Roles of multimerization and membrane association in the proteolytic functions of FtsH (HflB)

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FtsH (HflB) is an *Escherichia coli* ATP-dependent protease that degrades some integral membrane and cytoplasmic proteins. While anchored to the cytoplasmic membrane by the two transmembrane (TM) segments near the N-terminus, it has a large cytoplasmic domain. The N-terminal region also has a role in homo-oligomerization of this protein. To study the significance of the membrane integration and oligomer formation, we constructed FtsH derivatives in which the N-terminal region had been deleted or replaced with either the leucine zipper sequence from *Saccharomyces cerevisiae* GCN4 protein or TM regions from other membrane proteins. The cytoplasmic domain, which was monomeric and virtually inactive, was converted, by the attachment of the leucine zipper, to an oligomer with proteolytic function against a soluble, but not a membrane-bound substrate. In contrast, chimeric TM-FtsH proteins were active against both substrate classes. We suggest that the cytoplasmic domain has intrinsic but weak self-interaction ability, which becomes effective with the aid of the leucine zipper or membrane tethering, and that membrane association is essential for FtsH to degrade integral membrane proteins.

Keywords: AAA ATPase/membrane protein/oligomerization/protein degradation

Introduction

Membrane proteins serve as structural components as well as catalysts of substrate transport and signal transduction. Their quality control is critical for maintenance of the membrane functions. However, our knowledge about how aberrant membrane proteins are degraded has been limited until recently.

In *Escherichia coli*, FtsH, a membrane-bound and ATP-dependent metalloprotease, is involved in degradation of some integral membrane proteins (Kihara *et al.*, 1995; Akiyama *et al.*, 1996b). This is the only integral membrane protein among the ATP-dependent proteases of *E. coli* (Gottesman *et al.*, 1997). It has two transmembrane (TM) segments at the N-terminal region, which is followed by a large cytoplasmic domain (Tomoyasu *et al.*, 1993a). Like other ATP-dependent proteases, FtsH has a homo-oligomeric structure (Akiyama *et al.*, 1995). We showed that the N-terminal region is important for homo-oligomerization (Akiyama *et al.*, 1995). The cytoplasmic region contains two subdomains: an AAA ATPase domain

located just C-terminal to the membrane region and a protease domain with the HEXXH zinc-binding motif (Tomoyasu *et al.*, 1993b).

We also showed that FtsH is at least partly in complex with a pair of membrane proteins, HflK and HflC, which themselves form a complex (HflKC) (Kihara *et al.*, 1996). HflKC seems to regulate FtsH function (Kihara *et al.*, 1996, 1998). The short periplasmic domain between the TM segments of FtsH is essential for the interaction with the periplasmically exposed HflKC complex (Akiyama *et al.*, 1998b).

Membrane-bound substrates of FtsH include several multi-spanning membrane proteins, such as SecY (Kihara *et al.*, 1995), subunit *a* of the proton ATPase (*F₀a*) (Akiyama *et al.*, 1996a) and the product of the *yccA* open reading frame (Kihara *et al.*, 1998). Whereas the SecY subunit and the *F₀a* subunit are degraded rapidly when they fail to associate with their partner proteins, YccA is degraded more slowly and its cellular function is unknown.

The cytoplasmic localization of the enzymatic domains of FtsH raises a question of whether and how it hydrolyzes the membrane-integrated and periplasmically exposed regions of a substrate protein. We examined *in vivo* degradation profiles of YccA and SecY derivatives that had an alkaline phosphatase (PhoA) domain inserted into a periplasmic region of these proteins (Kihara *et al.*, 1999). The FtsH-mediated proteolysis seemed to propagate rapidly into the PhoA reporter region when the PhoA part remained unfolded. In contrast, the degradation stopped before the PhoA domain when it was tightly folded. From these results, we proposed that FtsH-mediated degradation of membrane proteins is processive and accompanied by dislocation of the periplasmic domains to the cytoplasmic side (Kihara *et al.*, 1999).

In the present work, we studied the significance of membrane integration and multimerization in the functions of FtsH. We constructed and analyzed several derivatives of FtsH in which the membrane region was deleted, or replaced either with a leucine zipper sequence or with TM regions of other membrane proteins. The results suggested that multimerization of the cytoplasmic domain is essential for the proteolytic activity of FtsH, but without a TM region it cannot work against membrane proteins. We propose that FtsH-catalyzed degradation of membrane proteins requires membrane integration of this enzyme.

Results

In vivo proteolytic functions of FtsH(Δ TM)-His₆-Myc and Zip-FtsH(Δ TM)-His₆-Myc

As a first step in our examination of the role of the membrane region, N-terminal residues 1–123 were deleted

Fig. 1. Schematic representations of FtsH-His₆-Myc, FtsH(ΔTM)-His₆-Myc, Zip-FtsH(ΔTM)-His₆-Myc, LacY-FtsH-His₆-Myc and EnvZ-FtsH-His₆-Myc used in this study. All the proteins carried the C-terminal His₆-Myc tag. Hatched regions are derived from FtsH. TM1 and TM2 indicate the first and second TM segments of either FtsH, LacY or EnvZ. The molecular mass of each protein calculated from the amino acid sequence is indicated in parentheses.

from FtsH-His₆-Myc [FtsH(ΔTM)-His₆-Myc in Figure 1]. We also constructed Zip-FtsH(ΔTM)-His₆-Myc in which the leucine zipper sequence from the *Saccharomyces cerevisiae* GCN4 protein (Karimova *et al.*, 1998) was attached to the N-terminus of FtsH(ΔTM)-His₆-Myc (Figure 1). Because the N-terminal region is required not only for membrane localization (Tomoyasu *et al.*, 1993a) but also for multimerization of FtsH (Akiyama *et al.*, 1995), FtsH(ΔTM)-His₆-Myc was expected to be monomeric and soluble. The leucine zipper sequence was thought to induce dimerization of FtsH(ΔTM)-His₆-Myc.

To assess the *in vivo* abilities of these proteins to degrade soluble and membrane-bound substrates, cellular accumulation of σ^{32} (chromosomally encoded) and SecY (overexpressed from a plasmid; Taura *et al.*, 1993) was examined by immunoblotting (Figure 2). We used *ΔftsH* cells, which allowed high level accumulation of these proteins (Figure 2, lane 1), and their reduction was taken as the proteolytic activity of FtsH-His₆-Myc derivatives expressed from a compatible plasmid. In contrast to FtsH-His₆-Myc, which reduced the amounts of σ^{32} and SecY markedly (Figure 2, lane 2), expression of FtsH(ΔTM)-His₆-Myc at a similar level did not significantly affect the levels of σ^{32} and SecY (lane 3), although its higher overproduction resulted in a slight reduction of σ^{32} (data not shown). Thus, the cytoplasmic domain alone is largely non-functional.

In contrast, Zip-FtsH(ΔTM)-His₆-Myc strikingly lowered the level of σ^{32} (Figure 2, lane 4). Therefore, proteolytic activity was fully regained by the attachment of the leucine zipper sequence. A mutant form of Zip-FtsH(ΔTM)-His₆-Myc with a mutation in the protease active site (*ftsH317*) (Figure 2, lane 5) or in the ATP-binding site (*ftsH41*) (lane 6) did not reduce the accumulation of σ^{32} appreciably even when they were expressed at higher levels. Thus, the activities observed with Zip-FtsH(ΔTM)-His₆-Myc were dependent on the normal activities of FtsH. Interestingly, accumulation of SecY was not affected by Zip-FtsH(ΔTM)-His₆-Myc (Figure 2, lane 4). These results were substantiated more directly by

Fig. 2. Accumulation of SecY and σ^{32} in cells expressing FtsH(ΔTM)-His₆-Myc or Zip-FtsH(ΔTM)-His₆-Myc. Strain AR5090 (*ΔftsH*) carrying pKY248 (*secY*) was transformed further with the plasmids indicated below. Cells were grown in L medium at 30°C and induced with 1 mM IPTG and 5 mM cAMP for 2.5 h. Proteins were precipitated by 5% trichloroacetic acid and analyzed by SDS-PAGE followed by immunoblotting using anti-FtsH, anti- σ^{32} or anti-SecY antibodies as indicated. The second plasmids carried were: pUC119 (vector; lane 1), pSTD113 (*ftsH-his6-myc*; lane 2), pSTD219 [*ftsH(ΔTM)-his6-myc*; lane 3], pSTD430 [*zip-ftsH(ΔTM)-his6-myc*; lane 4], pSTD432 [*zip-ftsH317(ΔTM)-his6-myc*; lane 5] and pSTD444 [*zip-ftsH41(ΔTM)-his6-myc*; lane 6]. FtsH⁺ indicates C-terminally self-processed product of FtsH-His₆-Myc (Akiyama, 1999) (lane 1).

pulse-chase experiments (Figure 3). Zip-FtsH(ΔTM)-His₆-Myc greatly enhanced the degradation of σ^{32} (Figure 3A, solid circles) but not of SecY (Figure 3B, solid circles). Both of these substrates were stable in the presence of FtsH(ΔTM)-His₆-Myc (solid triangles). These results indicate that: (i) the cytosolic domain of FtsH is barely active; (ii) it is activated by the N-terminal addition of the leucine zipper sequence with respect to proteolysis against σ^{32} ; and (iii) the leucine zipper sequence is still unable to confer proteolytic activity against SecY.

Oligomeric states of FtsH(ΔTM)-His₆-Myc and Zip-FtsH(ΔTM)-His₆-Myc

Upon cell fractionation, both FtsH(ΔTM)-His₆-Myc and Zip-FtsH(ΔTM)-His₆-Myc were recovered almost exclusively in the soluble cytoplasmic fraction (data not shown). When Zip-FtsH(ΔTM)-His₆-Myc (calculated molecular mass 65.5 kDa) was highly overproduced, it formed a doublet of ~70 and ~60 kDa upon electrophoresis (Figure 4B, lane 9). The lower band had almost the same mobility as FtsH(ΔTM)-His₆-Myc (calculated molecular mass 60.8 kDa) and reacted with both anti-FtsH (see Figure 4A) and anti-Myc (data not shown). Zip-FtsH317(ΔTM)-His₆-Myc did not produce the doublet (data not shown). These observations suggest that the lower band represented a product of self-cleavage around the junction between the leucine zipper and FtsH(ΔTM).

We purified the proteins using Ni-NTA affinity chromatography and examined their profiles by gel filtration chromatography (Figure 4A). FtsH(ΔTM)-His₆-Myc and the self-cleavage product of Zip-FtsH(ΔTM)-His₆-Myc were recovered at fractions corresponding to

Fig. 3. Degradation of σ^{32} and SecY in cells expressing FtsH(Δ TM)-His₆-Myc, Zip-FtsH(Δ TM)-His₆-Myc or LacY-FtsH-His₆-Myc. Plasmids pSTD113 (*ftsH-his6-myc*; open circles), pSTD430 [*zip-ftsH(ΔTM)-his6-myc*; solid circles], pSTD348 (*lacY-ftsH-his6-myc*; open triangles), pSTD219 [*ftsH(ΔTM)-his6-myc*; solid triangles] and pUC119 (vector; open squares) were introduced into AR5090 (*ΔftsH*) to examine SecY stability (A) or into AR5090 carrying pKY248 (*secY*) to examine σ^{32} stability (B). Cells were grown in M9 medium at 30°C, induced with 1 mM IPTG and 5 mM cAMP for 2 h, pulse-labeled with [³⁵S]methionine for 2 min and chased with unlabeled methionine for the indicated time. At each time point, a portion of culture was treated with trichloroacetic acid for subsequent immunoprecipitation of σ^{32} (A) or SecY (B). σ^{32} and SecY radioactivities were determined after SDS-PAGE, and are reported as a percentage of the initial (0 min chase) radioactivity for each culture.

~70 kDa. On the other hand, the intact Zip-FtsH(Δ TM)-His₆-Myc protein (upper band) was eluted earlier, at fractions corresponding to ~170 kDa.

The oligomeric states of the purified proteins were also examined by cross-linking experiments (Figure 4B). Purified preparations of FtsH(Δ TM)-His₆-Myc and Zip-FtsH(Δ TM)-His₆-Myc were treated with increasing concentrations of a disulfide-cleavable cross-linker, dithio-bis(succinimidyl propionate) (DSP) and subjected to SDS-PAGE before and after cleavage of the cross-link. DSP-treated Zip-FtsH(Δ TM)-His₆-Myc yielded slow migrating products with apparent molecular masses of ~155 and 175 kDa (Figure 4B, lanes 1–4), which disappeared upon reduction (lanes 9–12). No such cross-linked product was generated from FtsH(Δ TM)-His₆-Myc (Figure 4B, lanes 5–8).

Taken together, these results suggest that FtsH(Δ TM)-His₆-Myc is monomeric whereas Zip-FtsH(Δ TM)-His₆-Myc is at least dimeric. The generation of two cross-linked

Fig. 4. Oligomeric states of FtsH(Δ TM)-His₆-Myc and Zip-FtsH(Δ TM)-His₆-Myc. (A) Gel filtration. Purified preparations of FtsH(Δ TM)-His₆-Myc (upper panel) and Zip-FtsH(Δ TM)-His₆-Myc (lower panel) (12.5 μg each) were fractionated by Superose 6 gel filtration chromatography. Each fraction was examined for FtsH content by SDS-PAGE and anti-FtsH immunoblotting. The molecular mass markers (indicated at the top) used were: thyroglobulin (670 kDa), bovine γ -globulin (158 kDa) and chicken ovalbumin (44 kDa). (B) Cross-linking. Zip-FtsH(Δ TM)-His₆-Myc (lanes 1–4 and 9–12) and FtsH(Δ TM)-His₆-Myc (lanes 5–8 and 13–16) were treated with 0 (lanes 1, 5, 9 and 13), 62.5 (lanes 2, 6, 10 and 14), 187.5 (lanes 3, 7, 11 and 15) or 562.5 (lanes 4, 8, 12 and 16) μg/ml DSP at 4°C for 1 h. Samples were analyzed by SDS-PAGE, with (+ME) or without (no ME) 2-mercaptoethanol, and stained with Coomassie Brilliant Blue. Asterisks indicate a self-cleaved product of Zip-FtsH(Δ TM)-His₆-Myc.

products for Zip-FtsH(Δ TM)-His₆-Myc might have been due to cross-linking at different residues.

***In vivo* proteolytic functions of LacY-FtsH-His₆-Myc and EnvZ-FtsH-His₆-Myc**

To address the significance of membrane localization, the TM region of FtsH was replaced with those of two unrelated membrane proteins, EnvZ and LacY (Figure 1). EnvZ has two N-terminal TM segments of the same topology as FtsH (Forst *et al.*, 1987), whereas LacY contains 12 TM segments (Calamia and Manoil, 1990). The EnvZ-FtsH fusion protein consists of the entire TM region of EnvZ followed by the FtsH(Δ TM)-His₆-Myc sequence, whereas LacY-FtsH-His₆-Myc consists of the N-terminal region of LacY, including the first and the second TM segments, followed by the FtsH(Δ TM)-His₆-Myc sequence.

The proteolytic functions of these proteins were examined by expressing them in the *ΔftsH* cells as described above. As shown in Figure 5, LacY-FtsH-His₆-Myc (lane 3) and EnvZ-FtsH-His₆-Myc (lane 6) both lowered the levels of SecY as well as of σ^{32} . They were as effective as FtsH-His₆-Myc (Figure 5, lane 1). Fusion proteins with the *ftsH317* mutation were ineffective (Figure 5, lanes 2 and 4). Pulse-chase experiments

Fig. 5. Accumulation of SecY and σ^{32} in cells expressing LacY-FtsH-His₆-Myc or EnvZ-FtsH-His₆-Myc. Strain AR5090 ($\Delta ftsH$) carrying pKY248 (*secY*) was transformed further with the plasmids indicated below. Accumulation of SecY and σ^{32} was examined as described in the legend to Figure 2. The second plasmids carried were: pSTD120 (*ftsH-his₆-myc*; lane 1), pSTD319 (*ftsH317-his₆-myc*; lane 2), pSTD350 (*lacY-ftsH-his₆-myc*; lane 3), pSTD389 (*lacY-ftsH317-his₆-myc*; lane 4), pMW119H (vector; lane 5), pSTD310 (*envZ-ftsH-his₆-myc*; lane 6) and pTYE007B (vector; lane 7). Arrowheads indicate the cytoplasmic domain of FtsH-His₆-Myc generated by self-cleavage between the fusion junction of LacY-FtsH-His₆-Myc (lane 3) or EnvZ-FtsH-His₆-Myc (lane 6).

(Figure 3) showed that expression of LacY-FtsH was accompanied by rapid degradation of SecY (Figure 3B, open triangles) and significant degradation of σ^{32} (Figure 3A, open triangles). These results indicate that LacY-FtsH-His₆-Myc and EnvZ-FtsH-His₆-Myc are as active as FtsH-His₆-Myc in degrading the membrane-bound substrates. LacY-FtsH-His₆-Myc and EnvZ-FtsH-His₆-Myc underwent the cleavage around the fusion junction as indicated by the generation of anti-Myc- and anti-FtsH-reacting fragments of smaller sizes (Figure 5, lanes 3 and 6). This cleavage was due to self-processing since it was blocked by the *ftsH317* mutation (lane 4 and data not shown for EnvZ-FtsH-His₆-Myc).

In vivo degradation of YccA-(P3)-PhoA-His₆-Myc by Zip-FtsH(Δ TM)-His₆-Myc and LacY-FtsH-His₆-Myc

YccA spans the cytoplasmic membrane seven times with a cytoplasmic N-terminus and a periplasmic C-terminus (Kihara *et al.*, 1998). We previously examined the mode of the FtsH-dependent degradation of YccA-(P3)-PhoA-His₆-Myc, having the PhoA sequence inserted within the third periplasmic region (Kihara *et al.*, 1999). Folding of the PhoA domain depends on DsbA-dependent formation of its intramolecular disulfide bonds. Whereas the periplasmic PhoA domain was degraded completely in a *dsbA*⁻ background in an FtsH-dependent manner, the degradation stopped before the PhoA domain in a *dsbA*⁺ background. Thus, a product containing a segment from the PhoA domain to the C-terminus, termed I₆₀, was generated in the latter background. These and other observations led us to propose that FtsH degrades YccA-(P3)-PhoA-His₆-Myc processively in the N to C direction and that the periplasmically exposed regions are dislocated to the cytoplasmic side during the proteolysis (Kihara *et al.*, 1999).

Fig. 6. Degradation of YccA-(P3)-PhoA-His₆-Myc in cells expressing FtsH-His₆-Myc, Zip-FtsH(Δ TM)-His₆-Myc or LacY-FtsH-His₆-Myc. AR5090 ($\Delta ftsH$) carrying pKH412 [*yccA-(P3)-phoA-his₆-myc*] (A) and that carrying pSTD423 [*yccA-(P3)-phoA(SSSS)-his₆-myc*] (B) were transformed further with the plasmids indicated below. Pulse-chase experiments were carried out as described in the legend to Figure 3, except that the growth temperature was 28°C and immunoprecipitation was with anti-PhoA. Anti-Myc gave similar results (data not shown). I₆₀ indicates the degradation product of YccA-(P3)-PhoA-His₆-Myc, which consists of the PhoA domain and the C-terminal end. The second plasmids carried were: pSTD113 (*ftsH-his₆-myc*; lanes 1-4), pSTD430 [*zip-ftsH(Δ TM)-his₆-myc*; lanes 5-8], pSTD348 (*lacY-ftsH-his₆-myc*; lanes 9-12) and pTYE007B (vector; lanes 13-16).

We addressed the question of whether the degradation profile of YccA-(P3)-PhoA-His₆-Myc was affected in cells in which Zip-FtsH(Δ TM)-His₆-Myc or LacY-FtsH-His₆-Myc substituted for normal FtsH (Figure 6). Degradation of YccA-(P3)-PhoA-His₆-Myc was examined by pulse-chase experiments in $\Delta ftsH$ *dsbA*⁺ cells that were complemented with either FtsH-His₆-Myc (lanes 1-4), Zip-FtsH(Δ TM)-His₆-Myc (lanes 5-8) or LacY-FtsH-His₆-Myc (lanes 9-12). YccA-(P3)-PhoA-His₆-Myc was stable in the $\Delta ftsH$ cells (Figure 6A, lanes 13-16) as well as in those cells with Zip-FtsH(Δ TM)-His₆-Myc (Figure 6A, lanes 5-8). FtsH-His₆-Myc (lanes 1-4) and LacY-FtsH-His₆-Myc (lanes 9-12) led to rapid degradation of YccA-(P3)-PhoA-His₆-Myc with the concomitant appearance of a 60 kDa fragment of the same mobility as I₆₀, which reacted with both anti-PhoA (lanes 4 and 12) and anti-Myc (data not shown). To examine degradation of an unfolded PhoA domain, we constructed YccA-(P3)-PhoA(SSSS)-His₆-Myc, in which all four cysteines in PhoA had been replaced by serine. Different FtsH derivatives produced essentially the same effects on YccA-(P3)-PhoA(SSSS)-His₆-Myc as on YccA-(P3)-PhoA-His₆-Myc, except that degradation of the former substrate was not accompanied

Fig. 7. Dominant inhibition of LacY-FtsH-His₆-Myc by LacY-FtsH41. Strain AR5090 (*ΔftsH*) was transformed with combinations of two plasmids, one (1st plasmid) encoding a functional FtsH derivatives and the other (2nd plasmid) encoding a non-functional FtsH41 derivative. Cells were grown at 37°C and induced with 1 mM IPTG for 2.5 h. Total proteins were analyzed by 10% SDS-PAGE followed by immunoblotting using anti-FtsH or anti- σ^{32} as indicated. For the explanation of the closed arrowhead see legend to Figure 5. The second plasmids carried were: pSTD405 (*ftsH41*; lanes 1–3), pSTD406 (*lacY-ftsH41*; lanes 4–6) and pSTV28 (vector; lanes 7–9). The first plasmids carried were: pSTD120 (*ftsH-his6-myc*; lanes 1, 4 and 7), pSTD350 (*lacY-ftsH-his6-myc*; lanes 2, 5 and 8) and pMW119H (vector; lanes 3, 6 and 9).

by the production of the I₆₀ product (Figure 6B). These results suggest that LacY-FtsH-His₆-Myc is able to induce dislocation of a periplasmic domain that lacks tight folding. In contrast, Zip-FtsH(Δ TM)-His₆-Myc is unable to degrade the two membrane protein substrates examined.

Dominant interference with LacY-FtsH-His₆-Myc function by ATPase mutants

We showed previously that a number of non-functional mutant forms of FtsH dominantly inhibit the function of wild-type FtsH (Akiyama *et al.*, 1994). Thus, σ^{32} was stabilized by co-expression of FtsH41 in the presence of FtsH-His₆-Myc (Figure 7, compare lanes 1 and 7). We now find that FtsH41 exerted much weaker inhibition against LacY-FtsH (Figure 7, lanes 1 and 2). Interestingly, LacY-FtsH with the *ftsH41* mutation (LacY-FtsH41) dominantly inhibited LacY-FtsH itself in degrading σ^{32} (Figure 7, lanes 5 and 8), but not FtsH (lane 4). The combination-specific dominant interference suggests that the dominant effects were not due to sequestration of the substrate by the inactive FtsH molecules. The simplest explanation may be that they preferentially sequester the respective wild-type counterpart. Thus, FtsH molecules, whether wild-type or with the *ftsH41* mutation, oligomerize themselves in an exclusive manner. Thus co-existing LacY-FtsH molecules are left behind. Our results thus indicate that the latter molecules still interact mutually. Since LacY is monomeric (Sahin-Tóth *et al.*, 1994), it seems unlikely that its TM1–TM2 region oligomerizes. We suggest that the cytosolic domain of FtsH has intrinsic self-interaction ability, which becomes apparent in the LacY-FtsH configuration. In support of this notion, we found that FtsH41(Δ TM) interfered weakly but significantly with LacY-FtsH-mediated σ^{32} degradation, but not with that mediated by FtsH (data not shown).

Cross-linking analysis of LacY-FtsH-His₆-Myc

The self-interacting property of LacY-FtsH-His₆-Myc was studied by cross-linking experiments (Figure 8). Membrane fractions were prepared from pulse-labeled cells containing His₆-Myc-tagged and untagged versions of

Fig. 8. Cross-linking of LacY-FtsH. Membranes prepared from [³⁵S]methionine-labeled cells of AD21 (WT)/pSTD401 (*ftsH*)/pSTD113 (*ftsH-his6-myc*) (A) or AD21/pSTD393 (*lacY-ftsH*)/pSTD389 (*lacY-ftsH317-his6-myc*) (B) were treated with 0 (lanes 2 and 4) or 0.24 (lanes 1 and 3) mg/ml DSP at 4°C for 1 h as indicated. Immunoprecipitates with anti-FtsH or anti-Myc were subjected to SDS-PAGE after reduction with 2-mercaptoethanol. Proteins in the DSP-treated samples were electrophoresed slightly more slowly than those in the untreated samples because of modification by DSP.

FtsH or LacY-FtsH (LacY-FtsH317-His₆-Myc was used as a tagged protein to prevent self-processing) and they were treated with DSP. Proteins were solubilized with SDS, immunoprecipitated with anti-FtsH or anti-Myc and analyzed by SDS-PAGE following cleavage of cross-linkages. As reported previously (Akiyama *et al.*, 1995), FtsH without the tag was recovered with anti-Myc antibodies only when the sample had been treated with DSP (Figure 8A, lanes 3 and 4). Similarly, LacY-FtsH was precipitated with anti-Myc in a DSP-dependent manner (Figure 8B, lanes 3 and 4). These results indicate that LacY-FtsH molecules were in close proximity to LacY-FtsH-His₆-Myc on the membrane. Thus, LacY-FtsH can interact homotypically.

Interaction of LacY-FtsH with HflKC

We examined whether LacY-FtsH-His₆-Myc can interact with HflKC. Membranes prepared from cells expressing FtsH-His₆-Myc or LacY-FtsH-His₆-Myc were solubilized with NP-40 and subjected to Ni-NTA affinity chromatography. As shown previously (Kihara *et al.*, 1996), HflKC was co-eluted with FtsH-His₆-Myc using imidazole (data not shown). In contrast, LacY-FtsH-His₆-Myc brought down essentially no HflKC (data not shown). Thus, LacY-FtsH-His₆-Myc is defective in its interaction with HflKC, confirming our previous findings that the periplasmic region of FtsH is important for the association between FtsH and HflKC (Akiyama *et al.*, 1998b).

Complementation activities

Expression of the *lacY-ftsH-his6-myc* gene from a plasmid did not rescue the growth of AR3317 [the *ftsH1*(Ts) mutant] at 42°C or that of AK525 (carrying *zgj-525::IS1A*; Kihara *et al.*, 1995) at 20°C, whereas expression of *ftsH-his6-myc* did (data not shown). The lack of complementation was not due to a harmful effect of LacY-FtsH-His₆-Myc on cell growth, since growth of neither AR3319 (*ftsH1 sfhC21*) cells nor AR3291 (*ΔftsH3::kan sfhC21*) cells was affected by LacY-FtsH at any temperature. Thus, although LacY-FtsH-His₆-Myc is functional in degrading SecY and σ^{32} , it cannot carry out all the biological roles of FtsH.

Discussion

FtsH is similar to other *E. coli* ATP-dependent proteases in that it is multimeric, but is unique in that it is membrane integrated (Gottesman *et al.*, 1997). The membrane localization may be related to its involvement in membrane protein degradation, while the multimeric structure *per se* is required for the catalytic mechanism of the AAA ATPase (Karata *et al.*, 1999). The dual role of the N-terminal region in multimerization and membrane association makes it difficult to analyze these structural features separately. In this study, we overcame this difficulty by replacing the N-terminal region of FtsH with a domain of other proteins that functions specifically for either multimerization or membrane association.

It was found that the isolated cytoplasmic domain of FtsH was virtually inactive and that the addition of the leucine zipper sequence strikingly stimulated the proteolytic activity against σ^{32} . The oligomerization domain of the λ CI protein had a similar ability when N-terminally fused to FtsH(Δ TM)-His₆-Myc (our unpublished results). Gel filtration and cross-linking experiments showed that the leucine zipper sequence indeed induced multimerization of FtsH(Δ TM)-His₆-Myc. These results suggest that the proteolytic activity of FtsH requires multimerization. The importance of the multimerization for the ATPase and protease activities of FtsH has also been suggested from the studies of MBP-FtsH fusions (Makino *et al.*, 1999). In addition, it has been shown that Yta10 and Yta12, FtsH homologs in *S. cerevisiae*, form a (hetero-)oligomer before they are able to function (Arlt *et al.*, 1996).

Zip-FtsH(Δ TM)-His₆-Myc is at least dimeric *in vitro*. A dimer could be the minimum functional unit of FtsH. Recently, the crystal structure of the D2 domain, one of the two AAA cassettes of *N*-ethylmaleimide-sensitive factor (NSF), was determined (Yu *et al.*, 1998). Based on the structure of D2, Ogura and colleagues (Karata *et al.*, 1999) proposed that Arg315 in the conserved region (SRH region) of FtsH participates in the formation of the ATPase active site between the neighboring subunits. The leucine zipper-mediated dimerization may activate the cytoplasmic domains of FtsH by allowing them to form a functional ATPase active site. We consider a further possibility that dimerization of FtsH(Δ TM)-His₆-Myc induces higher order multimerization through interaction of the cytoplasmic domain (see below for the cytoplasmic interaction of FtsH). Self-compartmentalization is a common feature of multimeric ATP-dependent proteases (De Mot *et al.*, 1999).

FtsH41 and LacY-FtsH41 acted in a dominant-negative manner against FtsH and LacY-FtsH, respectively. Sequestration of the substrate (σ^{32}) by the ATPase-defective proteins is insignificant since the dominant effects were combination specific; neither mutant protein affected degradation of σ^{32} that was mediated by the active form of the other protein. Such effects can be explained by sequestration of the wild-type proteins by the respective mutant forms. Taken together with the results of the cross-linking experiments, it is likely that the LacY-FtsH molecules form homo-oligomers. Since LacY is monomeric (Sahin-Tóth *et al.*, 1994), it is unlikely that its TM1-TM2 region self-associates. The oligomerization of LacY-FtsH-His₆-Myc could be mediated by its cyto-

plasmic domain. The monomeric state of FtsH(Δ TM) suggests that such an interaction, if any, is weak and it alone is insufficient to form a functional oligomer. In the case of LacY-FtsH, the self-interaction of the cytoplasmic domain may be aided by the tethering to the membrane. Thus, increased local concentration and restricted movement favor the association. We propose that the cytoplasmic interaction is an intrinsic property of the AAA domain of FtsH. The precise region of interaction could be identified by the sites of intragenic suppressors against the dominant effect of LacY-FtsH.

LacY-FtsH-His₆-Myc has an important difference from Zip-FtsH(Δ TM)-His₆-Myc in that it is able to degrade a membrane protein. Thus, multimerization is crucial for proteolytic activity of FtsH but insufficient for degradation of membrane proteins. It is conceivable that membrane localization results in the correct positioning of the active site relative to a substrate, and hence enables proteolysis of the membrane protein. TM segments of FtsH could interact with those of substrate membrane proteins during the degradation process. However, efficient degradation of SecY and YccA-(P3)-PhoA-His₆-Myc by LacY-FtsH-His₆-Myc suggests that no specific primary sequence is required for the TM segments of FtsH. Thus, such an interaction, if any, may be of a more general kind, such as a hydrophobic interaction. LacY-FtsH-His₆-Myc and FtsH-His₆-Myc degraded YccA-(P3)-PhoA-His₆-Myc in an essentially identical fashion; when the PhoA domain of the fusion was tightly folded, a characteristic degradation product designated as I₆₀ was generated, whereas the fusion protein with the unfolded PhoA domain was totally degraded without any stable intermediates. According to our previous model (Kihara *et al.*, 1999), these results suggest that LacY-FtsH-His₆-Myc is capable of dislocating the extracytoplasmic domains of YccA. Our present finding, that the putative dislocation process does not require specific primary sequences in TM segments, raises an interesting question as to what might comprise the channel for dislocation. For instance, the FtsH TM segments may form a channel-like structure that is induced by the interaction in the cytoplasmic domain of FtsH. Alternatively, a channel may be formed by other membrane proteins, such as SecYEG.

Although LacY-FtsH-His₆-Myc was almost as functional as FtsH-His₆-Myc in degradation of σ^{32} and SecY, it lacked the complementation activity against the *ftsH* mutations. LacY-FtsH-His₆-Myc may have defects in efficient degradation of a subset of substrates including LpxC that is critical for cell viability (Ogura *et al.*, 1999).

Materials and methods

Bacterial strains and media

Escherichia coli K12 derivatives AD21 (*cya283/F'lacI^q*) (Akiyama and Ito, 1985), TYE024 (*ompT::kan/F'lacI^q*) (Akiyama *et al.*, 1995) and AK525 (*zgj-525::IS1A*) (Kihara *et al.*, 1995) were described previously. Isogenic strains AR3307 (*ftsH⁺, sfhC⁺*), AR3317 (*ftsH1, sfhC⁺*), AR3289 (*ftsH⁺, sfhC21*), AR3319 (*ftsH1, sfhC21*) and AR3291 (*Δ ftsH3::kan, sfhC21*) were also described previously (Ogura *et al.*, 1999). AR5090 (*Δ ftsH3::kan, sfhC21/F'lacI^q*) was a gift of K. Karata and T. Ogura. The *sfhC21* mutation is a suppressor of the *Δ ftsH3::kan* mutation (Ogura *et al.*, 1999).

L medium (Davis, 1980) and M9 medium (Silhavy *et al.*, 1984) were used. Ampicillin (50 μ g/ml) and/or chloramphenicol (20 or 100 μ g/ml) were added for growing plasmid-bearing strains.

Table I. Plasmids used in this study

Plasmid	Cloned gene	Vector (replicon)	Reference
pSTD113	<i>ftsH-his₆-myc</i>	pTYE007 [pBlueScript II SK(-)]	Akiyama <i>et al.</i> (1995)
pSTD120	<i>ftsH-his₆-myc</i>	pMW119 (pSC101)	Akiyama <i>et al.</i> (1995)
pSTD319	<i>ftsH317-his₆-myc</i>	pMW119	this study
pSTD401	<i>ftsH</i>	pHSG575 (pSC101)	Akiyama <i>et al.</i> (1995)
pSTD405	<i>ftsH41</i>	pSTV28 (pACYC184)	this study
pSTD219	<i>ftsH(ΔTM)-his₆-myc</i>	pUC119	this study
pSTD430	<i>zip-ftsH(ΔTM)-his₆-myc</i>	pUC119	this study
pSTD432	<i>zip-ftsH317(ΔTM)-his₆-myc</i>	pUC119	this study
pSTD444	<i>zip-ftsH41(ΔTM)-his₆-myc</i>	pUC119	this study
pSTD348	<i>lacY-ftsH-his₆-myc</i>	pTYE007	this study
pSTD350	<i>lacY-ftsH-his₆-myc</i>	pMW119	this study
pSTD389	<i>lacY-ftsH317-his₆-myc</i>	pMW119	this study
pSTD393	<i>lacY-ftsH</i>	pSTV28	this study
pSTD406	<i>lacY-ftsH41</i>	pSTV28	this study
pSTD310	<i>envZ-ftsH-his₆-myc</i>	pTYE007	this study
pKY248	<i>secY</i>	pKY238 (pACYC184)	Taura <i>et al.</i> (1993)
pKH412	<i>yccA-(P3)-phoA-his₆-myc</i>	pSTV29 (pACYC184)	Kihara <i>et al.</i> (1999)
pSTD423	<i>yccA-(P3)-phoA(SSSS)-his₆-myc</i>	pSTV29	this study
pMW119H	—	pMW119	Akiyama <i>et al.</i> (1998)
pTYE007B	—	pTYE007	this study

Plasmids

Plasmids used in this study are listed in Table I. For construction of pSTD219, a *Hind*III site was first introduced into the immediate 3' region of the second TM segment of FtsH (on pSTD178; Akiyama *et al.*, 1995) using a primer (CGCCGCCCTGCAAGCTTTGACGCATGA) (Kunkel *et al.*, 1987). Then, a *Hind*III–*Sac*I fragment of the resulting plasmid, encoding the cytoplasmic region of FtsH-His₆-Myc, was cloned into pUC119. pSTD348 was constructed as follows. An *Eco*RI–*Acc*I fragment of a pT7-5/*lacY* cassette (a gift of H.R.Kaback, DDBJ/EMBL/GenBank accession No. X56095) was inserted into *Bam*HI-digested pSTD113 (Akiyama *et al.*, 1995) after treatment with T4 DNA polymerase. From the resulting plasmid, the region between the LacY N-terminal region (from the N-terminus to the second TM segment) and the cytoplasmic domain of FtsH was deleted using the Quick change mutagenesis kit (Stratagene) and a pair of primers (CCGCTGTTTGGTCTGCTTTCTCGTCAAATGCAGGGCGCGCGG and CCGCCGCCCTGCATTGACGAGAAAGCAGACCAACAGCGG). pSTD350 was constructed by replacing an *Xba*I–*Mlu*I fragment of pSTD120 with an *Xba*I–*Mlu*I fragment of pSTD348. pSTD430 was constructed by cloning the *Kpn*I fragment of pT18-*zip* (Karimova *et al.*, 1998) into *Hind*III-digested pSTD219 after treatment with T4 DNA polymerase. pSTD310 was constructed by deleting the upstream region of *envZ-ftsH-his₆-myc* on pSTD117 (Akiyama *et al.*, 1995) by site-directed mutagenesis using the Quick change mutagenesis kit and a pair of primers (GAAACAGCTATGACCTATGACCATGAGGCGATTGCGC and GAAACAGCTATGACCTATGAGGCGATTGCGC) to place the *envZ-ftsH-his₆-myc* gene under the control of the *lac* promoter. The *ftsH317* mutation (causing a His417 to Tyr change) was first introduced into the *ftsH* gene on pSTD401 (Akiyama *et al.*, 1995) using the Quick change mutagenesis kit with a pair of primers (GAATCGACGGCTTACTACGAAGCGGGTC and GACCCGCTTCGTAGTAAGCCGTCGATTTC). The resulting plasmid was designated pSTD314. Other plasmids carrying the *ftsH317* mutation were constructed by replacing an appropriate fragment of each plasmid by a corresponding fragment from pSTD314. The *ftsH41* mutation was transferred similarly from pSTD41 (Akiyama *et al.*, 1994). All of the mutagenized genes were confirmed by DNA sequencing. pSTD423 was constructed by replacing the *Bgl*I–*Sph*I fragment of pKH412 by a corresponding fragment of pTY7 (Sone *et al.*, 1998). pMW119H (Akiyama *et al.*, 1998b) and pTYE007B were constructed by self-ligation of *Hind*III-digested pMW119 (Nippon Gene) or *Bam*HI-digested pTYE007 (Akiyama *et al.*, 1995) after treatment with T4 DNA polymerase, respectively.

Immunoblot examination of protein stability in vivo

Cells were grown in L broth at 30 or 37°C to mid-log phase and induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 2 h, and a portion of the cultures (containing $\sim 3 \times 10^7$ cells) was removed and

mixed with the same volume of 10% trichloroacetic acid. Denatured proteins were collected by centrifugation, dissolved in SDS sample buffer (Laemmli, 1970) and separated on a 10% acrylamide gel (for FtsH and σ^{32}) (Laemmli, 1970) or 16.1% acrylamide–0.12% *N,N'*-methylene-bis-acrylamide gel (for SecY) (Akiyama and Ito, 1985). They were then detected by immunoblotting with anti-FtsH (Akiyama *et al.*, 1998a), anti-Myc (A-14) (Santa Cruz Biotechnology, Inc.), anti- σ^{32} (a gift of M.Kanemori) or anti-SecY (Shimoike *et al.*, 1995) antibodies. Visualization and quantification were done by means of an ECL detection kit (Amersham Pharmacia Biotech) and a Fuji LAS1000 lumino-image analyzer.

Pulse-chase examination of protein stability in vivo

Cells were induced with 1 mM IPTG and 5 mM cAMP for 2 h, pulse-labeled with [³⁵S]methionine, and chased with unlabeled methionine as described previously (Akiyama *et al.*, 1995), except that the growth temperature was 28°C in the experiments described in Figure 6 to minimize the DegP-mediated degradation of unfolded PhoA (Spiess *et al.*, 1999). YccA-(P3)-PhoA-His₆-Myc, YccA-(P3)-PhoA(SSSS)-His₆-Myc, σ^{32} and SecY were immunoprecipitated essentially as described previously (Akiyama *et al.*, 1995) and separated by 10% polyacrylamide gel electrophoresis. Labeled proteins were visualized and quantified by means of a Fuji BAS2000 imaging analyzer.

Purification of FtsH(Δ TM)-His₆-Myc and Zip-FtsH(Δ TM)-His₆-Myc

Cells of TYE024/pSTD219 and TYE024/pSTD430 were grown at 30°C in 1 l of L medium containing 1 mM IPTG and 1 mM cAMP for 3 h. Cells were harvested, washed with wash buffer (10 mM Tris–HCl pH 8.1 and 10 mM 2-mercaptoethanol), suspended in buffer A (10 mM Tris–HCl pH 8.1, 300 mM KCl, 20 mM imidazole, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 10 mM 2-mercaptoethanol) and disrupted by French press (at 8000 p.s.i.). Samples were centrifuged (Beckman 70Ti rotor, 38 000 r.p.m. for 1 h), and supernatants were applied to an Ni-NTA agarose column, which was washed with buffer A and eluted with a linear gradient of 20–500 mM imidazole in buffer A. The peak fractions of FtsH, as detected by SDS–PAGE and Coomassie Brilliant Blue staining, were pooled and dialyzed against buffer B (20 mM HEPES–KOH pH 7.5, 50 mM KCl, 10% glycerol, 5 mM MgCl₂ and 10 mM 2-mercaptoethanol).

Cross-linking experiments

FtsH(Δ TM)-His₆-Myc and Zip-FtsH(Δ TM)-His₆-Myc preparations (in buffer B) were treated with the indicated concentrations of DSP (dissolved in dimethyl sulfoxide) at 4°C for 1 h. DSP was then quenched with 0.75 M ammonium acetate, and proteins were precipitated with 5% trichloroacetic acid. They were dissolved in SDS sample buffer with or

without 10% 2-mercaptoethanol and analyzed by 6% SDS-PAGE followed by Coomassie Brilliant Blue staining.

Cross-linking of labeled crude membranes was carried out as described previously (Akiyama *et al.*, 1995). Briefly, cells of AD21/pSTD401/pSTD113 and AD21/pSTD393/pSTD389 were grown in M9 medium, induced and pulse-labeled with [³⁵S]methionine. After cell disruption by sonication, membrane fractions were prepared and treated with 0.24 mg/ml DSP. Proteins were solubilized in SDS and immunoprecipitated with anti-FtsH or anti-Myc antibodies. Immunoprecipitates were analyzed by 10% SDS-PAGE after cleavage of the cross-linkage with 2-mercaptoethanol.

Acknowledgements

We thank K.Karata and T.Ogura for bacterial strains, M.Kanemori for anti- σ^{32} , H.R.Kaback for the pT7-5/*lacY* cassette, A.Kihara, S.Chiba, N.Saikaawa and H.Mori for discussion, and T.Yabe, Y.Shimizu and K.Mochizuki for technical assistance. This work was supported by grants from the Ministry of Education, Science, Sports and Culture, Japan and from CREST, Japan Science and Technology Corporation.

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Received March 16, 2000; revised May 24, 2000;
accepted June 5, 2000