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Second transmembrane segment of FtsH plays a role in its proteolytic activity and homo-oligomerization

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Abstract The FtsH (HflB) protein of *Escherichia coli* is a membrane-bound ATP-dependent zinc protease. The role(s) of the N-terminal membrane-anchoring region of FtsH were studied by fusion with a maltose-binding protein (MBP) at five different N-termini of FtsH. The MBP-FtsH fusions were expressed in the cytoplasm of *E. coli*, and were purified as soluble proteins. The four longer constructs, which have a second transmembrane segment and the C-terminal cytoplasmic region in common, retained ATP-dependent protease activity toward heat-shock transcription factor σ^{32}, and were found to be homo-oligomers. In contrast, the shortest construct which has the C-terminal cytoplasmic region but not the second transmembrane segment showed neither protease activity nor oligomerization. Therefore, the second transmembrane segment, which neighbors the C-terminal cytoplasmic region of the FtsH, participates in not only its membrane-anchoring, but also its protease activity and homo-oligomerization.

Key words: ATP-dependent protease; FtsH/HflB; Homo-oligomerization; Water-soluble MBP-FtsH fusion; AAA protein family

1. Introduction

In *Escherichia coli*, some ATP-dependent proteases, such as Lon [1], ClpAP [2], ClpXP [3,4], HslUV [5], and FtsH [6], are known. Among them, only FtsH is a membrane-bound ATP-dependent protease [6,7], and is essential for cell growth [8]. FtsH belongs to the AAA (ATPases associated with a variety of cellular activities) protein family [9], whose members are widely found in various organisms. The members have conserved 200 amino acid modules, which are called AAA modules. They are associated with diverse cellular functions, such as cell cycle control, protein degradation, transcriptional regulation, protein secretion, and the biogenesis of organelles [10]. FtsH seems to have to do with various cellular functions. The phenotypes of *ftsH* mutants are temperature-sensitive growth (Ts^-) [11], a high frequency of lysogenization (Hfl^-) [12], a stop-transfer defect (Std^-) [8], and mRNA stabilization [13]. Also, more severe *ftsH* mutants, *tolZ* (except for *tolZ10* and *tolZ60* [14]) and Δ*ftsH*, require suppressor mutation(s) for survival [14,15], and show pleiotropic phenotypes, i.e. colicin tolerance (Tol^-) and no growth on succinate or other non-fermentable carbon sources (Nfc^-), as well as Ts^-, Hfl^- and Std^- [16,17].

FtsH consists of a membrane-anchoring region (amino acids 1 to 123) in the N-terminus and a cytoplasmic region (amino acids 124 to 647) in the C-terminus [7]. The membrane-anchoring region consists of two transmembrane segments and a periplasmic region in between. The membrane-anchoring region participates in homo-oligomerization [18], especially the periplasmic region [19]. The cytoplasmic region includes active-site motifs for ATPase and zinc-dependent protease. FtsH degrades several membrane proteins, such as the uncomplexed SecY protein [20,21], unassembled subunit a of the proton ATPase F_0 sector [22], and YccA [23], and also degrades several cytoplasmic proteins, heat-shock transcription factor σ^{32} [6,24], λphage cII [12] and cIII [25], and some mutant form of the λphage cI [26]. The purified FtsH protein was found to degrade its substrates in an ATP-dependent manner in in vitro experiments [6,21,26].

To determine further the biochemical characteristics of FtsH in vitro, we previously purified a soluble form of FtsH which has only the cytoplasmic C-terminal region, there was little or no protease activity (unpublished data). Therefore, we assumed that the membrane-anchoring region might be involved in the expression of the protease activity, and constructed fused proteins of maltose-binding protein (MBP) with truncated FtsH. We investigated the effect of truncation of the N-terminal membrane-anchoring region on the ATPase and protease activities, and the oligomerization of the fused proteins.

2. Materials and methods

2.1. Bacterial strain and DNA manipulation

The *E. coli* K-12 strain used in this study was JM109 [*recA1, endA1, gyrA96, hsdR17, supE44, relA1*,Δ(*lac-proAB*)/F′ *traD36, proAB*$^+$, *lac*^{q}, lacZΔM15] [27]. The recombinant DNA techniques were essentially as described [28]. DNA sequencing was performed by the dideoxynucleotide termination method with an Applied Biosystems DNA sequencer (model 310).

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2.2. Plasmids

The plasmid, pJN340, which has a 1.8-kb *Bam*HI fragment containing the *ftsH* gene [16], was digested with *Eco*RI and *Pst*I, and then the fragment was inserted into the cloning site of pMAL-c2 (New England Biolabs, Inc), which had been digested with *Eco*RI and *Pst*I, giving pMFC0. This pMFC0 plasmid codes for only MBP since FtsH is not in-frame. An *Eco*RI site was introduced into pAR145R [14] to make pM1, and pJN340 to make pM4 and pM5, by means of a QuikChange mutagenesis kit (Stratagene) with the following mutagenic primers: 5′-CTTGAGTGACATGGCGGAATTCCTAATACTCTGGC-3′, 5′-CGGTGAACCGCCTGAATTCCCAAGCCTGCTGG-3′, and 5′-GTGTCTGGATCTCCGAATTCCGTCAAATGCAGG-3′ (*Eco*RI site is underlined), with each complementary oligonucleotide, respectively. The three resultant plasmids, pM1, pM4 and pM5, were cut with *Cla*I and *Eco*RI, and then the fragments were inserted into the corresponding sites of pMFC0, giving pMFC1, pMFC4 and pMFC5, respectively. pAR145R was digested with *Apa*I, filled-in with Klenow enzyme, and then digested with *Cla*I. This *Apa*I (filled-in)-*Cla*I fragment was inserted into pMFC0, which had been digested with *Eco*RI (filled-in) and *Cla*I, giving pMFC2. pJN340 was digested with *Pst*I and *Sac*I, and then treated with Klenow enzyme to make a blunt end. This blunt-ended *Pst*I-*Sac*I fragment was ligated into the blunt-ended *Pst*I site of pMAL-c2, giving pMFC3. The cloned fragment was confirmed to have no unexpected mutations by DNA sequencing.

2.3. Purification of MBP-FtsHs

E. coli JM109 cells harboring a recombinant plasmid were cultured at 37°C in 0.2 l of LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl, adjusted with NaOH to pH 7.0) containing ampicillin (100 μg/ml) with a 1% inoculum of an overnight culture, and then induced by the addition of 0.3 mM IPTG at $A_{660} = 1$ for 1 h. The cells were collected by centrifugation, and then washed with 20 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 10 mM 2-mercaptoethanol. The washed cells were suspended in 20 ml of the same buffer containing 1 mM phenylmethanesulfonyl fluoride, and then disrupted by sonication. The sonicated sample was centrifuged at $4000 \times g$ for 40 min. The supernatant was applied to an amylose resin column (3 ml) equilibrated with the same buffer. After washing the column with the same buffer, the enzyme was eluted with the same buffer containing 10 mM maltose. The eluate was concentrated by ultrafiltration using Ultrafree-15 (Millipore Corp.), exchanging the buffer with 20 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl, 10 mM 2-mercaptoethanol and 1 mM maltose. The protein concentrations were measured with the Bio-Rad protein assay reagent (Bio-Rad Laboratories), with bovine γ-globulin as a standard.

2.4. Assay for ATPase activity

The assay for ATPase activity was carried out according to the method of Akiyama et al. [21], except that the assays were performed without a detergent.

2.5. Assay for proteolytic activity toward σ^{32}-C-his

σ^{32}-C-his was purified as described previously [29], and used as a substrate for MBP-FtsH proteins. The reaction for the proteolytic activity was performed essentially as described by Tomoyasu et al. [6] with some modifications. The complete reaction mixture (50 μl) consisted of the following: 50 mM Tris-acetate (pH 8.0), 5 mM magnesium acetate, 12.5 μM zinc acetate, 20 mM KCl, 100 mM NaCl, 2 mM 2-mercaptoethanol, 4 mM ATP, 2 μg of BSA, 3.26 μg of the purified MBP-FtsH, and 4 μg of purified σ^{32}-C-his (the molar ratio of MF1 and σ^{32}-C-his being 1:7). Reactions were performed at 42°C. Portions (4 μl) of the reaction mixtures were subjected to SDS-PAGE on 12% polyacrylamide gels [30], and then stained with Coomassie brilliant blue.

2.6. Analytical ultracentrifugation

Sedimentation velocity experiments were performed with a Beckman Optima XLA analytical ultracentrifuge equipped with absorbance optics and an An50Ti rotor. The experiments were performed at 4°C and 30 000 rpm with protein concentrations of 1.04, 1.50, 1.63, 1.45 and 1.56 mg/ml, for MF1 to MF5, respectively, ranging in OD_{280} from 0.8 to 1.0. The data were collected at 280 nm and at a spacing of 0.003 cm in the continuous scan mode. The data were analyzed with DCDT software to determine the distribution of sedimentation coefficients, $g^*(s)$, using the differences of absorbance profiles at successive times averaged over many differences [31].

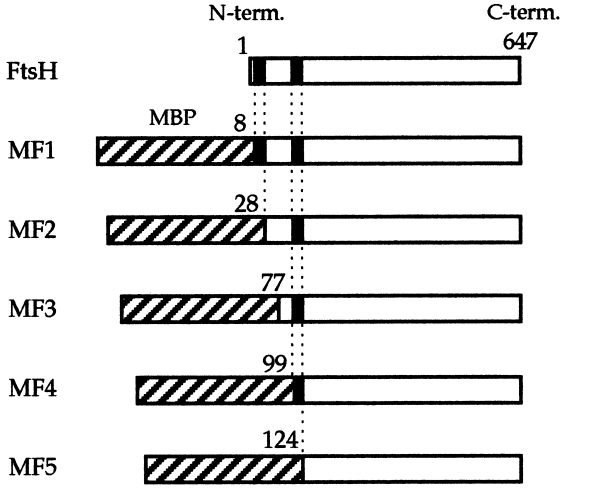

Fig. 1. Schematic representation of FtsH and the MBP-FtsH fusions (MFs). The MBP region is presented as a hatched box. Two membrane-spanning segments of FtsH are shown as filled boxes. Each fused point between MBP and FtsH is indicated as the number of amino acid residues from the N-terminus of FtsH.

2.7. Circular dichroism spectroscopy

Circular dichroism spectra were acquired with a Jasco Model 720 spectropolarimeter. Samples were prepared in 10 mM potassium phosphate (pH 8.0) with a protein concentration of 60 μg/ml. For helix content determination, the Protein Secondary Structure Estimation Program (Jasco, Japan) was used. This program is based on the method described by Yang et al. [32].

3. Results

3.1. Purification of MBP-FtsH fusions

Five MBP-FtsH fusions (MFs), named MF1 to MF5 (Fig. 1), were purified as soluble enzymes in a single step on an amylose resin column. On SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2), their polypeptide sizes corresponded well to those calculated from the coding sequence; 113 kDa, 110 kDa, 106 kDa, 102 kDa, and 99 kDa, for MF1 to MF5, respectively. We purified the MFs to more than 80% homogeneity, which was determined by densitometric scanning of a Coomassie brilliant blue-stained SDS-PAGE gel (Fig. 2). We assume that major contaminants are partially degraded MFs, because anti-FtsH reacted with the bands below the main bands on Western blotting (data not shown).

3.2. ATPase activity

All MFs retained ATPase activity (Table 1). The ATPase activity of MF1 was similar to that of intact FtsH [6] or FtsH-His_6-Myc [21] when compared per mole of protein. MF2 to

Table 1
ATPase activities of the MBP-FtsHs

Enzyme	ATPase activity (nmol/min/mg)
MF1	122
MF2	70.1
MF3	56.4
MF4	54.9
MF5	13.5

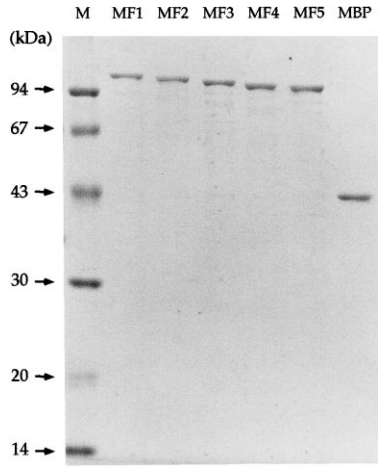

Fig. 2. SDS-PAGE of MFs. The proteins were fractionated by SDS-PAGE on a 12% polyacrylamide gel and then stained with Coomassie brilliant blue. M, the leftmost lane, indicates molecular mass markers.

MF4 showed reduced ATPase activity to some extent. MF5 showed rather low ATPase activity.

3.3. Degradation of σ^{32}-C-his

The four relatively large MFs, MF1 to MF4, degraded σ^{32}-C-his equally (Fig. 3). In contrast, MF5, which possesses only the cytoplasmic region, showed no proteolytic activity (Fig. 3). In the absence of ATP, none of the MFs degraded σ^{32}-C-his (data not shown). These results indicate the requirement of the second transmembrane segment for their proteolysis of σ^{32}-C-his. As can be seen in Fig. 3, the MFs having protease activity degraded σ^{32}-C-his without detectable intermediates.

3.4. Homo-oligomerization observed on analytical ultracentrifugation

To determine whether or not the MFs are in a homo-oligomeric state, all the purified proteins were subjected to sedimentation velocity analyses. $g^*(s)$ analysis showed a clear difference of MF5 from the others (Fig. 4). MF5 was supposed to be in a monomeric state since the molecular mass calculated from the roughly estimated sedimentation coefficient and diffusion coefficient was smaller than the size of a single polypeptide chain (data not shown). All the other four proteins, i.e. MF1 to MF4, formed large complexes with peaks from 12 S to 20 S. Faster sedimenting minor fraction seen as the peaks around 35 S would be an aggregated product for its very large

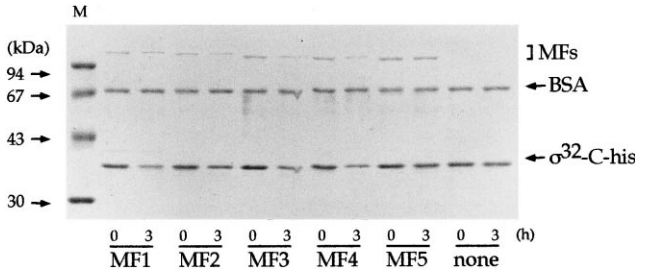

Fig. 3. Proteolysis of σ^{32}-C-his by MFs. Samples were analyzed by SDS-PAGE on a 12% polyacrylamide gel and then stained with Coomassie brilliant blue. The incubation times are indicated in the figure. M, the leftmost lane, indicates molecular mass markers.

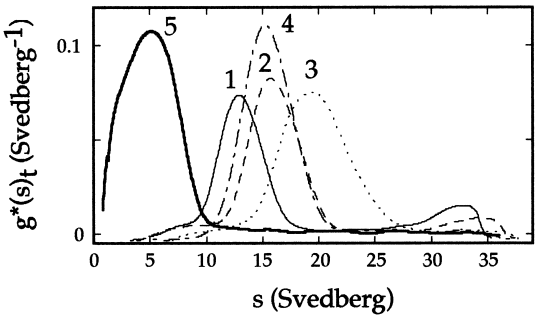

Fig. 4. Sedimentation coefficient distribution, $g^*(s)$, analyses of MFs. MF1 (thin line), MF2 (dashed line), MF3 (dotted line), MF4 (dot-dash line), and MF5 (thick line) were subjected to sedimentation velocity experiments as described in Section 2. The $g^*(s)$ plots were generated as described in Section 2 using DCDT software.

size and a small amount, considering from the report [33] of the tendency for FtsH-His_6-Myc to aggregate without ATP at 37°C. For the polydispersity and instability of MFs even in the storage at 4°C, we could not hold sedimentation equilibrium analysis to obtain accurate molecular masses.

3.5. Determination of secondary structure components

The estimated α-helix contents of the MFs and MBP on circular dichroism spectroscopy are shown in Table 2. For MBP, the α-helix content was in good agreement with that of the known three-dimensional structure [34]. For the MFs, the α-helix contents which originate from FtsH portion were also calculated (Table 2). All MFs had similar α-helix contents. This suggests that the loss of activity seen for MF5 is not due to a destruction of the secondary structure.

4. Discussion

ATP-dependent proteolytic systems have been widely found, and are probably essential for cells to survive. In eukaryotes, the 26 S proteasome is a major ATP-dependent protease, which consists of a number of proteins [35]. In *E. coli*, ATP-dependent proteases known so far, such as ClpAP [2], ClpXP [3,4], HslVU [5], Lon [1], and FtsH [6], each form a multimeric enzyme [2,18,36,37]. For example, HslVU consists of two different multimeric components, HslV and HslU. HslU (=ClpY) is the ATPase component, which belongs to the ClpY subfamily of the Clp family, and HslV is the protease component, which exhibits similarity to the β-type subunit of the 20 S proteasome [36]. Mutational analysis of HslU in the ATP-binding site revealed a functional relationship among ATP-binding, oligomerization, and peptide hydrolysis [38]. In the case of FtsH, mutations in the ATP-binding site reducing the ATPase activity affected the protease activity at

Table 2
α-Helix contents of the MBP-FtsHs and MBP

Construct	α-Helix content (%)	α-Helix content of FtsH portion (%)
MF1	30.6	25.0
MF2	32.5	27.9
MF3	30.4	24.1
MF4	32.3	27.0
MF5	31.5	25.3
MBP	39.4	–

the same time [21]. This implies the inseparability of a protease domain from an ATPase domain. Therefore, we suppose FtsH has an ATP-dependent protease domain.

The N-terminal transmembrane region is involved in the formation of a complex of FtsH in the cytoplasmic membrane [18], and FtsH solubilized with a non-ionic detergent can also be cross-linked to form an oligomeric structure [18], suggesting that FtsH molecules directly interact with each other. Moreover, the molecular mass was estimated to be about 1000 kDa on gel filtration chromatography of FtsH-His^6-Myc [19]. Recently, it was reported that the periplasmic region plays crucial roles in protein-protein interactions and modulates proteolytic functions [19].

In this study, five MBP-FtsH fusions, named MF1 to MF5, were constructed so as to have the full (MF1), partial (MF2 to MF4) or no (MF5) transmembrane region. We could obtain all the MFs as soluble enzymes without a detergent. The addition of MBP might increase solubility and inhibit membrane insertion. Probably transmembrane segments may interact with each other to minimize hydrophobic surface. The MFs having a second transmembrane segment, MF1 to MF4, formed homo-oligomers and showed ATP-dependent proteolytic activity toward σ^{32}-C-his. Such characteristics are the same as those of FtsH purified from membranes with a detergent [6]. Although MBP is known to form a dimer, we included the ligand, maltose, in the enzyme solution to prevent the dimerization of the MBP portion, for it is known that maltose provokes monomerization [39].

As for the ATP-dependent proteolysis of σ^{32}-C-his, the second transmembrane segment and the C-terminal cytoplasmic region were necessary and sufficient. Therefore we assume these constitute an ATP-dependent protease domain, and the rest, mostly the periplasmic domain, probably participate in its modulation. The ATPase activity of the MBP fusions was affected by the length of the retained N-terminal sequence of FtsH. This could be evidence that the periplasmic domain and the transmembrane segments participate in the modulation of ATPase activity.

Through analogy with the other ATP-dependent proteases [40], we suppose FtsH can interact with protein substrates and that its chaperone-like activity is promoted as to the unfolding or remodeling of substrates through ATP hydrolysis. At present it is known that a denatured alkaline phosphatase can associate with FtsH-His$_6$-Myc [33]. Moreover, an ATP-induced conformational change of FtsH-His$_6$-Myc itself was observed [33]. Our data suggested an intimate relationship among ATPase activity, protease activity and oligomer formation. Therefore we assume oligomer formation is needed for the ATP-driven structural change or the ability to extend substrate polypeptides. A recent study on Yme1, which is a mitochondrial homolog of FtsH, revealed chaperone-like activity for only its AAA domain [41], lacking a transmembrane region. They noted its low substrate affinity might be due to insufficient oligomerization. Here we showed that the second transmembrane segment of FtsH was indispensable for its proteolytic activity and its oligomerization.

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