Identification, Characterization, and Molecular Cloning of a Homologue of the Bacterial FtsH Protease in Chloroplasts of Higher Plants*§

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Marika Lindahl‡§, Sarit Tabak§§, Leland Cseke‖, Eran Pichersky‖, Bertil Andersson‡, and Zach Adam‡‡

From the ‡Department of Biochemistry, Archenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden, the ¶Department of Agricultural Botany, Faculty of Agriculture, The Hebrew University, Rehovot 76100, Israel, and the ‖Department of Biology, University of Michigan, Ann Arbor, Michigan 48109

In an attempt to identify and characterize chloroplast proteases, we performed an immunological analysis of chloroplasts using an antibody against Escherichia coli FtsH protease, which is an ATP-dependent metalloprotease bound to the cytoplasmic membrane. A cross-reacting protein of 78 kDa was found in the thylakoid membrane of spinach, but not in the soluble stromal fraction. Alkali and high salt washes, as well as trypsin treatment of thylakoid membranes, suggest that the chloroplastic FtsH protein is integral to the membrane, with its hydrophilic portion exposed to the stroma. The protein is not bound to any photosynthetic complex and is exclusively located in the stromally exposed regions of the thylakoid membrane. Its expression is dependent on light, as it is present in green pea seedlings, but absent from etiolated ones. An Arabidopsis cDNA was isolated, and the deduced amino acid sequence demonstrated high similarity to the E. coli FtsH protein, especially in the central region of the protein, containing the ATP- and zinc-binding sites. The product of this clone was capable of import into isolated pea chloroplasts, where it was processed to its mature form and targeted to the thylakoid membrane. The trans-bilayer orientation and lateral location of the FtsH protein in the thylakoid membrane suggest its involvement in the degradation of both soluble stromal proteins and newly inserted or turning-over thylakoid proteins.

Numerous examples of protein degradation in chloroplasts have been accumulated over the years. These include degradation of unassembled proteins, apoproteins lacking their prosthetic groups or pigments, photo- or otherwise-damaged proteins, and developmentally or environmentally regulated proteins, which can be imported into isolated chloroplasts, where it is targeted to the thylakoid membrane.

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§ These authors contributed equally to this research and should both be considered first authors.

**To whom correspondence should be addressed: Dept. of Agricultural Botany, The Hebrew University of Jerusalem, Rehovot 76100, Israel. Tel.: 972-8-948-1329; Fax: 972-8-946-7763; E-mail: zach@agri.huji.ac.il.

† The abbreviations used are: PSII, photosystem II; EST, expressed sequence tag; LHCII, light-harvesting complex of photosystem II; OEE33, the 33-kDa (psbO) subunit of the oxygen-evolving enzyme; PSI, photosystem I; CFI, coupling factor I.
FtsH Protease Homologue in the Thylakoid Membrane

Isolation and Fractionation of Chloroplasts—Spinach plant growth and chloroplast and thylakoid membrane isolation were performed as described previously (19). Intact chloroplasts were fractionated into stromal and thylakoid fractions by osmotic shock, followed by a 10-min centrifugation in a microcentrifuge. Pea seedlings were germinated in the light or dark to yield green or etiolated seedlings, respectively. Intact plastids were isolated from pea seedlings by centrifugation of homogenates through 40% Percoll cushion.

Thylakoid Treatments—To remove peripherally bound proteins, thylakoid membranes were washed with either 0.1 M NaOH or 2 M NaBr (20). Thylakoid membranes were treated with 10 μg/ml trypsin as described previously (20).

Fractionation of Thylakloid Membranes and Isolation of Protein Complexes—Thylakoid membranes were fractionated into appressed and stromally exposed regions either by mechanical disruption with a Yeda press, followed by separation in an aqueous two-phase partition system (21), or by digitonin fractionation followed by differential centrifugation (22). PSI, LHCII, cytochrome b6f, and PSI complexes were all isolated using established methods (23–26), respectively.

Western Blot Analysis—FtsH homologues were detected in the chloroplast with an antibody which was generously donated to us by Dr. T. Ogura of Kumamoto University, Japan. This antibody (17) has been generated against a synthetic peptide of 16 amino acids, which are highly conserved among FtsH-like proteins in different organisms. Other antibodies used as controls were against OEE33 and LHCII. All antibodies were used at a 1:1000 dilution. Immune complexes were reacted with a secondary antibody conjugated to alkaline phosphatase and visualized by developing the blots in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Cloning and Sequencing—The Arabidopsis ESTs data bank in EMBL was first searched for sequences with similarity to the aforementioned 16-mer antigenic peptide. The clone showing the highest similarity, designated 47FS77 (EMBL accession no. T14128), was obtained from the Ohio Arabidopsis Stock Center. A 1.3-kilobase SalI-XbaI fragment from this clone was used as a probe for isolation of a full-length cDNA clone from a size-fractionated (2–3-kilobase) cDNA library from Arabidopsis (27). Sequencing was done by the dideoxy method.

In Vitro Translation and Import into Isolated Chloroplasts—The cDNA encoding the FtsH protein was subcloned into psP72, and transcribed and translated using a coupled transcription-translation kit (Promega), with [35S]Met as a radiolabel. The radiolabeled precursor protein was imported into isolated pea chloroplasts as described previously (28).

RESULTS

Growing evidence for the existence of the FtsH metalloprotease family in different organisms led us to test whether this protein occurs in higher plant chloroplasts as well. Its possible existence in chloroplasts was supported by the recent identification of homologous genes in the chloroplast genomes of red and brown algae (9, 10), and by indications of homologous sequences among Arabidopsis ESTs. However, the occurrence of this protein in yeast mitochondria suggested that at least some of the Arabidopsis ESTs may encode a mitochondrial protein. We therefore chose to explore the possible existence of a FtsH-like protein in chloroplasts by using a specific antibody, rather than a DNA-based approach (Southern blot or polymerase chain reaction), which could lead to the identification of a gene that would eventually turn out to encode an extra-chloroplast protein. Moreover, this direct approach could pave the way for biochemical analysis of a potential protease in the complex and heterogeneous chloroplast organelle.

The antibody used in this study was generated against a synthetic peptide of 16 amino acids, which matches a sequence located in the hydrophilic region of the bacterial protein (17). Western blot analysis of intact and fractionated spinach chloroplasts revealed a single protein of ~78 kDa cross-reacting with the antibody (Fig. 1). This protein was found exclusively in the thylakoid-membrane fraction, but not in the soluble stromal one. No cross-reacting bands were observed when a pre-immune serum was used (data not shown).

Following the demonstration of the FtsH-like protein’s existence in the thylakoid membrane, we characterized its topological organization there. Membranes were washed with either 0.1 M NaOH or 2 M NaBr, both known to remove peripherally bound proteins from membrane surfaces. The effectiveness of such a treatment is demonstrated in Fig. 2B. While integral membrane proteins such as LHCII are impervious to these washes, peripheral proteins such as subunits of CFI are washed off. When the NaOH-treated membranes were analyzed with the specific antibody for FtsH, the 78-kDa protein remained associated with the membrane and no traces of it were found in the wash supernatant (Fig. 2A). Similar results were obtained with NaBr-washed membranes (data not shown). These results suggest that chloroplastic FtsH is indeed an integral membrane protein rather than a peripheral one. This finding is consistent with the hydrophobic nature, including two membrane-spanning helices, of FtsH in E. coli (17).

To further describe the topology of the protein in the membrane, thylakoids were treated mildly with 10 μg/ml trypsin for 5 min and then pelleted by centrifugation. This treatment is expected to digest proteins exposed to the stromal side of the membrane, which is analogous to the cytoplasmic side of the E. coli plasma membrane. The effectiveness of this treatment could be observed in the typical cleavage of the surface-exposed N terminus of LHCII (29), which shortened the protein by 2–3 kDa (Fig. 2B). When these trypsin-treated thylakoids were analyzed by Western blot, FtsH was no longer observed (Fig. 2C). However, a soluble cross-reacting fragment of ~40 kDa was recovered in the supernatant (data not shown). Control probing of the same membrane with an antibody against the luminal OEE33 subunit revealed that this protein was not affected by the trypsin treatment, indicating that the thylakoids remained sealed and retained their correct sidedness. Taken together, the results from the proteolysis experiment suggest that the major bulk of the FtsH protein, containing the hydrophilic region against which the antibody was generated, is exposed to the stromal side of the thylakoid membrane, consistent with the topology of the bacterial protein.

The well documented lateral heterogeneity of the thylakoid membrane, with PSI in the appressed region and PSII in the non-appressed one (30, 31), raised the question of where exactly the chloroplast homologue of FtsH was located. To answer this, the thylakoid membranes were mechanically fragmented by Yeda press and subsequently fractionated by centrifugation and phase partition (30). Western blot analysis of the mem-
brane subfractions revealed that, as compared with intact thylakoids, the protein was less abundant in grana stacks (Fig. 3, lanes 2 and 4) and almost totally absent from the appressed regions of the grana (Fig. 3, lane 5). In contrast, the stromal thylakoids were enriched with this protein (Fig. 3, lane 6). Similarly, stromal thylakoids obtained by digitonin fractionation (22) were highly enriched with FtsH protein, as compared to grana stacks (data not shown).

The possible association of the chloroplastic FtsH protein with photosynthetic complexes was also examined. PSI, LH-CII, cytochrome b_{6}f, and PSI complexes were isolated and immunologically analyzed, revealing that FtsH is not associated with any of them (data not shown).

Initial experiments to characterize the expression of FtsH were performed by investigating the effect of light on its accumulation. The protein was observed in plastids isolated from pea seedlings germinated in the light, but was absent from etiolated seedlings (Fig. 4). When etiolated seedlings were transferred to the light for 24 h, the FtsH protein accumulated to a level comparable with that found in light-grown seedlings (Fig. 4). Probing the lower portion of this blot with an antibody against LHCII revealed similar patterns for LHCII and FtsH accumulation (Fig. 4).

The above characterization of FtsH in chloroplasts prompted us to clone the corresponding gene. We first searched the Arabidopsis ESTs data bank for similarity to the 16-mer peptide against which the antibody was generated. The most similar clone was 47FST7, which deviated from the E. coli sequence only by one amino acid (Ser instead of Pro). The insert from this clone was used to probe an Arabidopsis cDNA library. The nucleotide and deduced amino acid sequences of the full-length cDNA encoding the FtsH protein are presented in Fig. 5. The protein, 709 amino acids long, contains two hydrophobic α-helices in its N-terminal region (residues 66–89 and 204–224), long enough to span a membrane, two motifs of a putative ATP-binding site (residues 298–309 and 357–362), and a zinc-binding domain (residues 524–528). Comparison of the deduced amino-acid sequence to the FtsH protein in E. coli shows that the two proteins are 49% identical and 68% similar. The ATP-binding site and the zinc-binding domain, as well as the antigenic peptide, are all highly conserved (Fig. 6A). In addition, the location of the second putative trans-membrane α-helix is also conserved. The similarity between the two proteins starts only at residue 111 of the plant protein (Fig. 6A), indicating that the plant protein is longer at its N terminus, consistent with the requirement for a transit peptide. The high abundance of Ser residues at the N terminus (Fig. 5) is also consistent with this observation. Comparison of the hydropathy plots of these two proteins shows that they are very similar beyond the second trans-membrane region (Fig. 6B), but the hydrophilic loop connecting the two membrane-spanning regions in the Arabidopsis protein is longer.

To confirm that the cloned gene does indeed encode a chloroplast protein, we tested for the capability of its product to import into isolated chloroplasts. The Arabidopsis FtsH cDNA
was transcribed and translated in vitro to yield a radioactive precursor. Following incubation with intact pea chloroplasts, stromal and thylakoid fractions were isolated, resolved by SDS-PAGE, and autoradiographed. The results presented in Fig. 7 demonstrate that the precursor protein (80 kDa) is imported into the chloroplast, where it is processed to a mature form (78 kDa) and targeted to the thylakoid membrane. These results are consistent with the localization of the FtsH protein as observed in the immunoblots (Fig. 1).

**DISCUSSION**

Intensive research into chloroplast function over the years has resulted, in addition to detailed descriptions of many bioenergetic processes (32), in the demonstration and characterization of several proteolytic processes of specific proteins (see, for example, Refs. 2, 3, 19, and 33–39). Surprisingly, little is known about the proteases involved in these processes. Taking a complementary approach to the identification of chloroplast proteases, we attempted to identify homologues to proteases known from bacterial systems among chloroplast proteins. We found this approach reasonable in light of the recent evidence that chloroplasts contain a homologue of the well-characterized, ATP-dependent bacterial Clp protease (1, 6–8, 39), and of the general prokaryotic characteristics of chloroplasts. In this study, we demonstrate both immunologically and by cloning the corresponding gene that plant chloroplasts contain a homologue of the bacterial ATP-dependent, membrane-bound FtsH protease, and characterize its membrane topology and expression. The structural predictions based upon the sequence data suggest that the folding of the plant FtsH is consistent with our biochemical/immunological analyses and with the characteristics of the bacterial enzyme.

The existence of an FtsH-like protein in a plastid other than chloroplasts has been demonstrated recently (40). In that case, a protein involved in vesicle fusion in red pepper chromoplasts exhibited a significant sequence homology to bacterial FtsH. However, in contrast to the bacterial protein which is bound to the cytoplasmic membrane (17), and to our present finding that the protein is an integral thylakoid-membrane protein (Figs. 2 and 7), the pepper protein was found in the soluble chromoplast stroma. This localization of the pepper protein to the stroma presents an ambiguity, because the reported sequence of the pepper gene (40) contains the two hydrophobic trans-membrane helices (dashed underline), two regions of a putative ATP-binding site (double underline), a region homologous to the bacterial 16-mer against which the antibody was generated (underline), and the zinc-binding site (box).

**Fig. 5.** Nucleotide sequence and deduced amino-acid sequence of Arabidopsis FtsH. Nucleotide sequence (upper line) of an Arabidopsis cDNA encoding FtsH protein is presented. Also indicated are putative trans-membrane helices (dashed underline), two regions of a putative ATP-binding site (double underline), a region homologous to the bacterial 16-mer against which the antibody was generated (underline), and the zinc-binding site (box).
brane helices that render the bacterial protein integral to the membrane.

The topology of the FtsH protein in the thylakoid membrane is defined both by its transverse orientation and its lateral heterogeneity (Fig. 8). It is excluded from the appressed regions, and found exclusively in the stromally exposed regions. The transverse orientation of the protein is consistent with that of bacterial FtsH. The major bulk of the protein extruding out of the membrane, in which the ATP-binding and catalytic sites are exposed, is exposed to the corresponding ATP-containing compartments: the cytosol of the bacterial cell and the stroma of the chloroplast (Fig. 8).

The two known substrates of FtsH in E. coli are a soluble protein (σ22; Ref. 12) and non-complexed forms of a membrane protein (SecY; Ref. 14). The trans-bilayer orientation and lateral location of the chloroplastic FtsH makes it ideally suited for a similar kind of function. It is in contact with stromal soluble proteins, and with all newly synthesized thylakoid proteins, which are initially integrated into the membrane in the stromally exposed regions (37, 41, 42). Moreover, PSII proteins undergoing turnover have been shown to migrate from appressed to non-appressed regions prior to degradation (2, 3).

Thus, one can expect to find both thylakoid and stromal proteins among the substrates of chloroplastic FtsH.

The FtsH protein could not be found in etioplasts of pea seedlings, suggesting that its expression is dependent on light. This is in contrast to the constitutive expression of Clp protease in chloroplasts (1, 6); illumination only increases the levels of the regulatory subunit pre-existing in the dark (7), but has no effect on the level of the catalytic subunit. The significance of these differences is not yet clear, but it may suggest that the FtsH protease takes part in degrading proteins whose synthesis and assembly are light-dependent. However, the physiological role and specific substrates of chloroplastic FtsH need to be further explored before any conclusions can be drawn.

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