Locations of Hook-Associated Proteins in Flagellar Structures of *Salmonella typhimurium*

MICHIKO HOMMA* and TETSUO IINO

Laboratory of Genetics, Faculty of Science, the University of Tokyo, Hongu, Tokyo 113, Japan

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Hooks of the flagella of *Salmonella typhimurium* were purified from an flaL mutant. Hook-associated proteins, namely HAP1, HAP2, and HAP3, were separated from them, and the antibody against each HAP was prepared. By immunoelectron microscopic observation, these three kinds of antiHAP antibodies were found to bind on the distal ends of hooks of filamentless mutants consistently with their composition of HAPs. The antiHAP2 antibody bound to the very tops of the claw-shaped ends of the hooks which contain all three HAPS. The antibodies against HAP1 and HAP3 bound to the basal areas and the middle areas, respectively, of the claw-shaped ends. The order of disassembly of the component proteins by heat treatment of the hook structure from the filamentless mutants was (HAP2, HAP3) > HAP1 > hook protein. These observations were consistent with our layered structure model: HAP1, HAP3, and HAP2 are assembled at the distal end of the hook in this sequence. All three HAPS were detected in the hook-filament complexes prepared from a flagellate strain.

When the hook-filament structure was treated with antibody against HAP1 and with the anti-rabbit immunoglobulin G antibody, the antibody aggregate was observed in the region corresponding to the boundary between filament and hook. This observation strongly suggests that HAP1 is the protein connecting filament with hook. The locations of HAP2 and HAP3 in the hook-filament structure were not clarified with the same procedure.

A bacterial flagellum consists of three distinct parts: a basal body, a hook, and a filament. The hook connects the filament, which extends from the cell body, with the basal body embedded in the cell surface layers (1-3, 5-8, 11, 12). Molecular compositions and arrangements of the hook and the filament have been studied intensively in *Salmonella typhimurium*, and their three-dimensional images have been reported (22, 23, 25). As a protein-protein interaction model of self-assembly, the assembly of flagellin, the component protein of the filament, to a flagellar filament has been investigated in detail by in vitro polymerization experiments (4, 19). The growth of in vivo flagellar filaments has been found to occur in their distal portion (9, 16), and their elongation rate decreases exponentially with increase of the length of the filament (17). From these observations, it has been inferred that the flagellin molecules, which synthesize in the cell, are transported through a central channel of the hook to its distal end and then polymerized sequentially.

Filamentless mutants, in which the final step of filament assembly is blocked, produce a hook basal-body structure missing the filament. The genes responsible for this step are flaV, flaU, flaW, flaL, and H1 H2 in *Salmonella* spp. (14, 24). In a recent report (14), we have shown that three kinds of minor proteins termed hook-associated proteins (HAPS)—HAP1 (molecular weight [MW], 59,000), HAP2 (MW, 53,000), and HAP3 (MW, 31,000)—contained in the hook structure are essential for filament formation, and that at least some HAPS might be located in the distal ends of the hooks. The compositions of HAPS have been analyzed in the hooks from the filamentless mutants (14). The hooks from the flaL and H1 H2 mutants contain all three HAPS, the hooks from the flaV mutant contain HAP1 and HAP3, the hooks from flaU mutant contain HAP1, and the hooks from the flaW mutant contain a very small amount of HAP3. When the hooks lose any one of these kinds of HAPS in the flaV, flaU, and flaW mutants, flagellin molecules transported to the tip of each hook are unable to polymerize there and consequently are excreted into the culture medium (13). Further studies on HAPS are expected to provide a clue for understanding the mechanism of filament formation. Therefore, we prepared the antibody against each of the HAPS and, using them, we examined the localizations of HAPS in the flagellar structure. As a result, we could identify the localization of each HAP on the distal ends of the hooks in the filamentless mutants. Furthermore, all of the HAPS were detected in the hook-filament structure of a flagellate strain.

**MATERIALS AND METHODS**

**Bacterial strains.** The strains used are listed in Table 1. All of the nonflagellate mutants are transductional derivatives of *S. typhimurium* LT2. The fla mutations of MH103 and MH111 were introduced from fla::Tn10 (kindly supplied by K. Kutsukake) by P22-mediated transduction, using the method of Kutsukake et al. (20).

**Media.** Media were described previously (14).

**Electrophoresis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (21). Sample solutions for SDS-PAGE were prepared by mixing 50 μl of protein solution with 10 μl of TDG buffer (0.4 M Tris-hydrochloride [pH 6.8], 6% SDS, 38% glycerol, 0.004% bromophenol blue) and 5 μl of 2-mercaptoethanol. The sample solutions were heated at 100°C for 10 min.

Proteins in the gel were stained with 0.1% Coomassie brilliant blue R250 as described previously (14), and the stained gel was scanned at 575 nm by a densitometer (Joyce-Loebl Chromoscan 200/201).

**Preparation of the antibody against each HAP.** Hooks were prepared from a 100-liter culture of SJW800 (FlaL−), and HAPS and hook protein were separated by SDS-PAGE as described in our earlier paper (14). Proteins in the gel were stained by 0.1% Coomassie brilliant blue R250 in water.
After 30 min, the gel was washed with water, and the stained band of each HAP was cut out from the gel. The gel piece containing each HAP was blended in 10 mM phosphate buffer (pH 7.2) containing 0.15 M NaCl with a Teflon homogenizer. Half of each homogenate was injected into a rabbit with Freund complete adjuvant, first into the footpads and, after 2 weeks, into the back. The rabbits were bled 2 weeks after the second injection. The antibody was obtained by salting-out, as described in our earlier paper (14).

**Immunodiffusion test.** Agar (Agarose-1, Dojindo Laboratories) was melted to 1% in 25 mM Veronal buffer (pH 8.6) and layered at ca. 1 mm thickness on a microslide. The agar plate was punched to produce wells of 3 mm diameter spaced 3 mm apart. Each well was filled with 7 μl of antibody or antigen solution and then incubated overnight at 4°C in a humid chamber.

For staining of precipitin bands, the gel was washed twice with 0.15 M NaCl over a period of 24 h and then washed with water for 1 h. The gel was dried on the slide at 37°C. The precipitin bands were stained by 0.1% Coomassie brilliant blue R250 in methanol-acetic acid-water (5:1:4), and the dye in the background was dissolved in the solvent.

**Immunoblotting.** After separation of proteins by SDS-PAGE, the proteins were transferred onto nitrocellulose sheets (Bio-Rad Laboratories) by the method of Howe and Hershey (15). The immunological protein detection on the blotted nitrocellulose sheets was carried out by the method of Howe and Hershey (15) with the following modifications. The blotted sheets were incubated with 2% bovine serum albumin in TBS (10 mM Tris-hydrochloride [pH 7.8], 0.9% NaCl) for 1 h at room temperature. After the sheets were rinsed with buffer B (0.2% SDS, 0.5% Triton X-100, 0.5% bovine serum albumin in TBS), they were incubated in antibody solution, which had been diluted 1:100 with buffer B at room temperature for 12 h with gentle shaking, and then washed in buffer B with four changes during 40 min. 125I-labeled *Staphylococcus aureus* protein A (Amersham) was diluted to ca. 0.1 μCi/ml with buffer B. The washed sheets were incubated with the diluted 125I-protein A solution for 6 h at room temperature with gentle shaking and then washed in buffer B with four changes during 40 min. The washed sheets were dried and exposed to Kodak X-Omat AR films with an intensifying screen (Lighting-Plus, Dupont).

**Preparation of hook-filament complexes of a flagellate strain.**

The hook-filament complexes of SJW1103 (Fla+) were removed from cells by blending and isolated by differential centrifugations under the conditions used for the isolation of hooks as described in our earlier paper (14).

The isolated hook-filament complexes were fragmented with a sonicator (Ohtake Works, Japan) 8 times for 5 s each at 80 W at 0°C. The resultant suspension was loaded onto a DEAE-cellulose (Whatman DE52) column, and the column was eluted with a linear gradient of 0.04 to 0.3 M NaCl as described previously (14).

**TABLE 1. *Salmonella* strains used**

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<th>Strain</th>
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<td><em>H</em>1 <em>H</em>2-1, 2 <em>vh</em>2+</td>
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<td><em>H</em>1-gt <em>H</em>2-exn <em>vh</em>2− (<em>H</em>2-off)</td>
<td>10</td>
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<td>10, 13</td>
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<td><em>Δ</em> (flaL- <em>flaL</em>) of SJW1103</td>
<td>14</td>
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<td><em>flaL</em>2087 of SJW1103</td>
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<td><em>flaV</em>2381 of SJW1103</td>
<td>14</td>
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<td><em>flaW</em>2391 of SJW1103</td>
<td>13, 14</td>
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<td><em>flaV</em>: <em>Tn</em>10 of LT2</td>
<td>14</td>
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<td><em>H</em>1-:: <em>Tn</em>10 of SJW1103</td>
<td>This study</td>
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**FIG. 1. Immunodiffusion test between HAPs in hooks of filamentless mutants and antibody against each HAP.** Hooks used as antigen were isolated from the filamentless mutants: A, SJW800 (Fla−); B, SJW2150 (Fla−); C, MH101 (FlaU−); D, SJW2160 (FlaU−). The hooks were dissociated by heating at 70°C for 10 min. Antibodies: 1, antiHAP1 antibody; 2, antiHAP2 antibody; 3, antiHAP3 antibody. The precipitin bands were stained by Coomassie brilliant blue R250.

**FIG. 2. Electron micrographs of hooks treated with antiHAP2 antibody.** Hooks were isolated from the filamentless mutants: A, SJW900 (H1− H2−); B, SJW800 (Fla−); C, SJW1448 (Fla−); D, MH111 (H1− H2−). Hooks were negatively stained with 1% potassium phosphotungstate. Arrows indicate antibody aggregates. Bar, 50 nm.
Immunoelectron microscopy. Antibody labeling was carried out as described in our earlier paper (14). The labeled samples were negatively stained with 1% potassium phosphotungstate (pH 7.2).

The specimens were observed in a JEM100C electron microscope (JEOL).

RESULTS

Reaction specificity of the antibody against each HAP. HAPs used for antibody production were prepared from hooks of a flaL mutant, SJW800, which contain all HAPs, namely, HAP1, HAP2, and HAP3 (14). HAPs were separated from the hook protein and each other on an SDS-PAGE gel. Then the stained bands of HAPs were cut out from the gel and used directly for immunization to minimize a loss of HAPs as much as possible, because the amounts of HAPs are very small, i.e., ca. one-tenth of the amount of hook protein in the hook structure (14).

The prepared antibodies were tested by immunodiffusion for examination of the specificity among them. Hooks from the flaV, flaU, flaW, or flaL mutant were dissociated by heating at 70°C for 10 min and used for double-diffusion tests in an agar gel. The compositions of HAPs in the hooks from each fla mutant were consistent with the precipitation specificities of the three kinds of antibodies (Fig. 1). This shows that the prepared antibodies have specific reactivities useful for the identification of three different HAPs.

Immunoelectron microscopic observation of hooks. We tried to show locations of HAPs in the hook structure. First, hooks of the various mutants were treated with the antibody against HAP2 and observed by electron microscopy (Fig. 2). Only the tips of the claw-shaped ends of hooks, i.e., their distal ends from the flaL or H1 H2 mutant, were covered with the antibody, which bound to the very tops of the hooks. Interestingly, the tips of hooks from some flaL or H1 H2 mutants were not covered with it. The reaction-positive mutants had HAP2 with an MW of 53,000 on SDS-PAGE, which was different from that of the reaction-negative mutants, i.e., 48,000, (data not shown). All of the reaction-negative mutants were isolated from SJW1103, which had the i-type flagellin gene, whereas the parent of the reaction-positive mutants had the gt-type flagellin gene introduced by P22-mediated transduction from Salmonella sp. SJ925. The phase 1 flagellin gene, H1, is closely linked to flaV (S. Yamaguchi, personal communication), which is inferred to be the structural gene for HAP2 (M. Homma, K. Kutsulake, and T. Iino, manuscript in preparation). Therefore, HAP2 of the reaction-negative mutants may have derived from Salmonella sp. SJ925, and its antigenic specificity might be different from that of the reaction-positive mutants from which the antibody was prepared.

When hooks were treated with the antibody against HAP1 (Fig. 3), the distal ends of hooks from the flaL, flaU, or H1 flaV flaL mutant were covered with the antibody. However, no parts of hooks from the flaW mutant covered with it because of the absence of HAP1.

When hooks were treated with the antibody against HAP3 (Fig. 4), the distal ends of hooks from the flaL, H1 H2, or flaV mutant were covered with the antibody but no parts of hooks from the flaU mutant were. These results show that
FIG. 5. Disassembly of HAPs by heat treatment of hooks. Hooks were isolated from (A) SJW800 (FlaL−); (B) SJW1411 (H1− H2−, FlaL−, FlaV−); (C) KK2078 (FlaU−). The hooks were incubated in a sample solution containing 0.1% SDS for 10 min at: lanes 1, 10°C; 2, 20°C; 3, 40°C; 4, 90°C. The resultant 50-μl suspensions were analyzed by electrophoresis in a 10% polyacrylamide gel at 10°C. The arrows on the right side show the positions of MW markers: bovine serum albumin (68,000 [68K]), ovalbumin (43K), and chymotrypsinogen (25.7K).

HAP1, HAP2, and HAP3 are all located on the distal ends of hooks.

Careful observations disclosed different profiles of antibody binding among the different filamentless mutants. On hooks of the flaL mutant containing all three HAPs, the antibody against HAP1 bound to their distal ends except for the very tops of their claw-shaped ends. On the other hand, the whole distal ends of hooks of the flaU or H1 flaV flaL

FIG. 6. SDS-PAGE of DEAE-cellulose fractions of hook-filament complex. Samples (50 μl) from DEAE-cellulose fractions were analyzed by electrophoresis in a 12% polyacrylamide gel.
mutant were covered with the antibody. The amount of antibody against HAP1 aggregated on each top was less in the flaL mutant than in the flaU or HI flaV flaL mutant. These different profiles of antibody binding in the distal end may reflect arrangements of HAPs on the distal end.

Disassembly of HAPs by heat treatment of hooks. Disassembly of HAPs by heat treatment of hooks was examined by analysis of SDS-PAGE. Because the hook is a supramolecular structure, it cannot move in a polyacrylamide gel. Conversely, the monomeric proteins dissociated under conditions of low pH or high temperature can move in the gel. HAPs were disassembled without a heat treatment in a sample solution for SDS-PAGE containing a strong detergent such as 1% SDS. Therefore, the concentration of SDS in the sample solution was decreased to 0.1%. Hooks were suspended in the sample solution, and the suspensions were incubated for 10 min at 10, 20, 40, or 90°C. The disassembled proteins were analyzed by SDS-PAGE in a chromatocam- ber at 10°C (Fig. 5). The results indicate that the hook structure was completely dissociated under heat treatment at 90°C and that HAP2 and HAP3 were disassembled from the hook structure under treatment at 10°C. Almost all of the HAPs and 19% of the hook protein, as compared with absorbances of the stained bands of the completely disassembled hook protein and HAPs at 90°C, were detected on the separation gel after treatment at 40°C.

Based on these results, the order of disassembly of the component proteins by heat treatment of the hook was inferred to be (HAP2, HAP3) > HAP1 > hook protein.

Detection of HAPs in the hook-filament complexes of a flagellate strain. Hook-filament complexes were isolated from SJW1103 (Fla+) and fragmented by sonication to decrease the filament length and viscosity. The resultant suspension was loaded on a DEAE-cellulose column and eluted with a linear gradient of NaCl. The eluted fractions were analyzed by SDS-PAGE (Fig. 6). The large amount of flagellin and the lesser amount of hook protein were detected on the SDS-PAGE gel. Moreover, two kinds of proteins that copurified with the hook protein were detected on the gel with the same mobilities as HAP1 and HAP3. The agar on the gel at which HAP2 was expected to be present was covered by the large amount of flagellin. We examined whether or not the proteins specifically reacted with the antibodies against HAPs. After proteins in fraction 17, which seemed to contain more HAP1 and HAP3 than the others, were separated by SDS-PAGE, the proteins were transferred onto a nitrocellulose sheet from the gel. The transferred proteins were incubated with each antibody against HAP1, HAP2, or HAP3. The antibodies were labeled with 125I-protein A, and the proteins specifically bound to the antibodies were detected by autoradiography (Fig. 7). The proteins with the same mobility as HAP1 and HAP3 on the SDS-PAGE gel were specifically reacted with the antibodies against HAP1 and HAP3, respectively. The antibody against HAP2 specifically reacted with the protein whose MW was equivalent to that of HAP2 as determined by SDS-PAGE. These results indicate that the hook-filament complexes from the flagellate strain contain all three HAPs.

Immunoelectron microscopic observation of hook-filament complexes. We showed above that all three HAPs were located on the distal ends of the hooks in the filamentless mutants. We examined the location of HAPs in the hook-filament structure of the Fla+ strain in which all three HAPs were detected. The hook-filament complexes in fraction 17 (Fig. 6) were treated with each of the antibodies against HAPs under the conditions used for the immunoelectron microscopy of the mutant hooks. However, the bound antibodies were not observed in the hook-filament structure by electron microscopy. Then the hook-filament structures treated with the antibody against HAP1 were incubated, after the excess antibody was washed, with the second antibody, i.e., anti-rabbit immunoglobulin G (IgG) antibody, to enhance the aggregation enough for electron microscopic observation. Owing to the second antibody, aggregates of the bound antibodies were observed by electron microscopy in the region corresponding to the boundary between filament and hook (Fig. 8). The aggregates were not observed in the hook-filament structure treated with the antibodies against HAP2 and HAP3, even after the treatment with the second antibody. Nor were they observed in the hook-filament structure of SJW797, which has flagellin with gt-type antigen and HAP2 with the same molecular weight as that used in the antibody preparation, after treatment with both antibody against HAP2 and the second antibody (data not shown). These observations indicate that at least HAP1 is located in the region between filament and hook; the locations of HAP2 and HAP3 in the hook-filament structure were not clear from our results.

DISCUSSION

We reported earlier (14) that hooks which contain all three HAPs, namely, HAP1, HAP2, and HAP3, have sharp, claw-like tips, whereas hooks lacking any HAPs have flat tips. When hooks were treated with antibody against hook
protein, each claw-shaped end was not covered with the antibody. The previous study suggested that all three HAPs, or at least some of them, are located in the distal ends of the hooks. In the present study, we prepared antibody against each HAP, which as antigen was separated on an SDS-PAGE gel and then cut out from the gel. The antibodies had specific reactivities useful for the identification of three different HAPs (Fig. 1). By immuno electron microscopic observation, these antibodies were found to bind on the distal ends of the hooks from the filamentless mutants. The antibody-binding profiles of the hooks from the filamentless mutants were consistent with their composition of HAPs (Fig. 2 to 4). This means that all of the HAPs are located in the distal ends of hooks. Thus, the presumption on the localization of HAPs in our previous study (14) was confirmed by the present study.

Then the question was raised of how the three kinds of HAPs are arranged on the distal end of the hook. Based on the following observations, we propose the layered structure model (Fig. 9): HAP1, HAP3, and HAP2 are assembled at the distal end of the hook in this sequence. When hooks of the flaL or H1 H2 mutant were treated with antibody against HAP2, the antibody bound to their very tops (Fig. 2). When hooks of the flaL mutant were treated with antibody against HAP1, the antibody bound to their distal portions except for the very tops of their claw-shaped ends. The aggregate of antibody against HAP1 on each top of the hook is less in the flaL mutant than in the flaU or the H1 flaV flaL mutant. Because hooks of the flaU or the H1 flaV flaL mutant do not contain HAP2, these different profiles of antibody binding may mean that the HAP2 molecules cover a part of the antigen determinants of HAP1. When hooks of the flaL or H1 H2 mutant were treated with antibody against HAP3, the antibody seemed to bind to the middle area of the claw-shaped portions. These observations may indicate that HAP3 is located in a region between HAP2 at the top and HAP1 at the bottom in the claw-shaped distal portion.

The order of disassembly of the component proteins by heat treatment of the hook structure from the filamentless mutants was (HAP2, HAP3) > HAP1 > hook protein. Based on our layered structure model (Fig. 9), the most probably explanation for this order is that HAP1, HAP3, and HAP2, which had assembled at the distal end of the hook in this sequence, were disassembled from the top of the claw-shaped tip of the hook by the heat treatment.

We reported previously (14) that hooks from the H1 H2 or the flaL mutant contained HAP1, HAP2, and HAP3; hooks from the flaV mutant contained HAP1 and HAP3; hooks from the flaU mutant contained HAP1; and hooks from the flaW mutant contained a very small amount of HAP3. From these observations, we inferred the process of hook morphogenesis and the responsible genes as follows: (i) the presence of HAP1 on the hook is essential for the attachment of HAP3 to the hook structure; (ii) HAP3 is essential for the attachment of HAP2; and (iii) flaV, flaU, and flaW are responsible for the production of HAP2, HAP3, and HAP1, respectively. This assembly process is entirely conformable with the layered structure model (Fig. 9).

All three HAPs were detected in the hook-filament complexes prepared from a Fla' strain (Fig. 6 and 7). Then the question arose of how the three kinds of HAP are arranged in the hook-filament structure. When the hook-filament structure was treated with antibody against HAP1 and then with the second antibody, i.e., anti-rabbit IgG antibody, the antibody aggregate was observed in the region corresponding to the boundary between filament and hook by electron microscopy (Fig. 8). This observation indicates that HAP1 is located in the region between filament and hook, and also strongly suggests that HAP1 is the protein connecting filament with hook. The locations of HAP2 and HAP3 in the hook-filament structure were not clarified with the same procedure. This may be because either the antigenic sites of HAP2 and HAP3 located at the distal ends of the hooks are concealed from the surface in the hook-filament structure, or the location of HAP2 or HAP3 or both is dispersed in the filament structure in the process of intact flagellum formation.

By the use of three-dimensional images from electron micrographs, the S. typhimurium hook and filament structure was investigated (22, 23, 25). From these investigations, Wagenknecht et al. (25) hypothesized that the two structures conjoin directly in the intact flagellum, although participa-

FIG. 8. Electron micrographs of hook-filament complex treated with antiHAP1 antibody and the second antibody, i.e., anti-rabbit IgG antibody. Complexes were negatively stained with 1% potassium phosphotungstate. Arrows indicate antibody aggregates. Bar, 100 nm.

FIG. 9. Layered structure model of HAPs in HOB (hook-basal body).
tion of a minor protein is not ruled out. Our results demonstrated that a minor protein actually participates in the conjoining.

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LITERATURE CITED


