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FtsH Recognizes Proteins with Unfolded Structure and Hydrolyzes the Carboxyl Side of Hydrophobic Residues[1]

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FtsH of *Escherichia coli* is an essential membrane-integrated ATP-dependent protease. We cloned a gene for an FtsH homolog (*T.*FtsH) from *Thermus thermophilus HB8*, expressed it in *E. coli*, and purified the expressed protein. ATPase activity of *T.*FtsH was activated by proteins with unfolded structure (α-casein and pepsin), and *T.*FtsH digested these proteins in an ATP-, Zn^{2+}-dependent manner. α-Lactalbumin was digested by *T.*FtsH when it was largely unfolded, but not in its native form. Analysis of the proteolytic products revealed that, in most cases, *T.*FtsH cleaved the C-terminal side of hydrophobic residues and produced a characteristic set of small peptides (<30 kDa) without releasing a large intermediate. Thus, *T.*FtsH recognizes the unfolded structure of the proteins and progressively digests them at the expense of ATP. A soluble domain of *T.*FtsH, which lacked the N-terminal two transmembrane helices, was also prepared but was found to retain neither ATPase nor protease activities. Thus, the membrane segment appeared to be indispensable for these activities of *T.*FtsH.

Key words: ATP,Zn^{2+}-dependent protease, chymotrypsin-like activity, FtsH, progressive degradation, unfolded structure.

Various ATP-dependent proteases are responsible for strictly regulated intracellular proteolysis in both prokaryotes and eukaryotes. *Escherichia coli* has at least five kinds of ATP-dependent proteases: Lon, ClpXP, ClpAP, ClpYQ (HslUV), and FtsH (*1*, *2*). Of these, only FtsH is an integral membrane protein and is essential for cell growth (*3*, *4*). FtsH has two transmembrane helices in the N-terminal region, and this is followed by a large cytoplasmic domain including an ATP-binding sequence, which is a conserved characteristic segment of members of the AAA-ATPase family (*5*, *6*). The cytoplasmic domain of FtsH also contains the HExxH sequence (x is variable), a characteristic motif of the zinc-binding site of metalloproteases (*5*, *6*). FtsH forms homo-oligomeric complexes through the interactions between the N-terminal membrane regions (*7*) and interacts with other membrane proteins referred to as HflK-HflC complex (HflKC), which has been suggested to regulate the activity of FtsH (*8*, *9*).

FtsH is required for degradation of the subunits of membrane protein complexes that fail to associate properly (SecY, subunit *a* of FoF_1-ATP synthase) (*10–12*), and thus it contributes to the quality control of membrane proteins. It has also been demonstrated *in vivo* and *in vitro* to degrade three soluble cytoplasmic proteins: the heat shock sigma factor (σ^{32}), of which degradation *in vivo* requires the DnaK chaperone system (*13–15*); a transcriptional regulator, λCII (*8*, *15–17*); and the *lpxC* gene product, which is involved in the committed step of lipid A synthesis (*18*). In all cases, the *in vitro* digestion is dependent on ATP hydrolysis and Zn^{2+}, and proceeds in a processive manner without releasing a large intermediate peptide (*19*). Considering that the cytosol is very crowded with numerous proteins, one can ask how FtsH recognizes only very few proteins as a substrate. However, the molecular basis of the apparently narrow substrate specificity of FtsH remains unclear. In this paper, we report that an FtsH homolog of *Thermus thermophilus* simply recognizes and digests proteins containing an unfolded structure.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions—*T. thermophilus* strain *HB8* (ATCC 27634) was used as a source of genomic DNA. *E. coli* strains used here were JM109 for preparation of plasmids, CJ236 for generating uracil-containing single-stranded DNA for site-directed

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; AMP-PNP, 5'adenylyl-β-,γ-imidodiphosphate; IPMDH, isopropylmalate dehydrogenase; LA, bovine α-lactalbumin; r-LA, reduced bovine α-lactalbumin; apo-LA, bovine α-lactalbumin deprived of bound Ca^{2+}; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate.

Data deposition: The nucleotide sequences reported in this paper have been submitted to the DDBJ/EMBL/GenBank databases with accession number AB032368

mutagenesis, and BL21 (DE3) for gene expression of plasmids carrying the T7 promoter. Plasmid pUC118 and helper phage M13KO7 were used to generate uracil-containing single-stranded DNA. Plasmid pET21c (Novagen) was used for the construction of the *T.*FtsH expression system. *E. coli* was grown aerobically at 37°C in 2×YT media. IPTG (final concentration 1 mM) was added to the medium for induction of the T7 RNA polymerase under the control of the inducible T7-lac promoter. Ampicillin (50–100 μg/ml) was added as a supplement to the growth media.

Cloning of T.ftsH Gene—DNA was handled by standard methods (*20*), and genomic DNA from *T. thermophulus* was isolated as described previously (*21*, *22*). Southern blot hybridization was performed using a 0.8-kbp *Bam*HI–*Nae*I fragment of a 5.5-kbp *Bam*HI clone as a probe to clone upstream of the 5.5-kbp *Bam*HI clone (*22*). A 2.4-kbp *Sac*I fragment was detected, and the 2.4-kbp *Sac*I clone were selected by colony hybridization. To clone the entire region of *T. thermophulus ftsH*, Southern blot hybridization was performed using a 1.1-kbp *Sac*I–*Bam*HI fragment of the 2.4-kbp *Sac*I clone, and a 3.5-kbp *Bam*HI fragment was obtained. Probe labeling and detection were performed by use of the ECL random prime labeling and detection system (Amersham Pharmacia Biotech).

T.FtsH Expression System—A 2.4-kbp *Sac*I–*Sac*I fragment of pFSI that carried N-terminal region of *T.*FtsH was cloned into the *Sac*I site of pFBI. pFBI contained a 3.5-kbp *Bam*HI fragment of the C-terminal region of *T.*FtsH inserted into the *Bam*HI site of pUC118. To express *T.*FtsH, an *Nde*I site and an *Eco*RI site were introduced into the translational initiation region of the *T.ftsH* gene in pFSI by the method of Kunkel *et al.* (*23*). The new plasmid carrying the *Nde*I site of the *T.ftsH* gene in the translational initiation region was digested with *Nde*I–*Bsp*EI, and the region of *T.ftsH* gene corresponding to the C-terminal region of *T.*FtsH was digested with *Bsp*EI–*Sal*I in pFU1. The obtained fragments were ligated into the corresponding *Nde*I–*Sal*I sites in pET21c, yielding plasmid pAFH1. Plasmid pAFH2 is identical to pAFH1 except that the 6-histidine tag was introduced into the C-terminal of *T.*FtsH (*23*). His6-tag was attached to avoid contamination of *E. coli* FtsH and to facilitate purification. The plasmid pAFS3 carrying the gene encoding the soluble region of *T.*FtsH which lacked 124 amino acids from N-terminus was made as follows. A *Nde*I site and a histidine tag were introduced at the end of the transmembrane region of *T.ftsH* gene in pFSI. The resultant gene was digested with *Nde*I–*Bam*HI, and the region corresponding to the C-terminal region of *T.*FtsH was digested with *Bam*HI–*Xho*I in pFU1. The obtained fragments were ligated into the corresponding *Nde*I–*Xho*I sites in pET21c. The N-terminal sequence of the soluble *T.*FtsH should be (His)6-MGA-^{125}RNGR·····. MGA is introduced as a linker.

Purification of T.FtsH—E. coli BL21 (DE3) harboring pAFH2 was grown at 37°C to mid-log phase in 6 liters of 2× YT medium supplemented with ampicillin (50–100 μg/ml), and expression of *T.*FtsH was induced by addition of IPTG (final concentration, 1 mM). The growth of expressing *E. coli* was markedly retarded. Culture was continued for another 3 h, and cells were harvested. Cells were suspended in the washing buffer (50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 300 mM NaCl, 10 mM imidazole) containing 1 mM EDTA and 1 mM PMSF, disrupted by French press (5501-M, Ohtake Works), and centrifuged at 100,000 ×*g* for 40 min at 4°C. The pellet was suspended with the washing buffer containing 1 M NaCl and centrifuged again. The pellet was resuspended with the washing buffer and solubilized by addition of a non-ionic detergent, 1-*O*-*n*-octyl-β-D-glucopyranoside (octylglucoside; final concentration, 2%). After stirring at 25°C for 30 min, remaining debris was precipitated by centrifugation at 100,000 ×*g* for 40 min at 4°C, and the supernatant was loaded onto a Ni-NTA Superflow column (Qiagen) (column volume, 15 ml) equilibrated with buffer A (50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 10 mM imidazole, 0.5% octylglucoside). The column was washed with 50 ml of buffer A, then with 50 ml of buffer B (buffer A plus 50 mM NaCl). Bound proteins were eluted with a gradient of 10 to 1,000 mM imidazole in buffer B. Fractions containing *T.*FtsH were combined and loaded onto a DEAE-Toyopearl column (Tosoh) equilibrated with buffer B. The proteins were eluted with a gradient of 50 to 400 mM NaCl in buffer C (50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 0.5% octylglucoside). The fractions containing pure *T.*FtsH were pooled and stored at –80°C. The N-terminal amino acid sequence of the obtained *T.*FtsH preparation was confirmed to be identical to the predicted one from the *T.*FtsH gene but not *E. coli* FtsH. Typically, 8 mg of purified *T.*FtsH was obtained from 4 liters of the culture (wet cell, 10 g). We used *T.*FtsH without removing the his-tag throughout this paper. The oligomeric state of the purified *T.*FtsH in octylglucoside was examined by gel permeation HPLC and equilibrium centrifugation. However, the results showed that purified *T.*FtsH was a mixture of homo-oligomers with different aggregation states.

Purification of the Soluble Domain of T.FtsH—E. coli BL21 (DE3) harboring pAFS3 was grown, harvested, disrupted, and centrifuged as described above. The supernatant was loaded onto a Ni-NTA Superflow column (15 ml) equilibrated with the washing buffer. The column was washed with 50 ml of the washing buffer, then with 50 ml of buffer D (50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 25 mM NaCl, 10 mM imidazole). Bound proteins were eluted with a gradient of 10 to 250 mM imidazole in buffer D. Fractions containing soluble His-tagged *T.*FtsH were pooled and loaded onto a DEAE-Toyopearl column equilibrated with buffer E (50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 25 mM NaCl, 20% glycerol). The proteins were eluted with a 25- to 500-mM gradient of NaCl in buffer E. Fractions containing soluble His-tagged *T.*FtsH were pooled and stored at –80°C.

ATPase Activity of T.FtsH—Unless otherwise stated, ATPase activity was assayed at 65°C in 100 μl of the ATPase assay mixture [50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 25 μM $Zn(CH_3COO)_2$, 12.5 mM Tris-CH_3COOH, pH 8.0, 300 mM NaCl, 20% glycerol, 0.5% octylglucoside, 2 mM ATP, and 2 μg *T.*FtsH]. The reaction was initiated by addition of ATP and terminated after 4, 8, and 12 min by addition of 25 μl of 20% perchloric acid (w/v). The reaction mixtures were centrifuged at 16,000 rpm for 5 min at 4°C and released P_i in the supernatant was quantified by the malachite green assay (*24*). As a control (0% ATPase activity), *T.*FtsH was injected into the reaction mixture containing 4% perchloric acid.

Degradation of Protein Substrates—Protein substrates (final concentration, 0.5 mg/ml) were incubated with 2 μM *T.*FtsH at 65°C in the protease buffer [25 mM Tris-Cl, pH 8.0, 2.5 mM $MgCl_2$, 12.5 μM $Zn(CH_3COO)_2$, 6.25 mM Tris-

CH_3COOH, pH 8.0, 0.25% octylglucoside, 10% glycerol, 150 mM NaCl] and 8 mM nucleotide. At indicated times, an aliquot (10 μl) was removed from the mixture, the reaction was terminated by addition of 5 μl of 6× SDS-sample solution of electrophoresis, and the solution was analyzed 13% SDS-PAGE (*25*). Gels were stained with Coomassie Brilliant Blue R-250.

Analysis of Peptides by Reverse-Phase HPLC—During digestion of α-casein and pepsin, aliquots were taken from reaction mixtures and mixed with an equal volume of 8.0 M guanidine HCl to stop the reaction. The aliquots were injected onto a C18 super ODS column (4.6 mm ID × 5.0 cm L; Tosoh) equilibrated with 0.05% trifluoroacetic acid. Peptides were eluted with a gradient of 0 to 80% acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1.0 ml/min. Peak fractions were subjected to the N-terminal amino acid sequencing (Shimadzu PPSQ20) and mass spectrometry. The molecular mass of peptides separated by a reverse phase HPLC was determined using a MALDI-TOF LASERMAT-2000 mass spectrometer (Finnigan MAT). The matrix used in all experiments was 4-hydroxy-α-cyanocinnamic acid (Aldrich). For calibration, bovine lactalbumin and substance P were used as standard.

Materials and Other Methods—Bovine α-casein, porcine pepsin, α-lactalbumin (Type I, holo-LA; Type III/Ca^{2+} depleted, apo-LA), and trypsin were purchased from Sigma. α-casein, pepsin, and r-LA were dissolved in buffer F (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT). RNase A was purchased from Boehringer Mannheim. Isopropylmalate dehydrogenase from *T. thermophilus* (IPMDH) was kindly provide by Dr. T. Oshima and F. Motojima (*26*). RNaseA, *T. thermophulus* IPMDH, apo- and holo-LA were dissolved in buffer F lacking 1 mM DTT. Glucose 6-phosphate dehydrogenase from *Bacillus stearothermophilus*, purchased from Unitika (Osaka), was dissolved in buffer G (50 mM MOPS, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$). Anti-*T.*FtsH antibody was prepared using purified soluble His-tagged *T.*FtsH. Anti-SRH antibody was prepared using a synthetic peptide (NH_2-TNRPDILDPALLRPGRC-COOH) which corresponded to amino acids 301–317 of *T.*FtsH. Western blotting was performed using polyvinyldenedifluoride membrane. The protein concentration was determined by BCA protein assay kit (Pierce).

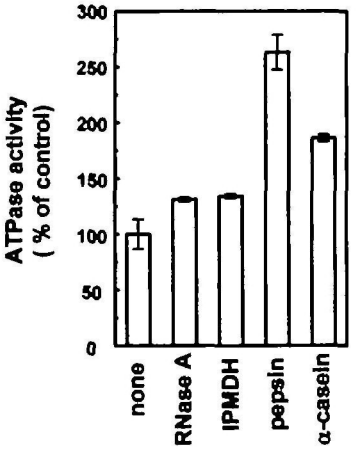

Fig. 1 **Effect of proteins with unfolded structure on *T.*FtsH ATPase activity.** ATP hydrolysis was determined by incubating 2 μg of *T.*FtsH with 2 mM ATP in the absence or presence of indicated proteins (1 mg/ml, final concentration) at 65 (see "MATERIALS AND METHODS").

RESULTS

ftsH Gene of T. thermophulus—Upstream of the *T. thermophulus dnaK* gene cluster (*dnaK-grpE-dnaJ-dafA-clpB*) (*22, 27, 28*), we found a new open reading frame (ORF). The ORF was oriented in the opposite direction to the dnaK gene cluster and encoded a protein with 624 residues. The overall amino acid sequence of the ORF showed similarity to that of *E. coli* FtsH (49% identity). In the ORF, two transmembrane segments were followed by Walker's ATP-binding motif A (GxxxxGKT, x can be varied; ^{196}GPPGVGKT^{203}) and B (zzzzD, z is a hydrophobic residue; ^{251}IVFID^{255}) (*29*). Further downstream were found the second region of homology [SRH motif (*30*); ^{296}IVVMAATNRPDILDPALLRPGRFD^{318}; conserved residues are underlined], which is characteristic of a group of AAA proteins, and the zinc-binding motif (HExxH; ^{418}HEAGH^{422}). Therefore, we concluded that this ORF encodes the *T. thermophilus* FtsH (*T.*FtsH). We cloned the *T.*FtsH gene and expressed it in *E. coli* in the membrane fraction from which *T.*FtsH was purified to homogeneity (see "MATERIALS AND METHODS"). To confirm the expression of *T.*FtsH in *T. thermophulus*, we prepared membrane fraction from this bacteria and ana-

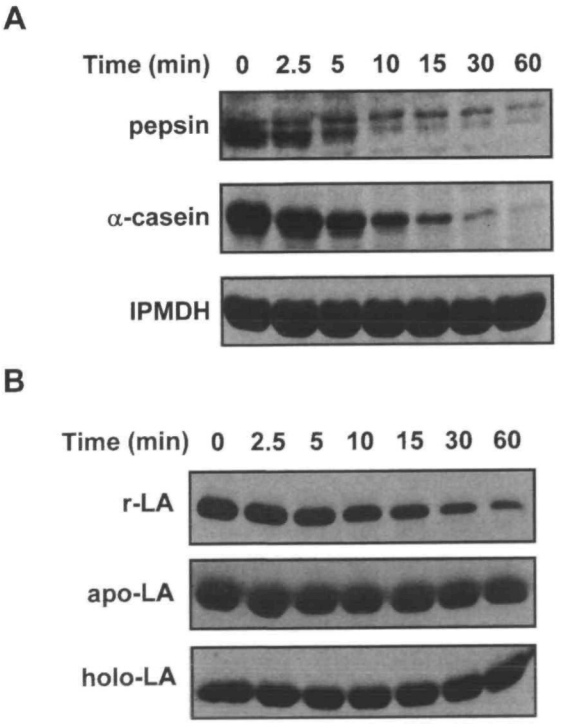

Fig. 2. **Digestion of protein substrates by *T.*FtsH.** A. Digestion of α-casein, pepsin, and IPMDH by *T.*FtsH. Substrates (final concentration, 0 5 mg/ml) were incubated in 80 μl of protease buffer with 10 μg of *T.*FtsH and 8 mM ATP. Aliquots of 10 μl were removed at the indicated times and analyzed by 13% SDS-PAGE. Commercially available pepsin contains an apparently larger unassigned polypeptide which was digested slowly by *T.*FtsH. B Digestion of r-, apo-, and holo-LA by *T.*FtsH Conditions were the same as A except that 15% SDS-PAGE was used

lyzed by immunoblotting using anti-*T.*FtsH antibodies raised against either the purified soluble domain of *T.*FtsH or a synthetic SRH motif peptide. Both antibodies gave a sharp single band at the expected position (not shown), and thus *T.*FtsH was confirmed to be expressed in membranes of *T. thermophilus*.

ATPase Activity of T.FtsH—Purified *T.*FtsH expressed in *E. coli* hydrolyzed ATP with an optimum temperature of 65°C. At 2 mM ATP, the turnover rate of ATP hydrolysis by *T.*FtsH (calculated as monomer) was ~0.65 s^{-1}. Compared with the activity at 65°C, activities at 36, 50, 60, and 70°C were 5, 17, 63, and 67%, respectively. To examine the effect of proteins with unfolded structure on the ATPase activity of *T.*FtsH, we used α-casein and pepsin as model unfolded proteins. It is known that the structure of α-casein is almost completely unfolded, and that pepsin at neutral pH is largely unfolded with some remaining structure (*31*, *32*). The effects of RNaseA and *T. thermophilus* IPMDH, both of which are stable and active at 65°C, on *T.*FtsH ATPase activity were also measured. As shown in Fig. 1, the two native proteins showed a small stimulative effect, while the model unfolded proteins, especially pepsin, stimulated *T.*FtsH ATPase to a greater extent. To achieve maximum activation, a large excess (~50-fold by weight) of unfolded proteins was required. Activation occurred without an apparent time lag when unfolded proteins were added to the reaction mixtures in which ATP hydrolysis was proceeding (data not shown). The optimum temperature of ATPase activity was not changed by the addition of proteins with unfolded structure, and similar degrees of activation were observed at all temperatures. Like *E. coli* FtsH (*13*), *T.*FtsH also hydrolysed CTP (74% of ATP) and, at a slower rate, GTP (19% of ATP). Only trace hydrolysis was observed for TTP and UTP. ADP and AMP were not hydrolyzed at all. Interestingly, ADP was a strong inhibitor: hydrolysis of ATP almost stopped when equimolar ADP was present. AMP did not inhibit ATP hydrolysis.

Digestion of Proteins with Unfolded Structure—As shown in Fig. 2A, α-casein and pepsin were digested by *T.*FtsH in the presence of ATP. Approximately, 0.5 and 1.2 mol of α-casein and pepsin, respectively, were digested per min per mol of monomer *T.*FtsH. *T.*FtsH did not digest proteins with native structures at 65°C, such as IPMDH from *T. thermophilus* (Fig. 2A), glucose 6-phosphate dehydrogenase from *B. stearothermophilus*, and bovine pancreas RNaseA (data not shown).

The preference of *T.*FtsH for unfolded protein as a substrate was supported by an experiment using the same protein in largely unfolded and folded forms. α-Lactalbumin (LA) is known to take three well-characterized conformations with different degrees of unfolding. Holo-LA is a native form of LA; LA deprived of bound Ca^{2+} (apo-LA) takes a typical molten globule structure which has native-like secondary structure but lacks the specific tertiary interactions of amino acid side-chains, and the disulfide reduced form (r-LA) is more unfolded and flexible, although it still has some secondary structure (*33–35*). These three forms of LA remained soluble at 65°C, and circular dichroism spectra were measured in the same buffer as that used for the protease reaction. The values of $[\theta] \times 10^3$ (deg cm/dmol) at 220 nm, an indication of the contents of the secondary structure, of holo-LA, apo-LA and r-LA at 65°C were –11.2, –9.8, and –7.5, respectively. These values were not much changed from the corresponding values at 25°C, –11.7, –10.1, and –8.8, indicating that essential structural features of three forms were maintained at 65°C. *T.*FtsH digested r-LA at a rate of 0.15 mol per min per mol of monomer *T.*FtsH but did not digest apo-LA and holo-LA (Fig. 2B). Thus, we conclude that *T.*FtsH recognizes and digests proteins with wholly or largely unfolded structure.

Nucleotide and Zn^{2+} Dependency of Digestion—Digestion did not occur when ATP was omitted from the reaction mixture, or when ADP, AMP, or AMP-PNP was substituted for

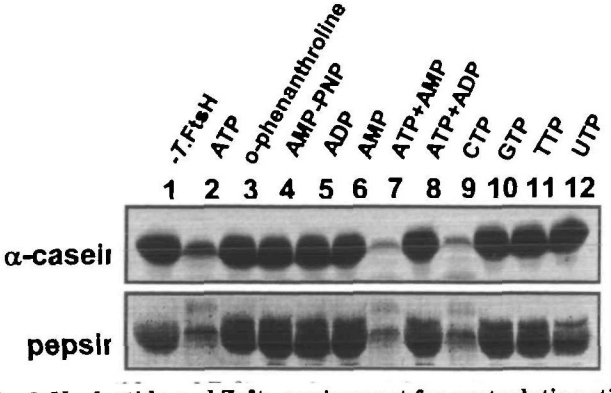

Fig. 3 **Nucleotide and Zn^{2+} requirement for proteolytic activity of *T.*FtsH.** *T.*FtsH protease activity was measured in the presence of several nucleotides and a metal-chelating agent, *o*-phenanthroline. Reactions were stopped at 90 min and analyzed by 13% SDS-PAGE Lane 1, -*T.*FtsH; lane 2, + 8 mM ATP; lane 3, + 2 mM *o*-phenanthroline; lane 4, + 8 mM AMP-PNP; lane 5, +8 mM ADP; lane 6, +8 mM AMP; lane 7, +8 mM ATP +8 mM AMP; lane 8, +8 mM ATP +8 mM ADP; lane 9, +8 mM CTP; lane 10, +8 mM GTP; lane11, +8 mM TTP; lane 12, +8 mM UTP.

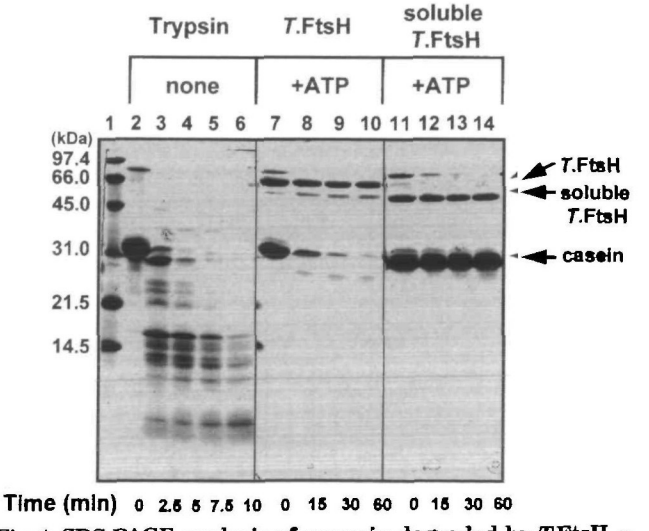

Fig 4. **SDS-PAGE analysis of α-casein degraded by *T.*FtsH.** α-Casein (final concentration, 0.5 mg/ml) was incubated in 100 μl of protease buffer containing 13 μg of *T.*FtsH or the soluble domain of *T.*FtsH As a control, digestion by trypsin (0.1 μg) is also shown For reactions with *T.*FtsH or soluble *T.*FtsH, samples of 10 μl were removed at 0, 15, 30 and 60 min, and the reaction was quenched by the addition of 6 × SDS-sample buffer For reactions with trypsin, incubation times were 0, 2.5, 5, 7.5, and 10 min. Samples were subjected to 13% SDS-PAGE

ATP (Fig. 3, lanes 2, 4–6), suggesting that protease activity of *T.*FtsH is dependent on hydrolysis of ATP. Of the several other nucleotide triphosphates tested, only CTP could substitute ATP (Fig. 3, lanes 9–12). The addition of equimolar ADP to ATP resulted in nearly complete inhibition of proteolysis (Fig. 3, lane 8). AMP had no such effect (Fig. 3, lane 7). Thus, both the ATPase activity and the protease activity of *T.*FtsH were inhibited by ADP. Similar inhibition by ADP has been reported for other ATP-dependent proteases, HslUV and Lon protease (*36*, *43*). The nucleotide specificity and dependency of protease activity correspond exactly to those observed for ATPase activity of *T.*FtsH, supporting the notion of coupling between ATP hydrolysis and proteolysis (*12*, *13*, *37*). Digestion was also dependent on Zn^{2+}, since proteins with unfolded structure were not digested when *o*-phenanthroline, a chelator for Zn^{2+}, was included in the mixture (Fig. 3, lane 3).

Many Small Peptides are Produced at Similar Rates—Digestion of α-casein with trypsin, a classic protease, produced many partially digested intermediates with molecular size larger than 10 kDa (Fig. 4, lanes 2–6). On the contrary, only a very small amount of intermediate appeared when α-casein was digested by *T.*FtsH (Fig. 4, lanes 7–10). Similarly, digestion of pepsin to *T.*FtsH proceeded without generating large intermediate (not shown). Because polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) usually did not separate the peptides smaller than 10 kDa, we analyzed peptide products by HPLC. Pepsin and α-casein were incubated with *T.*FtsH, and aliquots removed at 15, 60, and 120 min were analyzed by reverse-phase HPLC (Fig. 5, A and B). It is clear that the heights of most peptide peaks increased at similar rates as incubation continued. The large peak at 27 min in Fig. 5B is that of undigested α-casein, and its peak height decreased with time. Undigested α-casein was not eluted from the column. Thus, during digestion of α-casein and pepsin by *T.*FtsH, a characteristic set of small peptides steadily increased, while undigested full-length proteins concomitantly decreased. We determined the molecular sizes of the peptides present in the peaks separated by reverse-phase HPLC using mass spectrometry (Fig. 6). Although mass spectrometry does not always reflect the real population of various peptides in mixtures, it is still noteworthy that peptides of 1–2 kDa were most abundant, and 50 out of 59 peptides ranged below 3 kDa. Thus, we conclude that *T.*FtsH digests protein substrates in a processive manner: it cleaves polypeptides at multiple sites and produces small peptides without releasing partially digested intermediates into the bulk solution.

Cleavage Specificity of T.FtsH—We analyzed the N-terminal amino acid sequences of the peptides contained in the HPLC peaks, and cleavage sites were determined (Fig. 7, A and B). Residues at the N-terminal side of cleavage sites were leucine (8 sites), phenylalanine (5 sites), alanine (4 sites), tyrosine (4 sites), glycine (3 sites), tryptophane (2 sites), and others. Among 32 identified residues at cleavage sites, 30 are nonpolar residues and 20 are bulky hydrophobic residues (tyrosine is included in the hydrophobic group). It is clear that *T.*FtsH has a tendency to cleave peptide bonds next to hydrophobic residues. As to the C-terminal side amino acid residues of the cleavage sites, no obvious preference was found

Soluble Domain of T.FtsH—The soluble domain of *T.*FtsH, which lacked the transmembrane helices (124 amino acids of the N-terminal region), was expressed in the soluble fraction of *E. coli* and purified. The N-terminal amino acid sequence determined by peptide sequencing agreed with the sequence predicted from the DNA sequence. The depth of the trough in far-UV circular dichroism spectrum of the soluble *T.*FtsH domain was ~70% of the full-length *T.*FtsH (data not shown), and therefore the obtained soluble domain of *T.*FtsH contained a significant amount of secondary structure. Consistent with the previous report that FtsH aggregates into oligomers through the interaction between membrane regions (*38*), analysis by gel filtration HPLC indicated that the soluble *T.*FtsH domain

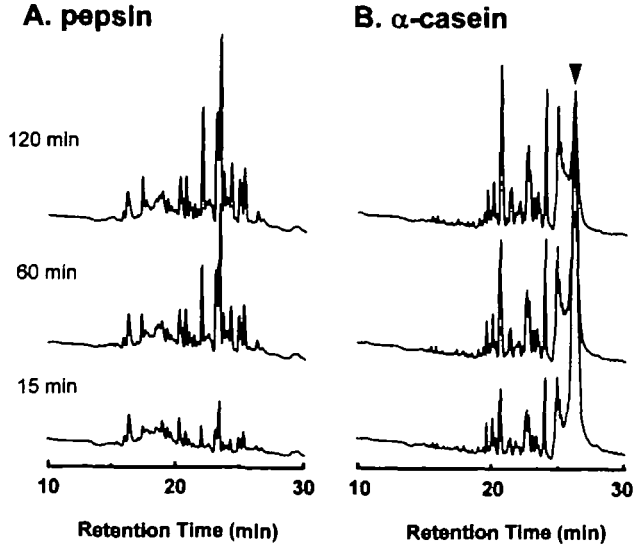

Fig. 5 **Production of a set of peptide fragments from α-casein and pepsin.** Pepsin (A) and α-casein (B) (1.25 mg/ml) were incubated in 100 μl of protease buffer with 6 μg of *T.*FtsH At 15, 60, and 120 min, samples of 20 μl were removed, and the reaction was quenched by the addition of equal volume of 8.0 M guanidine HCl The products were separated on a C18 reverse-phase column. The absorbance profile at 220 nm of peptides eluted in a gradient of acetonitrile is shown. Undigested α-casein at 27 min is indicated by an arrow.

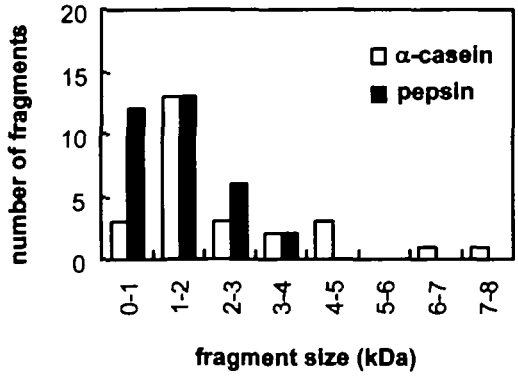

Fig 6 **Size distribution of the peptide fragments of pepsin and α-casein produced by *T.*FtsH.** The peptides present in the peak fractions of reverse-phase HPLC in Fig. 5 were analyzed by MALDI-TOF mass spectrometry The total number of peptides analyzed was 59

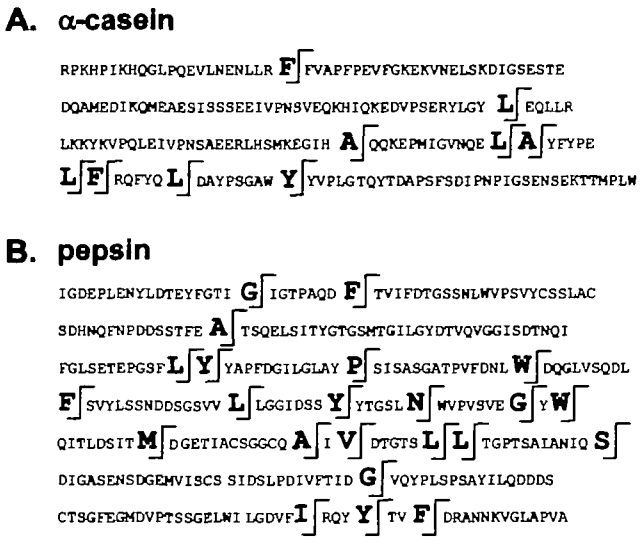

Fig. 7. **Map of sites cleaved by *T.*FtsH.** (A) α-casein, (B) pepsin. Cleavage sites identified from N-terminal sequencing are denoted by hooked marks.

had a molecular size of ~55 kDa, the size of a monomer (data not shown). Activity of the soluble *T.*FtsH domain was examined but neither degradation of α-casein (Fig. 4, lanes 11–14) nor hydrolysis of ATP (data not shown) was detected. Oligomeric organization of FtsH protomers seems essential for the activity. This contention is consistent with the model proposed by Karata *et al.*, in which a conserved arginine residue of the neighboring protomer has been demonstrated to be important for hydrolysis of ATP (*37*).

DISCUSSION

Probably by virtue of its low proteolytic activity at 36°C, expression of *T.*FtsH in *E. coli* was achieved, and the purified enzyme was subjected to biochemical study. The most significant finding of this study was that *T.*FtsH appears to be a protease which digests proteins with unfolded structure. *T.*FtsH digested model unfolded proteins, α-casein and pepsin, but not thermophilic IPMDH from *T. thermophilus*. (Fig. 2). It might be argued that our observation that one unfolded protein is digested while a different folded protein is not can be explained by recognition at the level of a primary sequence motif. However, using α-lactalbumin as a substrate, we demonstrated that a largely unfolded form, but not a native form, of the same protein is susceptible to digestion by *T.*FtsH. Digestion of proteins with unfolded structure by *T.*FtsH is not attributable to a side reaction that proceeds independently of the highly regulated *T.*FtsH function because, similar to other specific substrates, these proteins with unfolded structure (i) stimulate the ATPase activity of *T.*FtsH, (ii) are digested by *T.*FtsH in a Zn^{2+}-, ATP-dependent manner, (iii) are digested in a processive manner. It appears that *T.*FtsH simply recognizes flexible, partly unfolded proteins, brings them to a catalytic site, and digests them, as observed in other conventional proteases.

E. coli FtsH, as a protease, is thought to have a strict substrate specificity: it can digest only a very limited number of proteins such as σ^{32}, λCII, LpxC gene product, unassembled SecY, and Fo-*a* subunit. The biochemical basis of this strict substrate specificity has not been clearly understood. Our results raise the possibility that these proteins have an unfolded portion and display it to FtsH. These proteins might have a portion with limited stability, which, when the proteins need to be eliminated, becomes unfolded and recognized by FtsH. This unstable portion could be a terminal region of the protein (*37*, *39*); and FtsH would pull a polypeptide chain into the catalytic cavity with accompanying forced unfolding of the structured part of the protein at the expense of ATP.

Besides proteolytic activity, FtsH has been suggested to participate in several cellular processes such as protein assembly into the membrane (*4*, *40*) and protein export (*4*, *41*). These molecular chaperone-like activities of FtsH may be related to its ability to recognize unfolded protein structure.

Another finding of this study was that *T.*FtsH has a chymotrypsin-like cleavage specificity: that is, cleavage sites favored by *T.*FtsH are peptide bonds next to hydrophobic residues. This seems consistent with the fact that the *in vivo* substrates of FtsH are often membrane proteins. However, this rationale is weaked by the fact that other ATP-dependent proteases which degrade soluble proteins, such as Lon and HslUV, were reported to have chymotrypsin-like activities when they digested fluorogenic peptides (*42*, *43*). Eukaryotic proteasome has a chymotrypsin-like activity as well as trypsin-like activity (*44*, *45*). Recently, it was suggested from *in vivo* self-processing of *E. coli* FtsH that substrates may be cleaved at positively charged or hydrophobic side chains (*46*). Clarification of molecular and physiological bases of the characteristics of FtsH including the cleavage specificity awaits further study.

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