Chapter 3
FtsH Protease-Mediated Regulation of Various Cellular Functions

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Abstract FtsH, a member of the AAA (ATPases associated with a variety of cellular activities) family of proteins, is an ATP-dependent protease of ~71 kDa anchored to the inner membrane. It plays crucial roles in a variety of cellular processes. It is responsible for the degradation of both membrane and cytoplasmic substrate proteins. Substrate proteins are unfolded and translocated through the central pore of the ATPase domain into the proteolytic chamber, where the polypeptide chains are processively degraded into short peptides. FtsH is not only involved in the proteolytic elimination of unnecessary proteins, but also in the proteolytic regulation of a number of cellular functions. Its role in proteolytic regulation is achieved by one of two approaches, either the cellular levels of a regulatory protein are controlled by processive degradation of the entire protein, or the activity of a particular substrate protein is modified by processing. In the latter case, protein processing requires the presence of a stable domain within the substrate. Since FtsH does not have a robust unfolding activity, this stable domain is sufficient to abort processive degradation of the protein – resulting in release of a stable protein fragment.

Keywords FtsH • Processive degradation • Protein processing • Protein quality control • Regulation of lipid synthesis

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D.A. Dougan (ed.), *Regulated Proteolysis in Microorganisms*, Subcellular Biochemistry 66,
DOI 10.1007/978-94-007-5940-4_3,

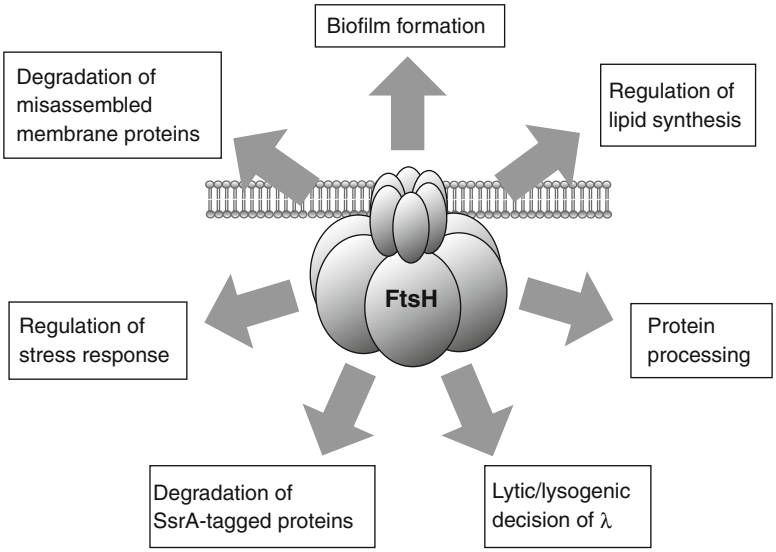

Fig. 3.1 Various cellular functions regulated by FtsH protease. FtsH, a membrane-bound AAA protease, is responsible for regulation of various cellular functions in *E. coli*. FtsH acts on both membrane and cytoplasmic substrate proteins. FtsH functions not only in the proteolytic elimination of unnecessary proteins but also controls the cellular levels of several regulatory proteins and the processing of specific substrate proteins (For further details refer to the main text)

Introduction

FtsH is an evolutionarily conserved protein that is present in all bacterial cells. It consists of transmembrane segment 1 (TM1), periplasmic domain, TM2, cytoplasmic ATPase and protease domains in this order from the N-terminus [1–6]. FtsH is a zinc-binding metalloprotease, which forms a homohexameric ring-shaped structure. It can degrade unstructured model substrates as well as structurally unstable substrate proteins, which can be easily unfolded. Unfolded polypeptide chains are translocated through the central pore of the ATPase domain into the proteolytic chamber. Proteolysis by FtsH is processive and most degradation products are short peptides of several to 20 amino acid residues in length [7]. In some cases, however, larger functional products containing at least one stable domain are released from FtsH as a result of incomplete processive proteolysis. This type of protein processing depends on domain stability and not sequence specificity. Eukaryotic homologs of FtsH have been identified in mitochondria and chloroplasts [8–11]. In mitochondria, there are two types of FtsH homologs, commonly referred to as *i*-AAA and *m*-AAA proteases depending on their topology in the mitochondrial inner membrane. Dysfunction of these proteins in humans has been related to a variety of diseases. In this chapter, however, we will mainly overview the cellular functions controlled by *Escherichia coli* FtsH (Fig. 3.1) and its regulatory mechanisms, and only briefly discuss some of the recent advances of other bacterial or mitochondrial homologs of FtsH.

Quality Control of Cytoplasmic Proteins

Regulation of the Heat Shock Response

Under normal cellular conditions, the heat shock transcriptional factor σ^{32} (which binds to the promoter region of heat shock genes) is rapidly degraded by FtsH. Upon shift to stress conditions, such as a temperature upshift, the cellular levels of σ^{32} increase ~15–20 fold in *E. coli*. This rapid increase in the cellular levels of σ^{32}, leads to an induction of stress response proteins, which is important for cell survival under these conditions. This increase in the level of σ^{32} is transient, which ensures the rapid and transient induction of stress response proteins. Sigma32 accumulates in *ftsH* mutant strains. The *ftsH* gene has been found to encode an inner-membrane anchored ATP-dependent AAA-type protease, which contributes to the efficient degradation of σ^{32} [12, 13]. Although the soluble cytoplasmic ATP-dependent proteases such as ClpAP, ClpXP and HslUV contribute to the degradation of σ^{32}, to some extent, the membrane-anchored FtsH recognizes and degrades σ^{32} preferentially. Currently, the precise amino acid sequence of σ^{32} responsible for recognition and degradation by FtsH has not yet been defined, however substitution of amino acid residues in region 2.1 and region C of σ^{32} affects FtsH-dependent stability of σ^{32} in the cell [14–16]. Since FtsH rapidly degrades destabilized σ^{32} *in vitro*, FtsH may only act on unstructured σ^{32}. Interestingly, to date, the efficient *in vivo* degradation of σ^{32} has not been reconstituted *in vitro* using purified FtsH. As such, it has been suggested that DnaK/DnaJ (DnaK/J) contributes to the stability of σ^{32} *in vivo*. Careful *in vitro* analysis has revealed that DnaJ binding to region 2.1 of σ^{32} destabilizes a distant region in close vicinity of the DnaK-binding site, and that DnaK destabilizes a region in the N-terminal domain [17]. If DnaK/J-induced destabilization of the N-terminal domain might facilitate degradation of σ^{32} by FtsH, it would be consistent with the fact that FtsH degrades σ^{32} from the N terminus to the C terminus [18]. So far, however, a DnaK/J-mediated stimulation of σ^{32} degradation by FtsH *in vitro* has not been reported. Indeed, the cooperative regulation of the FtsH activity by DnaK/J may be more complicated. A novel *in vitro* assay system, containing additional factors/components, needs to be developed to reveal the regulatory mechanism of FtsH-mediated degradation of σ^{32} by DnaK/J. It should also be noted that the transcriptional activity of σ^{32} is inhibited by binding of DnaK/J.

Degradation of SsrA-Tagged Proteins

SsrA RNA, also called tmRNA, is a specialized RNA that has properties of both a tRNA and an mRNA. When an mRNA lacks a stop codon, protein translation on the ribosome stalls, resulting in the production of an incompletely synthesized polypeptide. A short polypeptide "SsrA tag" is cotranslationally added to the C-terminus of the incomplete polypeptide in a reaction that is mediated by ribosome-bound SsrA

RNA. ATP-dependent proteases recognize and degrade these SsrA-tagged proteins to prevent accumulation of toxic incomplete polypeptides [18, 19]. The SsrA tag is attached to about 0.5% of newly synthesized polypeptides *in vivo*. Greater than 90% of SsrA-tagged polypeptides are digested by ClpXP protease [20] (for a recent review see [21]). The remaining 10% of the tagged polypeptides are removed from cells by ClpAP, Lon and FtsH. Therefore, FtsH partially but significantly contributes to degradation of SsrA-tagged polypeptides [22].

Quality Control of Membrane Proteins

FtsH is responsible for quality control of the inner-membrane environment. FtsH degrades membrane proteins, in an ATP-dependent manner, when they fail to form functional membrane protein complexes. For example, FtsH recognizes and degrades unassembled SecY, an integral membrane subunit of the protein translocation machinery (Sec translocase) in the inner membrane, and unassembled Fo subunit *a*, a membrane subunit of the ATP synthase [23, 24]. Similarly, when the Sec translocase becomes blocked with an inefficiently exported protein, the "jammed" SecY is degraded by FtsH [25]. The integral membrane protein YccA is also a proteolytic substrate of FtsH. Interestingly, YccA also modulates the FtsH-mediated degradation of membrane proteins. For example, YccA inhibits the FtsH-mediated degradation of blocked SecY. Nevertheless, the molecular mechanisms for the recognition of jammed SecY by FtsH and the inhibition of FtsH-mediated degradation by YccA remain elusive.

It has been proposed that FtsH cleaves polypeptide chains of substrate membrane proteins by extracting them from the inner membrane, releasing substrate-derived short peptides into the cytoplasm. To initiate degradation of membrane proteins, FtsH recognizes either N- or C-terminal cytoplasmic segments of a sufficient length (20 amino acid residues or more) [26, 27]. FtsH-mediated degradation of membrane proteins is processive, starting from one terminus, dislocating their transmembrane helices and periplasmic regions into the cytoplasmic side of the membrane, where the peptidase active site of FtsH is located. At present, the precise mechanism of this dislocation by FtsH has not been elucidated. Degradation by FtsH stops, when FtsH encounters the structurally stable domain [28]. Undigested fragments containing stable domains accumulate in the inner membrane [26].

It is conceivable that FtsH works cooperatively with an ATP-independent protease HtpX to remove substrate membrane proteins from the inner membrane [29]. HtpX is anchored to the inner membrane by the N-terminal transmembrane segment with overall topology similar to FtsH. The metal-binding protease active site of HtpX faces the cytoplasm. Although the physiological substrates of HtpX have not been identified yet, HtpX catalyzes the cleavage of casein and SecY polypeptide chains *in vitro* as well as the cleavage of overproduced SecY *in vivo*. HtpX is also responsible for endoproteolytic cleavage within cytoplasmic regions of membrane proteins. A plausible scenario for the collaboration of FtsH and HtpX is that HtpX cleaves the cytoplasmic

loops of substrate membrane proteins, generating a new cytoplasmic tail, which can be recognized by FtsH, resulting in the FtsH-mediated dislocation and degradation of the rest of the polypeptide chain [30].

Regulation of Lipid Synthesis

The composition and amount of lipid in the cell membrane is important for normal function. The balance of lipid composition in both inner and outer membranes of *E. coli* is tightly regulated. Since the biosynthesis of both phospholipids (PL) and lipopolysaccharides (LPS) are multistep pathways, which involve many different enzymes, the precise regulation (of amount and/or activity) of the enzymes that catalyze the committed steps in the pathway are critical for maintaining proper lipid composition. The cellular level of two key enzymes in biosynthesis of LPS is controlled by FtsH-mediated degradation. Loss of FtsH causes serious defects in the membrane function of *E. coli* and leads to cell death. The role of FtsH in the cell is not only restricted to proteolytic elimination of unnecessary proteins, but also to fine-tuning the cellular level of several critical proteins. Interestingly, the mitochondrial homolog of FtsH, Yme1, also participates in the regulation of lipid composition in the inner membrane of mitochondria as described below.

Synthesis of Lipid Molecules in E. coli

E. coli membranes are composed of two types of lipid molecules; PL and LPS. Both of which are synthesized by different pathways, and supplied to the membranes. The synthetic pathways and enzymes involved in lipid synthesis in *E. coli* are summarized in Fig. 3.2. For a detailed description of the biosynthesis of lipid molecules in *E. coli*, please refer to the following excellent reviews [31, 32]. The acyl donor, *R*-3-hydroxymyristoyl-ACP is an important branch point in the biosynthesis of both lipid molecules. In the synthesis of PL, *R*-3-hydroxymyristoyl-ACP is dehydrated by FabZ (*R*-3-hydroxy-acyl-ACP dehydrase), followed by elongation of short carbon units to produce long chain acyl-ACP species. Acyl-ACP is then transferred to lysophosphatidic acid (LPA) by either PlsX or PlsB, to produce phosphatidic acid (PA). Various types of PL are then synthesized from PA. In the synthesis of LPS, *R*-3-hydroxymyristoyl-ACP is attached to UDP-N-acetylglucosamine (UDP-GlcNAc) by LpxA to produce UDP-3-hydroxymyristoyl-GlcNAc. This reaction is the slowest step in LPS synthesis and the product of this reaction tends to return to the reactants. Therefore the next step in LPS synthesis, catalyzed by LpxC, is the rate-determining process [33]. Stimulation of LPS, but not PL, biosynthesis can exhaust the supply of the common intermediate, *R*-3-hydroxymyristoyl-ACP, causing an imbalance in biosynthesis of lipid molecules which prevents normal growth of *E. coli*. Therefore, for the correct maintenance of the PL:LPS ratios in the cell membrane, it is critical for the cell to balance the use of *R*-3-hydroxymyristoyl-ACP appropriately in both pathways.

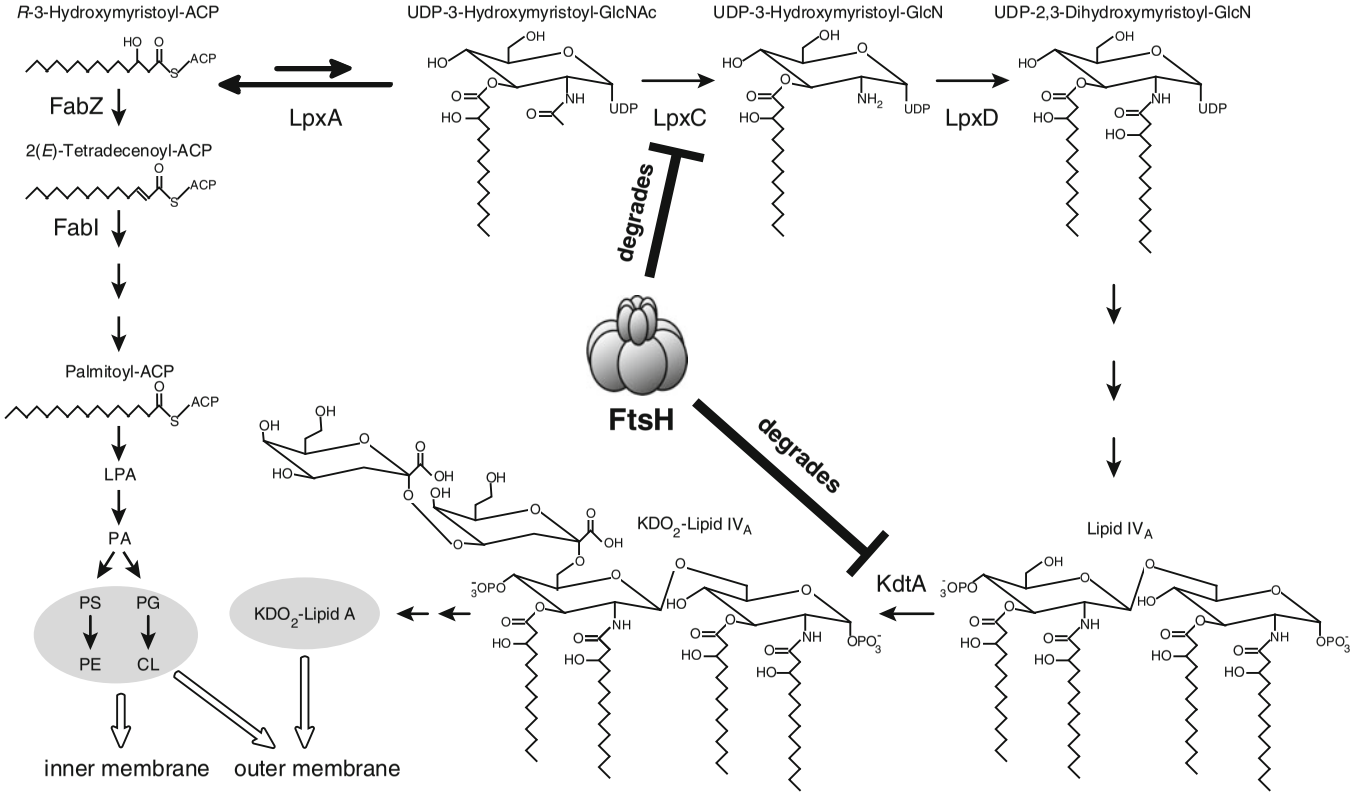

Fig. 3.2 Biosynthesis pathways of major membrane components in *E. coli* and regulation by FtsH. *Cartoon* illustrating the reaction steps in the biosynthesis of lipids that are regulated by FtsH. FtsH controls cellular levels of LpxC and KdtA by regulated degradation. *T-bars* represent negative control of the pathway, by the specific degradation of LpxC and KdtA. *ACP* acyl carrier protein, *GlcNAc* N-acetylglucosamine, *GlcN* glucosamine, *LPA* lysophosphatidic acid, *PA* phosphatidic acid, *PS* phosphatidylserine, *PG* phosphatidylglycerol, *PE* phosphatidylethanolamine, *CL* cardiolipin, *KDO* 3-deoxy-D-*manno*-octulosonic acids

Discovery of the Connection of FtsH to Lipid Synthesis

ftsH is an essential gene in *E. coli*. In the mid-1970s a thermosensitive mutant *E. coli* strain (*ftsH1*) was isolated [34]. Although this mutant strain was originally isolated as a cell division mutant, it was later (in the early 1990s) demonstrated to carry two mutations; *ftsH1* – responsible for lethality at high temperature and *ftsI372* – responsible for the cell division phenotype [35]. However, it wasn't until 1999, that the molecular basis of the lethal phenotype of the *ftsH1*(ts) mutation was clarified. The key finding to demonstrate the essential nature of the *ftsH* gene was the identification of a suppressor gene in another *ftsH* mutant, *tolZ21*. This mutant was isolated as a colicin tolerant strain, and it was found that *tolZ* was identical to *ftsH* [36]. The *tolZ21* mutation (H421Y) was located in a critical residue in the zinc-binding motif of FtsH essential for the metalloprotease activity, and thus it was expected that the mutant FtsH had no proteolytic activity. However, as the *tolZ21* mutant was viable, it suggests that, either the protease activity of FtsH is not required or that the *tolZ21* mutant carries a suppressor mutation. Precise genetic analysis revealed the presence of a suppressor mutation, and it was found that the suppressor mutation was an allele of *fabZ* [37]. Consistently, abnormal membrane structures accumulated in the periplasmic space of the *ftsH1* mutant at the non-permissive temperature. Biochemical analysis also showed an increase in the amount of LPS at non-permissive temperature [37]. Collectively, these data indicate that dysfunction of the FtsH protease causes alterations in lipid synthesis. Studies on two distinct *ftsH* mutant strains led to the discovery of a novel role of FtsH in the regulation of lipid synthesis.

Regulation of LpxC Levels by FtsH Protease

LpxC is a cytoplasmic enzyme responsible for catalyzing the committed step in LPS synthesis. It is composed of 205 amino acid residues and has a molecular mass of 33.9 kDa. A segment of the C-terminal sequence of LpxC (~20 amino acid residues) is required for degradation by FtsH [38, 39]. The C-terminus of LpxC, which resembles the SsrA tag, is rich in non-polar residues and mutation of which have been shown to stabilize LpxC [39]. Interestingly, although FtsH preferentially degrades LpxC, and hence regulates the amount of LpxC in enterobacteria [37], the FtsH-mediated degradation of LpxC is not conserved across all Gram-negative bacteria [40]. Accumulation of LpxC stimulates LPS biosynthesis, leading to a lethal imbalance in the PL:LPS ratio. Excess amounts of LPS in the cell result in the formation of abnormal membrane structures in the periplasmic space. The suppressor mutation *sfhC21* identified in the *tolZ21* mutant was shown to contain a point mutation in *fabZ*. This point mutation stimulates the activity of FabZ and compensates for the accumulation of LpxC, preventing the overproduction of LPS using *R*-3-hydroxymyristoyl-ACP for PL synthesis instead of LPS synthesis. Similarly, repression of LpxA or LpxD (two enzymes involved in LPS synthesis pathway, found before or after LpxC, in the pathway) also suppress the lethality

of *ftsH1*. Indeed, to date, all identified suppressor mutations of *ftsH1* repress LPS synthesis.

Here an interesting question arises. Does the *sfhC21* mutation alone cause any defects in cell growth? Although it is reasonable to assume that the *sfhC21* mutation in *fabZ* causes an acceleration of PL synthesis and hence an increase in the PL:LPS ratio, this is not the case. In fact the *sfhC21* mutation in FabZ stabilizes LpxC, independent of the presence or absence of FtsH, and in fact the normal ration of PL and LPS is maintained in the strain that only carries the *sfhC21* mutation. Moreover, the stabilization of LpxC by the *sfhC21* mutation is substrate-specific, as the FtsH-mediated degradation of σ^{32} is not affected. The molecular mechanism, however, of this substrate-specific inhibition of FtsH-mediated degradation remains to be elucidated. Perhaps, alterations in acyl-ACP pools might modulate FtsH activity in a substrate-specific manner.

Degradation of KdtA

FtsH also regulates a late step in the LPS biosynthesis, the transfer of two 3-deoxy-D-manno-octulosonate (KDO) residues to lipid IV_A, which is catalyzed by KDO transferase (KdtA). KdtA is the sole enzyme that catalyzes transfer of two KDOs to lipid IV_A and hence is an essential glycosyltransferase in oligosaccharide biosynthesis [31]. KdtA is an inner-membrane protein, which is tethered to the membrane through an N-terminal transmembrane segment and its catalytic residues are presumed to face the cytoplasm. The *in vivo* half-life of KdtA is very short (~10 min) and the protease primarily responsible for its rapid degradation is FtsH [41]. The site or domain in KdtA to be recognized by FtsH has not been identified. As describe above, FtsH regulates the biosynthesis of LPS by controlling the amount of LpxC in the cell. Taken together, membrane-anchored FtsH protease plays important roles in the lipid biosynthesis by regulating the amount of two critical enzymes involved in the biosynthesis pathway of LPS.

Regulation of Lipid Composition in Mitochondria

As briefly mentioned above, homologs of FtsH are also found in the chloroplast and mitochondrion of eukaryotic cells. In mitochondria, there are two different FtsH-like proteases; referred to as *m*-AAA and *i*-AAA. In yeast, *i*-AAA is a homohexamer of Yme1 while *m*-AAA is a heterohexamer composed of two different proteins (Yta10 and Yta12). In contrast, humans contain a single *i*-AAA homohexamer (composed of YME1L) and two different *m*-AAA proteases (a homohexamer of AFG3L2 and a hetero-oligomer composed of AFG3L2 and paraplegin). All mitochondrial FtsH-like proteases are located in the inner membrane, *i*-AAA has one transmembrane segment near the N-terminus and its proteolytic active site is exposed to the intermembrane space, whereas *m*-AAA protease contains two transmembrane segments and its active site faces the

matrix. Both *i*-AAA and *m*-AAA proteases are responsible for the proteolytic elimination of misfolded membrane proteins and protein processing in mitochondria. Dysfunction of *m*-AAA proteases in mitochondria causes neurodegenerative diseases [10, 42].

Although mitochondria have their own system for PL synthesis, several PLs, which constitute the inner and outer membranes of mitochondria, are supplied from the endoplasmic reticulum, as precursors. Cardiolipin (CL) and phosphatidylethanolamine (PE) are synthesized at the inner membrane, from phosphatidic acid and phosphatidylserine, respectively, and then transferred to the outer membrane through the intermembrane space. Homeostasis of CL and PE is regulated by two intermembrane space proteins, Ups1 and Ups2, respectively [43]. Although it is not yet clear how Ups1 and Ups2 regulate the concentration of CL and PE in the membrane [44]. Importantly, the level of Ups1 and Ups2 in the intermembrane space is regulated by rapid degradation by *i*-AAA protease [44]. Consistent with this, both Ups1 and Ups2 accumulate in mitochondria from the yeast *yme1* deletion strain. Overexpression of either Ups1 or Ups2 causes alterations in mitochondrial lipid composition, which leads to mitochondrial dysfunction [45]. Interestingly, the *i*-AAA-mediated degradation of Ups1 and Ups2 can be inhibited by binding of Mdm35 (a member of the twin Cx_9C protein family) [46].

Processing of Substrate Proteins

FtsH, in comparison to other AAA^+ proteases such as ClpXP, ClpAP, and HslUV has been demonstrated to have a "weak" unfolding activity [47, 48]. This distinguishing feature of FtsH plays an important role in its various *in vivo* functions. Although proteolysis by FtsH is processive, it is also abortive when FtsH encounters a tightly folded domain. Release of the stable polypeptide fragment, generated from abortive digestion by FtsH (and its homologs), is referred to as 'protein processing' and plays an important regulatory role in a number of cellular functions. In this section, 'protein processing' by FtsH and its homologs is summarised.

Processing of Colicins

Colicins are protein antibiotics that are released into the medium from *E. coli* cells carrying *colicin* genes, which kills other *E. coli* cells. Colicins released into the medium bind to receptors (BtuB, OmpF, FepA, etc.) on the outer membrane of *E. coli*, and are translocated, in cooperation with Tol and Ton translocators located in the inner membrane, into the periplasm. Then, colicins are imported into the cytoplasm, although the machineries for the translocation of colicins from the periplasm to the cytoplasm are not yet understood [49, 50]. Nuclease-type colicins must be imported into the cytoplasm of the target *E. coli* cell, where they disrupt DNA, tRNA and rRNA.

The *tolZ* mutant is tolerant to colicins E2, E3 and D. All of these colicins are nucleases, which, when translocated to the cytoplasm, act on either DNA (colicin E2), rRNA (colicin E3), or tRNA (colicin D). The *tolZ21* mutation, as mentioned previously, has been identified as a point mutation (H421Y) in the *ftsH* gene, which inactivates FtsH function [36]. Detailed analysis indicated that nuclease colicin toxicity is dependent on functional FtsH [51]. It has been shown that colicins D and E3, which are translocated by different machineries (BtuB/Tol and FepA/TonB, respectively), are processed by FtsH during their import into the cytoplasm [52]. Premature colicin D (75 kDa) is processed in an FtsH-dependent manner to yield a 12.4 kDa fragment containing the tRNase domain [52]. Production of the processed form of colicin E3 (15 kDa) was also found to be FtsH-dependent. Details of the processing of colicins by FtsH remain elusive [52]. Since FtsH leaves tightly folded domains of substrate proteins undegraded, it is possible that the processed form of colicin may result from abortive degradation by FtsH (see later).

Self-Processing of FtsH

The C-terminal seven amino acid residues of FtsH are removed auto-catalytically in a process that is, not only growth-phase dependent but also affected by mutations in *hflKC* [53]. Although the molecular mechanism of FtsH self-processing has not been elucidated, the processing site has been precisely determined. There is a clear preference for specific amino acid residues at the cleavage site. This is the sole example of FtsH-mediated site-specific cleavage. However, the biological significance of the self-processing of FtsH is unclear, since both the processed and full-length forms of FtsH are functionally indistinguishable. Although the self-processing of FtsH appears site-specific, the cleavage site may be determined by the combination of sequence preference and stability of the domain preceding the processing site. An example of the position-specific processing, whose cleavage site is simply determined by domain stability, has been reported for a mitochondrial FtsH homolog, *m*-AAA protease, and is discussed below (for a recent review see [54]). Such a possibility should be investigated for the self-processing of FtsH.

Molecular Mechanism of Substrate Processing

When FtsH encounters a stable domain within a substrate protein, processive degradation of polypeptide chain by FtsH is aborted, and the stable domain that cannot be unfolded for threading through the narrow pore of the FtsH ring, is released. SecY is an integral membrane protein, which contains ten transmembrane segments with both the N- and C-termini facing the cytoplasm. To better understand how FtsH processes integral membrane proteins, a number of SecY fusion proteins were generated. In one case, the eighth transmembrane helix (TM8) together with the following cytoplasmic region (30 amino acid residues) was fused to the C-terminal

end of a periplasmic enzyme; alkaline phosphatase A (PhoA) to produce a model protein, PhoA-TM8-C_{30}. Although the reduced form of PhoA-TM8-C_{30} (lacking disulfide bonds in the PhoA domain) was completely degraded by FtsH, the oxidized form of PhoA-TM8-C_{30} (stabilized by disulfide bonds in the PhoA domain) was incompletely degraded and the stable PhoA domain fragment was released [26]. Collectively these data indicate that FtsH can initiate processive proteolysis from the C-terminus of the model substrate (PhoA-TM8-C_{30}), but cannot dislocate a stably folded domain (PhoA) from the periplasm to the cytoplasm.

Flavodoxin is a small flavin mononucleotide-containing protein, which plays an essential role in electron transfer pathways. Apo-flavodoxin, but not holo-flavodoxin, is degraded by FtsH *in vitro*. Interestingly, when apo-flavodoxin was attached to glutathione *S*-transferase (GST) or green fluorescent protein (GFP) such that FtsH can initiate processive proteolysis from the apo-flavodoxin moiety of the different model fusion substrates, FtsH was able to unfold and degrade the attached GST, but not the attached GFP. These data suggest that FtsH cannot unfold or degrade the thermally stable GFP, while it can unfold and degrade, to some extent, the GST moiety [55]. Therefore, the susceptibility of a domain to degradation by FtsH depends on thermal stability of that domain [56]. Interruption of processive proteolysis by a stably folded domain shown for these model substrates may be the molecular mechanism for FtsH-mediated processing of substrate proteins such as colicins and FtsH itself.

Processing of Substrate Proteins by Mitochondrial FtsH Homologs

The mitochondrial *m*-AAA and *i*-AAA proteases, participate in processing of several mitochondrial regulatory proteins by limited proteolysis as well as general quality control of mitochondrial proteins by complete digestion of damaged proteins [57, 58]. Yeast *m*-AAA protease is responsible for the processing of MrpL32, a component of the mitochondrial ribosome. MrpL32 is synthesized as a precursor in the cytoplasm, imported to the mitochondrial matrix, and processed to its mature form before it is assembled into mitochondrial ribosomes. The *m*-AAA protease cleaves the protein between the 71st and 72nd residue of the MrpL32 precursor to produce the mature form. Careful analysis demonstrated that processing of MrpL32 by the *m*-AAA protease depends on the folding of MrpL32 rather than on the specific recognition of the cleavage site [59]. The *m*-AAA protease initiates proteolysis from the N-terminus of MrpL32, which is halted by a tightly folded domain. Mammalian *m*-AAA proteases can also act as processing enzymes *in vivo* [60]. In murine mitochondria, the repertoire of *m*-AAA proteases is further expanded by the presence of an additional FtsH homolog AFG3L1 which forms various other hetero-oligomeric *m*-AAA complexes. The mitochondrial processing peptidase MPP generates an intermediate form of AFG3L2. This intermediate form is matured autocatalytically. AFG3L1 or AFG3L2 is also required for maturation of imported paraplegin after its cleavage by MPP. It is of great interest that mutations in different protease subunits are associated with distinct neuronal disorders in human; mutations in AFG3L2 are associated with

spinocerebellar ataxia type 28 and spastic ataxia-neuropathy [41, 61], and those in paraplegin are associated with hereditary spastic paraplegia [62], respectively.

Mitochondrial *m*-AAA protease is also responsible for maturation of cytochrome *c* peroxidase (Ccp1) by rhomboid protease Pcp1 [63]. Premature Ccp1 is synthesized in the cytoplasm, translocated, and inserted into the inner membrane of mitochondria. The *m*-AAA protease mediates dislocation of the hydrophobic segment from the membrane in an ATP-dependent manner, making the processing site accessible for the rhomboid protease Pcp1. It should be noted that the maturation of Ccp1 depends only on the ATPase but not the proteolytic activity of the *m*-AAA protease.

Moreover, it has been shown that both *m*-AAA and *i*-AAA proteases are linked to the processing of the dynamin-like GTPase OPA1, a component of the mitochondrial fusion machinery [64, 65]. However, it is uncertain whether they act as processing enzymes or just assist processing by other proteases.

Biofilm Formation

Biofilm is an aggregate of microorganisms, in which cells stick to the surface of substrates and organisms. Development of the biofilm 'state' depends on the environments surrounding the bacteria, and is thought to be regulated by expression of multiple genes and operons [66, 67]. Because secretions, containing extracellular polysaccharide, wrap the aggregate of microorganisms, the microorganisms within the biofilm acquire physical strength and resistance to chemicals. Understanding the details of the biofilm 'life cycle' is important to develop procedures to control biofilm formation, which will be useful in medicine and food industry. At present, however, the precise processes that regulate the formation of biofilms are still unclear. Recently, the membrane anchored FtsH protease was implicated in the formation of biofilms. It was shown that a Δ*ftsH* mutant of *Lactobacillus plantarum* had a reduced capacity to form biofilms on abiotic surfaces [68]. Quantitative reverse transcription polymerase chain reaction (RT-PCR) studies revealed that expression of *ftsH* was upregulated during accretion of *Porphyromonas gingivalis* in heterotypic biofilms with *Streptococcus gordonii*. Contrary to the studies with *L. plantarum*, the Δ*ftsH* mutant of *P. gingivalis* formed more abundant biofilms with *S. gordonii* [69]. Taken together, it seems reasonable to assume that FtsH is involved in the biofilm formation in a variety of microorganisms. Yet, the roles of FtsH in the regulation of biofilm formation largely remain elusive.

Regulatory Proteins of FtsH

Following infection of an *E. coli* cell by a λ phage, the phage either enters the lytic or the lysogenic pathway. This choice is dependent on the physiological condition of the host cell and the decision is controlled by several key proteins encoded by the λ

genome. For example, the amount of the transcription factor CII plays a crucial role in deciding which pathway to take. The cellular level of CII is primarily controlled by FtsH [70]. Indeed the degradation of CII by FtsH is very rapid (half-life ~2 min) under normal conditions, leading to the lytic pathway. However, under certain conditions, the degradation of CII by FtsH is inhibited, and CII accumulates in the cell, favouring the lysogenic pathway. The other key λ phage protein is λCIII, as it has been identified as an inhibitor of FtsH-mediated λCII degradation [71, 72]. This is because λCIII competes with λCII for binding to FtsH and is very slowly degraded by FtsH, CII is stabilized in the presence of CIII.

The proteolytic activity of FtsH is also modulated by two inner membrane proteins; HflK and HflC, which are homologs of the eukaryotic prohibitins. Both proteins have transmembrane segments near their N-termini and large periplasmic domains. Together they form a stable complex (HflKC). The HflKC complex binds to FtsH to form the FtsH holoenzyme, an exceptionally large complex (~1,000 kDa) with a proposed *in vivo* composition of $FtsH_6$•$HflK_6$•$HflC_6$ [73]. Binding of HflK or HflC to FtsH inhibits the degradation of CII [74, 75]. However, because the rate of σ^{32} degradation is not affected by the addition of HflKC, the HflKC complex does not simply decrease the proteolytic activity of FtsH. It has also been proposed that HflKC is involved in the regulation of proteolysis of membrane substrates [75, 76]. Currently however, the details of the structure of the FtsH holoenzyme and the selective regulation of the proteolytic activity of FtsH by HflKC remain to be elucidated.

Prohibitins, the eukaryotic homologs of HflK and HflC, are highly conserved membrane proteins that are required for normal cell growth and development. Prohibitins localize to the inner membrane of mitochondria and form large, multimeric ring-shaped complexes with a diameter of 20–25 nm. The function of prohibitins in mitochondria is related to the regulation of various mitochondrial functions such as respiration, stability of mitochondrial DNA, and maintenance of mitochondrial morphology [77–79]. Prohibitins associate with *m*-AAA protease and modulate its proteolytic activity. The loss of prohibitins in mitochondria stimulates the degradation of unassembled inner membrane proteins by *m*-AAA protease. On the other hand, it has also proposed that the prohibitin ring complex performs a scaffolding function to recruit *m*-AAA protease to a specific functional site in the inner membrane of mitochondria. The complexes of *m*-AAA proteases and prohibitins were also identified in plant mitochondria [80].

Conclusions and Perspectives

Since the discovery of FtsH protease, a number of substrate proteins have been identified. FtsH recognizes a wide range of substrate proteins, and thus is involved in the regulation of a variety of cellular processes. In some substrates, an unstructured tail of ~20 amino acid residues, located at either the N- or C-terminus is recognized by FtsH to initiate processive proteolysis. Since FtsH lacks a robust

unfolding activity, it is primarily responsible for (a) the selective degradation of structurally unstable proteins or (b) processing of specific protein substrates as a result of encountering a stable domain, which aborts processive proteolysis by FtsH. This processing role has been more extensively studied in eukaryotic FtsH homologs present in mitochondria. Indeed, recent studies revealed that both substrate selectivity and weak unfolding ability of FtsH are crucial for its regulatory roles in diverse cellular activities. To date, however, the precise mechanisms of substrate recognition, unfolding, and processive degradation by FtsH are still largely unclear. Further investigations will be of importance to understand the molecular mechanism of FtsH, which executes the regulation of a variety of cellular processes.

Acknowledgements We are grateful to Yoshinori Akiyama for stimulating discussion and invaluable comments and to David Dougan for generous editorial suggestions to the manuscript. We also thank to Chiyome Ichinose for secretarial assistant. This work was supported in part by grants from the Ministry of Education, Culture, Science, Sports and Technology of Japan.

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