Reconstitution of Membrane Proteolysis by FtsH*

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Escherichia coli FtsH is a membrane-bound and ATP-dependent protease responsible for degradation of several membrane proteins. The FtsH action is processive and presumably involves dislocation of the substrate from the membrane to the cytosol. Although elucidation of its molecular mechanism requires an in vitro reaction system, in vitro activities of this enzyme against membrane protein substrates have only been assayed using detergent-solubilized components. Here we report on the construction of in vitro reaction systems for FtsH-catalyzed membrane protein degradation. A combination of two inverted membrane vesicles or of two proteoliposomes, one bearing the enzyme and the other bearing a substrate, was fused by polyethylene glycol 3350 treatment. Addition of ATP then resulted in degradation of the substrate. It was shown that FtsH can function in the process of membrane proteins degradation without aid from any other cellular factors.

Cells respond to accumulation of abnormal proteins not only in the cytosol but also in membranes. Thus, a certain class of abnormal membrane proteins must be degraded rapidly. Despite the importance of membrane protein degradation, our knowledge about these processes is limited because of difficulty arising from the membrane localization of both substrates and enzymes. For instance, a question of how a peptide bond that is embedded within the lipid bilayer can receive efficient hydrolysis must be answered.

Among the ATP-dependent proteases, ClpPX, ClpAX, HslU, Lon, and FtsH, of Escherichia coli, FtsH is unique in that it is membrane-integrated and that its substrates include integral membrane proteins (1, 2). The membrane localization of FtsH is crucial for its ability to degrade membrane proteins (3). Intriguingly, the proteolytic activity of FtsH is stimulated by the proton motive force (PMF) across the membrane (4). Three membrane proteins have been identified as native substrates of this enzyme. Two of them, the SecY subunit of protein translocase (5, 6) and subunit α of FfFo-ATPase (Foz) (7), are degraded rapidly in unassembled states. YcaA, a multi-spanning membrane protein of unknown function, may be constitutively degraded, although it is not known whether this protein forms any complex (8). In addition, FtsH is known to degrade several soluble proteins (1, 2).

FtsH contains two transmembrane segments located N-terminally and a large cytoplasmic domain, which consists of two subdomains, an AAA ATPase domain and a protease domain with a zinc metalloprotease motif (HEXXH) (9). FtsH forms a homo-oligomer, and this feature is essential for the ATPase and the protease activities (3, 10, 11). FtsH is also known to interact with a pair of membrane proteins, HiiK and HiiC, that forms a complex (HiiKC) (12). HiiKC may have a regulatory role in the proteolytic functions of FtsH (8, 13). In vivo, FtsH exists exclusively as a large complex (estimated molecular mass of more than 1000 kDa) with HiiKC in the cytoplasmic membrane.

We proposed that FtsH exerts proteolysis against the entire molecule of a membrane-integrated substrate by dislocating it to the cytosolic side of the membrane (3, 14). This was based on our in vivo analyses using model membrane proteins. A YccA derivative, YccA-(P3)-PhoA-His6-Myc, was one of them. It was a chimeric protein having a PhoA (alkaline phosphatase mature part) sequence inserted within the third periplasmic region of this protein, which also contains C-terminally attached His6 and Myc tags. The folding state of the PhoA domain can be manipulated as its tight folding depends on the formation of the intramolecular disulfide bonds. Degradation of this fusion protein by FtsH only occurred when it had an intact N-terminal cytoplasmic tail. Although the degradation went to completion when the PhoA moiety was unfolded, it stopped before the PhoA domain when it was folded, leaving a degradation product that contained the PhoA and its C-terminal regions up to the His6-Myc tag. These degradation processes were dependent on FtsH and took place only as continuation of proteolysis initiated at the N-terminal region of the substrate. Thus, the reaction must have been processive, which is aborted before a tightly folded domain, a probable obstacle for the dislocation process.

It was shown further that the degradation initiation requires cytoplasmic exposure of some 20 amino acids or longer N-terminal tail (15). FtsH is also able to recognize a C-terminal tail for initiation of membrane protein degradation (16). For our further understanding of the molecular mechanisms of polypeptide extraction from the membrane and presentation to the proteolytic active site, it is essential to develop an in vitro system, in which membrane protein degradation by FtsH can be studied using membrane-integrated enzyme and substrates.

Here we report on the use of polyethylene glycol 3350 (PEG3350) to induce fusion of vesicles bearing the enzyme and a substrate, and thereby to reproduce subsequent degradation of the substrate upon addition of ATP. We were thus able to reconstitute ATP-dependent proteolysis of lipid bilayer-inte-

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2 The abbreviations used are: PMF, proton motive force; DTT, dithiothreitol; Foe, subunit α of FfFo-ATPase; FRET, fluorescence resonance energy transfer; HA, hemagglutinin; IMVs, inverted membrane vesicles; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yi)-dioleoylphosphatidylethanolamine; PEG3350, polyethylene glycol 3350; PhoA, alkaline phosphatase mature part; Rh-PE, N-(lissamine rhodamine B sulfonyl) dioleoylphosphatidylethanolamine; MOPS, 4-morpholinopropesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

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18146
grated substrates using purified components. Our results indicate that the dislocation-accompanied proteolytic event can occur without involving other cellular factors such as the Sec translocation channel.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Media**—E. coli K12 strains used were as follows. TWEY024 (ompF::kan/F′ lacI) (10) and AD1434 (Δ(sfs H) sfc21 ΔuncF′ lacI) (4) were derivatives of CU141 [araD139 Δ(argF::lac)U169 rpsL150 relA1 fsb530 del1 fts52 rbd5/F′ lacI1877 proA1 lacZAM1897] (19). For construction of pAC1680 (CU141, sfc21 zhd-220::Tn10 [tcr334 kan::phc pret]) the prec:tcat maker was first introduced into AB1157 (20) carrying pkm2001 (pacman-red-gam) (a gift of K. Murphy) by linear transformation using a DNA fragment amplified from the chromosomal DNA of AK2168 (21) with a pair of primers (GAAGCCGCCGACGCATGAACTTTTGGACATCTATCGTACATTCGCGCATCTCATCCAG and GTCTGCCGAGATACGTGCGATA-TATCGCCATGCTACGATGTTAAGGCAGCCG). The then prec:cat maker was P1-transduced into AD1672 (CU141, sfc21 zhd-220::Tn10 [tcr334 kan::phc]) (22) that had been constructed by successive transductions of sfc21 (23) and tcr334 kan::phc (17) into CU141. AD1680 was used for purification of YccA-HA-Myb-Myc and YccA11-HA-Myb-Myc. Plasmids—pSTD113 (encoding FtsH-His6-Myc) (10), pKH303 (YccA-His6-Myc) (8), and pKH412 (YccA-(P3)-His6-Myc) (14) were described previously. pSTD537 (YccA-His6-Myc) and pSTD562 (YccA11-His6-Myc) were constructed in the following way. pKD379 (14), which had been constructed from pKH350 (YccA-His6-Myc) (8) by insertion of the SacI recognition sequence (ACTAGT) between 5′ and 3′ of C997 corresponding to the third periplasmic domain of YccA (14), was mutagenized using mutagenic primers (GGTTAGTGTGTTCTCGGCGCTTTGAATGTTGC and CAGCAGATATCGAGGCGGAAGAACTAAGCG) to replace Cys-119 and Cys-120 by alanine. Plasmids—pSTD113 (encoding FtsH-His6-Myc) (10), pKH303 (YccA-His6-Myc) (8), and pKH412 (YccA-(P3)-His6-Myc) (14) were described previously. pSTD537 (YccA-His6-Myc) and pSTD562 (YccA11-His6-Myc) were constructed in the following way. pKD379 (14), which had been constructed from pKH350 (YccA-His6-Myc) (8) by insertion of the SacI recognition sequence (ACTAGT) between 5′ and 3′ of C997 corresponding to the third periplasmic domain of YccA (14), was mutagenized using mutagenic primers (GGTTAGTGTGTTCTCGGCGCTTTGAATGTTGC and CAGCAGATATCGAGGCGGAAGAACTAAGCG) to replace Cys-119 and Cys-120 by alanine.

**Preparation of IMVs**—Preparation of IMVs and Proteoliposome Vesicles—IMVs or reconstituted vesicles were incubated in the presence of 50 mM MOPS (pH 7.0), 0.5 mM KCl, and 12.5% PEG3530 at 37 °C for 5 min. That this procedure indeed induced the vesicle fusion was shown by FRET cancellation and dynamic light scattering measurements. For fluorescence microscopy (27), liposomes were prepared as described above except that N-(2-nitro-1,3-benzoxadiazol-4-yl)-dioleoylphosphatidylethanolamine (NB-D-PE) and N-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (Rh-PE) were added to occupy 0.8% (w/w) of the liposomes. The fluorescent liposomes were then mixed with 4-fold weight excess of non-labeled proteoliposomes and treated with 12.5% PEG3530 at 0 or 37 °C. The extent of lipidosome fusion was assessed from the NB-D fluorescence in the outer leaflet of the liposome, NB-D- and Rh-labeled liposomes were preincubated with 20 mM sodium dithionite at 0 °C for 30 min, followed by removal of excess sodium dithionite by microspin S-400 HR column chromatography. Size distribution profiles of IMVs were determined by dynamic light scattering measurements using FPAR-1000 fiber optics particle analyzer (Photal Otsuka Electronics) and the CONTIN program according to the manufacturer’s instructions.

**Membrane Protein Degradation Reactions**—PEG3530-fused IMVs, which contained FtsH and a substrate membrane protein, were treated with or without 250 mM DTT at 37 °C for 10 min. They were diluted 10-fold with degradation assay buffer containing 50 mM Tris-HCl (pH 8.1), 5 mM MgCl2, 25 μM zinc acetate, 10 mM 2-mercaptoethanol, and 5 mM sodium succinate and incubated at 37 °C in the presence or absence of either 5 mM ATP or 5 mM ADP. YccA degradation reactions with purified FtsH to degrade membrane-integrated substrates, because such important features as the dislocation and the PMF stimulation cannot be addressed. Because it is difficult to prepare membrane vesicles carrying both FtsH and its degradation substrate, we attempted to prepare separate membrane vesicles, one carrying the enzyme and the other carrying a substrate. These membrane vesicles should then be fused to enable the enzyme-substrate interaction. Our initial trials of several conditions that had been reported to induce membrane fusion indicated that treatment of the enzyme vesicles and the substrate vesicles with PEG3530 resulted in an ATP-dependent degradation of the latter (see below).
We verified the PEG-induced fusion of membrane vesicles by two independent methods (Fig. 1). First, dynamic light scattering measurements (Fig. 1A) indicated that incubation of a mixture of inverted membrane vesicles with PEG3350 at 37 °C for 5 min resulted in a marked shift in the mean vesicle sizes, from 185 (± S.D. of 52.7) to 1322.9 nm (± 261.6) with no appreciable overlapping or shouldering of the two curves. Similar PEG3350-dependent size shift was observed with reconstituted proteoliposomes (22).

Second, fluorescence resonance energy transfer (FRET) experiments (Fig. 1, B and C) provided evidence for PEG-induced mixing of phospholipids. Liposomes were prepared in the presence of NBD-PE and Rh-PE. These liposomes contained sufficiently high concentrations of NBD-PE and Rh-PE such that NBD fluorescence was quenched by Rh-PE due to FRET. Liposome solubilization with Triton X-100 resulted in the elimination of the FRET and a marked increase in NBD fluorescence (data not shown). Liposomes doubly labeled with NBD-PE and Rh-PE were mixed with 4-fold excess amount of unlabeled liposomes. The mixture was incubated in the presence or absence of 12.5% PEG3350 (Fig. 1B). After 1 min of incubation at 0 °C, about 13% of the maximum fluorescence observed in the presence of Triton X-100 was observed with the PEG-treated sample. This value increased to more than 50% after prolonged incubation at 37 °C (for 10–30 min). PEG-untreated samples showed only negligible increase in fluorescence.

We then examined whether PEG-induced lipid mixing occurred not only for the outer leaflet but also for the inner leaflet of the liposomes (Fig. 1C). The fluor-labeled liposomes were treated with a membrane-impermeable bleach, dithionite, prior to the PEG3350 treatment. Dithionite treatment decreased the initial NBD fluorescence by 45%, indicating that the outer leaflet was mostly bleached. Essentially, the remaining fluorescence should have come from the inner leaflet. Upon PEG3350 treatment, the dithionite-treated liposomes exhibited similar levels of NBD fluorescence quenching as the untreated liposomes. Thus, PEG3350-induced lipid mixing occurs for both leaflets, indicating a complete fusion of the vesicles. Taken together, we have established an effective procedure for membrane vesicle fusion in vitro.

FtsH-dependent Degradation of Foa in IMVs upon PEG3350 Treatment—We applied this simple membrane fusion technique using PEG3350 to our FtsH studies. Inverted membrane vesicles were prepared from two E. coli strains, one overproducing FtsH and the other overproducing Foa, an FtsH substrate. These IMVs were mixed and incubated in the presence of 12.5% PEG3350 at 37 °C for 5 min. Subsequently, they were incubated further in the presence (Fig. 2, lanes 5–8) or absence (lanes 1–4) of ATP. Foa, as detected by anti-Foa immunoblotting, was degraded with time in the presence of ATP but not in its absence. When IMVs from FtsH-deleted cells were used instead of the FtsH-containing IMVs (lanes 9–12), no degradation of Foa was observed. Also, omission of the pretreatment with PEG3350 (lanes 13 and 14) resulted in no appreciable degradation of the substrate even after 4 h of incubation. YccA, another membrane-integrated substrate of FtsH, was also degraded in an ATP- and FtsH-dependent manner in PEG3350-treated IMV (data not shown). These results indicate that PEG3350 treatment induces membrane fusion, allowing FtsH to ATP-dependently hydrolyze membrane-integrated substrates in the fused vesicles.

Degradation of YccA-(P3)-PhoA in Vitro—The PhoA portion of the YccA-(P3)-PhoA-His6-Myc fusion protein can be either folded tightly or unfolded depending on the presence or absence of the intramolecular disulfide bonds (28, 29). We characterized IMVs carrying YccA-(P3)-PhoA-His6-Myc. YccA-(P3)-PhoA-

![Fig. 1. Membrane fusion is induced by PEG3350 treatment. A, size distribution of membrane vesicles. IMVs were prepared from cells of AD1434 (ΔftsH/pSTD425 (Foa)) and from cells of AD1434 (ΔftsH/pSTD113 (FtsH-His6-Myc)). They were mixed and incubated with (filled circles) or without (open circles) 12.5% PEG3350. The samples were diluted with 10 mM Tris-HCl (pH 8.1) and subjected to dynamic light scattering measurements. Results are shown as calculated vesicle sizes. B, effects on FRET between two fluorophores attached to phosphatidyethanolamine. Liposomes were prepared from E. coli phospholipids to which 0.8 weight % NBD-PE and Rh-PE were added. They were then mixed with 4-fold excess weight of non-labeled liposomes and treated with 12.5% PEG3350 at 0 or 37 °C for the indicated times. The NBD fluorescence intensity was measured and expressed as % fusion as described under "Experimental Procedures." C, lipid mixing in the inner leaflet of liposomes. NBD-PE- and Rh-PE-containing liposomes were incubated with or without dithionite and subsequently fused by PEG3350 treatment. Relative NBD fluorescence intensities of dithionite-treated and untreated vesicles (right) as well as estimated extents of vesicle fusion with these vesicles (left) are shown.
was withdrawn and mixed with 5% trichloroacetic acid. Proteins were
lanes 1–8, 13, and 14) or from cells of AR5090 (ΔftsH) (lanes 9–12). The mixtures were treated with (lanes 1–12) or without (lanes 13 and 14) 12.5% PEG3350 at 37 °C for 5 min. They were diluted and incubated further at 37 °C in the presence (lanes 5–14) or absence (lanes 1–4) of 5 mM ATP. Samples were withdrawn at the indicated time points and analyzed by 15% acrylamide, 0.12% N,N-methylenebisacrylamide gel electrophoresis and anti-PhoA immunoblotting.

His6-Myc decreased with time only in the presence of ATP (Fig. 3). Intensity of the intact band of YccA-(P3)-PhoA-His6-Myc was converted by trypsin to a slightly smaller trypsin-generated PhoA-sized fragment, respectively. Lane M is for molecular size markers (81.8, 68.4, 55.0, and 41.6 kDa from top to bottom).

His6-Myc was expressed in AD1434 (ΔftsH) cells, and IMVs were prepared. They were treated with or without 250 mM DTT at 37 °C for 10 min and then digested with trypsin (Fig. 3). Whether or not the DTT pretreatment was included, YccA-(P3)-PhoA-His6-Myc was converted by trypsin to a slightly smaller species that remained stable up to 120 min of incubation (lanes 1–12). Because YccA has the cytosolic N terminus and the periplasmic C terminus, trypsin must have removed the N-terminal tail of this chimeric protein in IMV. On the other hand, when the IMV was solubilized with Triton X-100 before trypsin digestion, a smaller degradation product corresponding to PhoA was produced (lanes 13–15 and 19–21). This PhoA product was further degraded almost completely for the DTT-pretreated sample (lanes 16–18); no degradation occurred without DTT (lanes 22–24). These results indicate that the PhoA portion of YccA-(P3)-PhoA-His6-Myc can be reduced and unfolded by DTT treatment. It was also shown that this fusion protein assumes the expected orientation in the IMV. The integrity of the IMVs was also confirmed. Essentially the same results were obtained after PEG3350-induced fusion of the IMVs (data not shown). Although these results suggest that DTT treatment disrupted the intramolecular disulfide bonds in the PhoA domain, neither DTT nor PEG3350 affected the integrity of the membrane vesicles. IMVs from FtsH-overproducing cells and those from YccA-(P3)-PhoA-His6-Myc-overproducing cells were subjected to PEG3350-mediated fusion. The fused vesicles were treated with or without DTT and incubated in the presence or absence of ATP (Fig. 4). Intensity of the intact band of YccA-(P3)-PhoA-His6-Myc decreased with time only in the presence of ATP (Fig. 4A, upper panel, lanes 4–6 and 10–12; lower panel, circles).

Without DTT treatment, this degradation was accompanied by the generation of a fragment of an identical electrophoretic mobility as I60 (Fig. 4A, upper panel, lanes 4–6; lower panel, open squares). This 60-kDa fragment contained both the PhoA and the Myc epitopes (Fig. 4B, lanes 1–9), indicating that it corresponded to the in vivo observed I60 product (14). In the DTT-treated sample, little accumulation of I60 was observed (Fig. 4A, upper panel, lanes 7–12; lower panel, solid squares). These results completely agree with the in vivo observations. It is thus concluded that our in vitro system can reproduce the in vivo mode of membrane protein degradation catalyzed by FtsH. Probably the dislocation mechanism is also taking place in vitro. This point was supported by the following experiments (Fig. 5).

The PEG3350-fused membranes were first incubated in the presence of ATP to accumulate I60. They were then treated...
with or without DTT, followed by the second incubation in the presence of ATP or AMP-PNP. Further incubation of the DTT-treated sample with ATP resulted in a gradual decrease in the I₆₀ intensity (Fig. 5, filled triangles). Such a decrease did not occur when AMP-PNP was used instead of ATP (filled squares). Without DTT treatment, I₆₀ accumulation continued (open triangles). These results suggest that I₆₀ was not in a dead-end state but was competent to receive further degradation by FtsH, which might resume proteolysis of the PhoA moiety after its unfolding. Thus, processivity continues over the chronological gap, when the steric obstacle has been removed.

When the fused membranes were solubilized with Triton X-100 and incubated with ATP, a new fragment of about 57 kDa, termed I₅₇, was produced (Fig. 4B, lanes 10–18). Like I₅₀, I₅₇ cross-reacted with anti-PhoA and anti-Myc (lanes 13–18), suggesting that it was an N-terminally shortened version of I₅₀. Degradation by FtsH might have been halted by the membrane at an earlier position than the position of steric hindrance by the folded PhoA domain itself. Even in the presence of Nonidet P-40 some I₅₀-like fragment was also observed. It might be possible that detergent micelles that are bound to the transmembrane regions acted like the membrane to partially arrest the processive degradation process.

Reconstitution of Membrane Protein Degradation from Purified Components—We then attempted to establish a more defined in vitro system that reconstitutes the FtsH-catalyzed membrane protein degradation. We constructed a model substrate, YccA-HA-His₆-Myc, having an HA tag sequence inserted between YccA and the His₆-Myc tag, as well as its derivative (YccA11-HA-His₆-Myc) having the yccA11 internal deletion of 8 amino acids in the N-terminal cytoplasmic region. The YccA11 alteration renders this protein resistant to FtsH in vivo. These YccA derivatives as well as FtsH were purified from membranes after their overproduction (Fig. 6).

Each of these preparations was mixed with E. coli phospholipids and diluted to lower detergent concentration to reconstitute proteoliposomes. Integral association of each protein with the liposomes was demonstrated by their resistance to extraction with 0.2 N NaOH or 1 M NaCl (data not shown). Protein orientation in the proteoliposomes was examined by proteinase K digestion. Reconstituted FtsH was mostly accessible by proteinase K (Fig. 7A), indicating that its ATPase and protease domains were exposed outside the vesicles. Proteinase K digestion of the reconstituted YccA derivatives removed the N-terminal segment (Fig. 7C), and this digestion pattern was similar to the proteinase K digestion pattern of YccA-HA-His₆-Myc in IMVs (Fig. 7F). The proteoliposome-associated YccA was completely degraded upon membrane solubilization with Triton X-100 (Fig. 7F), as was YccA-His₆-Myc in IMV (Fig. 7F). Proteoliposome-reconstituted YccA11-HA-His₆-Myc behaved similarly to the “wild-type” counterpart, but the proteinase K-removed part was smaller (Fig. 7, C and D), consistent with the shortening of the N-terminal tail by the yccA11 deletion mutation. This was thus indicated that each of the reconstituted FtsH proteins and the reconstituted YccA proteins had the same topology as in IMV.

One might argue that a simpler procedure would be to mix the enzyme and a substrate at low temperature and then to subject the mixture to reconstitution, making the vesicle fusion step unnecessary. To test this possibility, we carried out mixed reconstitution using YccA-HA-His₆-Myc and FtsH. However, YccA-HA-His₆-Myc in the resulting proteoliposomes was now completely digested by proteinase K even in the absence of a detergent (Fig. 7E). FtsH might have bound to YccA-HA-His₆-Myc in solution and prevented its correct integration into the liposomes. Thus, simultaneous reconstitution with the enzyme is not appropriate at least for this substrate.

The FtsH proteoliposomes and the YccA proteoliposomes were subjected to PEG3350-mediated fusion. The fused proteoliposomes were incubated at 37 °C in the presence of ATP or ADP, and YccA-HA-His₆-Myc was analyzed by anti-Myc (Fig. 8) and anti-HA (data not shown) immunoblotting. ADP was included in the control samples, because purified FtsH tended to aggregate when incubated at 37 °C without any nucleotide (data not shown). YccA-His₆-Myc (Fig. 8, A and F) and YccA-

![Image](image89x562to275x737)
coli phospholipids into proteoliposomes. His6-Myc were first mixed and then reconstituted simultaneously into proteoliposomes. FtsH was prepared from cells of AD1680/pSTD537 (YccA-HA-His6-Myc). The proteoliposomes and the IMV were treated with 0.5 mg/ml proteinase K at 0 °C for the indicated time in the presence or absence of 1% Triton X-100. Proteins were visualized by anti-Myc immunoblotting.

HA-His6-Myc (Fig. 8, B and F) were degraded with time in an ATP-dependent manner. Without prior PEG3350 treatment, YccA-HA-His6-Myc was not measurably degraded (Fig. 8, C and F). Also, no YccA-HA-His6-Myc degradation was observed when YccA-HA-His6-Myc were reconstituted separately with E. coli phospholipids into proteoliposomes. E. FtsH-His6-Myc and YccA-His6-Myc were first mixed and then reconstituted simultaneously into proteoliposomes. FtsH was reconstituted separately with 3 μl of those containing YccA-HA-His6-Myc (A), YccA-HA-His6-Myc (B and C), or YccA11-HA-His6-Myc (D). The mixtures were treated with A, B, and D or without (C) PEG3350 and then incubated at 37 °C in the presence or absence of 5 mM ATP. E, 1.2 μg of FtsH-His6-Myc and 0.14 μg of YccA11-HA-His6-Myc was incubated at 37 °C with 5 mM ATP or ADP in the presence of 0.5% Nonidet P-40. Samples were withdrawn at the indicated time points and analyzed by 12.8% Tricine gel electrophoresis and anti-Myc immunoblotting. F, quantitation of the results in B–D. The band intensity of each protein at time 0 was set at 100%, and the relative values are plotted.

Fig. 8. Degradation of the YccA derivatives in reconstituted proteoliposomes after fusion with FtsH-bearing proteoliposomes. A–D, 10 μl of the FtsH-His6-Myc proteoliposomes were mixed with 3 μl of those containing YccA-His6-Myc (A), YccA-HA-His6-Myc (B and C), or YccA11-HA-His6-Myc (D). The mixtures were treated with A, B, and D or without (C) PEG3350 and then incubated at 37 °C in the presence or absence of 5 mM ATP. E, 1.2 μg of FtsH-His6-Myc and 0.14 μg of YccA11-HA-His6-Myc was incubated at 37 °C with 5 mM ATP or ADP in the presence of 0.5% Nonidet P-40. Samples were withdrawn at the indicated time points and analyzed by 12.8% Tricine gel electrophoresis and anti-Myc immunoblotting. F, quantitation of the results in B–D. The band intensity of each protein at time 0 was set at 100%, and the relative values are plotted.

**DISCUSSION**

In this study, we succeeded in reproducing the FtsH-catalyzed membrane protein degradation in vitro, using the enzyme and substrates, both of which were integrated into the lipid bilayer of either IMVs or reconstituted proteoliposomes. Recently, much attention has been directed to the importance of proteolytic reactions that occur in the vicinity of a membrane. Such reactions not only serve as a quality control device to eliminate unwanted membrane proteins but also as a regulatory mechanism, in which a membrane protein is cleaved to liberate and activate a regulatory domain (30). However, biochemical and molecular characterization has only insufficiently been conducted for membrane-integrated proteases. Although their enzymatic reactions in solution can be studied in vitro in the presence of a detergent and such reactions will provide some important information on the reaction parameters of the protease, many pertinent questions can only be answered by examination of reactions in which both the enzyme and substrates are engaged in their membrane-integrated states.

We have shown here that PEG3350 treatment is a simple and effective method to induce fusion of membrane vesicles, and thereby to initiate interaction between a membrane-bound enzyme and a membrane-bound substrate. The size measurement results indicated that the PEG3350 treatment induces fusion of an average of ~50 vesicles. As this method proved...
Reconstitution of FtsH

effective for both native IMVs and artificial proteoliposomes, it can probably be used for membrane vesicles of diverse biological sources. However, for in vitro analysis to be possible, membrane vesicles should be in such an orientation, in which enzymatic active site of the protease faces the external milieu, whose composition can easily be manipulated. The substrate should also assume the compatible orientation in the membrane vesicles. Fortunately, in our present case, the availability of inverted membrane vesicles and the asymmetric reconstitution of both FtsH and YccA allowed the strategy described above.

The mode of YccA-(P3)-PhoA-His6-Myc degradation in the fused IMVs was very similar to that observed in vivo, including the degradation arrest in front of the folded PhoA domain. Unfolding of the PhoA domain allowed the degradation to continue beyond the PhoA moiety over the entire molecule. Furthermore, FtsH degraded YccA in reconstituted proteoliposomes without leaving a distinct degradation product. Because the YccA1 mutant protein with the shortened N-terminal tail did not receive degradation in the reconstituted liposomes, the proteolysis in this reconstituted system could only be initiated at the N-terminal region of the fusion protein. Thus, if degradation cannot continue beyond a transmembrane region, an end product lacking only the N-terminal tail should have been detected for the YccA-PhoA-His6-Myc protein. The lack of such product, together with the fact that this system consisted of purified components, strongly suggests that FtsH can continue degradation of YccA-PhoA-His6-Myc beyond the first transmembrane segment into the periplasmic and the following transmembrane regions. These results provide strong evidence for the dislocation mechanism of the FtsH-catalyzed membrane protein degradation.

Our results showed that the folded PhoA moiety of YccA-(P3)-PhoA-His6-Myc could at least partially be unfolded by treatment with a high concentration (250 mM) of DTT that should have reduced the intramolecular disulfide bonds in PhoA. Although the wild-type PhoA protein exhibits significant resistance to a reducing agent (31), the PhoA portion of the YccA-(P3)-PhoA-His6-Myc fusion protein may be distorted to some extent (14) and more susceptible to reduction, making the post-translational unfolding possible. In contrast to our previous in vivo experiments, in which the formation of the intramolecular disulfide bonds in PhoA was prevented in the first place by the host dsbA mutation or mutational absence of the responsible cysteine residues in PhoA (3, 14), the DTT treatment disrupts the structure once established. Our results thus indicate that post-translationally unfolded periplasmic domain can be dislocated and degraded by FtsH. It may be generalized that FtsH has the ability to degrade a periplasmic domain of a membrane protein when it was unfolded post-translationally by environmental stresses. However, for this event to happen, the cytosolic region of such a membrane protein should also be affected to be recognized by FtsH for degradation initiation. During the dislocation-unfolding events, FtsH appears to remain bound to the substrate protein, and this should provide a basis for the processive nature of the proteolysis. Indeed, the L60 intermediate was degraded when the PhoA moiety on the trans side was unfolded in our in vitro system. The PhoA domain within the YccA-(P3)-PhoA-His6-Myc fusion protein may not be completely unfolded by DTT as it exhibited some degree of trypsin resistance. The presumed ATP hydrolysis-coupled dislocation activity of FtsH may unfold further such a loose structure.

Our previous studies (4) showed that the in vitro proteolytic functions of FtsH against different substrate proteins were stimulated by PMF. This stimulation of degradation was observed both for soluble and membrane-integrated substrate proteins, the latter including YccA and Foa. Our in vitro attempts to reproduce the PMF-stimulation of membrane protein degradation has so far failed. Although direct comparison with the in vivo activity is difficult, the reaction efficiency in our system appears rather low. The same reaction mixture exhibited more rapid degradation of the substrates after solubilization of every component by a non-ionic detergent (data not shown). Although the inefficiency of the in vitro reaction could suggest that some stimulatory component is missing in the reaction system, the apparent degradation speeds were similar between the IMV-based and the proteoliposome-based reactions. Thus, slow reaction might be an inherent nature of the lipid bilayer-integrated reaction components in vitro. The slow reaction could be due either to a slow enzyme-substrate encounter, slow initiation of proteolysis, slow dislocation, slow catalysis, or a combination of these.

Whereas our results suggest that FtsH does not need any aid from other proteins for its ability to degrade membrane-integrated substrates, more efficient and physiological reactions could still require some additional factor(s). In the case of the endoplasmic reticulum-associated degradation in eukaryotic cells, the translocon (the Sec61 complex)-mediated dislocation has been suggested. It remains possible that the E. coli translocon (the SecYEG complex) plays a similar role for the FtsH-catalyzed membrane protein degradation to occur rapidly. However, it must be stressed at this point that our results point to the ability of the FtsH protein itself to mediate the dislocation/degradation events, even if inefficiently.

We feel that involvement of the SecYEG channel is unlikely for the following reasons. First, our preparations of FtsH and YccA contained undetectable levels of SecE and only minute amounts (less than 1.5% weight ratio) of SecY (data not shown). Second, IMVs from SecYEG-overproducing cells did not exhibit any enhanced activity of YccA degradation. Third, examination of several SecY mutants revealed none, in which in vivo degradation of YccA was retarded significantly. In addition, the m-AAA protease, a mitochondrial counterpart of FtsH, is also believed to dislocate substrate membrane proteins for degradation (32), whereas the mitochondrial inner membrane does not contain a Sec61/SecY homolog (35).

Whereas FtsH-catalyzed proteolysis of membrane proteins does not require the SecYEG translocon channel as an essential component, participation of some stimulatory factor is not excluded. It should be noted also that FtsH in wild-type cells exists exclusively as a complex, termed FtsH holoenzyme, with the HflKC complex. The roles played by the HflKC complex should also be elucidated for our understanding of regulation of the FtsH function in its proteolytic functions against membrane-integrated and soluble protein substrates. Our experimental approaches described in this paper can be extended further to address these important questions in membrane protein quality control.

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Reconstitution of FtsH

18153

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