Proteolytic Activity of HtpX, a Membrane-bound and Stress-controlled Protease from Escherichia coli

Machiko Sakoh, Koreaki Ito, and Yoshinori Akiyama

From the Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan

Escherichia coli HtpX is a putative membrane-bound zinc metalloprotease that has been suggested to participate in the proteolytic quality control of membrane proteins in conjunction with FtsH, a membrane-bound and ATP-dependent protease. Here, we biochemically characterized HtpX and confirmed its proteolytic activities against membrane and soluble proteins. HtpX underwent self-degradation upon cell disruption or membrane solubilization. Consequently, we purified HtpX under denaturing conditions and then refolded it in the presence of a zinc chelator. When supplemented with Zn\(^{2+}\), the purified enzyme exhibited self-cleavage activity. In the presence of zinc, it also degraded casein and cleaved a solubilized membrane protein, SecY. We verified its ability to cleave SecY in vivo by overproducing both HtpX and SecY. These results showed that HtpX is a zinc-dependent endoprotease member of the membrane-localized proteolytic system in E. coli.

It is vital for cells that membranes and membrane proteins retain their integrity. Malfolded and misassembled membrane proteins, produced under stressful conditions and in non-physiological situations, should receive proteolytic quality control. In Escherichia coli, FtsH, a membrane-bound and ATP-dependent zinc metalloprotease, is known to play a central role in the degradation of unstable membrane proteins (1, 2). Thus, it degrades SecY, a subunit of protein translocase (3), and Fo subunit of proton ATPase (4) when they have failed to assemble. It also degrades YeeA, the function of which is unknown (5), and some unstable cytosolic proteins (1, 2). A characteristic feature of FtsH is that it processively degrades membrane-protein substrates by recognizing their ends when they protrude sufficiently into the cytosol (6, 7). This degradation appears to be accompanied by dislocation of the substrate from the membrane, a process presumably mediated by the ATPase function of FtsH (8–10).

In E. coli, only a few other membrane-integrated proteases are known: DegS and RseP (YaeL), which introduce regulated cleavage into a membrane-bound substrate (11–13), and Lep, which cleaves off a signal peptide of secretory precursor proteins (14). HtpX was first described by Kornitzer et al. (15) as a heat-inducible protein from E. coli with sequence features of a membrane protein and a metalloprotease. They showed that its expression, in a form truncated at the C-terminal, enhanced degradation of cellular proteins in puromycin-treated cells (15). Subsequently, it was also shown that the htpX gene in Xylella fastidiosa was induced by an increase in temperature (16), whereas the Streptococcus gordonii htpX was not heat-inducible (17). Disruption of S. gordonii htpX caused changes in several properties of the cell surface, although the relationship of these changes with any protease activity of HtpX was unclear (17).

We rediscovered HtpX during the course of study of cellular responses to “membrane protein stress.” Our results showed that ftsH disruption led to the induction of the Cpx stress response, which was exaggerated further by overexpression of SecY or Fo subunit a in the absence of FtsH (18). It was suggested that an unknown Cpx-controlled factor might function to alleviate the membrane stress (18). Our screening for genes up-regulated by constitutively active Cpx mutation (cpxA24) resulted in the identification of htpX as a Cpx-controlled gene (18). We determined the cellular localization and topology of HtpX and showed that it has two transmembrane segments located at the N terminus and a large cytoplasmic domain, which includes two hydrophobic regions that probably interact with the membrane (18). Although disruption of htpX alone was silent, its combination with an ftsH disruption resulted in a synthetic growth defect (18). Complementation tests using the ∆ftsH ∆htpX strain indicated that the zinc metalloprotease active site motif in HtpX is essential for its functionality in vivo (18). These findings suggested that HtpX functions as a protease, having cellular roles that complement or overlap those of FtsH.

In this study, we have described some biochemical properties of HtpX and confirmed its proteolytic activity. Purified HtpX has the unusual ability to cleave itself autocatalytically in the presence of Zn\(^{2+}\). We have also described its ability to introduce endoproteolytic cleavages into SecY, in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media—E. coli K12 strains AD16 (F lacI) (19), AD202 (ompT::kan) (20), SC1060 (AD16, gbd21, zad-220::Tn10) (18), and AD1691 (SC1060, ftsH3::kan) (18) have been described previously; MA90 (SC1060, htpX::tet) and MA91 (AD1691, htpX::tet) were constructed as follows. First, the htpX::tet marker was introduced into AD16 carrying pKN201 (Ptet-red-gam) (gift from K. Murphy) by linear transformation (21) using a DNA fragment amplified from the chromosomal DNA of AD1735 (zag-220::Tn10) (22) with a pair of primers (5’-GGCATATTGCGTTTTTGTTAACTGAGTTAAGAAAAAGAAATCTCGGACCTCATTAAAGCAGCCTC-3’ and 5’-GGCGGTCGATCA-GGGCACGCGTTTATTGATATCTCATAAAGACTGACTTG-TCTTCTTG-3’). The htpX::tet marker was then P1-transduced into SC1060 and AD1691, respectively.

L (22) and M9 (23) were used as complete nutrient and minimal salt media, respectively. Ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), and kanamycin (50 μg/ml) were used to select for their respective resistances.

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Plasmids—pSTD343 was a derivative of pSTV29 (Takara) carrying the lacZ-gene. pSTD576, a derivative of pSTD568 (18), carried the htpX(H139F)-his6-myc gene, and was constructed by site-directed mutagenesis (24). pSTD588 (htpx(H139F)-his6-myc) was constructed by cloning a XbaI-BamHI fragment of pSTD576 between the same sites of pTYE007 (25). pSTD650 carried the htpx-his6-myc gene that was directly fused to an upstream lacZ SD sequence and constructed by site-directed mutagenesis of pSTD566 (18) using a primer (′-ATTTCCACACCGAAAACAGCTATGATGCGGAATCCGCTCTT-3′). pCH346, a derivative of pBAD33 (26), carried the secY gene under the control of ara promoter and was constructed by S. Chiba. Plasmids encoding truncated variants of SecY were constructed by PCR amplification of the corresponding DNA regions using appropriate primers and template plasmids and cloning into pBAD33, pTWV228, and pUC118. The N-terminally truncated SecY variants had an extra initiation methionine residue at their N terminus.

Cell Disruption Conditions—Cells were grown in L medium containing 1 mM IPTG and 1 mM cAMP at 37 °C for 2 h, washed with 10 mM Tris-HCl, pH 8.1, collected by centrifugation, and suspended in 10 mM Tris-HCl, pH 8.1, 10 mM 2-mercaptoethanol. When specified, samples were further supplemented with 5% protease inhibitor cocktail (Sigma), 1 mM phenylmethylsulfonyl fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM tosyl-L-lysine chloromethyl ketone, and 100 μg/ml soybean trypsin inhibitor. For sonication disruption, cells were treated with three cycles of brief (20 s) sonication with cooling in an ice-water bath. For mild cell lysis, AD16-derived cells were grown as above, washed with 10 mM Tris-HCl, pH 8.1, and suspended in 30 mM Tris-HCl, pH 8.1, 20% sucrose. Samples were mixed with 1/10 volume of 1 mg/ml lysozyme dissolved in 0.1 M EDTA (pH 7.0) and incubated at 0 °C for 30 min. Membranes were then solubilized by the addition of Nonidet P-40 to a final concentration of 1%, subsequent repeated pipettings, and incubation at 0 °C for 1 h.

Purification of HtpX-His6-Myc—Cells of AD16/pSTD650 and AD16/pSTD588 were grown in 8 liters of L medium containing 1 mM IPTG and 1 mM cAMP at 37 °C for 2 h, collected by centrifugation, washed three times with 10 mM Tris-HCl, pH 8.1, and suspended in 30 mM Tris-HCl, pH 8.1, 20% sucrose. Cells were treated with lysozyme as described above. Membranous materials were then collected by centrifugation, suspended in 6 M guanidine hydrochloride, and incubated at 0 °C for 1 h and then collected again by ultracentrifugation. The "denatured" pellets were suspended in buffer A (50 mM Tris-HCl, pH 8.1, 100 mM NaCl, 6 M urea, 10 mM 2-mercaptoethanol) and incubated with 1% Nonidet P-40 at 0 °C for 1 h. Samples were clarified by ultracentrifugation, and supernatants were mixed with Ni2+-nitrilotriacetic acid agarose pre-equilibrated with buffer B (buffer A + 1% Nonidet P-40) and stirred gently at 4 °C for 1 h. Then, the suspensions were poured into a plastic column, which was washed successively with 12.5 column volumes of buffer C1 (buffer B + 10% glycerol) and buffer C2 (buffer C1 + 10 mM imidazole) and eluted with 10 column volumes of buffer D (buffer C1 + 200 mM imidazole). The eluates were dialyzed against buffer E (20 mM Tris-HCl, pH 8.1, 1 M urea, 1 mM mercaptoethanol, 1% Nonidet P-40, 5% glycerol) and applied to a HiTrap Q HP column, which was developed with 20 column volumes of 0–300 mM NaCl linear gradient in buffer E. Peak fractions of HtpX, as detected by SDS-PAGE and Coomassie Brilliant Blue staining, were combined, concentrated 5-fold by centrifugation in UltraFree-0.5 (Millipore), and subjected to Superose 6 gel filtration with elution with buffer F (20 mM Tris-HCl, pH 8.1, 200 mM NaCl, 3 M urea, 10 mM mercaptoethanol, 1% Nonidet P-40, 5% glycerol). Finally, HtpX peak fractions were pooled and dialyzed extensively against buffer G (50 mM Tris-HCl, pH 8.1, 100 mM NaCl, 1 mM mercaptoethanol, 1% Nonidet P-40, 5% glycerol, 1,10-phenanthroline).

In Vitro Assays for HtpX-His6-Myc Protease Activity—Purified HtpX and a substrate protein were incubated at 37 °C in reaction buffer (50 mM Tris-HCl, pH 8.1, 100 mM NaCl, 1% Nonidet P-40, 5% glycerol) in the presence of either 5 mM zinc acetate or 10 mM 1,10-phenanthroline. Samples were withdrawn at intervals, mixed with a quarter volume of SDS sample buffer, incubated at 37 °C for 5 min, and subjected to SDS-PAGE. Preparations of purified SecY and YccA were gifts from N. Shimokawa and S. Chiba, respectively.

Pulse-Chase, Immunoprecipitation, and Immunoblotting Experiments—Cells were grown in M9 medium supplemented with 18 amino acids (20 μg/ml) other than Met and Cys, 2 μg/ml thiamine, 0.4% glucose, and 1 mM IPTG, and pulse-labeled with [35S]methionine for 1 min followed by chase with 200 μg/ml unlabeled methionine for the indicated periods. Immunoprecipitation and immunoblotting were carried out essentially as described previously (27). Rabbit anti-HtpX serum was prepared using a synthetic peptide (NH2-SVGGEVIEQPRNERERW) corresponding to amino acids 61–77 of HtpX, after conjugation with keyhole limpet hemocyanin. Rabbit anti-His (H15) antibodies were purchased from Santa Cruz Biotechnology, Inc.

RESULTS

Cell Disruption and Membrane Solubilization Result in Self-degradation of HtpX—To characterize HtpX, we attempted to overproduce and purify it. An HtpX derivative carrying a His6-Myc bipartite tag attached

\[ \text{HtpX-Protease} \]

\[ \mu g/ml, \text{and/or tetracycline (25 } \mu g/ml) \] were added for selecting transformants and transductants as well as for growing plasmid-bearing strains.
to the C terminus (HtpX-His₆-Myc) was expressed from a high copy number plasmid. Its cellular accumulation was examined by directly precipitating the whole-cell proteins with trichloroacetic acid. Immunoblotting analysis using anti-HtpX (Fig. 1) and anti-Myc (data not shown) antibodies showed that a protein of the expected size, about 35 kDa, accumulated after induction (Fig. 1A, lane 1). However, we consistently observed a marked reduction in the amount of HtpX present when cells were disrupted by sonication (Fig. 1, lane 2). When protease inhibitors were included in the sonication buffer, an additional, smaller fragment was detected (Fig. 1, lane 3, F1).

For more gentle cell disruption, we used strain AD16, which is known to undergo lysis on simple treatment with lysozyme. HtpX-His₆-Myc was expressed in this strain and examined for its accumulation before and after lysozyme treatment (Fig. 1B). Although lysozyme treatment of the HtpX-His₆-Myc-expressing cells released substantial amounts of cytoplasmic proteins, indicative of lysis (data not shown), HtpX-His₆-Myc remained intact without appreciable decrease in its accumulation level (Fig. 1B, lanes 1 and 2). We then proceeded to the solubilization of HtpX, a step required for purification. When the crude membranes obtained above were treated with Nonidet P-40, a non-ionic detergent, HtpX was almost totally converted to the smaller fragment that remained stable even in the absence of protease inhibitors (Fig. 1B, lane 3). These observations suggested that HtpX-His₆-Myc is degraded by cellular proteases after sonication or detergent solubilization of the membranes. This proteolysis also occurred in protease-deficient strains such as ΔftsH and ΔompT (data not shown).

We next expressed a derivative of HtpX-His₆-Myc with a mutation (H139F) in its metalloprotease active site motif (18). It was detected in its full-length form even after sonication (Fig. 1A, lanes 4–6) or Nonidet P-40 solubilization of membranes (Fig. 1B, lanes 4–6). Also, other HtpX mutants with abolished complementation activity (18) were found to remain stable after membrane solubilization (data not shown). This stability of the inactive variants suggested that the degradation is self-catalyzed. HtpX seems to undergo self-cleavage upon membrane disruption by sonication or detergents.

**Purification of HtpX-His₆-Myc under Denaturing Conditions—**We searched for conditions that allowed solubilization of HtpX without self-cleavage. Inclusion of metal chelators during solubilization did not prevent the cleavage (data not shown), suggesting that the zinc ion in HtpX was inaccessible under such conditions. Thus, it was essential to use protein denaturants to inactivate the enzyme. Lysozyme-treated cells were treated with 8 M urea before solubilization of the membranes with 1% Nonidet P-40, and HtpX was solubilized as the full-length form, which was expected to have been denatured by the urea (Fig. 1C). The use of 6 M guanidine hydrochloride instead of 8 M urea resulted in essentially the same result (data not shown). To purify HtpX in sufficient quantity for biochemical studies, cells of AD16 overexpressing the wild-type HtpX-His₆-Myc protein or its active site variant HtpX(H139F)-His₆-Myc were cultured on a large scale (8 liters), lysozyme-treated, and suspended in 6 M guanidine hydrochloride. Membranes were solubilized with 1% Nonidet P-40, and HtpX was purified by chromatography on a Ni²⁺-nitrilotriacetic acid affinity column, an anion exchange column, and a gel filtration column, successively.

The buffers used in the chromatographic procedures all contained 3 or 6 M urea to keep the proteins denatured and thereby to prevent self-degradation of HtpX, although a small amount of the self-degradation products was nevertheless generated during purification processes (see below). Finally, the urea was removed by extensive dialysis against buffer containing the metal-chelator 1,10-phenanthroline, allowing the proteins to refold. The addition of the chelator was essential to prevent
HtpX Protease

FIGURE 4. In vitro proteolysis of SecY by HtpX-His$_6$-Myc. A, requirements for SecY degradation. Purified SecY ($1 \text{ pmol} / \mu \text{l}$) was incubated with purified samples of HtpX-His$_6$-Myc ($1.55 \text{ pmol} / \mu \text{l}$; lanes 1–3, 6, and 7) at $37^\circ \text{C}$ in the presence of 5 mM zinc acetate (Zn$_2^+$, lanes 1, 3, 6, and 7) or 10 mM 1,10-phenanthroline (PT, lanes 4 and 5). Samples were withdrawn at the indicated time points and analyzed by 12.5% Tricine SDS-PAGE and immunoblotting using antiserum against the N-terminal region (lane 2) of SecY, as well as the mixture of these antisera (lane 1). Molecular size marker positions (in kDa) are shown on the left. Filled and open circles indicate fragments retaining the N terminus (n(I), n(II), and n(III)) and those retaining the C terminus (c(I) and c(II)) of SecY, respectively. WT, wild type.

![Image 316x627 to 566x744]

FIGURE 5. In vivo endoproteolytic degradation of SecY by HtpX-His$_6$-Myc. SecY degradation by co-expressed HtpX-His$_6$-Myc. Cells of MA91 ($\Delta$ftsH $\Delta$htpX/$lacZ$)/pCH346 (SecY under ara control)/pYER007 (empty vector) and MA91/pCH346/pSTD650 (HtpX-His$_6$-Myc under lac control) were grown in M9 medium supplemented with 20 µg/ml amino acids, 2 µg/ml thiamine, 0.4% glucose, and 1 µm IPTG at $30^\circ \text{C}$ for 9 h. Cells were then induced with 0.4% arabinose for 10 min and pulse-labeled with [${}^{35}$S]methionine for 1 min followed by chase with unlabeled methionine for the indicated periods. Samples were processed for immunoprecipitation using anti-SecY N + C antiserum (lanes 1–10). The sample used for lane 10 was also subjected to immunoprecipitation using antisera against the N-terminal region (lane 11) and the C-terminal region (lane 12) of SecY. Filled and open circles indicate fragments retaining the N terminus (n(I), n(II), and n(III)) and the C terminus (c(I), c(II), c(III)) and (cIV) of SecY, respectively. Molecular size marker positions (in kDa) are shown on the left. Ab, antibody; WT, wild type.

self-degradation of HtpX during dialysis (data not shown). Analysis of the purified proteins by 12.5% Tricine SDS-PAGE (Fig. 2) showed that the wild-type preparation contained two degradation products (F1 and F2) with estimated molecular masses of about 21.6 and 6.6 kDa (Fig. 2, lane 1), but the mutant preparation was free of these fragments (Fig. 2, lane 9).

HtpX Has Self-cleavage Activity—Purified and renatured preparations of the wild-type and mutant forms of HtpX were incubated at $37^\circ \text{C}$ in the presence of either 5 mM zinc acetate or 10 mM 1,10-phenanthroline (Fig. 2). In the presence of Zn$_2^+$ (Fig. 2, lanes 3–8), levels of the full-length HtpX-His$_6$-Myc decreased over time with concomitant increase in the degradation products F1 and F2. In contrast, the levels of intact protein and the already present degradation products remained almost constant in the presence of 1,10-phenanthroline even after 8 h of incubation at $37^\circ \text{C}$ (Fig. 2, lanes 1 and 2). The mutant enzyme HtpX(H139F)-His$_6$-Myc did not exhibit any degradation/cleavage even in the presence of Zn$_2^+$ (Fig. 2, lanes 9 and 10). Similar results were obtained at $42^\circ \text{C}$ (data not shown). These results showed that HtpX has zinc-dependent self-cleavage activity.

HtpX Deprecates Casein—We next examined whether HtpX can proteolyze a commonly used proteolytic substrate, $\beta$-casein (Fig. 3). When $\beta$-casein was incubated with HtpX in the presence of Zn$_2^+$ for 8 h at $37^\circ \text{C}$, its concentration decreased by more than 70% (Fig. 3, lanes 1–5). Self-degradation of HtpX also proceeded during this incubation (Fig. 3, lanes 1–5). Degradation of $\beta$-casein was negligible when it was incubated with HtpX-His$_6$-Myc in the presence of 1,10-phenanthroline (Fig. 3, lanes 8 and 9) or with HtpX(H139F)-His$_6$-Myc in the presence of Zn$_2^+$ (Fig. 3, lanes 6 and 7). Thus, HtpX not only shows self-cleavage activity but is also able to degrade other substrate proteins.

HtpX Degrades SecY—We have previously suggested a role for HtpX, like FtsH, in eliminating abnormal membrane proteins (18). We therefore examined whether it can proteolyze membrane proteins. We chose two E. coli membrane proteins as possible substrates, YccA and SecY, both of which are known FtsH substrates in vivo and in vitro (1). We observed virtually no degradation of YccA-His$_6$-Myc when its purified preparation was incubated with HtpX-His$_6$-Myc in the presence of Zn$_2^+$ at $37^\circ \text{C}$ for 4 h (data not shown). In contrast, HtpX-His$_6$-Myc was able to significantly degrade SecY (Fig. 4). When a mixture of detergent-solubilized and purified SecY preparation and HtpX-His$_6$-Myc was incubated in the presence of Zn$_2^+$, the amount of intact SecY decreased, and SecY-derived degradation products accumulated in a time-dependent manner (Fig. 4A, lanes 1–3). This decrease in the amount of intact SecY was partly a result of the formation of SDS-resistant aggregates (28), which we observed generally after the purification of SecY (Fig. 4, A and B). However, generation of the degradation products required both wild-type HtpX and Zn$_2^+$ (Fig. 4A, lanes 4–7), indicating that HtpX can cleave SecY endoproteolytically under the detergent-solubilized conditions.

The SecY sample incubated for 8 h with HtpX-His$_6$-Myc in the presence of Zn$_2^+$ contained at least four SecY-derived fragments that were reactive with a mixture of antisera against SecY N-terminal and C-terminal regions (Fig. 4B, lane 1, circles). Immunoblotting experiments using each antiserum separately identified two of the fragments (n(I) and n(II)) as having the N terminus and the other two (c(I) and c(II)) as having the C terminus (Fig. 4B, lanes 2 and 3). It should be noted that additional SecY fragments must have been produced that were not detected by the antibodies used in these experiments.

HtpX-dependent Cleavage of Overproduced SecY in Vivo—The previous results showed that HtpX degrades solubilized SecY in vitro. We next examined whether similar degradation of SecY could be observed in vivo. Overexpressed SecY is degraded rapidly in ftsH-disrupted cells (3, 9). We found that disruption of htpx did not appreciably affect this degradation (data not shown), suggesting that HtpX at its chromosomally encoded level played, if any, only a minor role in the degradation of overexpressed SecY. We then co-overexpressed HtpX and SecY in an ftsH HtpX double-deleted strain and followed the degradation of the latter by pulse-chase experiments.
The approximate SecY positions that received the HtpX-mediated cleavage were assessed by comparing the SDS-PAGE mobilities of the degradation products with those of genetically constructed SecY variants having either N- or C-terminal truncations (Fig. 6A). Several lines of evidence as well as its sequence homology with the Saccharomyces cerevisiae Ste24 protease suggested that E. coli HtpX is a protease. However, no direct evidence of its proteolytic activity was available. In this work, we purified and characterized HtpX to address this issue. HtpX self-cleaved when cells were broken by sonication or when the membrane was solubilized with a detergent, so we were forced to purify it under denaturing conditions. Fortunately, the denaturation appeared to be reversible, and we were able to refold the protein; the addition of the essential ligand, Zn$^{2+}$, restored protease activity to the preparation. The purified and reactivated HtpX exhibited self-cleavage activity and was capable of degrading a soluble protein, β-casein, and a membrane protein, SecY. These reactions were dependent on the presence of zinc and not observed for an HtpX variant that had an altered zinc-metalloprotease active site motif. These results established that HtpX is a protease, the substrates of which include membrane proteins. Indeed, we have shown that HtpX is able to proteolyze overproduced SecY in vivo in the absence of FtsH. Both in vivo and in vitro, HtpX introduced cleavages into SecY to produce discrete fragments. Also, it is
likely that internal cuts were introduced into HtpX itself in the self-cleavage reaction. These observations indicated that HtpX is an endopeptidase.

Both FtsH (1, 2) and HtpX are capable of degrading membrane proteins. A combination of htpX and ftsH disruptions causes synthetic cell growth defects (18). The loss of FtsH or HtpX activates the Cpx extracytoplasmic stress response, which is also induced by overexpression of membrane proteins (18). These results and observations strongly suggest that HtpX collaborates with FtsH to eliminate abnormal membrane proteins. Since the Cpx stress response is believed to be a cellular reaction to extracytoplasmic stresses (30), HtpX may be unique among the Cpx-controlled gene products in that its active site is located on the cytoplasmic side of the membrane (18). Our results suggested that HtpX cleaves the cytoplasmic regions of SecY in vivo, although it is apparently able to cleave both periplasmic and cytoplasmic regions of SecY in detergent extracts in vitro (data not shown for the cleavage points). These observations suggested that HtpX is involved in membrane-protein degradation as a component of the cellular machinery that acts from the cytosolic side. This was consistent with the notion of membrane stress responses; abnormal membrane proteins are expected to be attacked from both sides of the membrane. Kaser et al. (31) recently reported that a membrane-bound metalloprotease called Oma1 functions in the degradation of a mitochondrial membrane protein in conjunction with Yta10/12, a mitochondrial homolog of the FtsH protease. Unlike HtpX, Oma1 was reported to cleave a substrate membrane protein on both sides of the membrane. Kaser et al. (31) noted that the product of the E. coli gene, ygcC, has significant sequence homology to Oma1, and we noticed that HtpX also shows significant homology with Oma1 (see supplemental Fig. S1). Our Blast search results showed that E. coli has two other ygcC/htpX-related genes, ycaL, and yggG.2 However, the intracellular localization and topology are unknown for the ygcC, ycaL, and yggG gene products, and disruption of these genes alone or in combination with disruption of ftsH or htpX did not cause a growth phenotype.3 Thus, the cellular role of the HtpX paralogs in E. coli remains unknown. In any case, the HtpX/Oma1 class of proteases may play similar roles in membrane protein quality control along with the energy-utilizing AAA proteases.

Although HtpX and FtsH might have similar physiological roles, their mechanisms of action are expected to differ. Although the FtsH action is energy-dependent and processive (1, 2), HtpX is an energy-independent endopeptidase. Degradation of at least some membrane proteins by FtsH requires an N- or C-terminal cytoplasmic tail of sufficient length (6, 7); it is unknown to what extent FtsH can initiate degradation internally. Cleavages within the cytoplasmic loop of a membrane protein by HtpX will generate new cytoplasmic tails that could serve as an initiation point for FtsH to eliminate the entire polypeptide. It should be noted that HtpX might also be involved in the degradation or cleavage of some cytoplasmic proteins, like another S. cerevisiae homolog of HtpX, Ste24, which catalyzes proteolytic maturation of a mating pheromone a-factor (32).

We found that HtpX undergoes rapid self-cleavage upon disruption or solubilization of membranes and that the purified HtpX protein has intrinsic self-cleavage activity. Mass spectrometry analysis of in vitro-generated self-cleavage fragments of HtpX suggested that the cleavages occur between Leu-260 and Cys-261 and between Cys-261 and Ile-262 4 in the cytoplasmic domain. A C-terminally truncated variant of HtpX (residues 1–261), which mimics the self-cleaved product, did not complement the ftsH ΔhtpX defect, suggesting that the self-cleaved form is inactive. Moreover, we have no evidence that this reaction takes place in vivo, except for some unusual situations of partial cell lysis and extreme overcrowding.4 Therefore, it seems likely that the observed self-cleavage of HtpX is non-physiological and caused by artificial stresses during experimental manipulations, i.e. HtpX as a solubilized enzyme has intrinsic self-cleavage activity that is suppressed in the membrane-integrated in vivo state. Further genetic, biochemical, and structural studies will uncover the physiological function and regulation of this interesting membrane-bound protease.

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REFERENCES
1. Akiyama, Y., Ogura, T., and Ito, K. (2004) Handbook of Proteolytic Enzymes (Barret, A. J., Rawlings, N. D., and Woessner, J. F. eds) pp. 794–798, Second Ed., Academic Press, London
2. Ito, K., and Akiyama, Y. (2005) Annu. Rev. Microbiol. 59, 211–231
3. Kihara, A., Akiyama, Y., and Ito, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4532–4536
4. Akiyama, Y., Kihara, A., and Ito, K. (1996) FEBS Lett. 399, 26–28
5. Kihara, A., Kihara, A., and Ito, K. (1998) J. Mol. Biol. 279, 175–188
6. Chiba, S., Akiyama, Y., Mori, H., Matsu, E., and Ito, K. (2000) EMBO J. 1, 47–52
7. Chiba, S., Akiyama, Y., and Ito, K. (2002) J. Bacteriol. 184, 4775–4782
8. Kihara, A., Akiyama, Y., and Ito, K. (1999) EMBO J. 18, 2970–2981
9. Akiyama, Y., and Ito, K. (2000) EMBO J. 19, 3888–3895
10. Akiyama, Y., and Ito, K. (2003) J. Biol. Chem. 278, 18146–18153
11. Akiyama, Y., Kihara, A., and Ito, K. (2004) EMBO J. 23, 4434–4442
12. Akiyama, Y., Kanehara, K., and Ito, K. (2004) EMBO J. 23, 4434–4442
13. Grigorova, I. L., Chaba, R., Zhong, H. J., Alba, B. M., Rhodius, V., Herman, C., and Gross, C. A. (2004) Genes Dev. 18, 2686–2697
14. Paetzel, M., Karla, A., Strynadka, N. C., and Dalby, R. E. (2002) Chem. Rev. 102, 4549–4580
15. Kornitzer, D., Teff, D., Altuvia, S., and Oppenheim, A. B. (1991) J. Bacteriol. 173, 2944–2953
16. Coltr, P. P., and Rosato, Y. B. (2004) Curr. Microbiol. 48, 391–395
17. Vickerman, M. M., Matter, N. M., Minick, P. E., and Edwards, C. A. (2002) Oral Microbiol. Immunol. 17, 22–31
18. Shimohata, N., Chiba, S., Saikawa, N., Ito, K., and Akiyama, Y. (2002) Genes Cells 7, 653–662
19. Akiyama, Y., and Ito, K. (1985) EMBO J. 4, 3351–3356
20. Akiyama, Y., and Ito, K. (1990) Biochem. Biophys. Res. Commun. 167, 711–715
21. Murphy, K. C. (1998) J. Bacteriol. 180, 2063–2071
22. Davis, R. W., Botstein, D., and Roth, J. R. (1980) Advanced Bacteriology: A Manual for Genetic Engineering, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
23. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Savano, A., and Miyawaki, A. (2000) Nucleic Acids Res. 28, E78
25. Akiyama, Y., Yoshihisa, T., and Ito, K. (1995) J. Biol. Chem. 270, 23485–43490
26. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) J. Bacteriol. 177, 4121–4130
27. Shimoike, T., Taura, T., Kihara, A., Yoshihisa, T., Akiyama, Y., Cannon, K., and Ito, K. (1995) J. Biol. Chem. 270, 5519–5526
28. Akiyama, Y., Ehrmann, M., Kihara, A., and Ito, K. (1998) Mol. Microbiol. 28, 803–812
29. Ito, K. (1984) Mol. Gen. Genet. 197, 204–208
30. Dugan, A. R., and Silhavy, T. J. (2004) Biochim. Biophys. Acta 1649, 121–134
31. Kasar, M., Kambacheld, M., Kisters-Woike, B., and Langer, T. (2003) J. Biol. Chem. 278, 46414–46423
32. Tam, A., Fujimura-Kamada, K., and Michaelis, S. (1998) in Handbook of Proteolytic Enzymes, (Barret, A. J., Rawlings, N. D., and Woessner, J. F., eds) pp. 1094–1096, Academic Press, London
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Machiko Sakoh, Koreaki Ito and Yoshinori Akiyama

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