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Stabilization of FtsH-unfolded protein complex by binding of ATP and blocking of protease

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Abstract

The function of an ATP-dependent membrane protease FtsH was investigated using the enzyme from *Thermus thermophilus* HB8. An FtsH mutant with replacement of Glu-419 in the zinc-binding motif by Cys lost the activity to digest casein, a model unfolded protein, and the small ATPase activity of this mutant was no longer stimulated by casein. In the presence of ATP or ATPγS, but not ADP, a mutant FtsH-unfolded protein complex was isolated, indicating that ATP binding, but not ATP hydrolysis, is required for FtsH to form a stable complex with an unfolded protein. The FtsH without mutation at Glu-419 did not produce a stable complex with casein in the presence of any nucleotides tested and therefore it appears that blocking proteolysis also contributes to stabilization of the complex.

Keywords: FtsH; ATP-dependent protease; *Thermus thermophilus*

FtsH is an essential, membrane-bound ATP-dependent metalloprotease of *Escherichia coli* and its closely related homologs have been found in various bacteria, mitochondria, and chloroplasts [1]. FtsH is anchored to the inner membrane by two transmembrane segments at the N-terminal region and the rest large cytoplasmic region contains two domains; AAA ATPase domain, in which conserved Walker-A, Walker-B, and SRH (second region of homology) motifs exist, and the protease domain, in which a zinc-binding motif, HEXGH, exists. FtsH is responsible for degradation of certain cytoplasmic proteins such as the heat shock transcription factor σ^{32} [2,3], LpxC [4], λCII [5–7], CIII [8], and SsrA-tagged proteins [9], as well as certain integral membrane proteins such as SecY [10,11], $F_o a$ subunit of ATP synthase [12], and YccA [13]. The degradation proceeds in a processive manner without releasing large intermediate peptides. In vitro, FtsH digests unfolded proteins with specificity similar to that of chymotrypsin [14] or trypsin [15]. One of the crucial questions on the mechanism of ATP-dependent proteases is with regard to how the energy of ATP is used to regulate proteolytic activity. It has been known that ADP and ATP (and its analog) cause significant change in the conformation of FtsH [16,17]. FtsH is a homo-oligomeric enzyme and monomers of the soluble domain of FtsH do not show any catalytic activity [14], although they appear to be able to bind unfolded proteins and ATP [17]. Oligomer structure is thus crucial for the activity of FtsH and it is known that ATP is not necessary for oligomerization [18]. Here, we report characteristics of an FtsH mutant from a thermophilic bacterium *Thermus thermophilus* HB8 that lacks protease activity. The results indicate that a stable FtsH-unfolded protein complex is generated when ATP is bound and protease activity is blocked.

Materials and methods

Strains, plasmids, and reagents. Escherichia coli DH5α and BL21 (DE3) were used as a host strain for construction of plasmids and for gene expression of plasmids, respectively. Cells were grown in a 2× YT medium (16 g tryptone, 10 g yeast extract, and 5 g NaCl/liter) and ampicillin (80 μg/ml) was included for the culture of strains carrying plasmids. All plasmids for expression of FtsH used in this study were generated by inserting a segment of wild-type or mutant *ftsH* gene derived from pAFH1, an expression plasmid of the *ftsH* gene from *T. thermophilus* HB8 [14], into pET23a. The *ftsH* fragment was made by polymerase chain reaction with a T7 forward primer and another primer (5′-CCGAATTCTCAGGCCCCGCCCAGGGCGCCCCCCG GCTTC-3′) that contained the C-terminal region of FtsH and a new

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*Eco*RI site. This fragment was digested with *Nde*I and *Eco*RI and the *Nde*I–*Eco*RI fragment was cloned into pET23a (Novagen). Mutations were produced by the Quick-Change method (Stratagene). The whole sequences of *ftsH* were confirmed by DNA Sequencing. ATP, ADP, and ATPγS were purchased from Roche Molecular Biochemicals. Casein (α-casein from bovine milk) was from Sigma. Enzchek Protease Assay Kits that used BODIPY-casein as a substrate were purchased from Molecular Probes, Eugene, OR. A fluorescent, monofunctional reactive dye, Cy3, was purchased from Amersham Pharmacia Biotech.

Purification of FtsH. *Escherichia coli* BL21 (DE3) harboring the above plasmids was grown at 37 °C to a mid-log phase in 1 liter of 2× YT medium with ampicillin (80 μg/ml). Expression of FtsH was induced by addition of isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM). Culture continued for another 3 h and cells were harvested. Cells were suspended in buffer A (50 mM Tris–Cl, pH 8.0, 50 mM KCl, 20% glycerol, 1 mM EDTA, and 4 mM dithiothreitol), disrupted by sonication, and subjected to a centrifugation at 100,000*g* for 40 min at 4 °C. The precipitated fraction was again washed with buffer A and dissolved in buffer B (buffer A containing 1% 1-*O*-*n*-octyl-β-D-glucopyranoside) with stirring for 60 min at 25 °C. The solutions were centrifuged at 100,000*g* for 60 min at 4 °C and the supernatant fraction was loaded onto a Reactive Red column (Sigma). The column was washed with buffer B and FtsH was eluted with a 80–500 mM KCl gradient. Fractions containing FtsH were diluted and loaded onto a Super-Q column (Tosoh) with buffer B. FtsH was eluted with a 50–250 mM KCl gradient. Fractions containing FtsH were collected and dialyzed against buffer B. Thus, the obtained FtsH preparation contained pure and homogeneous FtsH as shown in Fig. 1. Purified FtsH was stored at 80 °C until use.

Assays. ATPase activities of FtsH were assayed by measuring the amount of liberated inorganic phosphate according to the malachite green method [19]. Purified FtsH (5 μg in 99 μl buffer B) was mixed with 100 μl of 100 mM Tris–Cl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 50 μM $Zn(COOH)_2$, 4 mM dithiothreitol, and 0.6% 1-*O*-*n*-octyl-β-D-glucopyranoside and the mixtures were incubated at 55 °C for 2 min. The reaction was started by addition of 1 μl of 200 mM ATP and terminated at indicated times by addition of perchloric acid on ice. The mixture was reacted with the malachite green reagent [19] and after 40 min at room temperature, absorbance at 630 nm was measured. Protease activities were measured by using fluorescently labeled casein [20]. BODIPY-casein (25 μl, 1 mg/ml) in 50 mM Tris–Cl was mixed with 1 ml of 50 mM Tris–Cl buffer, pH 8.0, containing 50 mM KCl, 5 mM $MgCl_2$, 25 μM $Zn(COOH)_2$, 2 mM dithiothreitol, 0.3% 1-*O*-*n*-octyl-β-D-glucopyranoside, and 4 μg purified FtsH. The mixture was incubated for 2 min at 55 °C and 1 mM ATP was added to start FtsH-mediated digestion. Fluorescence at 530 nm (excitation light at 485 nm) was monitored. Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard [21].

Gel filtration analysis. Casein was labeled by Cy3 in the mixture that contained 50 μM casein and 150 μM Cy3 in 9 mM HEPES buffer, pH 7.5. The mixture was incubated for 2 h in the dark at room temperature and excess reagents were removed by passing through a NAP-5 column. FtsH (2 μM) and Cy3-casein (2.5 μM) were dissolved in the buffer containing 50 mM Tris–Cl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 25 μM $Zn(COOH)_2$, 2 mM dithiothreitol, and 0.3% 1-*O*-*n*-octyl-β-D-glucopyranoside, and 1 mM ATP, ATPγS or ADP when indicated. The solution was preincubated for 20 min at 55 °C and then loaded onto a gel filtration column (Superdex 200 HR 10/30, Amersham Pharmacia Biotech) that was equilibrated with the same buffer used for preincubation. Proteins were eluted at 25 °C with the same buffer and elution was monitored with absorbance at 290 nm and fluorescence at 570 nm (excitation at 550 nm). The molecular size markers used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa).

Results

A single, conserved Cys of FtsH is not essential

FtsH from *T. thermophilus* HB8 has a single Cys residue at the position 250 between Walker A and B motifs. This Cys residue is conserved among all bacterial FtsHs and the eukaryotic homologs so far known. Intending to introduce a new, single Cys at desired positions for specific chemical labeling of FtsH for future study, we made a cysteine-less mutant FtsH by replacing Cys250 with Ala. Compared with the wild-type FtsH, a resultant mutant, termed as C250A, had a similar activity to digest casein in the presence of ATP (Figs. 2A

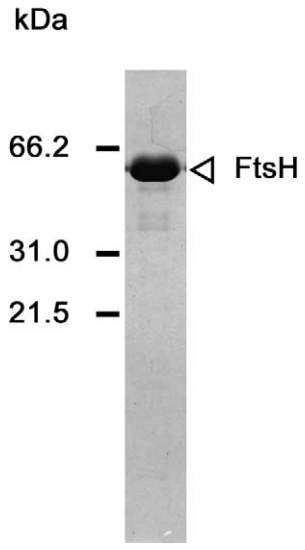

Fig. 1. Purity of *T. thermophilus* FtsH isolated from recombinant *E. coli* cells. Eight micrograms of proteins was loaded on 11% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

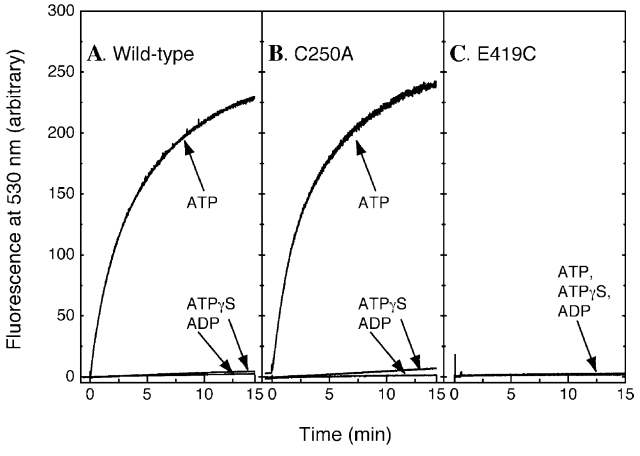

Fig. 2. Proteolytic activity of (A) the wild-type, (B) C250A mutant, and (C) E419C mutant FtsHs in the presence of 1 mM ATP, ATPγS, and ADP. During preincubation in the absence of nucleotide, no proteolysis occurred and nucleotide was added at the time zero. Proteolysis of fluorescently labeled casein was monitored by the increase of fluorescence. Other experimental conditions are described in Materials and methods.

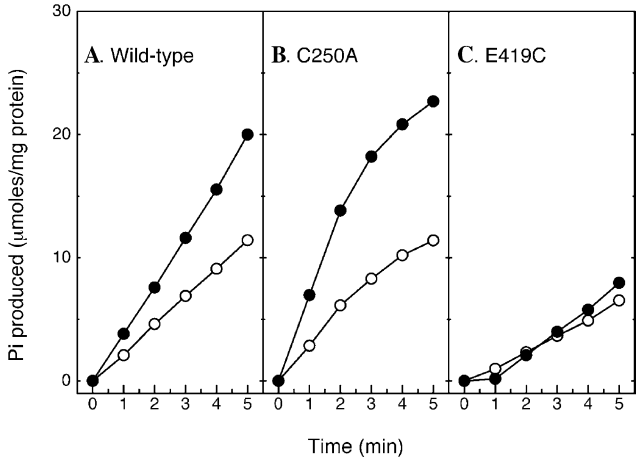

Fig. 3. ATPase activity of (A) the wild-type, (B) C250A mutant, and (C) E419C mutant FtsHs in the absence (open circle) or presence (closed circle) of casein. At the time zero, ATP (final concentration, 1 mM) was added to initiate the reaction and the amount of inorganic phosphate produced was measured. Other experimental conditions are described in Materials and methods.

and B). ATPγS, and ADP did not support efficient proteolysis. However, it was noticed that a very slow, but detectable digestion occurred when ATPγS was present (% of the protease activity in the presence of ATP; wild-type, 0.3%, C250A, 0.6%). ATPase activity of the C250A was also similar to that of the wild-type FtsH and was stimulated by casein (Figs. 3A and B). Thus, characteristics of the wild-type and C250A FtsH are similar to each other, suggesting that the conserved Cys250 is not essential for the function of FtsH.

A small ATPase activity of the E419 is not stimulated by casein

Second mutation, replacement of Glu at the position 419 in the zinc-binding motif (HEAGH) at the protease catalytic site by Cys, was introduced into the C250A background to generate a double mutant, termed as E419C. As expected, the E419C was unable to digest casein in the presence of ATP, ATPγS and ADP (Fig. 2C). Conversely, a small ATPase activity of this protease-deficient E419C mutant was not stimulated at all by casein (Fig. 3C). Therefore, it is clear that when there is no proteolysis of casein, there is no stimulation of ATPase by casein. These results imply that ATPase activity of the C250A (and wild-type) FtsH is composed of two fractions, the activity independent from proteolysis and the activity coupled with proteolysis.

E419 can form a stable complex with casein in the presence of ATPγS

The complex formation of FtsH-casein was analyzed with gel filtration chromatography. FtsH mutants, as well as the wild-type, were eluted as an apparently uniform oligomer (~410 kDa) under any conditions tested. FtsH was preincubated with Cy3-casein in the presence of ATPγS, ATP, ADP, or none and loaded onto a gel filtration column, which was equilibrated and eluted with a buffer containing the same nucleotide. The Cy3-casein was eluted alone and not associated with the C250A in the presence of any nucleotides (Fig. 4A). The same results were obtained for the wild-type FtsH (not shown). On the contrary, fluorescence of Cy3-casein was found at the position of the protease-deficient E419C mutant when ATP was present but not when ADP or no nucleotide was present (Fig. 4B). Noticeably, ATPγS also supports the stable complex formation. Other nonhydrolyzable ATP analog, AMP-PNP, was also examined and the result was similar to that observed for ATPγS (not shown). The analysis of the peak fraction at

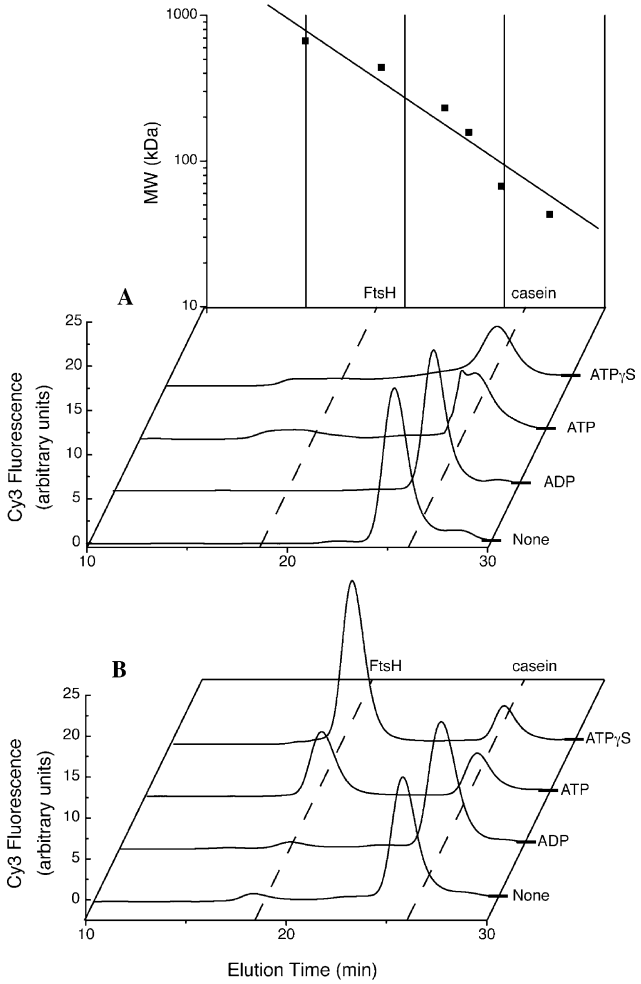

Fig. 4. Interaction of (A) the C250A and (B) E419C mutant FtsHs with Cy3-casein. FtsH was incubated with Cy3-casein in the presence of the indicated nucleotide and analyzed with gel filtration column chromatography eluting with the buffer containing the same nucleotide. Elution profiles of fluorescence at 570 nm of the Cy3-casein are shown. The positions of elution times of FtsH oligomer and free Cy3-casein are indicated by dashed lines. Other experimental conditions are described in Materials and methods.

18 min of the elution in the presence of ATPγS with polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed the presence of a casein band together with an FtsH band (not shown). Molar ratio of Cy3-casein and FtsH (as hexamer) in the preincubation mixture was 1.25 and about 60% of the casein was recovered as a complex with FtsH, indicating that a nearly stoichiometric complex (0.75 casein/FtsH) was formed. Thus, it appears that ATP binding to FtsH, but not hydrolysis, is necessary for the FtsH to tightly bind casein.

Discussion

It has been reported that a mitochondrial homolog of FtsH can bind unfolded protein in the absence of ATP [22]. Truncated monomer FtsH of *E. coli*, either a soluble domain of FtsH without N-terminal transmembrane segment or even a smaller AAA domain that contains conserved ATP-binding motifs, can bind unfolded proteins [15,17]. We also detected transient binding of casein to protease-active (wild-type and C250A) and protease-deficient (E419C) FtsHs of *T. thermophilus* in the absence of nucleotides (unpublished result). Therefore, FtsH by itself has an inherent ability to bind unfolded proteins. The results in this report indicate that this ability varies depending on nucleotide and protease activities. The protease-deficient FtsH, E419C, shows the ATP-dependent formation of the stable complex with casein. ATP binding, but not hydrolysis, is required and ADP destabilizes the complex. On the contrary, formation of the stable complex is not observed for the protease-active FtsH in the presence of ATP. Under this condition, bound casein is subjected to proteolysis and might be released from FtsH. The protease-active FtsH is also unable to produce the stable complex, even in the presence of ATPγS. Probably, only a trace activity of protease might be sufficient to cause dissociation of casein before or during gel filtration procedures. As mentioned, wild-type and C250A FtsHs showed very small, but detectable protease activities in the presence of ATPγS. It is possible that one of the intermediate steps in every catalytic cycle of proteolysis generates a conformational state in which FtsH only weakly binds its substrate protein. Thus, a speculation is possible that FtsH holds polypeptide segment(s) of substrate protein tightly upon occupation of nucleotide binding site(s) by ATP, sends it to the catalytic site(s) for proteolysis, cleaves peptide bond(s), loosens holding of the substrate protein when ATP is hydrolyzed, exchanges ADP by ATP at nucleotide binding site(s), and holds the next segment(s) of polypeptide tightly to initiate the next catalytic cycle. Obviously, further studies are needed to clarify the true mechanism of FtsH.

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