric assembly of HAP2 (FliD), having an annular structure of a fivefold rotational symmetry [8,9]. Flagellin monomers are synthesized in the cytoplasm, transported through the central channel of the flagellum, and incorporated into the growing structure at the tip [10]. The cap facilitates assembly of flagellin and prevents its excretion [11]. The HAP2 cap performs two apparently contradictory functions; it binds very strongly to the end of the flagellar filament, still allowing incorporation of newly exported flagellin subunits between the cap and the end of the filament. A model has been proposed to explain the molecular mechanism of capping [9,12]. Because of a mismatch in the rotational symmetry between the cap and the filament, individual subunits of the cap appear to bind to flagellin subunits in slightly different conformations [9,12,13].

Maki et al. [8] demonstrated the reconstitution of the filament–cap complex by mixing reconstituted filaments and purified HAP2 proteins in solution. HAP2-capped native filaments and reconstituted filament–HAP2 complexes appear morphologically indistinguishable in electron micrographs of negatively stained samples. High-resolution structural data are often obtained from reconstituted samples [9,13]. Therefore, it is important to know whether native and reconstituted HAP2–filament complexes are really identical to structural details. In this study, limited proteolysis and stability measurements were applied as sensitive tools to explore structural differences between HAP2-capped native filaments and reconstituted filament–HAP2 assemblies.

2. Materials and methods

2.1. Preparation of native filaments

Native filament samples were prepared in the following way: SJW1103 cells of *Salmonella typhimurium* were grown in 3% yeast extract overnight at 37 °C. The cells were collected by centrifugation at 4000 rpm for 30 min at 4 °C, suspended in 20 mM Tris–HCl containing 150 mM NaCl (pH 7.8) and vortexed for 3 min. After centrifugation at 10 000 rpm for 20 min (4 °C), detached filaments were recovered in the supernatant. The filaments were collected by high speed centrifugation at 40 000 rpm for 60 min and then gently suspended in 10 mM phosphate buffer (pH 7.0) containing 150 mM NaCl at a final concentration of 1 mg/ml.

To isolate filaments covered by an intact HAP2 cap from the mixture with uncapped ones, native filament samples were incubated at 45 °C (or 50 °C) for 12 h in the presence of trypsin (Boehringer) at a ratio of 50:1 (w/w). Proteolytic digestion was stopped by hen-egg trypsin inhibitor. The sample was ultracentrifuged at 200 000 × *g* for 10 min and the remaining filaments were gently resuspended in 10 mM phosphate buffer and 150 mM NaCl (pH 7.0).

Electron microscopic observation of the filaments was carried out on samples negatively stained with 1% (w/v) uranyl acetate by using a JEM1010 (JEOL) electron microscope. Samples were usually observed at a magnification of 50 000×.

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Abbreviations: AS, ammonium sulfate; CD, circular dichroism; HAP2, hook associated protein 2

0014-5793/$22.00
doi:10.1016/j.febslet.2004.05.029

2.2. Reconstitution of filament–HAP2 complexes

Flagellin was prepared from SJW1103 cells as described in [13]. Flagellar filaments were reconstituted from purified flagellin solutions by adding AS to a final concentration of 0.8 M. The filaments were washed twice by centrifugation and suspended in 10 mM phosphate buffer (pH 7.0) containing 150 mM NaCl at a final protein concentration of 3 mg/ml.

HAP2 was overproduced in *Escherichia coli* cells, strain BL21(DE3), carrying pLysS and pKOT134 plasmids, and was purified as described by [14]. The concentration of HAP2 protein was determined from absorption measurements at 280 nm using an extinction coefficient of $E^{1\%}_{280} = 3.45$ [12].

In capping experiments, reconstituted filaments at a protein concentration of 1 mg/ml were incubated with HAP2 at a ratio of 10:1 (w/w) at 20 or 37 °C, for 2 h or overnight in 10 mM sodium phosphate (pH 7.0). Excess HAP2 was removed by centrifugation at $200\,000 \times g$ for 12 min. To check the efficiency of HAP2 binding [12], HAP2-treated filament solutions were mixed with flagellin monomer solutions at a 1:20 (w/w) ratio. After 1 day of incubation at 25 °C, filament growth was checked by dark-field microscopy.

2.3. CD measurements

Temperature induced unfolding of HAP2-capped filaments was monitored by far-UV circular dichroism (CD) in a cylindrical quartz cell with a path length of 0.1 cm at 222 nm in 10 mM-phosphate buffer (pH 7.0) solution, over the temperature range of 20–70 °C with a heating rate of 50 °C/h. Measurements were done by a Jasco-720 spectropolarimeter equipped with a thermoregulated cell holder. The time scans to monitor depolymerization of filaments at elevated temperatures were also performed at 222 nm over a period of 100 min.

3. Results

3.1. Stability of native flagellar filaments

Native flagellar filaments were detached from bacteria and isolated by several cycles of centrifugation and resuspension. During the purification procedure, filaments are easily broken into smaller pieces. Thus, native filament samples are expected to contain both HAP2-capped and uncapped filaments.

Melting profiles of native filament samples exhibited a biphasic behavior (Fig. 1A; dash) as monitored at 222 nm by far-UV CD spectroscopy. This biphasic behavior is more clearly presented in Fig. 1B, which shows the derivatives of the curves in Fig. 1A. It was plausible to assume that the first transition around 57 °C corresponded to the melting of the uncapped filaments, while the second one around 61 °C appeared owing to the capped filaments. The obtained melting profiles indicated that about 20% of filaments contain intact caps, although this ratio varied somewhat from samples to samples.

Based on the assumption that HAP2-capped filaments are more resistant to heat-induced depolymerization than uncapped ones, a procedure was devised to separate filaments covered by an intact HAP2 cap: native filament samples were subjected to prolonged incubation at 45 °C in the presence of trypsin. While flagellar filaments are known to be rather resistant against proteolytic digestion, monomeric flagellin is degraded very quickly by proteolytic enzymes, since its terminal regions are disordered [15,16]. Incubation of uncapped filaments at elevated temperatures is expected to induce depolymerization. The equilibrium can be further shifted towards disassembly by adding proteases, since monomers are efficiently removed from the solution by proteolytic digestion. Thus, a tryptic treatment at elevated temperatures was assumed to promote complete depolymerization and destruction of uncapped filaments. On the other hand, filaments with intact caps were expected to survive this treatment.

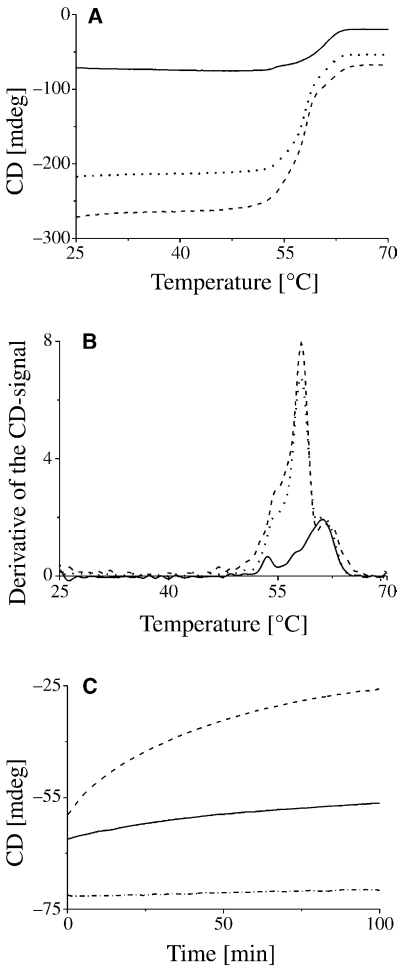

Fig. 1. Stability of the native filament samples against thermal denaturation and depolymerization as monitored by far-UV CD spectroscopy at 222 nm. (A) Melting profiles of the native filaments (dash), the native filaments incubated at 45 °C for 12 h (dot) and the HAP2-capped subpopulation of the native filaments (solid) isolated after a treatment by trypsin at 45 °C for 12 h at a protease to protein ratio of 1:50 (w/w). (B) Derivatives of the melting profiles of (A). (C) Depolymerization of the native filaments (dash), the HAP2-capped subpopulation purified after trypsin treatment (solid) and the tryptic digestion mixture without purification (dash-dot) as measured at 50 °C.

Almost 80% of the native filaments were degraded upon the tryptic treatment at 45 °C for 12 h. As expected, the remaining portion exhibited a melting profile corresponding to the second transition of the original sample (Fig. 1A and B; solid). Uncapped filaments are well-known to exhibit a fish-tail shaped end [17]. Electron micrographs of negatively stained samples demonstrated that our preparation contained nicely capped filaments (Fig. 2). In control experiments, prolonged incubation at 45 °C without proteolytic treatment resulted in only a slight decrease of the CD signal, and the biphasic behavior of the melting profile was preserved (Fig. 1A and B; dot). Our observations demonstrate that the HAP2-capped subpopulation of native filament samples can be efficiently separated by the procedure described above.

The protective effect of HAP2-capping against temperature-induced depolymerization was also investigated by monitoring spectral changes at 222 nm by CD spectroscopy. There is a

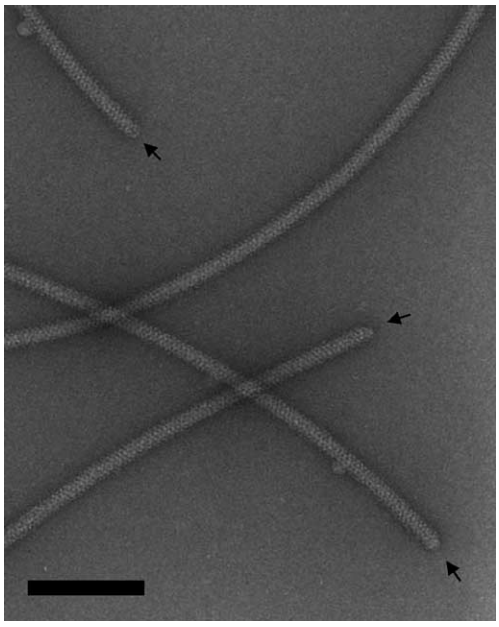

Fig. 2. Electron micrograph of negatively stained filament–cap complexes at a magnification of 50 000×. The arrows show the capped filament ends. Scale bar, 100 nm.

significant decrease in the intensity of the CD signal upon depolymerization [18], because the terminal regions of flagellin that form interlocking α-helical bundles in the inner core of the filament [3,4] acquire a disordered state in the monomeric form [15,16].

While native filament samples depolymerized rather quickly at 50 °C (Fig. 1C; dash), the filaments purified after the trypsin treatment exhibited a slowly changing CD signal (Fig. 1C; solid) at this temperature. As the isolation procedure of the HAP2-capped filaments from the digestion mixture by centrifugation and resuspension might have generated slight fragmentation, we checked whether the slow disassembly observed was really characteristic for the intact filament–HAP2 complexes or originated from broken filaments. When the digestion mixture containing both HAP2-capped filaments and fragmented flagellin peptides was directly measured, the rate of depolymerization was negligible (Fig. 1C; dash-dot), indicating that the native filament–HAP2 complexes are resistant against temperature-induced depolymerization at 50 °C. We found that HAP2-capping effectively prevented depolymerization of filaments almost up to 55 °C (data not shown).

3.2. Stabilization of reconstituted filaments by HAP2 capping

Helical filaments were reconstituted from flagellin by inducing polymerization with AS [6]. The filament–cap complex was reconstituted by mixing the reconstituted filaments with purified HAP2 in solution. Previous reconstitution trials [8] revealed that there was no significant difference in the efficiency of cap formation in a broad pH range (between pH 5 and 11) while changing the incubation temperature had a pronounced effect. We performed the reconstitution experiments either at 20 or 37 °C by varying the period of incubation.

Short filaments were prepared from purified flagellin by adding AS to a final concentration of 0.8 M. In all cases, HAP2 treatment completely inhibited further filament growth upon addition of flagellin monomers, as was judged by dark field microscopy [12]. This observation indicated that HAP2 bound firmly to the end of the filaments, and practically all the filaments were capped, at least partially, with HAP2.

HAP2 treatment of the reconstituted filaments resulted in a slight increase of their stability (Fig. 3A). Our short reconstituted filaments melted at 52.3 °C. Upon treatment with HAP2 for 2 h at 20 °C, the apparent denaturation temperature increased to 53.2 °C, whereas the same treatment at 37 °C resulted in a transition temperature of 53.9 °C. Prolonged (overnight) incubation of the reconstituted filaments with HAP2 at 37 °C increased the apparent melting temperature to 54.4 °C. However, even higher transition temperature, $T_m = 55.3$ °C, was observed when excess HAP2 was present in the solution, indicating that there was a dynamic equilibrium between the bound and free forms of HAP2. As a reminder, binding of the native HAP2 cap to the end of the filament was stable in this temperature range. Although relatively small, the reproducible increase of the thermal stability upon treatment of filaments with HAP2 at 37 °C instead of 20 °C suggests that cap formation occurs at 37 °C more efficiently.

Reconstituted (uncapped) filaments were unstable and underwent a temperature-induced depolymerization even at 45 °C (Fig. 3B; dash). Although HAP2 treatment clearly decreased the rate of depolymerization, all of the reconstituted filament–HAP2 samples were found to be much more sensitive to thermal depolymerization than the native ones. It was not possible to purify perfectly capped filaments from the reconstituted ones by employing proteolytic digestion at elevated temperatures. We tried filament reconstitution at various AS concentrations or by using seeds, and applied different HAP2

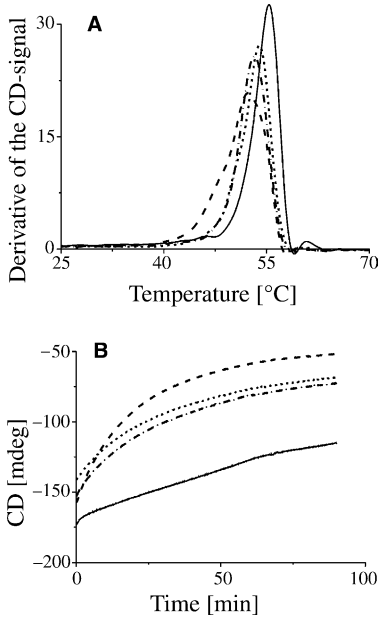

Fig. 3. Stabilization of the reconstituted filaments by HAP2 binding. (A) Thermal denaturation of the reconstituted filament–HAP2 complexes. Derivatives of the melting profiles monitored by CD spectroscopy at 222 nm are shown. (B) Temperature induced depolymerization of the reconstituted filament–HAP2 complexes at 45 °C as followed by CD at 222 nm. The codes are as follows: the reconstituted filaments without HAP2 (dash), the filaments treated by HAP2 at 20 °C for 2 h (dash-dot), the filaments treated by HAP2 at 37 °C for 2 h (dot). Excess HAP2 present in the solution (solid) had a pronounced effect on the filament stability.

incubation procedures, but our samples were always digested completely by trypsin at 45 °C.

4. Discussion

HAP2 plays an essential role in the in vivo assembly of flagellar filament by forming a cap at its end, which facilitates incorporation of flagellin subunits exported through the central channel of the flagellum [19]. Flagellar filaments are constructed from a few tens of thousands of flagellin molecules. Our experiments with isolated native filaments demonstrated that the tiny HAP2 cap exerts a significant stabilization effect on this huge macromolecular assembly by increasing its melting temperature by about 4 °C (from 57 to 61 °C) and efficiently preventing temperature-induced depolymerization even above 50 °C.

Monomeric flagellin undergoes heat-induced denaturation at 47 °C [20]. We found that the apparent denaturation temperature of the uncapped native flagellar filaments was 57 °C, indicating that flagellin subunits are stabilized in the filamentous state. Polymerization and depolymerization of flagellar filaments occur only at the distal end [10]. Depolymerization of the filaments above 47 °C is irreversible because of instant denaturation of detached subunits. The stabilization effect of HAP2-capping suggests that thermal destruction of the filaments starts with depolymerization, which is followed by unfolding of the subunits, and the HAP2 cap can effectively suppress the depolymerization. A sample containing shorter filaments is expected to exhibit a higher apparent depolymerization rate, and therefore a lower apparent denaturation temperature. This explains why our short reconstituted filaments showed significantly lower denaturation temperature than those much longer native ones. Indeed, we observed that reconstituted filament samples prepared at decreasing AS concentrations, thus containing longer filaments, exhibited an increasing denaturation temperature, approaching that obtained for the uncapped native filaments (data not shown).

Interaction with the HAP2 cap results in the stabilization of the filament. However, the stabilizing effect in not symmetrical. Thermal denaturation of HAP2 takes place around 61 °C [12], and the native filament–HAP2 complex unfolds at the same temperature where thermal denaturation of HAP2 occurs. Thus, it is the denaturation of HAP2 that leads to disassembly and denaturation of the native filament–cap assembly.

A procedure was developed to separate and purify the HAP2-capped native filaments based on the stabilizing effect by HAP2 capping. Electron micrographs demonstrated that the obtained sample contained nicely capped filaments. This native filament–cap complex was highly resistant against proteolytic degradation and depolymerization.

Although HAP2 bound readily to the end of reconstituted filaments, our studies revealed that the obtained filament–HAP2 complexes were always less stable than the native ones and underwent fast depolymerization at elevated temperatures. The stabilization effect was largely dependent on the method of incubation, e.g., prolonged incubation at 37 °C facilitated the cap formation as compared to that at room temperature. Previous reconstitution experiments suggested that the efficiency of cap–filament reconstitution may reach as high as 60% at 37 °C [21]. Still, it was not possible to isolate the HAP2-capped filaments by the procedure that worked well for the native filaments. The resistance of the reconstituted filament–HAP2 complex against proteolysis at elevated temperature was unexpectedly low. Whatever conditions and methods we used to reconstitute the filament–HAP2 complex, the samples were always digested completely.

The structures of the filament–cap complex and the isolated cap dimer complex have been deduced by electron cryomicroscopy and single particle image analysis [9,13]. These structures revealed that the cap is made of a pentagonal plate domain and five leg domains, which are thought to be responsible for the stable yet flexible binding of the cap to the filament end. The leg domains are composed of the terminal regions of HAP2 that are unfolded in the monomeric form and are likely to form an α-helical coiled coil to bind stably to the inner core of the filament made also by the terminal domains of flagellin subunits [3,4,9,12].

Microtubules and actin filaments in muscle and cytoskeleton are often capped by a variety of proteins regulating assembly and cellular location [22,23]. In many respects, HAP2 shows similarities to tight capping proteins, especially to CapZ [24], which binds to the barbed ends of actin filaments. Both HAP2 and CapZ stabilize the filament structure by preventing the loss of subunits at the end. Their binding regions are mobile and flexible involving amphipathic helical structures, and symmetry mismatch between the cap and filament structures plays an important role in binding [8,19,24]. While both proteins prevent incorporation of exogenously added subunits, the significant difference is that HAP2 is capable of promoting assembly of endogenous flagellin exported through the central channel of flagellar filament.

The structure analysis of the filament–cap complex was carried out on the reconstituted complex [9], which appears morphologically very similar to the HAP2-capped native filament in electron micrographs [8]. Exogenously provided HAP2 to a HAP2-null mutant actually restores the filament growth and cell motility, indicating that reconstitution of the functional complex occurs efficiently as far as endogenous flagellin molecules are provided afterwards through the central channel of the flagellum [25]. However, the reconstituted complex formed in vitro clearly contains structural defects that decrease the thermal stability significantly and making the complex susceptible to proteolytic degradation at elevated temperature. This suggests that the proper binding of the terminal regions of HAP2 to the filament core rarely occurs in the reconstituted complex. The structure of the natively capped filament would have to be analyzed to visualize the molecular interactions important for structural stability, and the procedure we developed to isolate natively capped filaments will help to carry out such analysis efficiently.

Acknowledgements: We thank S. Maki-Yonekura for taking electron micrographs and F. Oosawa for continuous support and encouragement. This work was partially supported by the Hungarian OTKA T034261 grant to F.V.

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