Self-Processing of FtsH and Its Implication for the Cleavage Specificity of This Protease†

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ABSTRACT: FtsH, a membrane-bound and ATP-dependent protease of *Escherichia coli*, is involved in degradation of some of uncomplexed integral membrane proteins and short-lived cytoplasmic proteins. It is composed of an N-terminal membrane-spanning region and a following large cytoplasmic domain that contains ATPase and protease active sites. In the present study, it was found that FtsH undergoes C-terminal processing in vivo. The processing was blocked by loss of function mutations of FtsH. Purified FtsH-His_6-Myc, a C-terminally tagged derivative of FtsH, was self-processed in vitro. This in vitro processing was observed only in the presence of ATP and not in the presence of adenosine 5′-(β,γ-imino)triphosphate (AMP-PNP). Moreover, such processing did not occur in the case of the ATPase motif mutant protein. These results indicated that this processing is a self-catalyzed reaction that needs ATP hydrolysis. Mutations in the *hflKC* genes that encode a possible modulator of FtsH, and the growth phase of the cells as well, affected the processing. Complementation experiments with genetically constructed variants suggested that both the processed and the unprocessed forms of FtsH are functional. The cleavage was found to occur between Met-640 and Ser-641, removing a heptapeptide from the C-terminus of FtsH. Systematic mutational analyses of Met-640 and Ser-641 revealed preferences for positively charged and hydrophobic amino acid residues at these positions for processing. This cleavage specificity may be shared by the self-cleavage and the substrate-cleavage reactions of this protease.

Intracellular protein degradation serves not only to eliminate potentially harmful abnormal proteins that are generated by, for example, mutational alterations or unbalanced subunit synthesis but also to modulate activities of authentic proteins such as short-lived regulatory proteins. Energy-dependent proteases have important roles in the above events in both eukaryotic and prokaryotic cells. In the cytoplasm of eukaryotic cells, the 26S proteasome is a major energy-dependent protease that is involved in degradation of a wide variety of cellular proteins (*1*). In contrast, prokaryotic cells have several energy-dependent proteases with different substrate specificities (*2*, *3*).

The *Escherichia coli* cell contains five ATP-dependent proteases, Lon, ClpAP, ClpXP, HslUV, and FtsH (*2*, *3*). FtsH, the only membrane protein among them, is anchored in the cytoplasmic membrane by means of the two N-terminal transmembrane segments (*4*). The large cytoplasmic domain of FtsH is composed of the AAA ATPase domain and a C-terminal region that contains the zinc metalloprotease consensus motif (HEXXH) (*5*). Like other ATP-dependent proteases, FtsH is an oligomeric enzyme. We showed that FtsH forms a homooligomer in which interaction of the subunits is mediated by the N-terminal membrane-anchor region (*6*, *7*). FtsH is also associated with other integral membrane proteins, HflK and HflC (*8*), which themselves form a complex termed HflKC (*9*, *10*). The exact number of FtsH molecules in the homooligomeric complex and the subunit composition of the heterooligomeric complex are unknown.

FtsH degrades unassembled subunits of integral membrane protein complexes such as the SecY subunit of protein translocase (*11*) and subunit *a* of the F_1F_o ATPase (*12*). Rapid degradation of these proteins by FtsH and their highly toxic nature when accumulated in the cell (*11*, *13*) suggest that FtsH plays a crucial role in quality control of the membrane. Substrates of FtsH also include cytoplasmic transcriptional factors, a heat shock σ factor (σ^{32}) (*14*, *15*), and the CII protein of phage λ (*16*−*18*). Thus, FtsH is important for regulation of gene expression as well.

Early studies suggested that HflKC is a protease that degrades CII (*9*). However, we showed that HflKC is mostly exposed to the periplasmic space and has no CII-degrading activity in vivo or in vitro (*8*, *10*). Our studies suggested that HflKC is a modulator of the activity of FtsH (*8*, *16*). We have identified missense mutations in the *hflK* and *hflC* genes (*hflK13* and *hflC9*) that inhibit degradation of membrane-bound substrates of FtsH (*8*). However, deletion of these genes (Δ*hflKC*) did not abolish the degradation of SecY but rather enhanced it (*8*). On the other hand, Δ*hflKC* retarded the degradation of CII but the missense mutations did not (*16*). We showed that removal of the periplasmic region from FtsH (the *ftsHΔ236* mutation) impaired the interaction between FtsH and HflKC and made FtsH defective in degradation of CII but not in degradation of SecY in

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vivo (*19*). Similar phenotypic consequences of the *ftsHΔ236* and Δ*hflKC* mutations suggest that the FtsH−HflKC interaction is important in regulation of the proteolytic functions of FtsH. We also showed that a mutant form of the YccA protein, a membrane-bound substrate of FtsH, selectively interfered with the degradation of other membrane-bound substrates by forming a complex with FtsH/HflKC and that HflKC is required for this inhibition (*20*). These findings indicate that the degradation pathways for the membrane-bound and the soluble substrates are in part different from each other and that HflKC is involved in this differentiation.

Despite the accumulating knowledge concerning the targets and regulations of FtsH, our understanding of the molecular mechanism of protein degradation by FtsH is still limited. For example, we do not know the cleavage specificity of FtsH. Nor do we know whether FtsH is subject to any kind of modification that might modulate its activities. Here, we report that FtsH undergoes C-terminal self-processing and that the processing is affected by *hflKC* mutations and the growth phase of the cells. Mutational analyses revealed preferences with respect to the amino acid residues at the processing site. This cleavage specificity may be shared by self-processing of FtsH and its degradation of substrate proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. AD367 (*ftsH*$^+$) and its isogenic strain, AD368 (*ftsH1*) (*21*) and TYE024 (*ompT*::*kan*/F′*lacI*^{q}) (*7*) were derivatives of MC4100. AR3291, a W3110 derivative, carried the Δ*ftsH3*::*kan* mutation and its suppressor *sfhC21* (*19*, *22*). AD16 (*ftsH*$^+$/F′*lacI*^{q}), AK865 (AD16, *hflK13*), and AK990 (AD16, Δ*hflKC*::*kan*) were also described previously (*8*).

The pSC101-based low-copy plasmids, pSTD233 (*ftsH-his$_6$-myc*) (*19*) and pSTD401 (*ftsH*) (*7*), and the pBluescript-based high-copy plasmids, pSTD113 (*ftsH-his$_6$-myc*) (*7*) and pSTD108 (*ftsH41-his$_6$-myc*) (*21*), were described previously. pSTD248 (*ftsHΔ7*) and pSTD331 (*ftsHΔ8*) were constructed from pSTD401 by using the Quick Change mutagenesis kit (Stratagene) with mutagenic primers CTGATGCGGGAACTTACATGGTGTTACCCGG/CCGGGTAACACCATGTAAGTTCCCGCATCAG and CTGATGCGGGAACTTAGGTGTTACCCGGGTTC/GAACCCGGGTAACACCTAAGTTCCCGCATCAG, respectively. pSTD258 [*ftsH(M640E)*] was constructed by mutagenesis of the Met-640 codon of *ftsH* on pSTD401 as described below.

Media. L medium (*23*) and M9 medium (*23*) were used. Ampicillin (50 μg/mL) or chloramphenicol (20 μg/mL) was added to culture media for growth of plasmid-bearing strains.

Pulse−Chase, Immunoprecipitation, and Immunoblotting Experiments. Cells were pulse-labeled with [^{35}S]methionine and chased with unlabeled methionine as described previously (*11*). FtsH and FtsH-His$_6$-Myc were immunoprecipitated with anti-FtsH or anti-Myc antibodies (*7*) and separated by SDS−7.5% or 6% PAGE[1] (*24*). Labeled proteins were visualized and quantified by means of a Fuji BAS2000 imaging analyzer.

Immunoblotting with anti-FtsH serum was carried out as described previously (*25*). Proteins were visualized and quantified by means of an ECL detection kit (Amersham Pharmacia Biotech) and a Fuji LAS1000 lumino-image analyzer. For separation of FtsHΔC7 and FtsHΔC8, proteins were electrophoresed on a "long gel" (6% Laemmli gel with a 14 cm separation gel) until a prestained 41.8 kDa marker went out of the gel.

Isolation and in Vitro Processing of [^{35}S]Methionine-Labeled FtsH-His$_6$-Myc. Cells of TYE024/pSTD113 or TYE024/pSTD108 were grown in M9 medium and pulse-labeled with 37 kBq/mL [^{35}S]methionine for 3 min. FtsH-His$_6$-Myc and FtsH41-His$_6$-Myc were purified from the membrane fraction by Ni−nitrilotriacetic acid affinity chromatography essentially as described previously (*21*). For the in vitro C-terminal processing assays, 10 μL of the purified proteins (in buffer containing 50 mM Tris-HCl, pH 8.1, 500 mM KCl, 250 mM imidazole, 10% glycerol, 0.5% Nonidet P-40, and 10 mM 2-mercaptoethanol) was mixed with 24 μL of 5× ATPase buffer (250 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, 50% glycerol, 50 mM 2-mercaptoethanol, 125 μM zinc acetate, and 12.5 mM Tris acetate), 30 μL of wash buffer (50 mM Tris-HCl, pH 8.1, 500 mM KCl, 20 mM imidazole, 10% glycerol, 0.5% Nonidet P-40, and 10 mM 2-mercaptoethanol), 52 μL of H_2O, and 4 μL of either 0.1 M ATP, 0.1 M AMP-PNP, or H_2O, and the mixture was incubated at 42 °C. At the indicated time points, a portion of the samples was withdrawn, mixed with 2× SDS sample buffer (*24*), and analyzed by SDS−7.5% PAGE.

Determination of the N- and C-Terminal Amino Acid Residues of FtsH′. For determination of the N-terminal amino acid residues of FtsH-His$_6$-Myc and FtsH′, a preparation containing FtsH-His$_6$-Myc and FtsH′ (approximately 1:1 and a total of 12 μg) was separated by SDS−7.5% PAGE, and separated proteins were electrophoretically transferred onto a poly(vinylidene difluoride) membrane. After Ponsau S (0.5%) staining, the FtsH-His$_6$-Myc and FtsH′ regions were excised, washed extensively with H_2O, and treated with 0.6 M HCl at 25 °C for 22 h to remove an N-terminal formyl group. They were washed again with H_2O and subjected to an automated protein sequencer, PPSQ-23 (Shimadzu). For determination of the C-terminal amino acid residues of FtsH′, purified FtsH-His$_6$-Myc and FtsH′ (a total of 50 μg) was separated by SDS−7.5% SDS PAGE and blotted onto a Teflon membrane filter. After Coomassie brilliant blue (0.1%) staining, a strip containing FtsH′ was excised from the filter, rinsed with 60% ethanol and then with H_2O, and subjected to analysis on an automatic C-terminal amino acid sequencer, HP241 (Hewlett-Packard).

Mutagenesis of the Met-640 and Ser-641 Residues. Mutagenesis of pSTD401 was carried out with the Quick Change site-directed mutagenesis kit (Stratagene). For simultaneous mutagenesis of the Met-640 and Ser-641 codons, a pair of degenerate primers with complementary sequences was used. One of the primers had the sequence CCCGGGTAACAC-CAMGSCAGAGCAGTTAGG where M stands for A and C (1:1 mixture) and S stands for G and C (1:1 mixture). The Met-640 and Ser-641 codons were also changed in prescribed ways, by using a pair of primers with complementary sequences. In each instance, one of the primers had the sequence CCCGGGTAACACCXXXTCAGAGCAGT-TAGG (for mutagenesis of Met-640) or CGGGTAACAC-

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

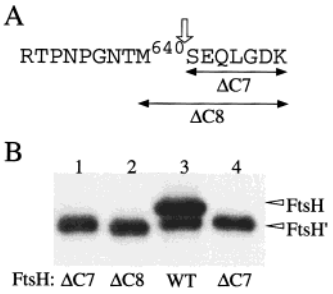

FIGURE 1: SDS−PAGE mobility of FtsH, FtsH′, FtsHΔC7, and FtsHΔC8. (A) C-Terminal amino acid sequence of FtsH. The residues deleted in FtsHΔC7 and FtsHΔC8 are indicated by the bidirectional arrows. The downward arrow indicates the self-cleavage site. (B) Cells of AR3291/pSTD248 (*ftsHΔ7*; lanes 1 and 4), AR3291/pSTD331 (*ftsHΔ8*; lane 2) and AR3291/pSTD401 (*ftsH*$^+$; lane 3) were grown in L broth and induced with 1 mM isopropyl β-D-thiogalactopyranoside and 1 mM cAMP for 3 h. Proteins were precipitated with trichloroacetic acid, separated by SDS−6% PAGE, and analyzed by means of LAS1000 lumino-image analyzer following immunoblotting with anti-FtsH serum.

CATGXXXGAGCAGTTAGGCG (for mutagenesis of Ser-641) where XXX was GCG (for replacement by Ala), TGC (Cys), GAT (Asp), GAG (Glu), TTC (Phe), GGC (Gly), CAC (His), ATC (Ile), AAG (Lys), CTG (Leu), AAC (Asn), CCG (Pro), CAG (Gln), CGT (Arg), TCG (Ser), CGT (Thr), GTG (Val), TGG (Trp), or TAC (Tyr).

RESULTS

In Vivo C-Terminal Processing of FtsH. FtsH was detected as two closely adjacent bands of about 71 and 70 kDa (the calculated molecular mass of FtsH is 71 037.63 Da) when total cell proteins of the *ftsH*-deleted strain expressing wild-type *ftsH* from a plasmid (Figure 1, lane 3) or those from the wild type (*ftsH*$^+$) strain (Figure 5A) were analyzed by immunoblotting with anti-FtsH antibodies.[2] Neither of these bands was detected from *ftsH*-deleted cells (data not shown), suggesting that both were derived from the chromosomal *ftsH* gene. Biosynthesis of the FtsH protein was analyzed by means of pulse−chase experiments (Figure 2). Cells of the wild-type strain were pulse-labeled with [^{35}S]methionine and chased with unlabeled methionine, and FtsH was immunoprecipitated with anti-FtsH serum (Figure 2, lanes 6−10). The [^{35}S]methionine-labeled FtsH protein initially appeared as a single band of about 71 kDa and was then converted gradually into a slightly smaller species (FtsH′) during the chase period. It was found that FtsH-His$_6$-Myc, a derivative of FtsH with a bipartite (His$_6$ and Myc) tag at the C-terminus, was also converted to a smaller species that showed mobility identical to that of FtsH′ on SDS−PAGE (lanes 11−15). The smaller species generated from FtsH-His$_6$-Myc was not immunoprecipitated with anti-Myc antibodies, indicating that it did not retain the C-terminal Myc epitope (lanes 16−20). N-Terminal amino acid sequence analyses of FtsH-His$_6$-Myc and the smaller species revealed the presence of the first 10 amino acids residues (MSDMAKNLIL) of FtsH (*26*) in both cases. These results strongly suggested that FtsH and FtsH-His$_6$-Myc were cleaved at the same site in the C-terminal portion to generate FtsH′. Previously we also noted that FtsH-His$_6$-Myc was converted into FtsH′ during purification (*21*).

[2] T. Tomoyasu and T. Ogura, unpublished results.

C-Terminal Processing of FtsH Is a Self-Catalyzed Reaction. The *ftsH1*(Ts) mutation, which is known to severely compromise the function of FtsH at 42 °C (*11*, *18*), was found to block the C-terminal processing (Figure 2, lanes 1−5). FtsH′ was not generated at the restrictive temperature 42 °C even after a 90 min chase (lane 5). Thus, normal FtsH activity is required for the C-terminal processing of FtsH.

The FtsH-His$_6$-Myc protein was pulse-labeled with [^{35}S]-methionine and purified by Ni−nitrilotriacetic acid affinity chromatography. The isolated ^{35}S-labeled purified FtsH-His$_6$-Myc protein was mostly in the unprocessed form (Figure 3, lane 1) because, as shown above (Figure 2, lane 11), little processing occurs during the short pulse-labeling period. When the labeled FtsH-His$_6$-Myc protein was incubated at 42 °C in the presence of ATP, it was slowly but quantitatively converted into FtsH′ (Figure 3, lanes 1−4). The in vitro generated FtsH′ was precipitated with anti-FtsH antibodies but not with anti-Myc antibodies (data not shown), confirming the removal of the C-terminal sequence. No appreciable processing was observed in the absence of ATP (Figure 3, lanes 9−12), or in the presence of AMP-PNP (lanes 5−8), even after 6 h of incubation. I also found that a species with an intermediate mobility between FtsH-His$_6$-Myc and FtsH′ was generated ATP-dependently (solid arrowhead). This species, also observed *in vivo* (Figure 2, lanes 11−15), seemed to have been generated by a cleavage within the His$_6$-Myc tag. FtsH41-His$_6$-Myc is a mutant form of FtsH-His$_6$-Myc that has an amino acid alteration (Lys-198 to Asn) in the ATP binding consensus sequence. We previously showed that FtsH41-His$_6$-Myc is inactive in ATP hydrolysis and proteolysis (*21*). FtsH′ was not produced even in the presence of ATP upon incubation of the purified and [^{35}S]methionine-labeled FtsH41-His$_6$-Myc (Figure 3, lanes 13−24). These results indicate that the C-terminal processing of FtsH is a self-catalyzed reaction that depends on ATP hydrolysis.

Effects of hflKC Mutations on the C-Terminal Processing of FtsH. The effects of the *hflKC* mutations on the C-terminal processing of FtsH in vivo were examined (Figure 4). We previously showed that the *hflK13* mutation specifically inhibited degradation of membrane-bound (SecY and Fo*a*) but not soluble (λCII and σ^{32}) substrates of FtsH (*8*, *16*). On the other hand, deletion of the *hflKC* genes retarded degradation of CII and enhanced SecY degradation (*8*, *16*). As shown in Figure 4, processing of FtsH was significantly retarded in the *hflKC*-deleted strain (○), whereas it was enhanced in the *hflK13* strain (△). The different effects of these two mutations on the C-terminal processing of FtsH were similar to the effects seen with these mutations on the degradation of soluble substrates of FtsH (*16*).

Growth-Phase-Dependent Accumulation of FtsH′. The accumulation of the processed and unprocessed forms of FtsH in cells in different phases of growth was examined (Figure 5). Stationary-phase cells were diluted into fresh L medium and cultured at 37 °C (growth curve is shown in Figure 5B). Samples were withdrawn at intervals, and the levels of accumulation of the processed and unprocessed forms of FtsH were examined by immunoblotting (Figure 5A; the quantitative data are shown in Figure 5B). The FtsH protein was found to be present almost exclusively in the processed form in the stationary-phase cells (Figure 5A, lane 1). The unprocessed form appeared shortly after the onset of growth (lane 2) and became predominant in the log-phase

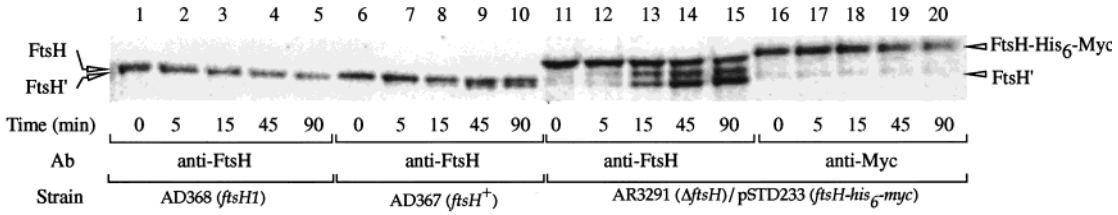

FIGURE 2: C-Terminal processing of FtsH and FtsH-His_6-Myc in vivo. Cells of AD367 (*ftsH*$^+$) (lanes 6−10), AD368 (*ftsH1*) (lanes 1−5), and AR3291 (Δ*ftsH*)/pSTD233 (*ftsH-his*$_6$*-myc*) (lanes 11−20) were grown in M9 medium at 42 °C for 2 h, pulse-labeled with [^{35}S]-methionine for 1 min, and chased for the indicated time. Cells of AR3291/pSTD233 were induced with 1 mM isopropyl β-D-thiogalactopyranoside and 5 mM cAMP for 10 min before the pulse-labeling. Proteins were precipitated with trichloroacetic acid and subjected to immunoprecipitation with anti-FtsH (lanes 1−15) or anti-Myc (lanes 16−20). Proteins were separated by SDS−PAGE and visualized by means of a BAS2000 Bioimaging analyzer. FtsH′ indicates the C-terminally processed form of FtsH and FtsH-His_6-Myc.

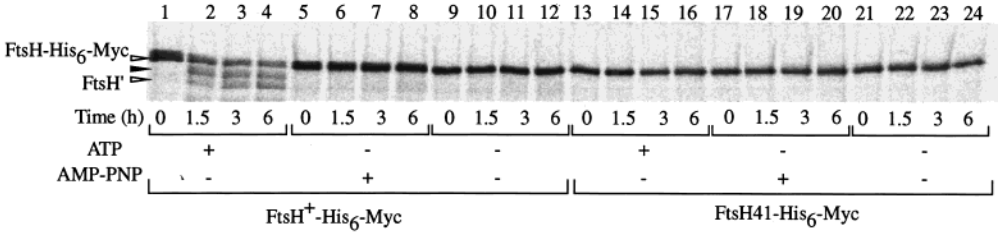

FIGURE 3: ATP-dependent in vitro processing of FtsH-His_6-Myc. [^{35}S]Methionine-labeled FtsH-His_6-Myc (lanes 1−12) and FtsH41−His_6-Myc (lanes 13−24) were incubated in the presence (lanes 1−4 and 13−16) or absence (lanes 9−12 and 21−24) of 3.3 mM ATP or in the presence of 3.3 mM AMP-PNP (lanes 5−8 and 17−20) for the indicated time. Proteins were separated by SDS−7.5% PAGE and visualized by means of a BAS2000 Bioimaging analyzer.

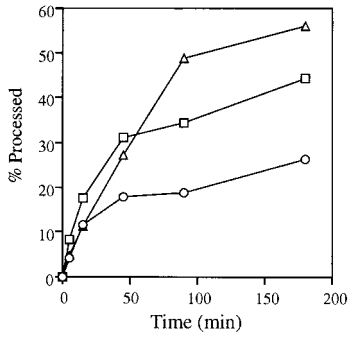

FIGURE 4: Effects of the *hflKC* mutations on the C-terminal processing of FtsH. Cells of AK990 (Δ*hflKC*, ○), AK865 (*hflK13*, △), and AD16 (wild type, □) were grown in M9 medium, pulse-labeled with [^{35}S]methionine for 1 min, and chased for the indicated time. FtsH was immunoprecipitated with anti-FtsH serum, separated by SDS−7.5% PAGE, and analyzed by means of a BAS2000 Bioimaging analyzer. The ratio of the processed form to total FtsH was plotted for each sample.

cells (lanes 2−5). Its proportion to total FtsH reached a maximum (78% of total FtsH) 1.5 h after the start of cultivation (lane 4). Then, the ratio decreased gradually to about 30% (6 h, lane 10). Thus, the processed form of FtsH accumulates in the late log to stationary phase of a culture.

Determination of the Cleavage Site. The C-terminal amino acid residue of FtsH′ was determined to be Met by use of an automated C-terminal amino acid sequencer, although no further residues could be determined (data not shown). Within the C-terminal 50 residues of FtsH, the only Met residue is Met-640. Cleavage of the protein between Met-640 and Ser-641 would remove seven amino acid residues from the C-terminus of FtsH, which is consistent with the observed difference in size between FtsH and FtsH′ (about 1 kDa). To verify the cleavage after Met-640, we constructed two FtsH derivatives, FtsHΔC7 and FtsHΔC8, in which seven and eight C-terminal amino acid residues of FtsH, respectively, had been deleted (Figure 1A). Wild-type FtsH,

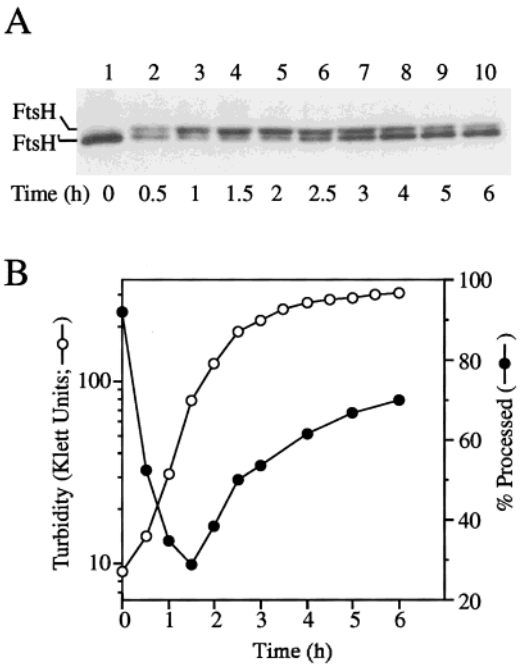

FIGURE 5: Growth-phase-dependent accumulation of the processed form of FtsH. Cells from an overnight culture of AD16 were transferred into fresh L medium and grown at 37 °C. At the indicated time points, a portion of the culture (about 6×10^7 cells) was removed and mixed with trichloroacetic acid. (A) Proteins were separated by SDS−7.5% PAGE and analyzed by means of a LAS1000 lumino-image analyzer following immunoblotting using anti-FtsH. (B) The turbidity of the culture (○) and the ratio of the processed form to total FtsH (●) were plotted against the time after the dilution.

FtsHΔC7, and FtsHΔC8 were expressed in AR3291 (Δ*ftsH*) and separated by means of a long gel SDS−PAGE (see Experimental Procedures). They were detected by immunoblotting with anti-FtsH serum (Figure 1B). Only a single band was detected in the case of FtsHΔC7 (lanes 1 and 4) and FtsHΔC8 (lane 2), whereas the intact and FtsH′ bands were

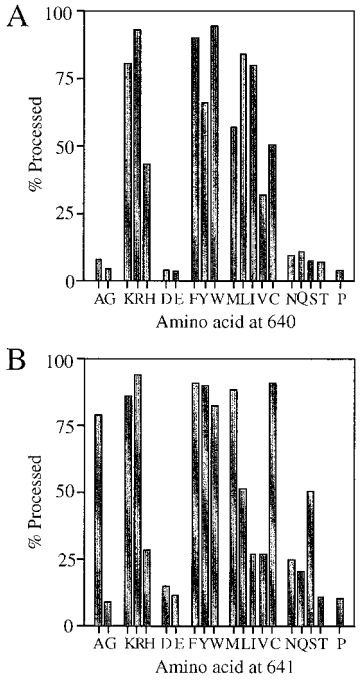

FIGURE 6: Effects of mutational alterations of Met-640 and Ser-641 on the processing of FtsH. Cells of AR3291 carrying pSTD401 (*ftsH*$^+$) or a pSTD401 derivative with a mutation that resulted in replacement of Met-640 or Ser-641 with one of 19 other amino acid residues were grown in L broth containing 1 mM isopropyl β-D-thiogalactopyranoside and 1 mM cAMP for 17 h. Proteins were precipitated with trichloroacetic acid, separated by SDS−PAGE, and analyzed by means of LAS1000 lumino-image analyzer following immunoblotting with anti-FtsH serum. The ratio of the processed form to total FtsH is shown for mutants with an amino acid substitution at residue 640 (A) or residue 641 (B). The replaced amino acid residue in each protein is indicated under the panels.

detected in the case of wild-type FtsH as expected (lane 3). The mobility of FtsHΔC7 was the same as that of FtsH′, whereas FtsHΔC8 migrated slightly faster than FtsH′ and FtsHΔC7. These results indicate that cleavage occurs between Met-640 and Ser-641. The inhibition of processing by mutational alterations of Met-640 or Ser-641 further supported this conclusion (see below).

Amino Acid Preference at the Cleavage Site. Residue 640 and/or residue 641 were changed to various amino acids to examine the cleavage specificity (Figure 6). First, I performed mutagenesis of the *ftsH* gene on a plasmid (pSTD401), using degenerate primers that were designed to introduce Lys or Thr at residue 640 and Ala or Pro at residue 641 (see Experimental Procedures for details). Screening for plasmids that produced only the unprocessed form of FtsH in the Δ*ftsH* cells gave three such clones. DNA sequencing revealed that the *ftsH* genes on these three plasmids carried the same mutation resulting in substitution of Thr for Met-640. These results indicated that certain amino acid residues are incompatible with the self-processing when placed at position 640.

Then, 18 kinds of FtsH mutants, each with an amino acid other than Met or Thr at position 640, were constructed. Each of the mutant proteins was expressed in the Δ*ftsH* strain and its C-terminal processing was examined at 17 h after induction. The processed and the unprocessed forms of FtsH were separated on a long gel and quantified following immunoblotting, and the ratio of the processed form to total FtsH was calculated in each instance. As shown in Figure 7A, substitution of E, D, A, G, N, Q, S, T, and P at residue

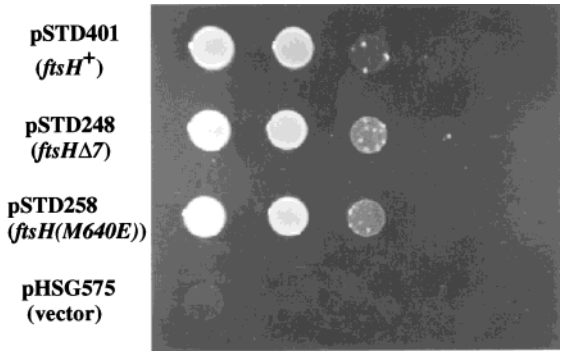

FIGURE 7: Complementation activity of the processed and the unprocessed forms of *ftsH*. Cells of AD368 (*ftsH1*) carrying pSTD401 (*ftsH*$^+$), pSTD248 (*ftsHΔC7*), pSTD258 [*ftsH(M640E)*], or pHSG575 (vector) were grown in L broth containing 0.4% glucose and 20 μg/mL chloramphenicol at 30 °C till the mid-log phase. The cultures (about 10^8 cells/mL) were subjected to 10-fold serial dilution (left to right), and 3 μL portions were spotted onto an L plate containing 20 μg/mL chloramphenicol and 1 mM isopropyl β-D-thiogalactopyranoside, and incubated at 42 °C for 8 h.

640 blocked the processing almost completely. On the other hand, K, R, H, F, Y, W, L, I, V, and C allowed cleavage with similar or enhanced efficiency as compared with M (wild type). It should be noted that although we did not determine the cleavage sites definitively for these and the following mutants by C-terminal amino acid analyses, mobility analyses on a long gel suggested that cleavage occurred between residues 640 and 641 in all of the processing-active mutants. Thus, amino acid residues with a negatively charged (E and D), hydrophilic (N, Q, S, and T), or small (A and G) side chain, as well as an imino acid (P), at residue 640 blocked the processing, whereas those with a positively charged (K, R, and H) or hydrophobic (F, Y, W, L, I, V, and C) side chain allowed it. I also replaced Ser-641 with each of 19 other amino acids and examined the processing of the mutant proteins (Figure 6B). Although the effects of amino acid substitutions at residue 641 on the processing were less clear than those in the case of residue 640, we observed similar amino acid preferences; negatively charged residues and most hydrophilic residues prohibited the processing, whereas positively charged and hydrophobic residues allowed it.

In Vivo Functioning of the Processed and Unprocessed Forms of FtsH. To examine biological significance of the self-processing of FtsH, complementation activities of FtsH$^+$, FtsHΔ7 (processed form), and FtsH(M640E) (uncleavable processing site mutant) were expressed from plasmids in *ftsH1*(Ts) mutant cells. As shown in Figure 7, both of the *ftsHΔC7* or the *ftsH(M640E)* genes supported the growth of the *ftsH1* mutant cells as well as the wild-type *ftsH* gene at the restrictive temperature (42 °C). All of the other mutant genes carrying a mutation affecting reside 640 or 641 retained the ability to complement the *ftsH1* mutation (data not shown). Pulse−chase experiments showed that overexpressed FtsH, FtsHΔC7, and FtsH(M640E) were active in degradation of the uncomplexed SecY protein or the CII protein (data not shown). These results suggested that both the processed and the unprocessed FtsH are functional.

DISCUSSION

The results of the present study show that the FtsH protease undergoes C-terminal processing in vivo. Several

lines of evidence indicate that it is a self-catalyzed reaction. First, it was inhibited by the temperature-sensitive *ftsH1* mutation at the restrictive temperature. Mutations in the ATP binding motif or the zinc-binding motif, which severely compromise the proteolytic activity of FtsH, also inhibited the processing *in vivo*[3] (*21*). These observations suggest that the normal activity of FtsH is required for the self-processing. Second, more directly, purified FtsH-His_6-Myc was processed in vitro in the absence of any other additional proteins. This in vitro processing of the wild-type FtsH-His_6-Myc protein was observed in the presence of ATP and not in the presence of AMP-PNP. Moreover, such processing did not occur in the case of the ATPase motif mutant protein (FtsH41-His_6-Myc). Thus, ATP hydrolysis seems to be required for self-processing as well as for the degradation of substrate proteins. It has been proposed that ATP hydrolysis is required for unfolding and/or presentation of substrate proteins in proteolysis by other ATP-dependent proteases (*3*). It will be possible that ATP hydrolysis has similar roles in FtsH-catalyzed proteolysis. Although we previously showed that FtsH undergoes ATP-dependent conformational changes (*27*), its correlation with the protein degradation activity of FtsH remains elusive. Further studies are needed to elucidate the roles of ATP binding and hydrolysis in the function of FtsH. The proteolytically inactive FtsH variants were not appreciably processed even when they were expressed in an *ftsH*$^+$ strain, indicating that the wild-type FtsH did not catalyze C-terminal processing of the mutant proteins in trans.[3] These observations suggest an intramolecular (cis) mechanism for the C-terminal processing, although the dominant inhibitory effects of the mutant proteins against the proteolytic activity of the wild-type protein (*6*, *30*) may have contributed partly to the absence of processing of the mutant FtsH.

Self-processing is also known to occur in the case of ClpP, the catalytic subunit shared by the *E. coli* Clp proteases (*28*). ClpP is synthesized as a precursor with an N-terminal extension of 14 amino acids and is self-catalytically converted into a "mature" form, but the physiological significance of the ClpP processing remains unknown (*28*). In eukaryotic cells, some catalytic subunits of the proteasome are also subject to N-terminal self-processing (*1*). It has been shown that the N-terminal extension of Doa3, one of the catalytic subunits, is required for incorporation of the subunit into the proteasome and its removal is needed for maturation of the active sites (*29*). In the case of FtsH, the C-terminal processing does not seem to be crucial for the homooligomeric interaction because our previous cross-linking experiments showed that the unprocessed FtsH-His_6-Myc could associate with FtsH (*7*). Our data indicate that both the processed and the unprocessed FtsH are functional. At present, I do not know whether the C-terminal processing of FtsH has any physiological role. However, it is possible that this processing affects the activity of FtsH to some extent. Indeed, our preliminary experiments suggested that FtsH(M640E)-His_6-Myc, an unprocessed mutant, had a weaker in vitro proteolytic activity against SecY as compared with the wild-type FtsH-His_6-Myc. The processing could have a role in fine-tuning of the activity of FtsH. The growth-phase-dependent accumulation of the processed form of FtsH might indicate that the activity of this enzyme is modulated in a growth-phase-dependent manner. The determinants of processed/unprocessed ratio of FtsH in the cell might either be some unidentified cellular factor(s) or simply the balance between the rates of synthesis and processing.

In vivo degradation of substrate proteins by FtsH is generally very rapid and no stable degradation intermediate accumulate (*30*). In such a case, it is difficult to analyze the cleavage specificity *in vivo*. The results described in this paper seem to provide information concerning the in vivo cleavage specificity of FtsH. Systematic mutagenesis resulting in substitutions of residues 640 and 641 revealed preferences for positively charged and hydrophobic residues at these positions for cleavage. Especially Lys and Arg (positively charged residues) and Phe, Tyr, and Trp (aromatic residues) allowed more efficient processing than the wild-type residue at either position. I propose that this cleavage specificity reflects the general cleavage specificity of FtsH when it acts against substrate proteins. We have shown that FtsH is involved in degradation of several membrane proteins (SecY, Fo*a*, and YccA) (*11*, *12*, *20*). Cytoplasmic regions of integral membrane proteins are generally rich in positively charged residues, while transmembrane regions are mainly composed of hydrophobic residues (*31*, *32*). The proposed cleavage specificity seems to be suitable for degradation of integral membrane proteins. However, it does not explain the high selectivity of FtsH in substrate recognition of this enzyme (*15*, *21*). We proposed that degradation of membrane-bound substrates by FtsH proceeds in processive manners (*30*). Selective substrate recognition must precedes the actual proteolytic steps with probable cleavage specificity as deduced in this study. Although it will be essential to analyze the cleavage specificity of FtsH with purified proteins and peptide substrates, the data from the present study will certainly guide further studies on cleavage specificity of this interesting protease.

[3] Y. Akiyama, unpublished results.

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