FtsH (HflB) Is an ATP-dependent Protease Selectively Acting on SecY and Some Other Membrane Proteins*

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The FtsH protein is a membrane-bound ATPase of Escherichia coli that was proposed to be involved in membrane protein assembly as well as degradation of some unstable proteins. SecY, a subunit of protein translocase, is FtsH dependently degraded in vivo when it fails to associate with its partner (the SecE protein). We constructed a series of mutants in which mutations were introduced into conserved residues in the two ATP binding consensus sequences or the zinc binding sequence of FtsH. We purified wild-type and mutant FtsH proteins by making use of a polyhistidine tag attached to their carboxyl termini. Complementation analysis and ATPase activity assays in vitro indicated that, of the two sets of ATP binding sequence motifs, the one located C-terminally (A1) is essential for ATPase activity and in vivo functioning of FtsH. Wild-type FtsH protein degraded purified SecY in an ATP hydrolysis-dependent manner in vitro. Mutant proteins without ATPase activity were inactive in proteolysis. A zinc binding motif mutant showed a decreased proteolytic activity. SecY and FtsH were cross-linkable with each other in the membrane, provided that FtsH had an ATPase-inactivating mutation. These results demonstrate that FtsH binds to and degrades SecY, its A1 motif and the zinc binding motif being important for the proteolytic activity. FtsH-dependent proteolysis was also demonstrated for SecY in crude membrane extracts, whereas a majority of other membrane proteins were not degraded, indicating that FtsH has high selectivity in protein degradation.

We previously reported that a membrane-bound ATPase, FtsH (HflB), is required for the degradation of the uncomplexed forms of SecY. In mutants with compromised FtsH functions, both overproduced SecY protein and the SecY24 mutant protein are stabilized (7). FtsH contains two transmembrane segments N-terminally located and a large cytoplasmic domain (8, 9). The cytoplasmic domain of FtsH includes a region that is homologous to the AAA ATPase family members involved in a variety of cellular processes (10). In addition, the cytoplasmic domain contains a region homologous with a zinc protease sequence motif (11). The transmembrane region is required for the oligomeric structure of FtsH (9). The hflB mutation, known to increase lysogenization frequency of bacteriophage λ by stabilizing the cII protein, proved to be an allele of the ftsH gene (12). Recently, an ATP-dependent protease activity of FtsH was described (11). It is involved in the degradation of an intrinsically unstable heat shock transcription factor, σ32 (11, 13). It was shown that FtsH homologs (Yta10, Yta12, and Osd1/Yme1) in the yeast mitochondria also participate in degradation of some inner membrane proteins (14–18). However, their proteolytic activities have not been characterized in vitro using purified proteins.

In this work, we characterized FtsH with respect to its activities to hydrolyze ATP and to degrade the SecY protein. The results indicate that FtsH has an ATP-dependent protease activity with high substrate specificity.

MATERIALS AND METHODS

Bacterial Strains and Media—CU141 (MC4100 carrying F lacP) (19, 20) and its ompT::kan derivative, TYE024 (9), were described previously. AD367 (MC4100, zhi-6198::Tn10kan zhd-33::Tn10 F lacP) and AD368 (MC4100, ftsH1::Tn10 zhi-6198::Tn10kan zhd-33::Tn10 F lacP) were constructed by introducing F lacP from CU141 into AR796 and AR797 (7), respectively, by conjugation. AD373 was constructed by introducing the ftsH1 mutation into AD202 (MC4100, ompT::kan) (21) by joint P1 transduction with zhu-6::Tn10. An E. coli B strain, AD432, was constructed by introducing hflA::kan (22) into E. coli (BL21(DE3) lacZ::Tn10 malP534a::P5lacZ::RBS+::ams) (23) by P1 transduction.

L medium (19) and M9 medium (19) were used. Ampicillin (50 µg/ml) and chloramphenicol (20 µg/ml) were included in media for growing plasmid-bearing strains.

Plasmids—The ftsH variants on plasmids are summarized in Fig. 1. pSTD113 and pSTD101 were derivatives of pBlueScript SK− (Stratagene) and carried ftsH−his6-myc and ftsH40-his6-myc, respectively, under the lac promoter-operator control (9). pSTD108 (carrying ftsH41) and pSTD129 (ftsH51) were constructed by replacing the 1.3-kb1 SpaI fragment or the 1.1-kb ApaI-MluI fragment of pSTD113 by the corresponding fragment of the ftsH41 gene on pSTD41 (24) or the ftsH51 gene on pSTD51 (24), respectively. pSTD131 (ftsH61) and pSTD132 (ftsH71) were constructed by replacing the 1.5-kb KpnI fragment of pSTD129 by the corresponding fragment of pSTD108 or pSTD113, respectively.

pSTD143 (ftsH81) and pSTD128 (ftsH62) were constructed

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1 The abbreviations used are: kb, kilobase pair(s); NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; STD, stop transfer-defective; DTT, dithiothreitol.
by introducing a mutation causing a Thr199 to Asn change to pSTD113 or a 1.5-kb PstI fragment of pSTD128 by the corresponding fragment of pSTD143 or pSTD113, respectively, were grown in 1 liter of L broth supplemented with 50 μg/ml ampicillin, isopropyl-D-thiogalactopyranoside (1 mM), and cyclic AMP (1 mM) at 37°C for 6 h. The membrane fraction was prepared by sonically disrupting cells followed by ultracentrifugation (29) in which all the buffers contained 10 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. Membranes were suspended in 25 ml of 0.1 M Tris/HCl, pH 8.1, 300 mM KCl, 50 mM NaCl, 1 mM DTT, 10% glycerol, a trace amount of bromphenol blue). The SecY protein was solubilized at 0°C for 1 h. After removal of insoluble materials by centrifugation, 45 μl of supernatant was mixed with 5 μl of 1 M ATP (final concentration, 2.5 mM), and cyclic AMP (1 mM) at 37°C for 1 h. After incubation, the sample was mixed with 2× SDS-PAGE sample buffer (50 mM Tris/HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, a trace amount of bromphenol blue) and subjected to SDS-PAGE and a BAS2000 imaging analyzer.

RESULTS

Phenotypes of ATP Binding Site Mutants—We noted previously (9) that FtsH has two sets of ATP binding consensus sequences (Walker A and B motifs) in its cytoplasmic region, one (A1 and B1) within the region homologous to the AAA family members (10) and the other (A2 and B2) located more N-terminally (Fig. 1). A mutation that changes Lys198 in A1 (K198N; see Ref. 24) plasmids did not complement the weakly. Thus, the importance of the A1 motif for the FtsH ATPase activities of the wild type and mutant forms of FtsH-His6-Myc preparation (220 or 930 ng of proteins were used as indicated in the legends to Figs. 3 and 4), 1 μl of 0.1 M ATP or other nucleotides (final concentration, 3.3 mM), and 13 μl of 2 × ATPase buffer. The mixture was incubated at 42°C. Samples (8.5 μl) were mixed with the same volume of concentrated SDS sample buffer (125 ml Tris/His, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, a trace amount of bromphenol blue). The SecY protein was separated by SDS-PAGE and detected by immunoblotting using a mixture of antisera against N-terminal and C-terminal regions of SecY (32) and the ECL detection kit (Amer sham Corp.) as described previously.

In Vitro Degradation of SecY by FtsH

In vitro degradation of SecY by FtsH was assayed according to Chan et al. (30). 10 μl of a sample (containing 0.2−2 μg of purified protein) were mixed with 13 μl of 0.5% Nonidet P-40, 4 μl of H2O, 3 μl of 20 mM ATP, and 30 μl of 2 × ATPase buffer (100 mM Tris/His, pH 7.5, 5 mM Tris acetate, 10 mM MgCl2, 50 μM zinc acetate, 2 mM DTT) and incubated at 37°C for 1 h. Then, it was mixed with 240 μl of the malachite green/polyvinyl ammonium molybdate reagent and 50 μl of 34% Na2 citrate/EDTA. After 30 min at room temperature, absorbance at 650 nm was measured.

In Vitro Degradation of Purified SecY Protein by FtsH-His6-Myc—Purified SecY in 50 mM potassium phosphate, pH 7.5, containing 150 mM NaCl, 2.5% 1-O-n-actyl-b-glycosylceramide, and 10% glycerol (475 μg/ml (31)) was diluted 100-fold with 1 × ATPase buffer (50 mM Tris HCl, pH 7.5, 2.5 mM Tris acetate, 5 mM MgCl2, 50 μM zinc acetate, 1 mM DTT). Typically, 4 μl of diluted SecY (containing 19 ng of protein) was mixed with 2.5 μl of purified FtsH-His6-Myc preparation (220 or 930 ng of proteins were used as indicated in the legends to Figs. 3 and 4), 1 μl of 0.1 M ATP or other nucleotides (final concentration, 3.3 mM), and 13 μl of 2 × ATPase buffer. The mixture was incubated at 42°C. Samples (8.5 μl) were taken at 0, 30, and 60 min and mixed with the same volume of concentrated SDS sample buffer (125 mM Tris/HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, a trace amount of bromphenol blue). The SecY protein was separated by SDS-PAGE and detected by immunoblotting using a mixture of antisera against N-terminal and C-terminal regions of SecY (32) and the ECL detection kit (Amer sham Corp.) as described previously.

Cross-linking—Cross-linking of membrane proteins with 0.25 mg/ml dithiobis(succinimidylpropionate) was carried out essentially as described (9). Anti-SecY, anti-FtsH (8), and anti-c-Myc (Ab-1) (Oncogene Science, Inc.) antibodies were used for immunoprecipitation.
In Vitro Degradation of SecY by FtsH

**FIG. 1. Summary of the FtsH variants used.** The FtsH protein is schematically represented, with A (A1 and A2) and B (B1 and B2) indicating Walker’s ATP-binding motif A and B, and TM1 and TM2, indicating transmembrane segments. One unit of ATPase activity corresponds to release of 1 μmol of P_i/mg protein/hour. ND indicates not determined. +, –, and (+) in the column of complementation activity indicate normal, no, and slow growth of the ftsH1 mutant cells at 42°C. The nomenclatures of the mutated ftsH genes are given at the left.

ftsH84-bearing cells was slightly slower than normal. When the weak A1 mutation, ftsH81, was combined with the ftsH84 mutation, the resulting gene, named ftsH83 (T199N and S137N), proved to totally lack the complementation activity. The synthetic effects of the A1 and A2 mutations may suggest that the A2 region has some functional role in FtsH. The mutant forms of ftsH that did not complement the ftsH1 mutant all exhibited a dominant Std phenotype (data not shown), suggesting that they interfered with the functioning of the wild-type FtsH protein.

**ATPase Activities of the Mutant Proteins**—We introduced each of the ATP binding site mutants of ftsH into the His<sub>6</sub>- and Myc-tagged version of the ftsH gene (on pSTD113). Immunoblotting experiments (data not shown) showed that these FtsH derivatives accumulated in the membranes, except for FtsH51 and FtsH 61 that proved to be toxic to the cells under the induction conditions. For purification of accumulated FtsH-His<sub>6</sub>-Myc proteins, membranes were solubilized with a non-ionic detergent, Nonidet P-40, and subjected to affinity purification using Ni<sup>2+</sup>-NTA-Sepharose or Ni<sup>2+</sup>-NTA silica, as described under “Materials and Methods.” Preparations of 50–80% purity (with respect to the full-length proteins) were obtained (Fig. 2). Some of them contained a slightly lower molecular mass protein, which should have been generated by proteolytic cleavage around the junction between FtsH and the bipartite tag during purification. We previously characterized a similar product termed FtsH<sup>TM1</sup> (9). The proteolytic product without the tag was recovered presumably through its interaction with FtsH-His<sub>6</sub>-Myc proteins (9). The preparations also contained several C-terminally truncated low molecular mass products, which cross-reacted with anti FtsH antibodies (data not shown). Taking this into account, the purity can be regarded as higher. In addition, they should have contained the contaminating wild-type FtsH protein (see above). On the other hand, the A2 mutants, FtsH71 (K136N) and FtsH84 (S137N), had only moderately reduced or wild type level of ATPase activity. Thus, the A1 motif region is mainly responsi-

**FIG. 2. SDS-PAGE profiles of the purified FtsH-His<sub>6</sub>-Myc proteins.** About 1 μg of proteins were subjected to 10% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, FtsH-His<sub>6</sub>-Myc (wild type); lane 2, FtsH40-His<sub>6</sub>-Myc; lane 3, FtsH41-His<sub>6</sub>-Myc; lane 4, FtsH71-His<sub>6</sub>-Myc; lane 5, FtsH81-His<sub>6</sub>-Myc; lane 6, FtsH82-His<sub>6</sub>-Myc; lane 7, FtsH83-His<sub>6</sub>-Myc; lane 8, FtsH84-His<sub>6</sub>-Myc; lane 9, FtsHd2-His<sub>6</sub>-Myc. FtsH<sup>TM1</sup> indicates the C-terminally cleaved product of FtsH-His<sub>6</sub>-Myc.

ble for the total ATPase activity of FtsH. Interestingly, FtsH81, with weak but significant complementation activity, had only very weak ATPase activity although the contamination by wild-type protein precluded the accurate assessment.

**Demonstration of SecY Degradation Activity of FtsH**—Our mutational studies strongly suggested that FtsH participates in degradation of SecY<sub>in vivo</sub> (7). We examined whether the purified FtsH-His<sub>6</sub>-Myc proteins can proteolyze SecY<sub>in vitro</sub>. Purified preparations of SecY and FtsH-His<sub>6</sub>-Myc, both in non-ionic detergent solutions, were mixed and incubated at 42°C in the presence or absence of ATP. SecY was evidently degraded when incubated for 1 h in the presence of both ATP and FtsH-His<sub>6</sub>-Myc (Fig. 3A, lanes 4–6). The purified SecY preparation used contained an N-terminal fragment of SecY (SecY<sup>NT</sup>) (confirmed by antiserum against the N-terminal region of SecY, data not shown) that was generated presumably by artificial cleavage during purification. The SecY fragment was degraded more rapidly than intact SecY (Figs. 3 and 4). Degradation of SecY and SecY<sup>NT</sup> was more efficient at 42°C than at 37°C or 30°C (data not shown). Several degradation intermediates were observed upon longer exposure, but none of them substantially accumulated during shorter periods of incubation (data not shown), indicating that the proteolysis, once initiated, was very rapid.

Without ATP (Fig. 3A, lanes 1–3) or without FtsH-His<sub>6</sub>-Myc (lanes 7 and 8), degradation of SecY or SecY<sup>NT</sup> was negligible. CTP and UTP substituted for ATP but ineffectively (Fig. 3B, lanes 8–11; see particularly the SecY<sup>NT</sup> band). GTP and ATP<sub>γ</sub>S
FtsH40-His6-Myc (plasmic domain (H414EAGH), and Tomoyasu region is important for the proteolytic activity. ATPase activity (Fig. 1). These results suggest that the Glu\textsuperscript{115} to Lys mutation markedly decreases the proteolytic activity of FtsH but does not totally inactivate it.

**Proteolytic Activity of FtsH Is Selective**—We examined how generally membrane proteins serve as a substrate for FtsH. Cells of the temperature-sensitive ftsH\textsuperscript{1} mutant strain that carried a SecY-overproducing plasmid (pKY6) were pulse-labeled at 42 °C with \textsuperscript{35}S-methionine. Membranes were solubilized with Nonidet P-40, and incubated with purified wild-type FtsH-His\textsubscript{8}-Myc protein at 42 °C in the presence or absence of ATP (Fig. 5). Labeled SecY in the detergent extract, as detected by immunoprecipitation, was degraded in a manner dependent on the exogenous FtsH and ATP (Fig. 5, lanes 13–16). Total electrophoretic profile of the labeled proteins (Fig. 5, lanes 19–28) showed that only a few of them (marked by arrowheads) were degraded upon incubation with FtsH-His\textsubscript{8}-Myc and ATP. Concomitantly, low molecular weight materials were generated (see the region ahead of the dye front). Many proteins, especially those of high molecular weights, remained undigested (Fig. 5, lane 28). In other words, FtsH degrades only a selected set of E. coli membrane proteins. As most E. coli membrane proteins are stable in vivo, the selectivity of the FtsH action appears to be preserved at least partially in vitro. In a similar experiment, we found that subunit \(\alpha\) of the F\textsubscript{0} part of the proton ATPase can be a substrate of FtsH.\textsuperscript{2}

**In Vivo Interaction between FtsH and SecY**—In vivo interaction between SecY and FtsH was examined by cross-linking experiments, using cells overproducing SecY and either FtsH-His\textsubscript{8}-Myc, FtsH40-His\textsubscript{8}-Myc, or FtsH41-His\textsubscript{8}-Myc protein. Membranes were prepared from pulse-labeled cells and treated with a membrane-permeable cross-linker, diethoxy(succinimidylpropionate). After solubilization with SDS, labeled proteins that were cross-linked with FtsH or SecY were isolated by immunoprecipitation using anti-FtsH or anti-SecY, respectively. The cross-linking was cleaved with 2-mercaptoethanol before visualization by SDS-PAGE and autoradiography. When cells overproducing FtsH40-His\textsubscript{8}-Myc or FtsH41-His\textsubscript{8}-Myc were used, an FtsH-sized protein was recovered as a cross-linked partner of SecY (Fig. 6, lanes 6 and 12), and an SecY-sized protein was recovered as a cross-linked partner of FtsH (lanes 2 and 10). The SecY-sized protein was also recovered when a portion of the sample for lane 2 was precipitated with anti-Myc antibodies (data not shown). Cross-linking was negligible or very weak when cells overproducing wild-type FtsH and SecY were used (lanes 4 and 8). This result may have been due to stabilization of the bound state of SecY by the ATPase mutations. In addition, SecY might have been degraded by the active FtsH.

**DISCUSSION**

FtsH is required in vivo for rapid elimination of the uncomplexed forms of SecY (7). Biochemical characterization described in this study established that FtsH directly degrades SecY. FtsH-dependent degradation of SecY was demonstrated using purified SecY protein as well as using detergent extracts of membranes. The proteolysis was ATP-dependent and not observed with the ATPase-deficient mutants of FtsH. It was demonstrated previously (11) that \(\alpha^{32}\), the heat shock \(\alpha\) factor, was degraded by FtsH in vitro. Although SecY and \(\alpha^{32}\) are apparently remote in nature, they are substrates of a common FtsH protease.

The results of our mutational analyses indicate that the ATP binding motif A1 in the region homologous to the AAA family members is essential for the ATPase and proteolytic activities.

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\[\text{Y. Akiyama, A. Kihara, and K. Ito, unpublished results.}\]
proteins are substrates of FtsH (11–13) suggests that the active site of FtsH for proteolysis is located within the cytosolic domain, which includes the ATPase region and zinc binding motif. This model, given the evidence, is likely that FtsH attacks cytosolic regions of SecY. Our genetic analysis of SecY-SecE interaction suggested that the fourth cytoplasmic region (C4) is important for the SecY-SecE interaction with SecE (3). An essential region of SecE has also been localized to its cytoplasmic domain (35). The interaction between the cytoplasmic domains of SecY and SecE may induce a structural change in the cytoplasmic regions of SecY, such that they become refractory to the attack of FtsH. We found that SecY-SecE complex in detergent extracts is far more resistant to even a non-specific protease (proteinase K) than singly overproduced SecY. The thermal stability of the SecY-SecE complex in detergent extracts (36) precluded the direct examination of its resistance to FtsH, but it is conceivable that overall conformation of the cytoplasmic domains determines the FtsH-resistance of SecY. We are in the process of identifying a SecY region that is primarily attacked by FtsH in a SecE-free state.

As reported previously (7), the physiological significance of the SecY degradation by FtsH should lie in the elimination of unassembled SecY molecules that are harmful to the membrane functions. Taken together with our finding that subunit $\alpha$ of the proton ATPase F$_0$ segment is also a substrate of FtsH, FtsH seems to comprise an important quality control machine in the $E. coli$ cell.

Our recent results (21) that FtsH can associate with the hflK and hflC gene products and that this association negatively regulates the SecY-degrading activity of FtsH suggest that the proteolytic activity of FtsH is subject to regulation by other proteins. The FtsH-His$_{6}$-Myc preparations used in this study contained a trace of HflK/HflC proteins that could be detected by immunoprecipitation with anti-FtsH or anti-SecY antisera as indicated. The cross-linking was cleaved before electrophoresis. Proteins were analyzed by 15% SDS-PAGE. Lane 1 was for molecular mass markers (see legends to Fig. 5).
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