FtsH, a Membrane-bound ATPase, Forms a Complex in the Cytoplasmic Membrane of Escherichia coli*

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The FtsH (HflB) protein of Escherichia coli is integrated into the membrane with two N-terminally located transmembrane segments, while its large cytoplasmic domain is homologous to the AAA family of ATPases. The previous studies on dominant negative ftsH mutants raised a possibility that FtsH functions in multimeric states. We found that FtsH was eluted at fractions corresponding to a larger molecular weight than expected from monomeric structure in size-exclusion chromatography. Moreover, treatment of membranes or their detergent extracts with a cross-linker, dithiobis(succinimidylpropionate), yielded cross-linked products of FtsH. To dissect possible FtsH complex, we constructed an FtsH derivative with c-Myc epitope at its C terminus (FtsH-His$_6$-Myc). When membranes prepared from cells in which FtsH-His$_6$-Myc was overproduced together with the normal FtsH were treated with the cross-linker, intact FtsH and in vitro degradation products of FtsH-His$_6$-Myc without the tag were cross-linked with the tagged FtsH protein. Co-immunoprecipitation experiments confirmed the interaction between the FtsH molecules. To identify regions of FtsH required or sufficient for this interaction, we constructed chimeric proteins between FtsH and EnvZ, a protein with a similar topological arrangement, by exchanging their corresponding domains. We found that only the FtsH-EnvZ hybrid protein with an FtsH-derived membrane anchoring domain and an EnvZ-derived cytoplasmic domain caused a dominant ftsH phenotype and was cross-linked with FtsH. We suggest that the N-terminal transmembrane region of FtsH mediates directly the interaction between the FtsH subunits.

Escherichia coli FtsH (HflB) protein belongs to a novel ATPase family whose members are widely found among eukaryotic and prokaryotic organisms (1). They all have one or two copies of the conserved regions of about 200 amino acid residues that include a set of ATP binding consensus motifs (2). They are suggested to be involved in diverse cellular functions such as regulation of cell cycle, vesicular transport in protein secretion, biogenesis of organelles, nuclear division, regulation of transcription, and protein degradation (2). This protein family is called AAA (ATPases associated with a variety of cellular activities) (3). However, their modes of involvement in the above mentioned cellular processes are mostly unclear. Even ATPase activities have been demonstrated only for a few of them (4–6). Their localizations in the cell are also diverse; some are bound to the plasma or the organella membrane, but many others are soluble proteins (2).

We previously showed that mutational impairments of the ftsH gene of E. coli caused an Std phenotype in which a normally cytoplasmic reporter PhoA$^*$ domain of a model membrane protein (SecY-PhoA) was exported to the periplasmic space (7, 8). Since the Std phenotype signifies insufficient anchoring of the transmembrane segment that precedes the reporter domain, we suggested that FtsH is involved in the process of protein assembly into the membrane. We also found that a decreased cellular content of the FtsH protein resulted in a strong Std phenotype and an impaired translocation of some secreted proteins (Sec phenotype) (7). Therefore, FtsH might have a role in protein export as well. Additionally, we found that the expression of C-terminally truncated forms of FtsH or ATP binding site mutants of FtsH from a plasmid caused the Std and Sec phenotypes dominantly (8). The existence of dominant negative alleles of ftsH raises a possibility that FtsH may function in multimeric states.

This study was aimed at clarifying the quaternary structure of FtsH in the cell. We showed that FtsH in the wild-type cells exists as a complex. Co-immunoprecipitation and cross-linking experiments using a Myc epitope/His$_6$-tagged FtsH revealed that the FtsH molecules interact with each other. A series of chimeric proteins between FtsH and EnvZ were constructed, and cross-linking experiments using them showed that the FtsH-EnvZ association required the N-terminal membrane association region but not the cytoplasmic domain.

MATERIALS AND METHODS

Bacterial Strains and Media—E. coli K12 strains AD21 (9) and MC4100 (10) were described previously. AD202 (11) was an ompT$^+$::kan derivative of MC4100, and CU141 (7) was an F$^+$ lac$^+$ derivative of MC4100, respectively. TYE024 (MC4100, ompT$^+$::kan$^+$ lacI$^q$) was constructed by introducing F$^+$ lacI$^q$ of CU141 into AD202 by conjugation. L medium (12), peptone medium (13), and M9 medium (10) were used. Media containing ampicillin (50 µg/ml) and/or chloramphenicol (20 µg/ml) were used for growing plasmid-bearing strains.

Construction of the ftsH-his$_6$-myc Plasmids—pSTD101 carrying ftsH-his$_6$-myc was constructed as follows. pSTD40 in which a mutant ftsH gene (the ftsH40 allele) was placed under the lac promoter/operator was described previously (7). The 2.7 kb EcoRI-PstI fragment of pSTD40 which was blunt-ended by treatment with T4 polymerase and cloned into Smal site of a pBlueScript SK($-$) (Stratagene) derived vector, pTVE007, which carried a sequence encoding a bipartite His$_6$/c-Myc tag

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1. The abbreviations used are: PhoA, alkaline phosphatase; kb, kilobase pair(s); DSP, dithiobis(succinimidylpropionate); OG, 1-0-0ctylβ-D-glucopyranoside.

2. Although we described previously that pSTD40 carried the wild-type ftsH gene (7), and subsequently found that the ftsH gene in pSTD40 and, as a result, that in pSTD101 contained a spontaneously introduced mutation (ftsH40) that causes a Thr$^{199}$ → Ala substitution. The ftsH40 form of the gene can complement ftsH$^+$::kan$^+$ (7) but not ftsH1(ts) (20), suggesting that this mutation lowers the FtsH activity to a small extent.
of 30 amino acid residues (EIEGRHHHHHHIDEEKLIQSEEDLLRKR) following its multiclone site.\textsuperscript{3} The ftsH gene and the his-s\textsubscript{myc} sequence on the resulting plasmid were fused in frame by site-directed mutagenesis according to Kunkel et al. (14) using a mutagenic primer (5'-TGTCAGACGCTGGCATGGAAGGAAATTGTCAGACGCTGGCAGCGCTGACCA-3'). pSTD113 (carrying ftsH-his-s\textsubscript{myc}) was constructed by replacing the 1.5-kb Saml fragment of pSTD101 by that of pSTD401, which had the same structure as pSTD400 except that it carried the wild-type ftsH gene. pSTD120 was constructed by inserting the 2.8-kb Xbal-KpnI fragment that contained the entire region of ftsH-his-s\textsubscript{myc} into the Xbal-KpnI site of pMW119 (Nippon gene), a pSC101-derived low copy number vector.

Constructions of Hybrid Genes between ftsH and envZ—envZ hybrid gene was constructed by site-directed mutagenesis as follows. First, a 0.8-kb Xbal-EcorF fragment of pAT2005S (15) carrying the envZ gene was ligated with pSTD113 that had been digested with BamH1, blunt-ended by treatment with T4 polymerase, and then digested with Xbal. Then, the region encoding the membrane anchoring domain (from the amino terminus to the 179th amino acid residue) of EnvZ was prepared by disruption of cells by sonication followed by ultracentrifugation. Cells were grown in M9 medium and pulse-labeled for 5 min. Membrane proteins suspended in buffer C and solubilized with OG in the presence of 5\% Triton X-100 were precipitated with 5\% trichloroacetic acid, separated by 15\% acrylamide, 0.12\% bisacrylamide polyacrylamide gel electrophoresis (9) and subjected to immunoblotting with anti-FtsH. The products of FtsH were also generated when solubilized membrane proteins were treated with 15\% acrylamide, 0.4\% glucose, and appropriate antibiotics. After 10 min of induction with isopropyl-1-thio-\beta-D-galactopyranoside (1 mM) and cAMP (5 mM), cells were pulse-labeled for 30 s with about 0.37 MBq/ml [35S]methionine followed by chasing with 200 \mu Ci/ml of [\textsuperscript{35}S]methionine for 15 min. Cross-linking of FtsH in Membranes and in Detergent Extracts—Cells were grown in M9 medium supplemented with 18\% glucose (10\% more than M2), 100 \mu g/ml chloramphenicol, and 0.4\% glucose, and appropriate antibiotics. After 10 min of induction with isopropyl-1-thio-\beta-D-galactopyranoside (1 mM) and cAMP (5 mM), cells were pulse-labeled for 30 s with about 0.37 MBq/ml [\textsuperscript{35}S]methionine followed by chase with 200 \mu Ci/ml of [\textsuperscript{35}S]methionine for 15 min. Cross-linking of Membrane Proteins with DSP—Total membranes were prepared as above. For solubilization, total membranes were treated with OG as described above except that Tris was not included and that pH of Hepes-KOH was 7.5 instead of 7.0. For cross-linking, either total membranes or their OG extracts were treated with 0.25 mg/ml DSP, a membrane permeable cross-linker, at 4°C for 1 h, and the reaction was terminated by the addition of 0.2 M ammonium acetate followed by incubation at 4°C for 10 min. Control samples received 0.2 M ammonium acetate prior to the addition of DSP. Samples were adjusted to 1\% SDS, incubated at 37°C for 10 min and subjected to immunoprecipitation as described above. Precipitated proteins were dissolved in SDS sample buffer (20) without 2-mercaptoethanol at 37°C for 10 min before electrophoresis. For cleavage of the cross-linker, 10\% 2-mercaptoethanol was included in SDS sample buffer.

Trypsin Digestion and Immunoblotting—Cells were grown in peptide medium supplemented with appropriate antibiotics; rapidly chilled by mixing with Na\textsubscript{2}CO\textsubscript{3} (0.02\%), chloramphenicol (100 \mu g/ml), and a small piece of ice, and disrupted by lysozyme freezing-thawing (7). The cell lysates were treated with trypsin as described previously (7). Proteins were separated by 10\% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-PhoA (obtained from 5 Prime→3 Prime, Inc.), anti-FtsH or anti-EnvZ as described previously (7).

RESULTS
Size-exclusion Chromatography of the FtsH Protein—Our previous findings that FtsH can be mutated to dominant negative with respect to the Std and Sec phenotypes (8) suggested that FtsH may function in multimeric states. To directly examine higher order structures of FtsH, we solubilized the cytoplasmic membrane with OG and subjected the solubilized proteins to size-exclusion chromatography using Superose 6. FtsH was eluted with a peak at fractions 45–47 that corresponded to a molecular mass of about 280 kDa (Fig. 1, A and B), while its monomeric molecular mass should be 71 kDa. Although the value of 280 kDa determined by the calibration using soluble proteins should not be regarded as accurate, FtsH was eluted far faster than SecY, a major part of which had the molecular mass of about 50 kDa (Fig. 1, A and B). This form of SecY could either be a monomer (the molecular mass is 49 kDa) or in a form of SecY-SecE-SecG complex of estimated molecular mass of about 74 kDa.

Cross-linking of FtsH in Membranes and in Detergent Extracts—We addressed the subunit structure of FtsH by cross-linking experiments. The membranes prepared from wild-type cells were treated with DSP and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-FtsH. Treatment with DSP yielded products with molecular masses of about 240 and 140 kDa (Fig. 2A, lane 4) that were not observed without DSP treatment (lane 3) or after cleavage of the cross-linker with 2-mercaptoethanol (lane 2). Cross-linked products of FtsH were also generated when solubilized membrane proteins were treated with DSP (Fig. 2B, lane 4). Under the latter condition, however, the intensity of the 240-kDa species was much less than when intact membranes were cross-linked.

Cross-linking between the FtsH Proteins with and without an EpiTope Tag—To dissect the putative FtsH complex, we constructed an FtsH derivative with two tandemly located molec-
Ethanol and analyzed by 4% (ular tags, oligohistidine residues (His6), and a c-Myc-derived epitope at the C terminus (Fig. 3). The FtsH-His6-Myc protein was specifically isolated, and detected by nickel-nitrilotriacetic acid-agarose and anti-Myc antibodies, respectively. Cells were treated with anti-FtsH or anti-Myc antibodies. Anti-FtsH serum brought down two species of proteins (Fig. 4, lane 2). The upper band represented the tagged FtsH, since it was precipitated by anti-Myc antibodies as well (Fig. 2, lane 5). The lower band represented the normal FtsH, since it comigrated with the chromosomally encoded FtsH (lane 1) and did not cross-react with anti-Myc (lanes 4 and 5). FtsH-His6-Myc was stable in vivo; no degradation was observed during a 16-min chase period examined (lanes 2, 3, 5, and 6). The FtsH-His6-Myc protein was functional, since pSTD120 (a low copy plasmid carrying ftSH-his6-myc) complemented the temperature-sensitive ftsh1 mutation (20). It did not interfere with the cell growth. When pulse-labeled cells were disrupted by sonication and fractionated, most of FtsH-His6-Myc, like normal FtsH, was recovered in the membrane fraction (data not shown). We found that a fraction of FtsH-His6-Myc was cleaved in vitro by unknown proteases to a product (FtsH') slightly smaller than the authentic FtsH during the process of membrane preparation (see Figs. 5 and 6). The cleavage seemed to occur around the junction between FtsH and the His6-Myc tag, since FtsH' lost the Myc epitope (Fig. 5B, lane 5).

Cells of CU141 (F' lacIq) carrying both the ftSH-his6-myc plasmid (pSTD113) and the ftsh plasmid (pSTD401) were induced and pulse-labeled, and total membrane fractions were prepared. To minimize possible artificial effects resulting from overaccumulation of plasmid-encoded proteins, their synthesis was induced only for a short period (10 min) before pulse labeling in this and the following experiments. Membranes were treated with DSP, solubilized with SDS, and subjected to immunoprecipitation using anti-Myc or anti-FtsH antibodies. Samples were analyzed by SDS-polyacrylamide gel electrophoresis without (Fig. 5A) or following (Fig. 5B) cleavage of the cross-linker by 2-mercaptoethanol. Treatment of the membranes with DSP yielded high molecular weight cross-linked products that were immunoprecipitated with anti-FtsH (Fig. 5A, lane 1). Such cross-linked products were not detected when the cross-linker had been quenched by ammonium acetate (lane 2). When DSP was cleaved by 2-mercaptoethanol before electrophoresis, FtsH and FtsH' were recovered with anti-Myc antibodies (Fig. 5B, lane 1), whereas they were never recovered with anti-Myc without cross-linking (lane 3). The identities of FtsH and FtsH' were confirmed by recovery of these proteins by the second immunoprecipitation with anti-FtsH serum (Fig. 5C). These results suggested that more than two molecules of FtsH form a complex.

Immunoprecipitation of FtsH with FtsH-His6-Myc—We carried out immunoprecipitation under non-denaturing condi-

4 Although we used pSTD101 in the experiments described in Fig. 2 and Fig. 3, essentially the same results were obtained when pSTD113 (ftsH'-his6-Myc) was used.
Precipitated proteins were solubilized in SDS sample buffer with 6-mercaptoethanol. Precipitation with anti-FtsH antibodies (lanes 1-3) or anti-Myc (lanes 4-6), and analyzed by SDS-polyacrylamide gel electrophoresis.

Cross-linking (Fig. 2) and co-immunoprecipitation (Fig. 6) after precipitation with anti-FtsH or anti-Myc antibodies (see Introduction). Proteins were treated with SDS, and subjected to immunoprecipitation with anti-FtsH serum (lanes 1 and 2), anti-Myc antibodies (lanes 3 and 4), or normal serum (lane 5) in the presence or absence of the FtsH (lane 2) or Myc (lane 4) epitope peptides. Proteins were separated by SDS-polyacrylamide gel. FtsH* indicates the C-terminally-cleaved product of FtsH-His6-Myc.

Identification of the FtsH•FtsH Interaction Region Using Chimeras between ftsH and envZ—We then examined the roles of the two regions, the membrane-associated N-terminal region and the cytoplasmic C-terminal region, in the FtsH•FtsH interaction. We previously showed that an N-terminal fragment of FtsH caused a dominant Std effect. Thus, the N-terminal region of FtsH may be important for the subunit interaction of FtsH. To examine this possibility, we constructed chimeric genes between ftsH-his6-myc and envZ. The EnvZ protein is an E. coli inner membrane protein with FtsH-like topology (21). We constructed two kinds of chimeric genes encoding FtsH•EnvZ and EnvZ•FtsH-His6-Myc (Fig. 3). The FtsH•EnvZ chimeric protein consists of the FtsH-derived transmembrane domain and the EnvZ-derived cytoplasmic domain, whereas EnvZ•FtsH-His6-Myc has the EnvZ membrane domain followed by the tagged FtsH cytoplasmic domain.

These chimeric genes did not complement the ftsH1 mutation, indicating that both the membrane-bound and the cytoplasmic regions of FtsH are important for the FtsH functions. Cell fractionation experiments showed that these hybrid proteins are membrane-associated (data not shown).

We then examined whether the chimeric proteins cause a dominant Std phenotype (see Introduction). As the high level overexpression of these proteins from the plasmids used in the cross-linking experiments was found to be deleterious to cells, the fusion genes were recloned into a low copy number vector that is also compatible with the plasmid (pKY221) carrying the reporter secY-phoAC6 gene. Extracts of cells expressing either the chimeras, FtsH, or envZ, in addition to SecY-PhoAC6 fusion, were treated with trypsin and analyzed by immunoblotting with anti-PhoA antibodies (Fig. 7). The PhoA domain of SecY-PhoAC6 from the cells expressing FtsH•EnvZ resisted trypsin (Fig. 7A, lanes 7 and 8), indicating that it was exported to the periplasmic space. On the other hand, expression of the other three proteins, EnvZ•FtsH-His6-Myc (lanes 1 and 2), FtsH (lanes 3 and 4), or EnvZ (lanes 5 and 6) did not cause the Std phenotype. All of the above proteins evidently accumulated in the cells as shown by Western blotting with anti-FtsH or anti-EnvZ (Fig. 7B). These results suggested that among the above proteins, only FtsH*•EnvZ could interact with the chromosomally-encoded FtsH to interfere with its function.

Cells overexpressing FtsH and either FtsH•EnvZ, EnvZ•FtsH-His6-Myc, or EnvZ were pulse-labeled, and membranes were treated with DSP. Cross-linked products were examined by immunoprecipitation. Fig. 8A shows results of an experiment with FtsH•EnvZ. The anti-FtsH serum used in this study had been directed against a sequence in the cytoplasmic domain of FtsH (17). Thus, without cross-linking, the FtsH•EnvZ protein was immunoprecipitated with anti-EnvZ serum but not with anti-FtsH serum (lanes 5 and 6). The anti-EnvZ antibodies did not cross-react with FtsH (lane 6). After cross-
linking with DSP, FtsH-His6-EnvZ was recovered with anti-FtsH (lane 1), and FtsH was recovered with anti-EnvZ (lane 2). Quenching of DSP before cross-linking abolished these cross-reactions (lanes 3 and 4). On the other hand, EnvZ-His6-Myc was not cross-linked with FtsH, since FtsH was not precipitated with anti-Myc antibodies even after DSP treatment (Fig. 8B). As expected, no cross-linking was observed between FtsH and EnvZ (Fig. 8C). These results confirmed that FtsH-EnvZ can interact with FtsH but EnvZ-FtsH-His6-Myc cannot. The interaction between FtsH molecules is likely to be mediated by its membrane-associated region.

**DISCUSSION**

FtsH has been implicated to have diverse cellular activities. We suggested previously that FtsH is involved in integration/assembly of proteins through and/or into the membrane (7, 8). It was also found recently that FtsH is involved in rapid degradation of at least three short-lived proteins, ClpA and ClpX proteins, regulatory subunits of the Clp protease and distantly related to FtsH, do not have any proteolytic activity themselves. Instead, they are proposed to target substrate proteins for ATP-dependent degradation (26–28). It was also shown that ClpA functions as a molecular chaperone in replication of P1 plasmid or in vitro protein folding reactions (28, 29). Similarly, the AAA family includes some of regulatory ATPase subunits of proteasomes. They have been proposed to function in presentation of substrate proteins to the protease subunits, the process in which energy of ATP hydrolysis is somehow used (30). FtsH may be a multifunctional protein that exerts chaperone-like activities in the assembly or translocation of some cell surface proteins and degradation of some unstable proteins.

Oligomeric structure seems to be a common feature among the above mentioned ATPase subunits as well as other members of the AAA family. For example, (N-ethylmaleimidesensitive) functions as a homotrimer that interacts with many other proteins including SNAPs and SNAREs during process of vesicular transport in eukaryotic cells (31). P97 has also been proposed to be a homohexameric, although its function is not known (5). We have shown here that FtsH is in a complex that includes more than one molecules of FtsH. FtsH remains in high molecular mass state after solubilization in nonionic detergent. The solubilized FtsH could be cross-linked to form oligomeric structure and could be co-immunoprecipitated with the epitope-tagged version of FtsH. It is possible that the FtsH molecules are directly interacting with themselves.
The FtsH-EnvZ chimeric protein is cross-linkable with FtsH and causes dominant Std phenotype. We suggest that the dominant phenotype is at least partly a result of the formation of a nonfunctional FtsH complex containing wild-type and mutant molecules. The results with the hybrid proteins suggested that possible interaction between the FtsH molecules is mediated by direct association of their transmembrane regions. Several examples have been reported for inter- or intramolecular association of transmembrane segments (32–34). The ftsH101 mutation causes a change of Val32 to Met in the periplasmic region of FtsH (7). It did not affect the interaction between the FtsH molecules, implicating that the membrane domain is not only important for the oligomerization but may itself have some role in the FtsH functions.

It is not known how many FtsH molecules are present in the FtsH complex and whether any other proteins are associated with it. The major cross-linked products of 140 and 240 kDa might represent dimer and tetramer of FtsH. In addition, no other major proteins were found in the preparation of FtsH that was purified from overproducing strains (6). These results, however, do not exclude the possibility that the physiological complex of FtsH contains additional components. Preliminarily, two proteins of 27 and 16 kDa were found to co-immunoprecipitate with anti-FtsH antibodies. The 27-kDa protein was co-immunoprecipitated even after treatment of the membrane with urea.

Elucidation of the complete structure of the FtsH complex awaits purification of the physiological complex from wild-type cells. The present results showing that FtsH molecules can associate with each other even when they are exclusively over-produced (Figs. 5 and 6) will provide an important guidance for further biochemical characterization of this intriguing membrane protein.

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