Identification and Characterization of DegP, a Serine Protease Associated with the Luminal Side of the Thylakoid Membrane

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The proteases involved in proteolytic degradation in the thylakoid lumen are largely unknown. Western analysis with an antibody against the *Escherichia coli* periplasmic serine protease DegP suggested that pea chloroplasts contain a homologue of this protease. This homologue was peripherally bound to the luminal side of the thylakoid membrane and could only be removed by a combination of high salt and non-ionic detergent. Its level increased almost 2-fold in pea seedlings exposed to elevated temperature for 4 h, suggesting this protease's role in the chloroplast's heat response. Isolated thylakoid membranes containing the chloroplastic homologue of DegP degraded β-casein, an in vitro substrate of the bacterial protease. This activity was partially inhibited by a serine protease inhibitor, suggesting that at least part of the casein-degrading activity in the thylakoid membrane is attributable to DegP. The existence of chloroplastic DegP was further supported by isolating a full-length *Arabidopsis* cDNA (designated *AtDegP*) encoding a protein that is 37% identical and 60% similar to the *E. coli* protease. The amino terminus of the deduced amino acid sequence contained a bipartite transit peptide, typical of proteins targeted to the thylakoid lumen, and the mature portion of the protein contained the highly conserved serine protease catalytic triad His-Asp-Ser. The possible physiological roles of chloroplastic DegP protease are discussed.

The chloroplast, the photosynthetic organelle of eukaryotic cells, is composed of six compartments: three different membranes and the three aqueous compartments delimited by them. The chloroplast envelope, composed of an outer membrane, an inner membrane, and an intramembrane space, surrounds the stroma, the soluble compartment where most carbon metabolism reactions take place. The third membrane is the extensive network of thylakoid membranes, harboring the photosynthetic antennae, the photosynthetic electron transport system, and the ATP synthesis machinery. The sixth compartment is the thylakoid lumen, into which protons are pumped to form a proton gradient across the thylakoid membrane. This gradient is the driving force for ATP synthesis. Proteins found within the lumen, either soluble or bound to the inner side of the thylakoid membrane, include the oxygen-evolving complex of photosystem II, components of the photosynthetic electron transport system, and many as yet unidentified proteins. As in other biological systems, maintenance of the lumen is expected to require protein degradation to remove damaged or otherwise nonfunctional proteins. However, the proteolytic machinery in this compartment has never been detailed.

Many examples of protein degradation in the chloroplast have been documented (for review, see Ref. 1). Specific degradation of luminal proteins has also been demonstrated, with plastocyanin being the best characterized example. When *Chlamydomonas* cells are grown in a Cu²⁺-deficient medium, apoplasocyanin is synthesized, imported into the chloroplast, and translocated across the thylakoid membrane; however, it fails to accumulate due to rapid degradation (2). A similar observation was made recently in an in vitro system. Apoplastocyanin was sensitive to proteolytic digestion, whereas the native or reconstituted holoproteins were not (3). This increased sensitivity of the apoprotein probably resulted from its lacking the characteristic secondary structure displayed by the holoprotein, as revealed by circular dichroism spectroscopy (3).

In another recent study, truncated forms of a 23-kDa subunit of the oxygen-evolving complex, OE23, were correctly targeted to the lumen and processed to their mature size, but then rapidly degraded (4). These results suggest that the lumen contains its own proteolytic machinery, capable of degrading unassembled or damaged soluble proteins. It also implies that luminal proteases participate in the degradation of thylakoid membrane proteins exposed to the lumen.

Limited proteolytic processing also occurs in the lumen. Nuclear encoded thylakoid proteins, targeted to the lumen by N-terminal “thylakoid transfer” signals, are processed to their mature forms by a membrane-bound thylakoid-processing peptidase (5–7) that has not yet been purified or cloned. The plastid-encoded D1 protein of the photosystem II reaction center is also processed in the lumen. It is synthesized with a C-terminal extension of 8–16 amino acids that is removed within the thylakoid lumen immediately after synthesis (8, 9). Why this protein is synthesized with this extension is not clear, but its proteolytic removal is necessary to establish active photosystem II (10). The enzyme involved in this activity has been partially purified (8, 11) and characterized as a monomeric protein of 45 kDa (12). Recently, the gene encoding this peptidase was isolated from cyanobacteria (13) and spinach (14) and was found to be homologous to the periplasmic protease Tsp (tail-specific protease) of *Escherichia coli*, which is responsible for C-terminal processing of at least two bacterial proteins (13–16). Interestingly, it is also responsible for complete degradation of some proteins in *E. coli* (16). However,
whether the luminal homologue of Tsp can also participate in complete degradation or, alternatively, its activity is limited to C-terminal processing of the D1 protein is unknown.

The occurrence of a homologue of the bacterial periplasmic protease Tsp in the lumen suggests the existence of other periplasmic protease homologues there. This idea is also supported by the notion that the lumen is functionally and evolutionarily equivalent to the bacterial periplasm. Both are compartments into which protons are pumped by the respective ATPases, and the machinery responsible for protein translocation into the lumen resembles that into the bacterial periplasm (7, 17).

To identify other homologues of bacterial periplasmic proteases in the lumen, we initiated an immunological screening and data base search for a chloroplastic homologue of the bacterial periplasmic serine-type heat-shock protease DegP (also known as Htra or Protease Do) (18–22). A DegP homologue was found in the thylakoid, strongly associated with the inner side of the thylakoid membrane. This suborganellar fraction also exhibited the ATP-independent, serine-type β-casein-degrading activity typical of bacterial DegP. Moreover, expression of the chloroplastic homologue was rapidly stimulated by high temperature. Isolation of a cDNA encoding this protein and features of its sequence support its localization and proposed activity.

EXPERIMENTAL PROCEDURES

Plant Growth—Pea seedlings (Pisum sativum var. Alaska) were germinated and grown at 25 °C for 10–12 days under a 12-h photoperiod, at a light intensity of 150 μmol einstein m−2 s−1. To test the effects of high temperature, seedlings were thoroughly watered and then transferred to an incubator kept at 40 °C.

Chloroplast Isolation and Fractionation—Intact chloroplasts were purified on Percoll gradients (23). They were subfractionated by osmotic shock in 10 mM Hepes, pH 8.0, followed by a 10-min centrifugation in an Eppendorf centrifuge at 4 °C. The supernatant, containing stromal extract, was separated from the pellet containing intact thylakoids; the latter was then washed twice and resuspended in 110 mM sorbitol and 17 mM Hepes-KOH, pH 8.0, to 2 mg of chlorophyll/ml. Intact thylakoids were further fractionated by sonication on ice, three times for 10 s each, with 1-min cooling intervals. The sonicated thylakoids were then centrifuged for 5 min in an Eppendorf centrifuge at 4 °C to remove intact thylakoids, and thylakoid membranes were separated from the lumen extract by ultracentrifugation of the supernatant at 200,000 × g for 1 h.

The pellet containing thylakoid membranes was resuspended in 10 mM Hepes-KOH, pH 8.0, to 2 mg of chlorophyll/ml.

Intact Thylakoid and Thylakoid Membrane Treatments—Intact thylakoids, as well as thylakoid membranes, were incubated in 0.1 mg/ml thermolysin on ice for 15 min. The reaction was terminated by adding a 5 mM EDTA. Intact thylakoids were then collected by a 5-min centrifugation in an Eppendorf centrifuge at 4 °C; washed once in 10 mM Hepes-KOH, pH 8.0; and resuspended in the same medium. Thylakoid membranes were collected by ultracentrifugation at 200,000 × g for 30 min. Intact thylakoids and thylakoid membranes were finally resuspended in 10 mM Hepes-KOH, pH 8.0, to a concentration of 1 mg of chlorophyll/ml.

To characterize the association between the proteins and thylakoid membranes, they were washed in high salt and/or low detergent concentrations. The membranes were resuspended in 1 mM NaBr, 1 mM NaCl, or 1 mM NaCl supplemented with 0.05% Triton X-100 and incubated on ice for 30 min prior to ultracentrifugation at 200,000 × g for 30 min. The pellets were resuspended in 10 mM Hepes-KOH, pH 8.0, to a concentration of 1 mg of chlorophyll/ml.

Polyacrylamide Gel Electrophoresis and Immunoblot Analysis—Proteins were resolved on polyacrylamide gels (23) and blotted onto nitrocellulose membranes. DegP was detected with an antibody against the E. coli protein (18), generously donated by B. Lipinska (University of Gdańsk, Gdańsk, Poland). This antibody was used at a 1:100 dilution. Antibodies against the luminal 33-kDa subunit of the oxygen-evolving complex, OE33 (donated by N. Hoffman, Carnegie Institution), were used at a dilution of 1:10,000. Samples contained 7 and 0.1 μg of chlorophyll/lane (or their equivalent for stroma and lumen samples) for detection of DegP and OE33, respectively. Antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL), and bands were quantified by laser densitometry.

Casein Degradation Assay—Casein-degrading activity was determined using a method previously described to assay bacterial DegP activity (18). Reaction mixtures for the proteolytic activity assay contained 0.3 mg/ml β-casein (Sigma), 0.4 mg of chlorophyll/ml of thylakoid membranes, and 50 mM Tris-HCl, pH 6.8, and the reaction was carried out at 37 °C. Where indicated, 1 mM phenylmethylsulfonyl fluoride and 4 mM o-phenanthroline were included in the reaction mixture. Proteins were then resolved by SDS-polyacrylamide gel electrophoresis on 15% acrylamide gels, and the relative amount of casein in each sample was quantified by laser densitometric scanning of the Coomassie Blue-stained gel.

Cloning and Sequencing of AtDegP cDNA—Arabidopsis expressed sequence tag 125C11T7 showing homology to E. coli DegP and the Arabidopsis cDNA library λ-PRL2 were obtained from the Arabidopsis Biological Resource Center at Ohio State University. A 680-base pair BamHI fragment was isolated from the expressed sequence tag clone, radiactively labeled, and used as a DNA probe to screen the cDNA library. Isolated phages were excised in vivo, plasmid DNA was prepared, and the longest clones were further analyzed. Automated sequencing of both strands was performed in an Applied Biosystems Model 373 DNA Sequencer. DNA and deduced amino acid sequences were analyzed with the Genetics Computer Group program.

RESULTS

Thylakoid Membranes Contain a Homologue of DegP Protease—On preliminary immunoblots of pea chloroplasts with an antibody against bacterial DegP, we detected a single band with apparent molecular mass of ~40 kDa (Fig. 1A), suggesting that this protease has a homologue in chloroplasts. When chloroplasts were fractionated, the cross-reacting protein was detected in the intact thylakoid fraction, protected from external protease treatment (Fig. 1B). Similar results were obtained using an antibody against OE33, localizing chloroplastic DegP to within the thylakoid. To further characterize the intragranellar location of DegP, intact thylakoids were sonicated and subjected to ultracentrifugation to separate the thylakoid membranes from their soluble lumen contents. DegP was associated exclusively with the thylakoid membrane, with no traces detectable in the lumen, whereas OE33 was equally distributed between the two fractions (Fig. 1B). These results
suggested that DegP is strongly associated with the inner side of the thylakoid membrane.

The association of DegP with the thylakoid membrane was further characterized by subjecting membranes recovered after sonication to different treatments followed by immunoblot analysis. High salt washes with a 1 M concentration of either NaBr or NaCl, treatments that were effective in removing a considerable amount of OE33 from the membrane, failed to remove DegP (Fig. 1C). DegP could only be washed off the membrane when a low concentration of detergent was added to the salt. The low concentration of Triton X-100 used in this experiment (0.05%) was not sufficient to solubilize the membrane, suggesting that DegP is peripherally associated with the thylakoid membrane via hydrophobic rather than electrostatic interactions. Consistent with the contention that DegP is a peripheral membrane protein was its high sensitivity to proteolytic treatment (Fig. 1C). The residual signal left after protease treatment suggested that following sonication, most of the thylakoid membranes are in an inside-out orientation.

Expression of Chloroplastic DegP Is Stimulated by Heat—Bacterial DegP is a heat-shock protein, necessary for survival at elevated temperatures (18). To test the effect of heat on chloroplastic DegP expression, we transferred pea seedlings grown at 25 °C to 40 °C. Increased levels of DegP were observed after 2 and 4 h, with a decrease after longer exposures to high temperature (Fig. 2). The transient increase in DegP levels in response to elevated temperature was in contrast to the constant level of OE33 observed throughout the experiment. Similar to OE33, levels of the stromally exposed thylakoid protease FtsH (24) also remained unchanged (data not shown). Thus, it appears that in addition to a constitutive role, DegP may play a role in the chloroplast’s response to transient elevated temperatures.

Thylakoid Membranes Contain a β-Casein-degrading Serine Protease—β-Casein is the preferred substrate to assay bacterial DegP activity under in vitro conditions (18). We therefore tested whether the chloroplast fraction containing the DegP homologue also exhibits β-casein-degrading activity. When thylakoid membranes prepared by sonication followed by centrifugation were incubated with β-casein, >50% of the casein was degraded within the first 60 min (Fig. 3A). Addition of 0.2% Triton X-100 only slightly increased the degradation rate. Together with the aforementioned conclusion that the thylakoid membranes were for the most part inside-out (Fig. 1C), this suggested that most of the casein-degrading activity resides on the inner side of the thylakoid membrane and that no additional molecules of the enzyme are exposed by solubilizing the membrane.

To determine the class of protease involved in β-casein degradation by thylakoid membranes, protease inhibitors were included in the reaction mixture. Phenylmethylsulfonyl fluoride and o-phenanthroline, inhibitors of serine proteases and metalloproteases, respectively, showed considerable inhibition (Fig. 3B), whereas inhibitors of cysteine proteases had no effect (data not shown). A mixture of phenylmethylsulfonyl fluoride and o-phenanthroline almost totally inhibited the degradation of β-casein (Fig. 3B). These results suggest that both metalloproteases and serine proteases are involved in this degradation activity and that the serine-type proteolytic activity may be attributed to DegP. It should also be noted that the casein-degrading activities were independent of ATP (data not shown).

A Plant cDNA Encodes a Homologue of DegP—A search of the Arabidopsis data base revealed an expressed sequence tag showing considerable homology to bacterial DegP, including its active serine region. Since this cDNA was only a partial clone, we used it as a DNA probe to isolate the full-length homologue from an Arabidopsis cDNA library. The resultant 1477-base
indicated by ma- and lumen-targeting domains of the bipartite transit peptide are
sequence (Arabidopsis lower line residues are boxed).

The nucleotide sequence (DegP.

be either at Ser-43 or at Ser-51, based on the occurrence of Ile
loosely conserved among different chloroplastic proteins (25),
Since the recognition site of the stromal peptidase is only
enough to span the membrane (data not shown). However,
interaction since it is destabilized by low concentrations of
association appears to be mediated by hydrophobic
this protein has recently been shown to interact with phospho-
this protein has recently been shown to interact with phospho-
membrane (22), with the thylakoid lumen being considered the
membrane, making it available for cleavage of luminal soluble
proteins as well as hydrophilic portions of thylakoid membrane
proteins exposed to the lumen. The localization of DegP to the
inner side of the thylakoid is consistent with the occurrence of
its bacterial homologue on the periplasmic side of the inner
membrane (22), with the thylakoid lumen being considered the
functional and evolutionary equivalent of the bacterial
periplasm. In this respect, it is also interesting to note that
members of the α-lytic endopeptidase family (also designated as the S2 family
of serine proteases (26).

The DegP-membrane association is fairly strong, as evidenced by its resistance to high salt washes that remove OE33, another peripheral thylakoid membrane protein. Similar resistance to such washes has been observed with bacterial DegP (22). This association appears to be mediated by hydrophobic interactions since it is destabilized by low concentrations of detergent. Hydrophobic interactions between bacterial DegP and the inner membrane can also be implied by the fact that this protein has recently been shown to interact with phospholipid membranes (22). Hydrophyt plots of both the Arabidopsis and E. coli proteins did not reveal any hydrophobic sequence long enough to span the membrane (data not shown).

However, the occurrence of shorter (~10 residues) hydrophobic stretches may explain their strong interactions with membranes.

Comparison of the amino acid sequence of chloroplastic DegP with the Arabidopsis data base revealed two related genomic clones (FSI14.16 and FSI14.17) found on chromosome 1. These genes were clearly different from the cDNA isolated in this work and are unlikely to encode chloroplastic proteins because they lack putative transit peptides. However, since their N termini are similar to the bacterial signal peptide, they may be

Fig. 4. Nucleotide and deduced amino acid sequences of At-
DegP. The nucleotide sequence (upper line) and deduced amino acid sequence (lower line) of an Arabidopsis cDNA are presented. The
stroma- and lumen-targeting domains of the bipartite transit peptide are indicated by single and double underlining, respectively. Catalytic
residues are boxed.
translocated through the secretory pathway. Localization of the products of these two genes to cellular sites other than plastids can also be envisioned, as gene homologues of DegP have been recently identified in mammalian cells (27).

Although the physiological substrates of chloroplastic DegP are unknown, one may speculate that it participates in the degradation, or at least partial cleavage, of either soluble or membrane proteins. For example, proteolytic degradation of the photosystem II reaction center D1 protein involves two initial ATP-independent cleavage events in luminal loops connecting transmembrane helices, and the protease(s) involved are of the serine type (for review, see Ref. 28). The possible involvement of DegP in D1 protein degradation awaits direct examination. Efficient degradation of proteins extruding from both sides of the membrane, such as D1, is expected to require protease activity at both membrane surfaces. In this respect, DegP may be an important addition to the FtsH protease found in plastids can also be envisioned, as gene homologues of DegP have been recently identified in mammalian cells (27).

Of particular interest was the observation that the steady-state level of chloroplastic DegP increases rapidly upon exposure of seedlings to elevated temperatures. This finding is consistent with the heat-stimulated expression of the bacterial gene (18) and a homologous gene in algae (27). Under these conditions, proteins may undergo conformational changes, resulting in irreversible aggregation. One of the roles of DegP may be to prevent this by degrading heat-denatured proteins in the thylakoid lumen. Our findings provide tools for studying the physiological roles of this newly discovered chloroplastic protease.

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