Degradation of unassembled and damaged thylakoid proteins

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Abstract
To study protein degradation in thylakoid membranes we identified, characterized and cloned thylakoid proteases, and then linked them to known proteolytic processes. Several families of chloroplast proteases were identified and characterized to different extents. FtsH, an ATP-dependent metalloprotease that belongs to the AAA-protein family, was found to be integral to the thylakoid membrane, facing the stroma. It is involved in both the degradation of unassembled subunits of membrane complexes, such as the Rieske Fe–S protein of the cytochrome complex, and the degradation of oxidatively damaged proteins such as the D1 protein of the photosystem II (PS II) reaction centre. Plant genomes contain multiple isomers of this protease but the functional significance of this multiplication is not clear yet. A second protease, the serine ATP-independent DegP, was found to be strongly associated with the luminal side of the thylakoid membrane. Although a specific role has not yet assigned for it, its location suggests that it can degrade luminal soluble proteins as well as luminally exposed regions of thylakoid membrane proteins.

Introduction
Degradation of different chloroplast proteins, especially of thylakoid proteins, has been extensively documented (reviewed in [1,2]). Chloroplast proteins, like proteins in any other biological compartment, are prone to degradation owing to mutations, imbalances in the stoichiometry of multi-subunit complexes, heat denaturation and oxidative damage. In chloroplasts, the risk of oxidation and photo-damage is more pronounced owing to the high capacity of thylakoids for absorbing light energy and the high rates of photosynthetic electron transport. Thus proteolysis is essential to the quality control of chloroplast proteins. Moreover, protein degradation serves as an important mechanism for acclimation of the photosynthetic machinery to changes in light intensity when quantities of certain proteins are changed. Despite many examples of protein degradation in chloroplasts, the identity of chloroplast proteases has remained obscure until recent years. To study protein degradation in thylakoid membranes, we identified, characterized and cloned thylakoid proteases, and then linked them to known proteolytic processes. Several families of chloroplast proteases were identified and characterized to different extents. Their characteristics and possible role in the degradation of thylakoid proteins are described below.

FtsH protease
Bacterial FtsH and its related mitochondrial homologues are membrane-bound, ATP-dependent metalloproteases that belong to the AAA-protein family [3]. Chloroplast FtsH was first identified by immunoblot analysis with antibodies against Escherichia coli FtsH [4]. It is integral to the thylakoid membrane; its catalytic and ATP-binding domains face the stroma and it is found exclusively in stroma-exposed regions of the membrane. A cDNA encoding this protein was cloned from Arabidopsis and showed a high degree of similarity to the bacterial protein, especially in and around the functional regions of the protein [4]. When in vitro-translated Rieske Fe–S protein (RISP) of the cytochrome b₆–f complex was imported into isolated chloroplasts, we found that up to 75% of the imported protein remained associated with the stromal side of the thylakoid membrane instead of being translocated across [5]. Moreover, this protein was unstable, with a half-life of approx. 60 min, unlike other imported proteins that remained stable for at least 2 h. Thus it could serve as a model substrate for the degradation of unassembled proteins on the surface of the thylakoid membrane. Interestingly, when post-import incubations were performed in darkness, degradation of the imported RISP was slower. Increased degradation rates were correlated with increasing light intensities [5]. After exposure to a high light intensity at 4 °C, the imported protein remained stable. However, when the temperature was raised to 25 °C, degradation proceeded at equal rates in both light or darkness.
These results suggested that light resulted in photo-damage to the protein, and under proper conditions the damaged protein could be further degraded enzymically.

Further characterization of this degradation process in lysed chloroplasts demonstrated that it was sensitive to inhibitors of metalloproteases. To test the possible involvement of FtsH protease in the process, stability assays were performed in the presence of antibodies against native FtsH from E. coli. Whereas increasing amounts of preimmune serum had no effect on the degradation of RISP, increasing amounts of anti-FtsH gradually inhibited degradation [5], suggesting a role for FtsH protease in the process. Further support for this suggestion came from column chromatography experiments. When detergent-solubilized thylakoid membranes were fractionated on ion-exchange, hydrophobic or size-exclusion columns, two peaks of RISP-degrading activity were resolved. When column fractions were subjected to immunoblot analysis, at least one of these peaks coincided with the presence of FtsH, suggesting that at least part of the RISP-degrading activity can be attributed to FtsH protease (O. Ostersetzer and Z. Adam, unpublished work).

Photoinhibition (decreased photosynthesis rates in response to increasing light intensities) occurs primarily because of oxidative damage to the D1 protein of the photosystem II (PS II) reaction centre [6,7]. A repair mechanism of PS II involves rapid degradation of the D1 protein followed by the incorporation of newly synthesized copies into the PS II complex. Although degradation of the D1 protein has been a subject of thorough characterization for many years, proteases involved in the process were identified only recently. The D1 protein is an integral thylakoid membrane protein containing five membrane-spanning α-helices. Its N-terminus is facing the stroma, whereas its C-terminus extrudes into the lumen. Degradation of the D1 protein is a two-step process. In the first step, a single endoproteolytic cleavage event occurs at the stromal loop connecting the fourth and fifth transmembrane helices, to yield 23 and 10 kDa fragments [8]. In the second step these fragments are completely degraded. The initial cleavage was recently attributed to DegP2 protease [9] (see below). Complete degradation of the 23 kDa fragment seems to be mediated by FtsH protease.

When thylakoid membranes were subjected to photoinhibitory illumination, the 23 kDa fragment of the D1 protein accumulated. However, we observed lower amounts of the fragment in the presence of ATP. Moreover, in the presence of adenosine 5′-([γ-thio]triphosphate, the levels of the fragment resembled those found in the absence of ATP [10]. These results suggested the involvement of an ATP-dependent enzyme in the disappearance of the 23 kDa fragment. To test the possible involvement of FtsH protease in the process, we overexpressed the Arabidopsis cDNA in E. coli cells as a glutathione S-transferase fusion protein. The purified protein was proteolytically active on a model substrate in an ATP-dependent manner and was sensitive to inhibitors of metalloproteases. Thus it was suitable for further testing for possible involvement in the degradation of the fragment. For that purpose, core complexes of PS II devoid of FtsH were isolated. When these were illuminated, the 23 kDa fragment accumulated. When photoinhibited core complexes were incubated with the recombinant FtsH protease and ATP, degradation of the fragment was observed. No degradation was observed in the absence of FtsH or in the presence of adenosine 5′-([γ-thio]triphosphate, suggesting the involvement of FtsH protease in the process [10]. To support this conclusion further, we stripped FtsH from the membrane by mild treatment with trypsin. When such membranes were exposed to photoinhibitory illumination, the 23 kDa fragment accumulated to a higher level than in membranes that contained FtsH. When recombinant FtsH was added back to these membranes, degradation of the fragment was restored [10]. These results supported our claim for the involvement of FtsH protease in the degradation of the 23 kDa fragment of the D1 protein.

Recent analysis of Arabidopsis mutant lines revealed the existence of a second FtsH protein. Mutations in this protein resulted in a variegated phenotype, suggesting a role for this protein in the biogenesis of thylakoid membranes [11,12]. As more DNA sequence data have accumulated in databases, it has become evident that FtsH appears in multiple isomers in plant cells. Recent compilation of available data from the Arabidopsis genome-sequencing project revealed at least eight different FtsH genes. Because cDNA species or expressed sequence tags for at least seven of them were identified, it seems that they are expressed in plant cells. Thus the corresponding proteins were named FtsH1–FtsH8 [13]. The first two FtsH forms described above, now designated FtsH1 and FtsH2, were located to chloroplasts by import experiments [4,11]. Another, FtsH6, was recently

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428
found in chloroplasts by two-dimensional PAGE followed by MS analysis (O. Ostersetzer and Z. Adam, unpublished work). Prediction programs for cellular locations suggest that FtsH5, FtsH7 and FtsH8 are targeted to chloroplasts, whereas FtsH3 is likely to be mitochondrial [13]. Thus it is possible that chloroplasts contain six different FtsH proteases; however, the functional significance of this multiplicity is not yet known.

**DegP protease**

Bacterial DegP belongs to a family of serine proteases [14]. It is a peripheral membrane protein bound to the periplasmic side of the inner membrane in *E. coli* [15]. Chloroplast DegP was identified by immunoblot analysis with antibodies against *E. coli* DegP [16]. It is found in thylakoids, tightly associated with the luminal side of the membrane. Its interaction with the membrane is mediated by hydrophobic interactions, because it is resistant to washes with high concentrations of salt; its removal from the membrane requires low concentrations of detergent. Transient 2-fold increases in DegP protein levels were observed on the transfer of seedlings to high temperature, suggesting a role for the protein in the response of chloroplasts to heat. A cDNA encoding this protein was cloned from *Arabidopsis* and showed high degree of similarity to the bacterial protein [16]. The N-terminus of this protein, designated DegP1, contains a bipartite transit peptide that is typical of proteins translocated across the thylakoid membrane. In the central region of the protein, a catalytic triad of serine proteases composed of His-Asp-Ser residues is found. The C-terminus contains a PDZ-like domain that is implicated in protein–protein interactions in other biological systems [17]. Using β-casein as a substrate, we found a serine-type proteolytic activity on the luminal side of the thylakoid membrane that could be attributed to DegP1 [16]. However, no physiological substrate for this protease has yet been found. It is suggested that DegP1 degrades soluble luminal proteins as well as lumen-exposed regions of thylakoid membrane proteins. It is tempting to speculate that the cleavage of luminal loops connecting transmembrane helices of thylakoid membrane proteins facilitates their complete degradation by ATP-dependent proteases such as FtsH on the stromal side of the membrane. This suggestion still needs to be tested experimentally.

Recently, a second DegP cDNA was cloned and designated DegP2 [9]. This protein is very similar to DegP1, with the exception that it is associated with the stromal side of the thylakoid membrane. As described above, it is implicated in the initial stage of the degradation of the D1 protein of PS II. Similarly to FtsH, DegP in *Arabidopsis* is encoded by a gene family: 13 genes have been identified and their cellular locations predicted with bioinformatic tools [13]. Four are predicted to be located in chloroplasts, five are likely to be mitochondrial, one could be either chloroplastic or mitochondrial, one is cytosolic, one could be nuclear and one is either in the endoplasmic reticulum or the plasma membrane. This diverse localization still needs to be substantiated experimentally but it should already be noted that corresponding cDNA species or expressed sequence tags were found only for seven of these genes. Thus it could well be that not all of these genes are expressed in the plant cell.

**Other thylakoid proteases**

The first identified chloroplast protease was Clp protease (reviewed in [1,18]). Clp is a soluble ATP-dependent serine protease that is found in the stroma. Its intra-organellar location suggests that it can degrade both soluble proteins in the stroma and membrane proteins in the stroma lamellae of the thylakoid membrane. The involvement of Clp protease in degradation of thylakoid membrane proteins was inferred from experiments in *Chlamydomonas* [19]. Expression of the plastid-encoded gene was attenuated by mutating the translation initiation codon. A decreased level of ClpP was correlated with a decreased degradation rate of the cytochrome *b$_{6}$*–f complex in two situations: in wild-type cells that grew in nitrogen-limiting conditions and in a mutant deficient in RISP [19]. Whether this process is mediated by soluble or membrane-bound Clp protease is not clear yet; however, certain amounts of Clp could be observed on the stromal side of the thylakoid membrane. Recently, ten different ClpP-related subunits were found in a 350 kDa complex that is peripherally attached to the membrane [20], suggesting that it could participate in the degradation of thylakoid membrane proteins.

Database searches and cellular location predictions suggest that chloroplasts might contain another protease, Lon protease. Bacterial Lon is an ATP-dependent serine protease that also has a homologue in mitochondria. Three Lon genes were found in the *Arabidopsis* genome [13]. The product of one of them was found in mitochondria [21] but the products of the other two are predicted
to be located in chloroplasts [13]. Interestingly, immunoblot analysis with an antibody against Lon protease revealed a cross-reaction with protein extracts from both mitochondria and chloroplasts (O. Ostersetzer and Z. Adam, unpublished work). Moreover, the cross-reacting protein was tightly associated with the stromal side of thylakoid membranes. If the existence of Lon in chloroplast is indeed confirmed, it is likely that it also participates in the degradation of thylakoid proteins.

**Conclusion**

The recent discovery of a number of chloroplast proteases has provided us with the opportunity of starting to identify the proteases involved in the degradation of several chloroplast proteins whose degradation has been documented previously. Within the context of thylakoid membranes, two enzymes were implicated in the degradation of the photo-damaged D1 protein of the PS II reaction centre: DegP2 and FtsH. FtsH was also linked to the degradation of unassembled RISP on the stromal surface of the thylakoid membrane; degradation of subunits of the cytochrome b$_{6}$–f complex was shown to be mediated by Clp protease. The discovery of multiple isomers of these proteases raises the question of whether they perform redundant functions or whether specific isomers have their own specific substrates. Because all these proteases are known to function as large oligomers or hetero-oligomers.

The degradation of D1 protein suggests that degradation of integral thylakoid membrane proteins might require more than a single protease. Only after the initial cleavage of D1 by DegP2, which yields a fragment with a C-terminus facing the stroma, can FtsH degrade this 23 kDa fragment completely. It is highly likely that proteases residing on the luminal side of the membrane also participate in the process. By cleaving luminal exposed loops that connect transmembrane helices, the processing activity of ATP-dependent proteases such as FtsH is likely to be facilitated. These assumptions will have to be challenged experimentally in the near future.

**References**


Received 26 February 2001