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Dislocation of membrane proteins in FtsH-mediated proteolysis

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Escherichia coli **FtsH degrades several integral membrane proteins, including YccA, having seven transmembrane segments, a cytosolic N-terminus and a periplasmic C-terminus. Evidence indicates that FtsH initiates proteolysis at the N-terminal cytosolic domain. SecY, having 10 transmembrane segments, is also a substrate of FtsH. We studied whether and how the FtsH-catalyzed proteolysis on the cytosolic side continues into the transmembrane and periplasmic regions using chimeric proteins, YccA-(P3)-PhoA-His_6-Myc and SecY-(P5)-PhoA, with the alkaline phosphatase (PhoA) mature sequence in a periplasmic domain. The PhoA domain that was present within the fusion protein was rapidly degraded by FtsH when it lacked the DsbA-dependent folding. In contrast, both PhoA itself and the TM9-PhoA region of SecY-(P5)-PhoA were stable when expressed as independent polypeptides. In the presence of DsbA, the FtsH-dependent degradation stopped at a site near to the N-terminus of the PhoA moiety, leaving the PhoA domain (and its C-terminal region) undigested. The efficiency of this degradation stop correlated well with the rapidity of the folding of the PhoA domain. Thus, both transmembrane and periplasmic domains are degraded by the processive proteolysis by FtsH, provided they are not tightly folded. We propose that FtsH dislocates the extracytoplasmic domain of a substrate, probably using its ATPase activity.**

Keywords: AAA family/dislocation/FtsH/membrane protein/protease

Introduction

Proteolytic processes are important for diverse aspects of cellular function. Recent studies highlight the particular importance of ATP-dependent proteases in various organisms. In *Escherichia coli*, we find Lon, ClpAP, ClpXP, HslUV and FtsH (for reviews see Gottesman and Maurizi, 1992; Gottesman, 1996), whereas in eukaryotic cells the proteasome complexes comprise the major energy-dependent proteolytic system (for a review, see Coux *et al.*, 1996). Many of the ATP-dependent proteases are structurally related in that they form large oligomers of ring-shaped (top view) or barrel-like (side view) structures (Kessel *et al.*, 1995; Löwe *et al.*, 1995; Groll *et al.*, 1997; Rohrwild *et al.*, 1997; Grimaud *et al.*, 1998). The roles of ATP or ATP hydrolysis in the proteolysis catalyzed by these enzymes have been the subject of intensive research interest in recent years. It is believed that the ATPase subunits of some of these enzymes confer processivity to the proteolytic reaction. Thus, once an ATP-dependent protease recognizes a substrate, it catalyzes successive endo-proteolysis of the entire region of the substrate without releasing specific high molecular weight intermediates (Maurizi, 1987; Thompson *et al.*, 1994; van Melderen *et al.*, 1996; Akopian *et al.*, 1997). The ATP-utilizing steps may be concerned with recognition of the substrate proteins as well as their unfolding and translocation into the proteolytic compartment of the protease (Gottesman and Maurizi, 1992; Hoskins *et al.*, 1998). Experimentally, molecular chaperone activities have been established for the ClpA and ClpX ATPase subunits (Wickner *et al.*, 1994; Levchenko *et al.*, 1995; Wawrzynow *et al.*, 1995; Pak and Wickner, 1997). It is likely that these activities are closely related to their roles in substrate-specific proteolysis by the ClpAP or the ClpXP protease. Hoskins *et al.* (1998) demonstrated that a substrate protein first binds to ClpA and then it is translocated to the proteolytic ClpP subunit, resulting either in its degradation (without competition by the excess of other substrate molecules) or in its association with the proteolysis-defective forms of the ClpP subunit.

Among ATP-dependent proteases in *E.coli*, FtsH is unique in that it is integrated into the cytoplasmic membrane by two N-terminal transmembrane segments (Tomoyasu *et al.*, 1993; Akiyama and Ito, 1998). The transmembrane region is required for homo-oligomerization (Akiyama *et al.*, 1995), whereas the small periplasmic domain is important not only for homo-oligomerization but also for FtsH's association with the HflK–HflC membrane protein complex, a factor which modulates FtsH function (Kihara *et al.*, 1996, 1998; Akiyama *et al.*, 1998a). The large cytosolic domain of FtsH includes a region that belongs to the AAA ATPase family (Confalonieri and Duguet, 1995) as well as a region with a zinc metalloproteinase signature sequence (HEXXH). FtsH is conserved among members of the prokaryotic kingdom as well as in the mitochondrial and the thylakoid membranes of eukaryotic cells (for reviews see Suzuki *et al.*, 1997; Schumann, 1999). *Escherichia coli* FtsH degrades both cytosolically localized soluble proteins, such as the σ^{32} RNA polymerase subunit, λ CII protein and the *lpxC* gene product (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995; Kihara *et al.*, 1997; Shotland *et al.*, 1997; Ogura *et al.*, 1999), and integral membrane proteins, such as the SecY subunit of the protein translocase, subunit *a* of the F_0 component of H^+-ATPase and YccA, whose function is unknown (Kihara *et al.*, 1995, 1998; Akiyama *et al.*, 1996a,b).

Previously, we identified YccA as a new substrate for FtsH, as well as its non-degradable mutant form, YccA11, which has an internal deletion of eight amino acids within the N-terminal tail. Expression of this mutant protein interfered with the *in vivo* degradation of membrane-integrated substrates of FtsH but not with that of the soluble substrates (Kihara *et al.*, 1998). YccA itself contains multiple hydrophobic regions. Some mutations in *hflK* or *hflC* also have differential effects against degradation of membrane-integrated and soluble substrates. Thus, these two classes of substrate proteins seem to be degraded via distinct pathways by FtsH (Kihara *et al.*, 1998). In order to understand more about the different modes of action of FtsH, it is important to address some basic questions about the processes in which membrane-integrated proteins are degraded. For instance, it is important to know whether the substrate protein can be static or should undergo dynamic changes in order to be degraded. A simple version of the ATP-dependent unfolding and presentation mechanism may meet with difficulties when the proteolysis has to continue into a membrane-integrated region or even toward the domain exposed to the other side of the membrane. Recently, it has been proposed that abnormal membrane proteins of the endoplasmic reticulum are dislocated to the cytosol where they are captured by the proteasome system (Biederer *et al.*, 1996; Hiller *et al.*, 1996; Wiertz *et al.*, 1996; Pilon *et al.*, 1997; Plemper *et al.*, 1998). However, molecular mechanisms underlying this dislocation process are elusive. In the present work, we studied *in vivo* the processes of FtsH-dependent degradation of two membrane proteins, YccA and SecY, making use of alkaline phosphatase (PhoA) fusion derivatives. The PhoA domain was attached to a periplasmic region of these proteins and used to monitor whether degradation proceeds into the periplasmic side. This domain was also used as a controlled folding module; in a folded state, it may interfere with the hypothetical process in which it is dislocated to the cytoplasmic side. The results obtained suggest that the transmembrane movement of periplasmic domains accompanies the FtsH-dependent degradation of membrane proteins.

Results

Topology of YccA

YccA is an integral membrane protein, which is unstable *in vivo* (Kihara *et al.*, 1998). We previously showed that this protein is a substrate of the FtsH protease *in vivo* and *in vitro*. The TopPred II 1.1 program (Claros and von Heijne, 1994) predicts that YccA has seven transmembrane segments (TMs), four cytoplasmic segments (C1–C4 from the N-terminus) and four periplasmic segments (P1–P4) (Figure 1). Segments C1, C2, C3 and C4 contain 23, six, five and 18 amino acid residues, respectively, whereas segments P1, P2, P3 and P4 contain two, ten, six and two residues. We reported previously that an internal deletion of eight amino acids (residues 5–12; Figure 1) rendered YccA resistant to FtsH-mediated degradation (Kihara *et al.*, 1998). The mutant form of YccA, termed YccA11, associates with FtsH and interferes with FtsH's proteolytic activity against the membrane-embedded substrates (but not the cytosolic substrates). We constructed a plasmid

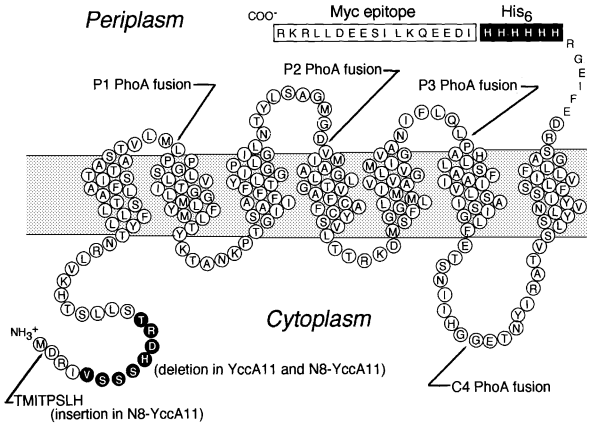

Fig. 1. YccA and its derivatives. The YccA amino acid sequence is shown by one letter notations in circles followed by a linker region and the His_6 and Myc tags. Its orientation in the cytoplasmic membrane is shown schematically. The residues deleted in the YccA11 form are indicated by solid circles. The positions of PhoA insertions in YccA-(P1)–PhoA-His_6-Myc, YccA-(P2)–PhoA-His_6-Myc, YccA-(P3)–PhoA-His_6-Myc and YccA-(C4)–PhoA-His_6-Myc, as well as the insertion in N8-YccA11, are indicated by arrows.

encoding a derivative of YccA and YccA11 (YccA-His_6-Myc and YccA11-His_6-Myc) in which the C-terminal P4 segment is followed by His_6 and the Myc epitope sequences (Kihara *et al.*, 1998; Figure 1).

Proteinase K digestion of the intact spheroplasts abolished the antigenicity of the Myc epitope attached to YccA and YccA11 (Figure 2, upper panel). The spheroplasts used were intact, since a cytosolic protein, GroEL, remained unaffected unless membranes were solubilized with Triton X-100 (Figure 2, lower panel). Similar results were obtained using trypsin, instead of proteinase K (data not shown). These results indicate that YccA-His_6-Myc and YccA11-His_6-Myc molecules have their C-termini facing the periplasm.

To ascertain further the topology of YccA-His_6-Myc, we constructed a series of PhoA sandwich fusion proteins. In these chimeras, the PhoA mature sequence was inserted in-frame into the carboxy end of P1, P2 and P3 or into C4 (Figure 1). Ehrmann *et al.* (1990) showed that the PhoA domains in sandwich configurations can serve as faithful reporters of the disposition of the membrane protein segment where the fusion has taken place. The fusion proteins constructed were named YccA-(P1)–PhoA-His_6-Myc, YccA-(P2)–PhoA-His_6-Myc, YccA-(P3)–PhoA-His_6-Myc and YccA-(C4)–PhoA-His_6-Myc, where the domains of PhoA insertions are indicated in parentheses (see Figure 1). All the periplasmic fusions gave high enzymatic activities of alkaline phosphatase, 87.0, 75.0 and 62.8 U for fusions at P1, P2 and P3, respectively. In contrast, the cytoplasmic fusion at C4 exhibited only 6.3 U of enzyme activity. These results confirm the periplasmic localizations of the P1, P2 and P3 regions of YccA, whereas C4 is indeed cytoplasmic.

We examined further whether the PhoA domain sandwiched within the P3 segment assumes the protease-resistant conformation, a characteristic of the exported PhoA (Akiyama and Ito, 1989, 1993; Derman and Beckwith, 1991). Trypsin digestion of cell lysate from wild-type PhoA-expressing cells yielded a trypsin-resistant

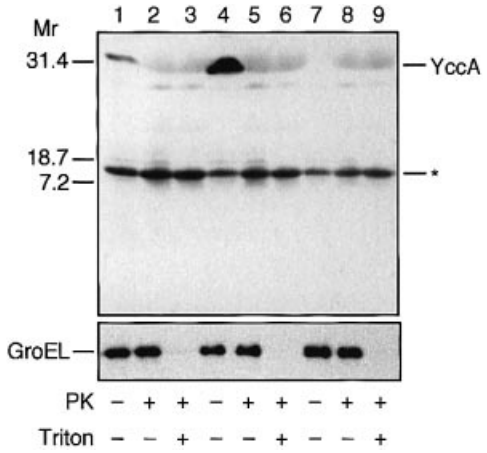

Fig. 2. The C-terminus of YccA is exposed to the periplasm. Cells of AD202 harboring pKH330 (p*lac-yccA-his*$_6$*-myc*; lanes 1–3), pKH331 (p*lac-yccA11-his*$_6$*-myc*; lanes 4–6) or pTWV229 (empty vector; lanes 7–9) were grown in L medium containing 0.1% glucose and 50 μg/ml ampicillin. Plasmid-encoded YccA-His$_6$-Myc and YccA11-His$_6$-Myc were induced with 1 mM cAMP for 2 h. Spheroplasts were prepared and treated with proteinase K (PK; 500 μg/ml) for 2 h at 0°C in the presence or absence of 1% Triton X-100 (Triton) as indicated. Proteins were separated by SDS–PAGE for visualization of YccA-His$_6$-Myc and YccA11-His$_6$-Myc (upper panel), as well as GroEL (lower panel) by immunoblotting using antisera against Myc and GroEL. The asterisk indicates a non-specific background.

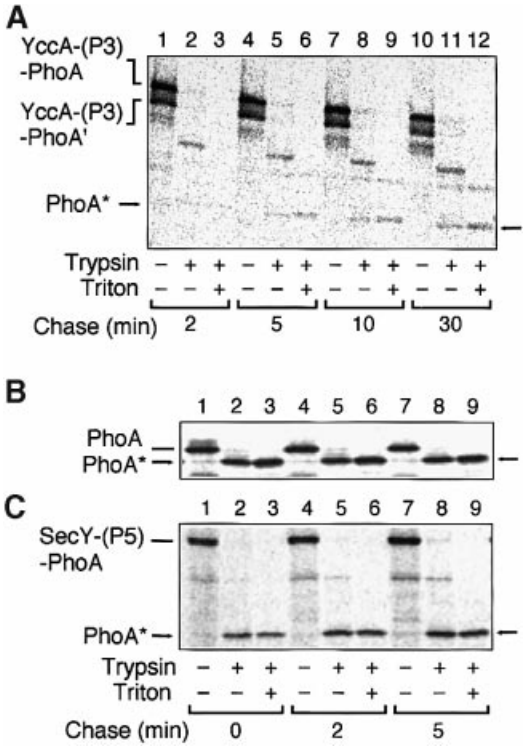

Fig. 3. Translocation and folding of the PhoA domain in different constructions. (**A**) Cells of KS474/pKH412 [p*lac-yccA-(P3)-phoA-his*$_6$*-myc*] were grown at 30°C in amino acid-supplemented M9–glucose medium and induced with 3 mM cAMP for 10 min, followed by pulse labeling with [^{35}S]methionine for 1 min and chase with unlabeled methionine for 2–30 min as indicated. Cells were converted to spheroplasts and treated with trypsin (50 μg/ml) in the presence or absence of 1% Triton X-100 as indicated. Labeled proteins precipitated with anti-PhoA were separated by SDS–PAGE and visualized using a BAS1800 imager (Fuji film). The band indicated as YccA-(P3)–PhoA′ was produced artificially during the sample manipulations. The trypsin-resistant PhoA fragment (PhoA*) is indicated by an arrow. KS474/pMS102 (p*lac-phoA*) (**B**) and KS474/pKY4::*phoA*12-1 [p*lac-secY-(P5)-phoA*] (**C**) were grown at 30°C and the *lac* transcription was induced with 3 mM cAMP for 10 min. Cells were pulse-labeled with [^{35}S]methionine for 2 min, and chased with unlabeled methionine for 0, 2 and 5 min as indicated. Cells were converted into spheroplasts, and treated with trypsin (50 μg/ml) in the presence or absence of 1% Triton X-100, as indicated. Anti-PhoA immunoprecipitates were separated by SDS–PAGE and visualized by a BAS1800 imager.

fragment of ~46 kDa (PhoA* in Figure 3B). As reported previously (Akiyama and Ito, 1989, 1993), this folding is very rapid *in vivo*, as wild-type PhoA molecules were already trypsin resistant when pulse-labeled for 2 min at 30°C (Figure 3B, lanes 1–3). YccA-(P3)–PhoA-His$_6$-Myc also produced, after trypsin digestion, a 46 kDa fragment that reacted with anti-PhoA antibodies (Figure 3A, lanes 11 and 12). Pulse–chase analysis showed, however, that the folding of the PhoA moiety required more time than the authentic PhoA (Figure 3A, lanes 2 and 3). It is possible that folding of the PhoA region is retarded by the presence of the flanking YccA sequences. At any rate, the full accessibility of trypsin to the pulse-labeled PhoA region of the fusion protein without disruption of the spheroplasts (Figure 3A, lane 2) indicates that translocation of the P3 domain occurred very rapidly. We conclude that YccA assumes the topology shown in Figure 1 in the membrane.

Degradation of YccA-His$_6$-Myc is initiated at the N-terminal cytoplasmic tail

The YccA11 mutant protein, with a deletion of eight amino acids in the cytosolic tail at the N-terminus, can associate with FtsH but is no longer susceptible to proteolysis (Kihara *et al.*, 1998). All the cytoplasmic regions of YccA are small in size, with the N-terminal region being the largest (Figure 1). We thought it possible that the size of the N-terminal region may be critical for YccA to be a substrate of FtsH. We constructed a variant protein, N8-YccA11-His$_6$-Myc, in which an unrelated sequence of eight amino acids was inserted between Met1 and Asp2 of YccA11 (Figure 1). Pulse–chase experiments showed that N8-YccA11-His$_6$-Myc was as unstable as YccA-His$_6$-Myc in wild-type cells (Figure 4A, circles and triangles). Degradation of N8-YccA11-His$_6$-Myc was FtsH dependent since it was stabilized in the *ftsH1* temperature-sensitive mutant cells at 42°C (Figure 4B, open circles). Since the N8 sequence added back was unrelated to the original YccA sequence, the original sequence deleted by the *yccA11* mutation is not itself essential for the initiation of proteolysis by FtsH. These results are consistent with the notion that initiation of YccA degradation requires an N-terminal tail of a length sufficient to be recognized by the cytoplasmic ATPase and/or protease active sites of FtsH. Whether the degradation initiation requires certain features in amino acid sequence or certain structural features (such as unfoldedness) in addition to the size of the cytosolic tail awaits further studies. At any rate, the properties of YccA, YccA11 and N8-YccA11 clearly indicate that the N-terminal cytoplasmic tail is crucial for the initiation of the FtsH-mediated degradation of YccA. The degradation of YccA proceeds in the N- to C-terminal direction.

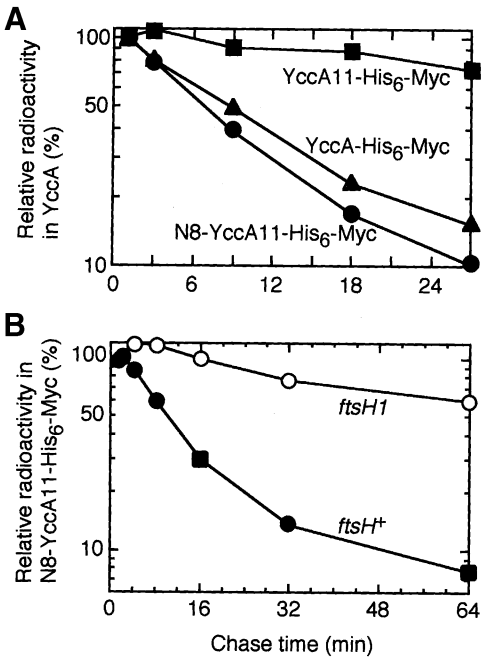

Fig. 4. *In vivo* sensitization of YccA11-His_6-Myc to FtsH by addition of eight amino acids. (**A**) AD16 carrying pKH330 (p*lac-yccA-his*$_6$-*myc*; ▲), pKH331 (p*lac-yccA11-his*$_6$-*myc*; ■) or pKH356 (p*lac-N8-yccA11-his*$_6$-*myc*; ●) were grown at 37°C in amino acid-supplemented M9–glucose medium containing ampicillin, and induced for *lac* transcription with 1 mM IPTG and 3 mM cAMP for 10 min. Cells were pulse-labeled with [^{35}S]methionine for 1 min followed by chase with unlabeled methionine for 1, 3, 9, 18 and 27 min as indicated. (**B**) AR796 (*ftsH*$^+$; ●) and AR797 (*ftsH1*; ○), each carrying pKH356 (p*lac-N8-yccA11-his*$_6$-*myc*), were grown first at 30°C and then shifted to 42°C. After 1 h, cells were induced with 1 mM cAMP for 10 min, and pulse-labeled with [^{35}S]methionine for 1 min followed by chase with unlabeled methionine for 1, 2, 4, 8, 16, 32 and 64 min. In both (A) and (B), labeled proteins were immunoprecipitated with anti-Myc and separated by SDS–PAGE. Radioactivities associated with YccA-His_6-Myc, YccA11-His_6-Myc and N8-YccA11-His_6-Myc were quantitated using a BAS1800 imager. Values relative to that at the 1 min chase point are shown for each experiment.

The periplasmic PhoA insertion acts to stop the FtsH-mediated degradation of YccA-(P3)–PhoA-His$_6$-Myc at a specific region

If FtsH only digested the cytosolically exposed portions of YccA-His_6-Myc, a fragment spanning TM1 to the C-terminus should have remained undigested. Even if FtsH can somehow reinitiate proteolysis at each cytosolic part, several TM–periplasmic region fragments, including an anti-Myc-reactive TM7–P4-His_6-Myc fragment, are expected to be generated. However, so far we have been unable to detect any short fragments, labeled or in steady state, by either immunoblotting or pulse–chase experiments (data not shown; see Figure 2, upper panel, lanes 1 and 4 for immunoblotting patterns). These observations raised an interesting possibility; once FtsH initiates degradation of YccA-His_6-Myc it may digest the entire region of the protein so rapidly that no intermediates of specific sizes accumulate. An alternative possibility would be that such fragments are produced but are digested immediately by other proteases. In addressing these questions, YccA-His_6-Myc is not very useful, since the fragments in question are expected to be too small for easy detection.

We therefore used the PhoA sequence either inserted into a periplasmic region of YccA-His_6-Myc (see above) or attached to periplasmic region 5 of the SecY protein (see below) for examining the presence or absence of degradation intermediates under different conditions. It should be noted that one interesting possibility is that YccA, captured by FtsH for degradation, is somehow dislocated to make its transmembrane and periplasmic regions accessible by the cytoplasmic active site domain of FtsH. Attachment of the PhoA sequence is suited to address this specific possibility, since the tight folding of the PhoA domain could interfere with the hypothetical dislocation process.

We first used the PhoA sandwich fusion with the PhoA moiety inserted into the P3 region of YccA-His_6-Myc, and asked whether the folded PhoA domain on the periplasmic side affected the degradation of YccA. The PhoA fusion protein was expressed in strains carrying the null mutation in *degP* encoding the major periplasmic protease. This was to minimize the DegP-mediated periplasmic protein degradation (Strauch and Beckwith, 1988). Steady-state accumulation of YccA-(P3)–PhoA-His_6-Myc was examined by immunoblotting using anti-Myc. A close doublet of ~60 kDa bands (termed I-60 in Figure 5A) was detected in addition to the full-length YccA-(P3)–PhoA-His_6-Myc protein of ~75 kDa (Figure 5A, lane 1). When FtsH was overproduced, the proportion of I-60 to the intact protein increased markedly (Figure 5A, lane 2). Conversely, when the FtsH function was compromised by the expression of FtsH41, a dominant-negative variant (Akiyama *et al.*, 1994b), the proportion of I-60 decreased dramatically (Figure 5A, lane 3). Thus, the appearance of I-60 depended on the presence of FtsH activity. Since I-60 retained Myc antigenicity as well as PhoA antigenicity (see below), it must have been generated by the loss of the N-terminal portion of YccA. Its apparent molecular mass suggested that the N-terminal end of I-60 was close to the PhoA sequence. The sizes of I-60 are indeed consistent with this doublet containing fragments of the PhoA–TM6–C4–TM7-Myc region of the fusion protein. We verified that degradation of YccA-(P3)–PhoA-His_6-Myc depended on the N-terminal tail, since the same fusion protein but with the shortened N-terminal tail of the YccA mutant [YccA11-(P3)-His_6-Myc] was stable and did not produce any detectable I-60 fragments (Figure 6).

Degradation of YccA-(P3)–PhoA-His_6-Myc was followed by pulse–chase experiments. [^{35}S]methionine-labeled proteins were immunoprecipitated using either anti-Myc serum or anti-PhoA serum, both of which gave essentially identical results (Figure 5B). Thus, the protein species discussed below all retained the intact C-terminus and the PhoA region. Results of two different experiments are shown as autoradiograms (Figure 5B) and relative radioactivities (Figure 5C). In *ftsH*$^+$ cells, YccA-(P3)–PhoA-His_6-Myc initially was labeled as the full-length product (Figure 5B, lane 1) and then degraded over the chase period of 120 min (Figure 5B, lanes 2–4; Figure 5C, solid circles). During the chase, I-60 appeared (Figure 5B, upper panel). The disappearance of the full-length protein (Figure 5C, solid circles) and the appearance of I-60 (Figure 5C, open circles) were nearly a mirror image, consistent with their precursor–product relationships. Upon expression of the dominant-negative FtsH41 protein

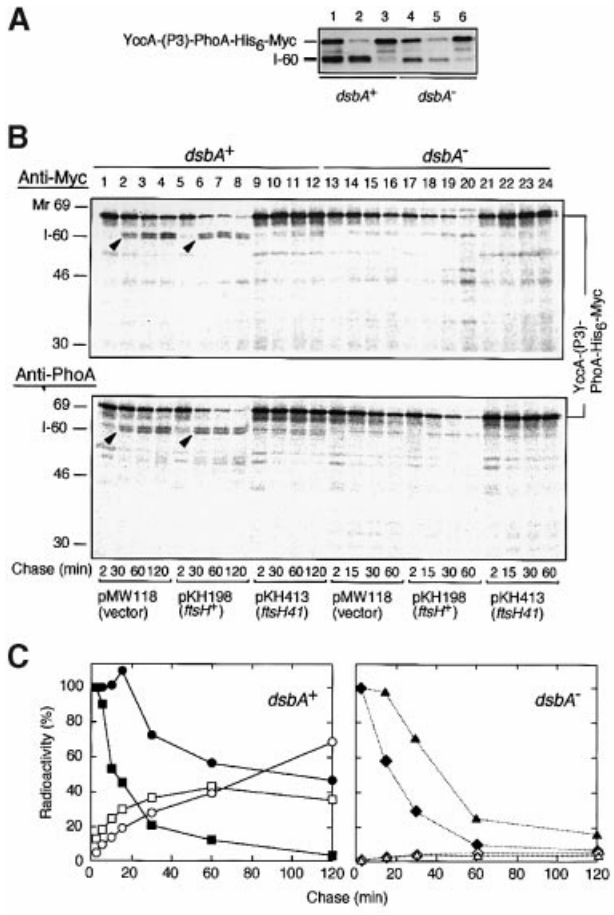

Fig. 5. Effects of *ftsH* and *dsbA* on degradation patterns of YccA-(P3)–PhoA-His_6-Myc in the absence of DegP. (**A**) Steady-state accumulation. KS474 (*degP*::Tn*5* *dsbA*$^+$ Δ*phoA*) carrying pKH412 [p*lac-yccA-(P3)-phoA-his*$_6$*-myc*] was transformed further with pMW118 (vector; lane 1), pKH198 (p*lac-ftsH*$^+$; lane 2) or pKH413 (p*lac-ftsH41*; lane 3). Similarly, AK2055 (*degP*::Tn*5* *dsbA*::*tet* Δ*phoA*) carrying pKH412 was transformed further with pMW118 (lane 4), pKH198 (lane 5) or pKH413 (lane 6). The *ftsH41* allele was dominant negative. Cells were grown in L-broth–glucose supplemented with ampicillin (50 μg/ml) and chloramphenicol (20 μg/ml) at 30°C. Plasmid-encoded proteins were induced with 1 mM IPTG and 3 mM cAMP for 2 h. Anti-Myc-reacting proteins were visualized after SDS–PAGE of whole-cell proteins and immunoblotting. (**B**) Degradation of newly synthesized protein. KS474 (*degP*::Tn*5* *dsbA*$^+$ Δ*phoA*) carrying pKH412 [p*lac-yccA-(P3)-phoA-his*$_6$*-myc*] was transformed further with pMW118 (vector; lanes 1–4), pKH198 (p*lac-ftsH*$^+$; lanes 5–8) or pKH413 (p*lac-ftsH41*; lanes 9–12). Similarly, AK2055 (*degP*::Tn*5* *dsbA*::*tet* Δ*phoA*) carrying pKH412 was transformed further with pMW118 (lanes 13–16), pKH198 (lanes 17–20) or pKH413 (lanes 21–24). Cells were grown at 30°C to an early log phase as described in the Materials and methods, and plasmid-encoded proteins were induced with 3 mM cAMP for 30 min. They were then pulse-labeled with [^{35}S]methionine for 1 min and chased with unlabeled methionine for the indicated periods. Radioactive proteins were isolated with anti-Myc and anti-PhoA immunoprecipitation, as indicated, separated by SDS–PAGE and visualized. (**C**) Conversion of full-length YccA-(P3)–PhoA-His_6-Myc to PhoA–TM6–C4–TM7-Myc fragment (I-60). The experiments shown in (B) were repeated with additional chase points (2, 5, 10, 15, 30, 60 and 120 min). After SDS–PAGE of anti-Myc precipitates, radioactivities associated with the full-length YccA-(P3)–PhoA-His_6-Myc protein (solid symbols) and the I-60 species (open symbols) were quantitated. Values relative to the full-length product at the 2 min chase point are shown. Circles, *dsbA*$^+$ strain with normal level of FtsH; squares, *dsbA*$^+$ with overproduced FtsH; triangles, *dsbA*::tet strain with normal level of FtsH; diamonds, *dsbA*::tet with overproduced FtsH. Values were corrected for the predicted methionine contents of the full-length and the I-60 products.

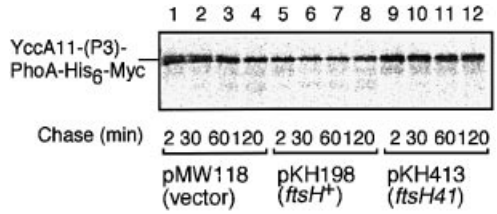

Fig. 6. YccA11-(P3)–PhoA-His_6-Myc is FtsH-insensitive. KS474 carrying pKH447 [p*lac-yccA11-(P3)-phoA-his*$_6$*-myc*] was transformed further with pMW118 (vector; lanes 1–4), pKH198 (p*lac-ftsH*$^+$; lanes 5–8) or pKH413 (p*lac-ftsH41*; lanes 9–12). Cells were grown at 30°C in amino acid-supplemented M9–glucose medium containing ampicillin (50 μg/ml) and chloramphenicol (20 μg/ml), and plasmid-encoded proteins were induced with 3 mM cAMP for 30 min. Cells were pulse-labeled with [^{35}S]methionine for 1 min and chased with unlabeled methionine for 2, 30, 60 and 120 min as indicated. Radioactive YccA11-(P3)–PhoA-His_6-Myc was immunoprecipitated with anti-Myc antibodies, separated by SDS–PAGE and visualized.

to inhibit the FtsH function, YccA-(P3)–PhoA-His_6-Myc was stabilized completely and no I-60 appeared (Figure 5B, lanes 9–12). Thus, FtsH was primarily responsible for the conversion. We will show below that impaired folding of the PhoA domain allows the FtsH-dependent degradation to continue all the way to the C-terminus of YccA-(P3)-His_6-Myc. Taken together, these results indicate that the presence of a folded PhoA prevented the continuation of processive proteolysis, which was initiated at the N-terminal cytosolic tail of YccA-(P3)–PhoA-His_6-Myc.

Complete degradation stop by the rapidly folding PhoA domain in a SecY–PhoA fusion protein

When FtsH was overproduced, the full-length product of YccA-(P3)-His_6-Myc disappeared more rapidly (Figure 5B, lanes 5–8; Figure 5C, solid squares). Quantitation of radioactivities showed, however, that this accelerated degradation was not accompanied by a corresponding increase in I-60 (Figure 5C, open squares). Thus, the final yield of I-60 was significantly lower than that expected for a quantitative conversion from the full-length product. In this fusion construction, the slow folding property of the sandwiched PhoA domain (see Figure 3A) may have undermined the degradation stop in the presence of an increased activity of FtsH. Thus, in the presence of excess FtsH, a greater fraction of substrate molecules may become committed for degradation before the establishment of the folded PhoA domain.

We then examined the effects of a more rapidly folding PhoA moiety on the FtsH-dependent degradation, using the SecY-(P5)–PhoA fusion protein. Previous studies established that SecY is a substrate of FtsH when it is produced in excess over SecE (Kihara *et al.*, 1995; Akiyama *et al.*, 1996a), and that the SecY-(P5)–PhoA protein has its PhoA domain exposed to the periplasmic space (Akiyama and Ito, 1987, 1989). The PhoA region, not sandwiched in this construct, is folded very rapidly upon translocation to the periplasmic side (Akiyama and Ito, 1989). Pulse labeling and trypsin treatment experiments shown in Figure 3C confirmed the rapid folding of the SecY–PhoA fusion protein. Unlike PhoA in YccA-(P3)–PhoA-His_6-Myc (Figure 3A), most of the PhoA domain in SecY-(P5)–PhoA was already folded into the

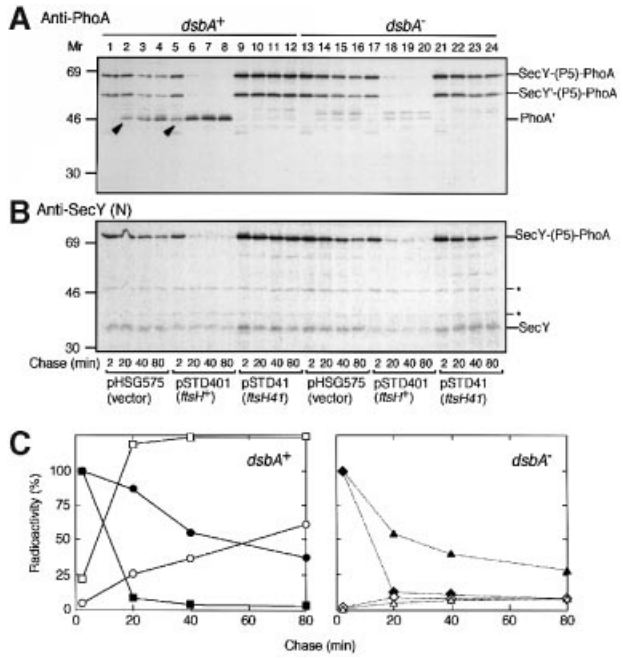

Fig. 7. Degradation of newly synthesized SecY-(P5)–PhoA: effects of *ftsH* and *dsbA*. KS474 (*degP*::Tn*5* *dsbA*$^+$ Δ*phoA*) carrying pKY4::*pho*A12-1 [p*lac-secY-(P5)-phoA*] was transformed further with pHSG575 (vector; lanes 1–4), pSTD401 (p*lac-ftsH*$^+$; lanes 5–8) or pSTD41 (p*lac-ftsH41*; lanes 9–12). Similarly, AK2055 (*degP*::Tn*5* *dsbA*::*tet* Δ*phoA*) carrying pKY4::*pho*A12-1 was transformed further with pHSG575 (lanes 13–16), pSTD401 (lanes 17–20) or pSTD41 (lanes 21–24). Cells were grown at 37°C to early log phase and plasmid-encoded proteins were induced with 3 mM cAMP for 10 min. Cells were then pulse-labeled with [^{35}S]methionine for 1 min and chased with unlabeled methionine for 2, 20, 40 and 80 min as indicated. Radioactive proteins were isolated by immunoprecipitation using antibodies against PhoA (**A**) or an N-terminal segment of SecY (**B**), separated by SDS–PAGE and visualized. Arrowheads indicate the degradation stop product containing PhoA, whereas asterisks indicate non-specific background bands. Chromosomally encoded SecY is also visible in (B). The band indicated as SecY′-(P5)–PhoA, which lacked an N-terminal region of SecY, was produced by either biosynthetic or proteolytic truncation of the N-terminal part. (**C**) Radioactivities associated with the intact SecY-(P5)–PhoA protein and those of the SecY′-(P5)–PhoA were summed and plotted as solid symbols, whereas those associated with the PhoA′ fragment were plotted as open symbols. Values were normalized to the total radioactivity [in SecY-(P5)–PhoA and SecY′-(P5)–PhoA] at the 2 min chase point for each strain. Circles, *dsbA*$^+$ strain with normal level of FtsH; squares, *dsbA*$^+$ with overproduced FtsH; triangles, *dsbA*::tet strain with normal level of FtsH; diamonds, *dsbA*::tet with overproduced FtsH. Values were corrected for the predicted methionine contents of SecY-(P5)–PhoA, SecY′-(P5)–PhoA and PhoA′.

trypsin-resistant conformation after pulse labeling for 2 min (Figure 3C).

Pulse–chase experiments revealed that degradation of SecY-(P5)–PhoA was also accompanied by the appearance of a fragment (48 kDa; designated PhoA′ in Figure 7) that reacted with anti-PhoA (Figure 7A, lanes 2–4 and 6–8), but not with antibodies against the N-terminal region of SecY (Figure 7B, lanes 2–4 and 6–8). Overproduction of FtsH greatly accelerated not only the disappearance of the intact SecY-(P5)–PhoA protein (Figure 7C, compare solid circles and solid squares) but also the kinetics of generation and the final yield of the PhoA′ fragment (Figure 7C, compare open circles and open squares). This was in contrast to what we observed with YccA-(P3)–PhoA-His$_6$-Myc (Figure 5C). In the FtsH-compromised cells, the full-length SecY-(P5)–PhoA protein was stabilized completely and no PhoA′ was produced (Figure 7A, lanes 9–12). These results suggest that PhoA′ was produced by the action of FtsH.

In the above experiments, a protein band of ~60 kDa [SecY′-(P5)–PhoA in Figure 7A] was labeled and precipitated with antibodies against PhoA (but not those against the N-terminus of SecY; Figure 7B). Whereas the initial appearance of this fragment was independent of FtsH (Figure 7A, lanes 1, 5 and 9), it was degraded subsequently in an FtsH-dependent fashion (Figure 7A, lanes 1–4, 5–8 and 9–12). SecY′-(P5)–PhoA may have been produced by either an internal initiation of translation or some extremely rapid cleavage of nascent SecY. In any case, quantification of the PhoA′ band indicated that it was produced by quantitative conversion from both the full-length protein and SecY′-(P5)–PhoA (Figure 7C, compare solid and open circles as well as solid and open squares). Thus, the degradation stops at a point of close N-terminal proximity of the periplasmic PhoA domain for both the YccA–PhoA and the SecY–PhoA fusion protein. The efficiency of this arrest in their degradation correlated well with the rapidity of the folding of the PhoA domain.

The lack of periplasmic disulfide bond formation allows continuous degradation of the PhoA fusion proteins

We studied degradation of YccA-(P3)-His$_6$-Myc and SecY-(P5)–PhoA in the absence of a tight folding of the PhoA domain. It is established that DsbA-dependent disulfide bond formation is essential for PhoA to fold into its native structure (Bardwell *et al.*, 1991; Derman and Beckwith, 1991; Kamitani *et al.*, 1992; Akiyama and Ito, 1993; Sone *et al.*, 1997a). Examination of the steady-state accumulation of YccA-(P3)–PhoA-His$_6$-Myc showed that the ratio of the I-60 species to the full-length product decreased markedly in the *dsbA*-disrupted mutant cells, in the presence of either normal (Figure 5A, compare lane 4 with lane 1) or increased (Figure 5A, compare lane 5 with lane 2) activity of FtsH. The decrease in I-60 was particularly striking under the latter conditions.

In the above immunoblotting experiments, broth medium was used for growing the cells, in which some low molecular weight disulfide compounds such as cystine may have supported a low level formation of disulfide bonds (Sone *et al.*, 1997b). We therefore used minimal salt medium for a detailed comparison of degradation patterns in the *dsbA*$^+$ and the *dsbA*$^-$ strains. Pulse–chase experiments showed that YccA-(P3)–PhoA-His$_6$-Myc in the *dsbA*-disrupted cells was degraded in an FtsH-dependent manner, but only very little I-60 was produced in the presence of either normal (Figure 5B, lanes 13–16; Figure 5C, open triangles) or overproduced (Figure 5B, lanes 17–20; Figure 5C, open diamonds) levels of FtsH. Whereas degradation of the full-length YccA-(P3)–PhoA-His$_6$-Myc was somewhat faster in the *dsbA*$^-$ cells than in the *dsbA*$^+$ cells (Figure 5C, compare solid circles and solid triangles), it was completely stabilized by the loss of FtsH function, even in the *dsbA*$^-$ cells (Figure 5B, lanes 21–24).

We also examined the degradation of SecY-(P5)–PhoA in the *dsbA*$^-$ genetic background (Figure 7, lanes 13–24).

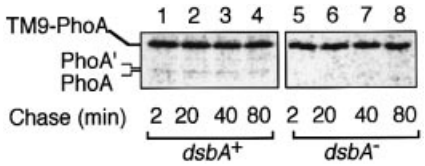

Fig. 8. Stability of independently expressed TM9–PhoA segment. KS474 (*degP*::Tn*5* *dsbA*$^+$ Δ*phoA*; lanes 1–4) and AK2055 (*degP*::Tn*5* *dsbA*::*tet* Δ*phoA*; lanes 5–8), each carrying pKH472 (p*lac-TM9-phoA*), were grown at 30°C and induced for *lac* transcription with 3 mM cAMP for 10 min. Cells were then pulse-labeled with [^{35}S]methionine for 1 min and chased with unlabeled methionine for 2, 20, 40 and 80 min, as indicated. Radioactive proteins were immunoprecipitated with anti-PhoA, separated by SDS–PAGE and visualized.

Unlike the case in *dsbA*$^+$ cells (Figure 7, lanes 1–8), the protein synthesized in the *dsbA*$^-$ mutant cells generated only insignificant levels of the PhoA′ fragment during degradation in the presence of either normal (Figure 7A, lanes 13–16; Figure 7C, open triangles) or overproduced (Figure 7A, lanes 17–20; Figure 7C, open diamonds) levels of FtsH.

The results presented above show that the modes of degradation of both YccA-(P3)–PhoA-His$_6$-Myc and SecY-(P5)–PhoA are affected strikingly by the folding states of the PhoA domain attached to the periplasmic region. Whereas efficient PhoA folding resulted in the stop of degradation at a site N-terminal to PhoA, inefficient folding leads to the protein being degraded past this point and into the C-terminal domains.

A TM9–PhoA fragment is stable when produced as an independent polypeptide separately from the N-terminal region of SecY

The PhoA′ fragment was only slightly larger in size than the authentic PhoA mature domain (data not shown). We constructed a plasmid that expressed the C5 (C-terminal 11 residues)–TM9–PhoA region of the SecY-(P5)–PhoA fusion protein. The migration of this product in SDS–PAGE was clearly slower than that of PhoA′. Whereas the intact SecY-(P5)–PhoA protein and the TM9–PhoA protein were fractionated as spheroplast-bound, a fraction of PhoA′, which was produced from SecY-(P5)–PhoA, was released by EDTA–lysozyme treatment (data not shown). These results suggest that PhoA′ does not retain the entire TM9 sequence and can be released into the periplasmic space.

If one is to explain the degradation of the entire fusion protein by means other than an FtsH-dependent dislocation process, it would be necessary to assume that, after degradation of the cytosolic portion by FtsH, some other protease takes over the job of rapidly degrading the rest of the protein. In this case, the TM9–PhoA construction is expected to be a substrate of the latter hypothetical proteolytic system. We examined the stability of the independently expressed TM9–PhoA fusion protein in either *dsbA*$^+$ or *dsbA*$^-$ background. It was confirmed by protease digestion experiments that the PhoA region in TM9–PhoA is translocated to the periplasmic side as rapidly as that in the intact SecY-(P5)–PhoA fusion protein (data not shown). Pulse–chase experiments in the *dsbA*$^+$ cells showed that TM9–PhoA was not converted to a PhoA′-sized product (Figure 8, lanes 1–4). TM9-PhoA was completely stable even when it was expressed in the *dsbA*-disrupted cells (Figure 8, lanes 5–8). The TM9–PhoA segment, which is supposed to mimic a hypothetical intermediate in which the N-terminal cytosolic regions of SecY have been degraded, is not actually degraded when it is produced separately from the N-terminal SecY regions. It should be noted that periplasmic PhoA protein is also stable in *degP*-disrupted strains even without the disulfide bond-dependent tight folding (Sone *et al.*, 1997a; our unpublished results). Rapid degradation of unfolded PhoA only occurs when it is attached to a substrate of FtsH. In the case of SecY-(P5)–PhoA, the processive action of FtsH, beginning at degradation initiation site(s) located elsewhere on SecY, is required for the rapid degradation of the TM9 and the PhoA segments.

Discussion

Certain forms of integral membrane proteins can be deleterious to the integrity of the membrane, and should be eliminated rapidly. FtsH is the protease responsible for the degradation of unassembled forms of the SecY subunit of protein translocase. While SecY's normal function is to facilitate protein translocation by forming, together with SecE and SecG, a translocation 'channel' in the membrane, its failure to associate with the SecE subunit may result in the formation of some incomplete or unregulated channel (Kihara *et al.*, 1995). Similar membrane-disrupting functions can also be imagined for another substrate of FtsH, the subunit *a* of the F_0 sector of the proton ATPase, which normally mediates proton transport across the membrane (von Meyenburg *et al.*, 1985). Since transmembrane regions of these proteins will be crucial for their toxicity, elimination of the membrane-embedded segments must be of particular importance.

Little is known, however, about how the transmembrane regions of a membrane protein can be hydrolyzed. Can it occur within the lipid environment or after extraction out of the membrane? Probably the best-studied fate of membrane-spanning segments is that of signal peptides of pre-secretory proteins. After cleavage by the signal peptidase, signal peptides of bacterial cells are degraded further by signal peptide peptidase (Ichihara *et al.*, 1984), although the molecular mechanisms of this proteolysis are unclear. In the endoplasmic reticulum of eukaryotic cells, the cleaved signal peptide undergoes an additional cleavage followed by release of the N-terminal segment to the cytosol (Martoglio and Dobberstein, 1998).

The FtsH-mediated elimination of the SecY subunit and the *a* subunit of F_0 is very rapid such that the half-lives of the intact molecules are only a few minutes. Since the ATPase and the protease active sites of FtsH reside in its large cytoplasmic domain, questions remain as to how rapidly the transmembrane and the periplasmic domains of its membrane-integrated substrates are degraded.

Several possibilities can be considered for the modes of the FtsH-mediated degradation of membrane proteins. For instance, FtsH hydrolyzes only a particular cytoplasmic domain(s), at which it initiates proteolysis. If this is the case for degradation of YccA, only the C1 domain can be degraded directly by FtsH. Alternatively, once FtsH has initiated proteolysis at the primary initiation site (within C1 in the case of YccA), it may somehow be able

to continue proteolysis of successive cytoplasmic domains. This is conceivable if secondary initiation sites have been sequestered by interactions among cytoplasmic domains until one of them is degraded. As already discussed, the possibility that transmembrane and periplasmic regions remain undigested does not make much sense in terms of the quality control functions of the degradation system. We therefore addressed whether the extracytoplasmic regions actually get degraded. Assuming that they do, the next question is how this degradation is possible. One formal possibility is that, after cleavage of the cytoplasmic domains, some other proteases on the periplasmic side or within the membrane are activated, although it seems difficult to imagine a simple mechanism for this coordination. Another possibility is that FtsH itself is able to catalyze proteolysis of not only the cytoplasmic but also the extracytoplasmic domains. In order to accomplish this task, FtsH should gain access to the transmembrane and periplasmic domains of the substrate protein. Logical solutions for this problem would be that either FtsH or the substrate moves across the membrane.

We used the YccA-(P3)–PhoA-His_6-Myc and the SecY-(P5)–PhoA fusion proteins to monitor the degradation of the periplasmic domains of these proteins. The PhoA sequence attached was expected to enable detection of possible degradation intermediates or initial cut products of these proteins. We previously studied degradation of HflK, a membrane protein mostly exposed to the periplasm (Kihara and Ito, 1998). When this protein was produced in the absence of the partner subunit (HflC), it was degraded with concomitant production of multiple intermediate fragments. In this degradation, at least two proteases, DegP and Prc, appeared to act successively. The subunit state of HflK was highly sensitive to trypsin, indicating that it is not folded as tightly as the normal PhoA protein. Thus, even polypeptides without tight folding can survive, at least transiently, in the periplasm of *E.coli* cells. It might be generalized that protein degradation in this compartment is of low processivity. In accordance with this notion, no energy-dependent protease is known in the periplasmic space.

When PhoA was produced in the *degP*-defective strain, it was not degraded measurably even if it was devoid of a tight folding due to the lack of disulfide bonds. In sharp contrast, when PhoA was attached to a periplasmic domain of YccA or SecY, it was degraded rapidly in *dsbA*$^-$ cells. This degradation was completely dependent on the function of FtsH, since the protein was stabilized in the absence of FtsH activity. The rate of disappearance of the intact fusion molecules correlated with the cellular abundance of FtsH. The degradation of the fusion proteins, whether accelerated by overproduced FtsH or not, was not accompanied by any detectable production of fragments that contained the PhoA sequence or the C-terminal Myc sequence. Thus, once degradation of the fusion protein is initiated by FtsH, it rapidly goes to completion, and the rate of degradation of the PhoA region is determined by the activity of FtsH. Although it has not been ruled out that the degradation of the PhoA region may be catalyzed by some periplasmic protease immediately after the FtsH-catalyzed degradation of the cytoplasmic domains, such degradation, if any, should be extremely rapid, such that the FtsH action is the rate-limiting step in overall degradation. Our findings that the TM9–PhoA protein is stable even without tight folding of the PhoA domain argue against the possibility that hydrolysis of the cytoplasmic domains induces a structural change that serves as a signal for the periplasmic protease to begin its function. It is highly likely that FtsH itself catalyzes proteolysis of both the PhoA moiety and the TM9 segment.

We controlled the folding states of the PhoA domains by expressing the fusion proteins in the presence or absence of DsbA. In contrast to the fusion proteins expressed in the *dsbA*-defective cells, those expressed in the presence of DsbA produced PhoA-containing fragments. In other words, the FtsH-initiated degradation stopped at a site N-terminal to a folded PhoA. It is noteworthy that the cytoplasmic C4 domain in YccA-(P3)–PhoA-His_6-Myc was stabilized when PhoA in the preceding P3 domain was folded. Such a transmembrane effect is not easily explained in terms of successive actions of different proteases. Successive degradation of cytoplasmic domains by FtsH is also unlikely. These results, taken together, support the notion that the PhoA domains in the fusion proteins are degraded by FtsH itself.

The kinetics and efficiency of folding of the PhoA domain were different between the two proteins examined; since YccA-(P3)–PhoA-His_6-Myc contained PhoA that was tethered to membrane-spanning segments at both ends, the PhoA domain of this fusion protein is folded more slowly and inefficiently than the same domain in SecY-(P5)–PhoA. Degradation stop occurred more efficiently for the latter than for YccA-(P3)–PhoA-His_6-Myc. Provided that the PhoA domain is proteolyzed by FtsH, the above results are fully consistent with a model in which the PhoA domain moves to the cytoplasmic side in order to get degraded. The folded state of PhoA is incompatible with this movement. The size of the PhoA-containing fragment produced *in vivo* from SecY-(P5)–PhoA was close to that of the normal PhoA, which migrated significantly faster than the TM9–PhoA protein upon electrophoresis. Thus, the *in vivo* proteolysis of the fusion protein, presumably FtsH-catalyzed, stops around the SecY–PhoA junction. Since the short segment at the N-terminus of PhoA is not folded into the protease-resistant conformation (Figure 3B; Roberts and Chlebowski, 1984), it is conceivable that the dislocation event occurred up to this segment, which transiently assumed a transmembrane configuration and was then released to the periplasm after degradation of the N-terminal cytosolic sequences. This is consistent with the observed localization of the product as well as with the concept that the folded domain prevents the dislocation process.

We have shown that degradation of the YccA protein is initiated at the N-terminal cytoplasmic tail of this protein. The behaviors of the YccA11 and the N8-YccA11 derivatives raised the tempting possibility that the N-terminal segment should have a certain length in order to be the initiation site for the FtsH-dependent proteolysis. This length may be required for the N-terminal tail to be captured by the cytoplasmic domain of FtsH for initiation of dislocation and proteolysis. It can be speculated that the initial recognition for proteolysis is by the ATPase domain of FtsH, which will then translocate the substrate to the protease domain.

Our results do not necessarily indicate that FtsH initi-

ation sites should generally be located at the N-termini. Recent re-examinations of the topology of subunit *a* (F_0 ATPase) indicate that the N-terminus of this FtsH substrate faces the periplasm (Long *et al.*, 1998; Valiyaveetil and Fillingame, 1998). Herman *et al*. (1998) reported that attachment of the SsrA tag sequence to the C-terminus of λ CI protein rendered this protein sensitive to FtsH. Degradation of σ^{32} by FtsH also depends on the C-terminal region (Blaszczak *et al.*, 1999). However, C-terminal ends cannot be the FtsH initiation site at least for the YccA protein and the SecY-(P5)–PhoA protein, both having periplasmically oriented C-termini. Our studies suggest that soluble and membrane-bound substrates differ in the way in which they are presented to FtsH (Kihara *et al.*, 1998). Although we have not identified the degradation initiation site(s) in SecY, the observation that the product of an internal translation initiation or a co-translational cleavage of SecY is a substrate of FtsH may mean that the newly created N-terminal region can be recognized by FtsH for degradation. In view of the observation that FtsH possesses an activity to bind to some unfolded proteins (Akiyama *et al.*, 1998b), a possibility remains that some unstructured region, not necessarily at the N-terminus, can be recognized by FtsH.

Although the cytosolic recognition of the substrate will be important for the initiation of degradation, FtsH may also recognize its substrates by interaction between membrane-embedded regions. This is supported by the fact that even the proteolysis-resistant YccA11 protein was associated with FtsH (Kihara *et al.*, 1998). In the ubiquitination- and proteasome-dependent degradation of abnormal endoplasmic reticulum proteins, the Sec61 translocation channel seems to be involved, possibly in the retrograde translocation process (Wiertz *et al.*, 1996; Pilon *et al.*, 1997; Plemper *et al.*, 1998). We have no evidence that the SecYEG translocation channel is involved in the proposed dislocation process. We suppose it is possible that FtsH itself serves as a channel for dislocation. The YccA11 protein may stay inside the putative FtsH channel since it cannot interact with the cytosolic ATPase portion of FtsH, thereby interfering with the entry of other membrane-integrated substrates into the channel. FtsH is known to be multimeric (Akiyama *et al.*, 1995). Moreover, Shotland *et al*. (1997) observed a ring-like structure of FtsH *in vitro*. Such structure might indeed suggest FtsH itself as a dislocation channel. Since the HflKC complex is associated with FtsH (Kihara *et al.*, 1996), there is a possibility that this pair of single-spanning membrane proteins participate in the formation of a channel. HflKC protein can also interact with FtsH substrates. At least this is true for the YccA protein (Kihara *et al.*, 1998). However, HflKC is not essential for the FtsH-catalyzed proteolysis and it is rather inhibitory to degradation (Kihara *et al.*, 1996). Thus, it is possible that HflKC binding to the substrate negatively controls the dislocation process.

A basic question regarding polypeptide translocation channels is how many polypeptide chains they can accommodate. For the dislocation process of polytopic membrane proteins, at least a pair of polypeptide segments should be accommodated such that a connecting periplasmic loop can be dislocated through a single channel. A number of additional questions remain about the dislocation mechanism. Does ATP hydrolysis catalyzed by FtsH solely provide the energy for the movement of the substrate polypeptide? Is the dislocation process coupled with peptide bond hydrolysis? Undoubtedly, these questions await *in vitro* studies using reconstituted components.

Materials and methods

Escherichia coli strains

Escherichia coli K-12 strains AD202 (*ompT*::*kan*; Akiyama and Ito, 1990), AR796 (*zhd-33*::Tn*10 zhj-3198*::Tn*10kan*; Kihara *et al.*, 1995), AR797 (*zhd-33*::Tn*10 zhj-3198*::Tn*10kan ftsH1*; Kihara *et al.*, 1995) and KS474 (Δ*phoA degP41*::Tn*5*; Strauch and Beckwith, 1988) were derivatives of MC4100 [*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*; Casadaban, 1976]. AK1924 was a derivative of KS474 carrying F′ *lac*$^+$ *lacI*^{Q}. AD16 (Δ*pro-lac thi* /F′ *lacI*^{q} *Z*^{M15} *Y*$^+$ *pro*$^+$) has been described previously (Kihara *et al.*, 1995). AK2055 (KS474, *dsbA*::*tet*) was constructed as follows. A plasmid carrying *dsbA* (named pKH400) was constructed by subcloning a 6.5 kb *Sph*I fragment of pSK140 (Kamitani *et al.*, 1992) into the *Sph*I site of pMW118, a pSC101-based *lac* promoter vector (obtained from Nippon Gene). A 1.5 kb blunt-ended *Xba*I–*Ava*I fragment of pACYC184 was inserted into the blunt-ended *Bgl*II site of pKH400, yielding pKH410, whose *Sac*II–*Xcm*I fragment (6.8 kb) was used to transform strain FS1576 (*recD*$^-$; Stahl *et al.*, 1986) to tetracycline (6 μg/ml) resistance. Finally, the *dsbA*::*tet* mutation in one of the transformants was introduced by P1 transduction into KS474.

Plasmids

pKH330 and pKH331 carried *yccA-his*$_6$*-myc* and *yccA11-his*$_6$*-myc* respectively, under *lac* promoter control (Kihara *et al.*, 1998). pKH198 (Kihara *et al.*, 1996) and pSTD401 (Akiyama *et al.*, 1995) carried *ftsH*$^+$ under *lac* promoter control. pSTD41 (Akiyama *et al.*, 1994b) and pKH413 carried *ftsH41*, a dominant-negative allele of *ftsH (*Akiyama *et al.*, 1994b), under the *lac* promoter. The latter plasmid was constructed by cloning a 2.5 kb *Eco*RI–*Hin*dIII fragment of pSTD41 into pMW118. pKY4::*pho*A12-1 carried *secY-(P5)-phoA* under the *lac* promoter (Akiyama and Ito, 1987). pMS102, carrying *phoA*$^+$ under the *lac* promoter, was constructed by Y.Takahashi (see Sone *et al.*, 1998). pHSG575 was a *lac* promoter vector derived from pSC101 (Takeshita *et al.*, 1987).

Plasmids encoding YccA–PhoA-His$_6$-Myc sandwich fusion proteins were constructed as follows. pKH371, in which a 0.8 kb *Kpn*I–*Hin*dIII *yccA-his*$_6$*-myc* fragment of pKH330 had been cloned into M13mp18, was subjected to site-directed mutagenesis (Kunkel *et al.*, 1987) such that the *Spe*I recognition sequence (ACTAGT) was inserted at the G563–C564 (for the P1 domain of YccA), C736–G737 (for P2), G896–C897 (for P3) and T986–G987 (for C4) boundaries of the *yccA* gene (numbering according to Tamura *et al.*, 1984). A 0.8 kb *Kpn*I–*Hin*dIII fragment was prepared from each of the constructions of confirmed sequence and cloned into pTWV229 (a pBR322-based *lac* promoter vector from Takara Shuzo). Finally, a *phoA* fragment was amplified from pMM1 (Ehrmann *et al.*, 1997) using primers 5′-CTGACTCTTATACACACTAGTGAAAACCTGTACTTC-3′ and 5′-CACGAGGCAGAACTAGTCGCCCCCCCCCC-3′ (the *Spe*I sequence underlined), digested with *Spe*I and inserted into each of the pTWV229 *yccA-his*$_6$*-myc* derivatives with the inserted *Spe*I sequence. Plasmids thus constructed were confirmed for their sequences, and named pKH383 [p*lac-yccA-(P1)-phoA-his*$_6$*-myc*], pKH384 [p*lac-yccA-(P2)-phoA-his*$_6$*-myc*], pKH385 [p*lac-yccA-(P3)-phoA-his*$_6$*-myc*] and pKH388 [p*lac-yccA-(C4)-phoA-his*$_6$*-myc*]. A derivative of pSTV29 (a pACYC184-based *lac* promoter vector from Takara Shuzo) carrying the *yccA-(P3)-phoA-his*$_6$*-myc* insert from pKH385 was named pKH412.

Plasmid pKH356 encoding N8-YccA11-His$_6$-Myc protein was constructed as follows. A *yccA11-his*$_6$*-myc* fragment was amplified from pKH331 using primers 5′-TGCGGAAGCTTGCATGATCGTATTTCACTGCTAGC-3′ (*Hin*dIII recognition sequence underlined) and 5′-GTGAATTCGAGCTCGGTACC-3′ (*Kpn*I recognition sequence underlined), digested with these enzymes and cloned into pTWV229 (Takara Shuzo). Thus, the N-terminal nine residues (Met-Thr-Met-Ile-Thr-Pro-Ser-Leu-His) encoded by the *lacZ*α multi-cloning region of this vector substituted for the initiation codon of YccA11-His$_6$-Myc.

Plasmid pKH472 encoding TM9–PhoA was constructed as follows. A fragment encoding the C-terminal 502 residues of the SecY-(P5)–PhoA protein was amplified from plasmid pKY4::*pho*A12-1 using primers 5′-GGGGTACCAGGAGGAAGAGCAAATGGCGAAGTATATCC-3′

(*Kpn*I recognition sequence underlined) and 5′-AAAAAAACCAGAC-CGAAAAGCTTGGTAACC-3′ (*Hin*dIII recognition sequence underlined), digested with these enzymes and cloned into pMW118.

Media

Media used were L (Davis *et al.*, 1980) and M9 (Silhavy *et al.*, 1984), with ampicillin (50 μg/ml) and/or chloramphenicol (20 μg/ml) included for growing plasmid-bearing cells. For induction of *lac* promoter-controlled genes, cells were first grown in the presence of glucose (0.1% for L and 0.4% for M9) to an exponential phase, then 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 1 mM cAMP were added. For Δ*lacI* strains, only cAMP was added.

Pulse–chase and immunoprecipitation

Degradation of the YccA and SecY derivatives was followed by pulse–chase and immunoprecipitation experiments. For pulse labeling *dsbA*$^+$ strains, cells were grown at 37°C with shaking to an exponential phase in M9 medium supplemented with amino acids (20 μg/ml, other than methionine and cysteine), thiamine (2 μg/ml), 0.4% glucose and appropriate antibiotics. The *dsbA*$^-$ *degP*$^-$ double mutant strains carrying one of the *phoA* fusion plasmids required some low molecular weight disulfide molecules for growth. Therefore, the latter strains were grown first in the presence of 10 mM glutathione to an early exponential phase, washed twice with the same medium without glutathione by repeated centrifugation/resuspension, followed by additional shaking at 37°C for 30 min. Procedures for pulse labeling with [^{35}S]methionine and chasing with unlabeled methionine were described by Taura *et al.* (1993). Whole-cell proteins were precipitated by direct treatment of a portion of culture with trichloroacetic acid, washed with acetone, solubilized in SDS and subjected to immunoprecipitation as described by the same authors, except that dilution of SDS-solubilized sample was with 50 mM Tris–HCl (pH 8.1) containing 0.1 mM EDTA, 0.15 M NaCl and 2% Triton X-100. Antibodies used were anti-Myc (A-14) (Santa Cruz Biotechnology, Inc.), anti-PhoA (5 Prime 3 Prime, Inc.) and anti-N-terminal peptide of SecY (Shimoike *et al.*, 1995). Anti-Myc and anti-PhoA sera had been pre-incubated with an excess of cell lysate prepared from KS474 to reduce backgrounds. Labeled and immunoprecipitated proteins were separated by SDS–PAGE, and visualized and quantitated by a BAS1800 imager (Fuji film).

Immunoblotting

Myc-tagged proteins and PhoA fusion proteins as well as their degradation fragments were detected by separating total cell proteins by SDS–PAGE, followed by their blotting onto a polyvinylidene difluoride membrane filter (Millipore) and immunological detection (Shimoike *et al.*, 1995) using the antibodies described above. Antiserum against GroEL was described previously (Akiyama *et al.*, 1994a). An ECL detection kit (Amersham) and a Luminescence Image Analyzer (LAS-1000, Fuji Film) were used for visualization.

Determination of alkaline phosphatase activities

Cells of AK1924 (Δ*phoA degP41*::Tn*5*) carrying either pKH383 [p*lac-yccA-(P1)-phoA-his*$_6$*-myc*], pKH384 [p*lac-yccA-(P2)-phoA-his*$_6$*-myc*], pKH385 [p*lac-yccA-(P3)-phoA-his*$_6$*-myc*] or pKH388 [p*lac-yccA-(C4)-phoA-his*$_6$*-myc*] were grown at 30°C in L-broth containing 0.1% glucose. Plasmid-encoded YccA derivatives were then induced with 1 mM IPTG and 1 mM cAMP for 3 h. Alkaline phosphatase activities were measured as described by Michaelis *et al.* (1983) and expressed in the units defined by Brickman and Beckwith (1975).

Spheroplast formation and protease treatment

Cells were resuspended in 30 mM Tris–HCl (pH 8.1)–20% sucrose, mixed with 1/10 vol. of 1 mg/ml lysozyme that had been dissolved in 0.1 M EDTA (pH 8.0) and incubated on ice for 30 min. Samples were then incubated further with trypsin or proteinase K of specified concentration at 0°C for 30–60 min. Reactions were terminated by addition of 1 mM phenylmethylsulfonyl fluoride, 3 mM N^{α}-*p*-tosyl-L-lysine chloromethylketone and 100 μg/ml soybean trypsin inhibitor. Proteins were precipitated with trichloroacetic acid for detection of specific components by immunoblotting or immunoprecipitation (Akiyama and Ito, 1989).

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