Induction of Acclimative Proteolysis of the Light-Harvesting Chlorophyll a/b Protein of Photosystem II in Response to Elevated Light Intensities

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Most plants have the ability to respond to fluctuations in light to minimize damage to the photosynthetic apparatus. A proteolytic activity has been discovered that is involved in the degradation of the major light-harvesting chlorophyll a/b-binding protein of photosystem II (LHCII) when the antenna size of photosystem II is reduced upon acclimation of plants from low to high light intensities. This ATP-dependent proteolytic activity is of the serine or cysteine type and is associated with the outer membrane surface of the stroma-exposed thylakoid regions. The identity of the protease is not known, but it does not correspond to the recently identified chloroplast ATP-dependent proteases Clp and FtsH, which are homologs to bacterial enzymes. The acclimative response shows a delay of 2 d after transfer of the leaves to high light. This lag period was shown to be attributed to expression or activation of the responsible protease. Furthermore, the LHCII degradation was found to be regulated at the substrate level. The degradation process involves lateral migration of LHCII from the appressed to the nonappressed thylakoid regions, which is the location for the responsible protease. Phosphorylated LHCII was found to be a poor substrate for degradation in comparison with the unphosphorylated form of the protein. The relationship between LHCII degradation and other regulatory proteolytic processes in the thylakoid membrane, such as D1-protein degradation, is discussed.

The main focus of photosynthesis research has been the functional and structural aspects behind the energy-transducing process. The progress has been significant, and the photosynthetic protein complexes can now be described at a very refined molecular level (Barber and Andersson, 1994). Conversely, relatively little is known about the acclimating, regulatory, and protective processes that maintain high photosynthetic efficiency during ever-fluctuating and even stressful environmental conditions.

The identification of such auxiliary processes associated with thylakoid membranes and the characterization of the enzymes involved are therefore central tasks of current photosynthesis research. Examples of auxiliary enzymes or components are kinases and phosphatases responsible for reversible protein phosphorylation (Gal et al., 1997), de-saturases controlling the lipid composition and dynamics of thylakoid membranes (Wada et al., 1990), stress-induced proteins such as the ELIPs (Adamska, 1997), and several enzymatic activities involved in various forms of regulatory proteolysis (Adam, 1996; Andersson and Aro, 1997). The knowledge of chloroplast proteases involved in regulatory proteolysis is still very limited. The presence of homologs to bacterial proteases in chloroplasts, such as the Clp and FtsH proteins, has provided new insights and possibilities (Gray et al., 1990; Moore and Keegstra, 1993; Adam, 1996; Lindahl et al., 1996; Ostersetzer et al., 1996).

In a previous study we discovered a regulatory proteolytic activity involved in the degradation of LHCII during acclimation of spinach (Spinacia oleracea) leaves from low- to high-intensity light (Lindahl et al., 1995). LHCII is the most abundant protein in the thylakoid membrane of higher plants (Jansson, 1994). It is not only a key component for light harvesting, but is also essential for regulation and distribution of excitation energy within the photosynthetic apparatus and responds to both short- and long-term fluctuations in light intensity and quality (Anderson and Andersson, 1988; Melis, 1991, 1996). This acclimation of the light-harvesting apparatus is generally thought to optimize photosynthetic efficiency and minimize light stress and photoinhibition to PSII (Andersson and Barber, 1996; Melis, 1996; Park et al., 1997).

The identified proteolytic activity was associated with degradation of the so-called “outer” or “peripheral” pool of LHCII enriched in the rapidly phosphorylated 25-kD subunit (Larsson et al., 1987a, 1987b; Anderson and Andersson, 1988), thereby reducing the antenna size of PSII in...
spinach leaves by 20% to 30% in response to elevated light intensities (Lindahl et al., 1995). The protease involved was found to be of the Ser or Cys type and extrinsically bound at the outer surface of the stroma-exposed regions of thylakoid membranes. Notably, the proteolytic process did not occur in the absence of ATP; therefore, it can be added to the list of the few known examples of ATP-dependent proteolysis associated with the thylakoid membrane (Adam, 1996). Another feature of the acclimative proteolysis of LHClII is that it requires a relatively long period of induction; therefore, no change in the size of the PSII antenna was seen during the first 2 d after transfer of spinach plants to increased light intensities (Lindahl et al., 1995).

To gain an understanding of the mechanism and regulation of the acclimative reduction of the LHClII antenna, we studied the induction of LHClII degradation and investigated the activation/deactivation pattern of the proteolytic process after transfer of spinach plants from low- to high-intensity light. The results revealed a light-dependent induction at the enzyme level and a regulatory control at the substrate level. Evidence was also provided that the newly discovered chloroplast ATP-dependent proteases Clp and FtsH were not responsible for the acclimative LHClII degradation.

MATERIALS AND METHODS

Growth and Acclimation of Plants

Spinach (Spinacia oleracea) plants were grown for 4 weeks at an intensity of 30 μE m⁻² s⁻¹ (low-intensity light). The light/dark cycle was 10 h of light followed by 14 h of darkness. The mature, low-light-grown spinach plants were transferred to an intensity of 600 to 800 μE m⁻² s⁻¹ (high-intensity light) for studies of the acclimative responses to high light.

Isolation and Subfractionation of Thylakoid Membranes

Thylakoid membranes were isolated according to the method of Andersson et al. (1976) from control plants grown at low light and from acclimating plants after the indicated times at the high-light regime. The isolated thylakoids were suspended in 15 mM Tricine-NaOH, pH 7.8, 15 mM NaCl, 3 mM MgCl₂, 100 mM sorbitol, 1 mM ascorbate, and 0.1% (w/v) BSA (incubation buffer). Chlorophyll concentration and the chlorophyll a/b ratio were determined spectrophotometrically according to the method of Lichtenthaler and Wellburn (1983).

Digitonin fractionation of isolated thylakoid membranes (Carlberg et al., 1992) was performed at a chlorophyll concentration of 0.2 mg mL⁻¹, and a final digitonin concentration of 0.1% (w/v) at room temperature for 30 s. The solubilization was terminated by a 10-fold dilution with ice-cold incubation buffer. Nonappressed thylakoid membranes (stromal lamellae vesicles) were isolated by differential centrifugation. The supernatant remaining after centrifugation at 40,000g for 30 min was centrifuged at 100,000g for 1 h. The final pellet containing the nonap-pressed membranes was resuspended in incubation buffer and stored at −80°C.

High-Salt Washings of Thylakoid Membranes

For studies of enzyme induction in vivo, thylakoid membranes were isolated from control leaves and from leaves at various stages in the acclimation process. Isolated thylakoid samples were washed three times in incubation medium supplemented with 0.5 mM NaCl to release the extrinsically bound proteolytic activity from the membrane (Lindahl et al., 1995). Control washings were made in incubation buffer without the addition of extra NaCl. The wash supernatants were concentrated, dialyzed against incubation buffer, and used for assays of proteolytic activities and in reconstitution experiments. Protein concentration was determined according to the method of Lowry et al. (1951).

Reconstitution of LHClII Proteolysis in Situ

In situ degradation of LHClII in low- and high-light-acclimating thylakoid membranes was followed after high-salt washing and homologous or heterologous reconstitution with an appropriate desalted supernatant. The reaction mixture contained washed thylakoids (1 mg of chlorophyll) resuspended in incubation buffer supplemented with 0.5 mM ATP. A concentrated and dialyzed wash supernatant obtained from thylakoids corresponding to 1 mg of chlorophyll was added in darkness at 22°C. As a control, unwashed thylakoids were incubated under the same conditions. Aliquots were frozen in liquid nitrogen and kept at −80°C before analysis of LHClII degradation by SDS-PAGE and western blotting.

Effect of Phosphorylation on LHClII Degradation

Thylakoids were incubated at 1 mg chlorophyll mL⁻¹ in incubation buffer in the presence of 12.5 μCi [γ-³²P]ATP mL⁻¹ and 0.5 mM unlabeled ATP. Different extents of phosphorylation of thylakoid membranes were induced in vitro by illumination at 160 μE m⁻² s⁻¹ at 25°C for various lengths of time. Residual radioactive ATP was removed by centrifugation, and the thylakoids were resuspended in fresh incubation buffer containing 0.5 mM ATP and 10 mM NaF to a concentration of 2 mg chlorophyll mL⁻¹. Thylakoids were then incubated in the dark at 25°C, and samples were removed after 0, 2, 4, and 6 h. LHClII degradation was analyzed by two-dimensional gel electrophoresis (Larsson and Andersson, 1985; Lindahl et al., 1995). The relative protein phosphorylation was determined by autoradiography.

Assay of Proteolytic Activity Using Isolated LHClII or Fluorescein Thiocarbamoyl-Casein as a Substrate

LHClII was isolated from high-light-grown spinach according to the method of Burke et al. (1978). The purified LHClII was added to the wash supernatants in incubation buffer in the presence of 0.1% (w/v) Triton X-100. The ratio
of LHClII protein to supernatant protein was 1:5. The samples were incubated at 25°C for 2 h, and then assayed for LHClII degradation by SDS-PAGE and western blotting. The antibody used was raised against both the LHClII-27 and LHClII-25 subunits.

Proteolysis of fluorescein thiocarbamoyl-casein was monitored by fluorescence emission spectra (500–600 nm) using an excitation wavelength of 495 nm (Twining, 1984).

Separation and Quantification of the LHClII-27 and LHClII-25 Polypeptides

Thylakoid proteins were separated either by two-dimensional SDS-PAGE, as described previously (Larsson and Andersson, 1985; Lindahl et al., 1995), or by SDS-PAGE according to the method of Laemmli (1970) using a 12% to 22.5% acrylamide gradient. Quantification of the LHClII-27 and LHClII-25 polypeptides was performed by Coomassie blue staining of the second-dimension gel or western blot, followed by laser-densitometry scanning.

Analyses of Clp and FtsH Proteins

Intact chloroplasts were isolated according to the method of Bartlett et al. (1982) and thylakoid membranes were prepared from them. The Clp protease was detected by western-blot analysis using antibodies raised against the rice clpP and pea clpC gene products expressed in Escherichia coli (Ostermiller and Adam, 1996; Ostermiller et al., 1996).

For FtsH analysis isolated thylakoid membranes were washed in either 2 M NaBr or 0.1 M NaOH; the suspensions were left on ice for 30 min, and then centrifuged at 10,000g for 5 min. Membrane preparations and wash supernatants were assayed for the presence of FtsH by western-blot analysis using an antibody raised against the E. coli enzyme, as described by Lindahl et al. (1996).

RESULTS

Regulation of LHClII Degradation at the Enzymatic Level

The acclimative reduction of the LHClII antenna is not an immediate response after transfer of low-light-grown spinach plants to high light (Lindahl et al., 1995). As illustrated in Figure 1A, no or very little decrease in the LHClII antenna occurred during the first 2 d of the high-light regime (including two night periods), as judged by the relatively constant chlorophyll a/b ratio and the unchanged proportion between LHClII and the PSII core (CPa). Only during d 3 to 4 was the reduction of the LHClII antenna complete, as revealed by a decrease of the LHClII/CPa ratio from 6.0 to 3.5 (Fig. 1A) and by the reduced level and changed proportion between the LHClII-27 and LHClII-25 subunits (Fig. 1A, inset). There was also an increase in the chlorophyll a/b ratio from 2.7 to 3.1.

The lag period of the acclimative response suggests that the proteolysis of LHClII requires some mechanism of induction. Such an induction could be controlled at the enzyme level and/or at the substrate level. To discriminate between these possibilities we took advantage of the fact that the LHClII protease is extrinsically located at the outer membrane surface of the stroma-exposed thylakoids and can be readily washed off with high-salt buffers (Lindahl et al., 1995). The wash supernatants were obtained from thylakoid membranes isolated from control low-light leaves and from leaves at various stages of the acclimation process, and used for different assays of proteolytic activities.
As a first approach, washed thylakoid membranes isolated from acclimating spinach leaves were used as a substrate. As illustrated in Figure 1B, the different supernatants showed various abilities to catalyze LHCII degradation. As judged by immunodetection, no proteolysis could be detected using the supernatant obtained from thylakoids derived from low-light-acclimated leaves. Only after 2 d (including dark periods) at the elevated light regime did the wash supernatant possess a proteolytic activity toward LHCII, particularly LHCII-25. The wash supernatant with maximal proteolytic activity (28% degradation of LHCII-25) was obtained after 3 d of exposure to high light (Fig. 1B).

After activation the proteolytic activity could either remain active or be deactivated once the new reduced level of the LHCII antenna size had been reached. The presence of proteolytic activity in the wash supernatant was therefore investigated after the amount of LHCII had reached a new stable value at the reduced level. The results revealed a rapid reduction in proteolysis during d 4 (Fig. 1B), suggesting a deactivation of the enzymatic activity.

These in situ degradations were complemented with experiments in which isolated LHCII was used as a substrate for the various wash supernatants. As judged by immunodetection, no or very little degradation occurred during the first 27 h after transfer of the dark-adapted leaves to the high-intensity light (not shown). Only after approximately 2 d could a significant degradation be observed, particularly for LHCII-25. Using the artificial protease substrate fluorescein thiocarbamoyl-casein (Twining, 1984), a similar increase of proteolytic activity was found for the various wash supernatants (not shown).

These data imply that LHCII degradation is regulated at the enzymatic level, which could involve expression and degradation of the protease (turnover) or a posttranslational activation/deactivation process.

**Regulation at the Substrate Level**

The fact that the initiation of the LHCII degradation involves a regulation at the enzymatic level does not exclude activation at the substrate level. For this reason, a series of reconstitution experiments was designed in which high-salt-wash supernatants from low-light controls or high-light-acclimating leaves were homologously or heterologously reconstituted with washed high-light thylakoids, particularly LHCII-25. The wash supernatant derived from high-light-acclimating leaves was heterologously reconstituted with highly activated high-light thylakoids (Fig. 2) to confirm the presence of active protease and determine the requirement for an enzymatic induction of the proteolytic process as discussed above. A crucial observation was made in the fourth reconstitution experiment, when the wash supernatant was added to the washed high-light control thylakoids (Fig. 2). This heterologous reconstitution resulted in only about 15% of the maximal LHCII degradation. Therefore, although the high-light-wash supernatant contained the active protease, it could not degrade LHCII in the low-light thylakoids.

The homologous and heterologous reconstitution experiments provide evidence that induction of the enzyme is not sufficient for the acclimative LHCII degradation, but that some additional activation at the substrate level is required. In the search for the molecular basis of such a substrate activation, two previous observations with respect to the long-term acclimation of LHCII have been considered. First, there is a lateral segregation between the LHCII substrate and the responsible protease along the stacked thylakoid membrane. The outer pool of LHCII is normally associated with the PSI centers located in the appressed thylakoid regions (Larsson et al., 1987a, 1987b; Mäenpää and Andersson, 1989; Melis, 1991), whereas the proteolytic activity is confined to the nonappressed thylakoid regions (Lindahl et al., 1995). Second, the outer pool of LHCII is not only a substrate for proteolysis during long-term acclimation (Lindahl et al., 1995), but is also readily subjected to protein phosphorylation (particularly the 25-kD subunit) and rapid lateral migration from the appressed to the nonappressed thylakoid regions (Kyle et al., 1983; Larsson and Andersson, 1985; Larsson et al., 1987b). The latter events are of significance for the so-called state transitions involved in the short-term regulation of the photosynthetic antenna (Gal et al., 1997). Consequently, the
substrate activation could involve protein phosphorylation and lateral migration of phospho-LHCII from its original location in the appressed thylakoid regions to the nonappressed thylakoid regions, where it would come in contact with the protease.

The relative content of LHCII in the stroma-exposed regions of thylakoid membranes isolated from spinach leaves during their acclimation from low- to high-intensity light. Stromal lamellae vesicles were isolated by digitonin incubation of thylakoid membranes at different stages in the acclimation process, followed by differential centrifugation. The relative amounts of LHCII were determined by mild SDS-PAGE. The light/dark cycle was 10 h of light followed by 14 h of darkness. The 100% value represents the initial amount of LHCII in the stromal thylakoids before exposure of plants to high light. Results are the means of three separate experiments.

Possible Relation between LHCII Degradation and the Clp and FtsH Proteases

Chloroplast homologs to bacterial proteases have recently been identified in higher plants (Adam, 1996). One of these proteins, Clp, is an ATP-dependent Ser-type protease composed of two subunits, ClpA and ClpP. Because the proteolytic activity degrading LHCII is both ATP dependent and of the Ser type (Lindahl et al., 1995), this prompted us to test whether Clp could be involved in this particular process. In Figure 5A, the presence of the Clp
subunits in chloroplasts isolated from acclimating as well as low-light control spinach leaves is shown by immunoblotting using ClpA and ClpP antisera. Quantification revealed no changes in the relative levels of the Clp subunits between the two chloroplast samples. Furthermore, the two Clp subunits were absent from thylakoid membranes isolated from both of these particular chloroplast samples and were recovered in the soluble stromal fraction. This demonstrates that the Clp protease does not associate with the membrane under the applied physiological conditions and that it cannot be responsible for the acclimative proteolysis of LHCII, since this enzymatic activity remained bound to the thylakoid membrane unless it was removed by high-salt treatment.

Another recently identified ATP-dependent protease of higher plant chloroplasts homologous to a bacterial protein is the FtsH protease (Lindahl et al., 1996). To find a relationship between this protease and the LHCII-degrading activity, we subjected the isolated thylakoid membranes to acclimation was investigated by washings with either NaBr or NaOH. Analysis was with SDS-PAGE and western blotting. Lane 1, Unwashed thylakoids; lane 2, NaBr pellet; lane 3, NaBr-wash supernatant; lane 4, NaOH pellet; lane 5, NaOH-wash supernatant.

Figure 5. Analysis of the chloroplast proteases Clp and FtsH in connection with LHCII degradation. A, The presence of ClpA and ClpP in chloroplasts (lanes 1 and 2) and thylakoids (lanes 3 and 4) isolated from high-light (lanes 2 and 4) and low-light (lanes 1 and 3) spinach leaves was analyzed by SDS-PAGE and western blotting. B, Association of FtsH with thylakoid membranes during acclimation was investigated by washings with either NaBr or NaOH. Analysis was with SDS-PAGE and western blotting. Lane 1, Unwashed thylakoids; lane 2, NaBr pellet; lane 3, NaBr-wash supernatant; lane 4, NaOH pellet; lane 5, NaOH-wash supernatant.

DISCUSSION

The overall changes of the PSII antenna in response to variations in the light environment for different plant species have been thoroughly described (Osmond, 1994; Melis, 1996). Despite the wealth of physiological information, the underlying molecular mechanisms for the differences in antenna composition and the dynamic acclimations are still not understood. The expression of the nuclear cab genes encoding LHCII is light regulated at both the transcriptional and translational levels (Green and Salter, 1996), and the hierarchy of Cab protein assembly during biogenesis has been characterized (Melis, 1996). Aside from the recently discovered proteolytic process (Lindahl et al., 1995; Lindahl, 1997), the process of reduction of the LHCII antenna during acclimation to elevated light intensities is basically unknown. An important feature of this acclimation is that it shows a pronounced lag in its response, so a major aim of the present investigation was to address the molecular control of this process.

It can be demonstrated that initiation of the acclimative LHCII proteolysis requires regulation at both the enzyme and the substrate levels. The lag period in the physiological response could be attributed mainly to an expression or activation of the responsible protease at high light. We have not been able to discriminate between these two mechanistic alternatives. In the case of a posttranslational activation, redox control via plastoquinone or regulation via thiol groups, as has been shown to occur for the enzymes involved in reversible thylakoid protein phosphorylation (Gal et al., 1997; Vener et al., 1998), are interesting possibilities for future experimental investigations.

What is the mechanism that triggers LHCII degradation at the substrate level? We conclude from the thylakoid-subfractionation experiments that the induction of degradation involves a lateral movement of the outer pool of LHCII, which is enriched in the rapidly phosphorylated 25-kD subunit, from the appressed to the nonappressed thylakoid regions where the protease is located (Lindahl et al., 1995). It is known that phosphorylation of LHCII is a driving force behind its lateral migration during the state-1 to state-2 transitions of the short-term acclimation of light harvesting (Barber, 1982; Gal et al., 1997). Therefore, it seems reasonable to assume that the lateral migration of LHCII seen under the present long-term acclimation to increased irradiance (Fig. 3) also involves protein phosphorylation. If that is the case, a likely mechanism for the transformation of the short-term, reversible reduction of the LHCII antenna (state transition) to the long-term, irreversible reduction of the LHCII antenna (degradation) would be the induction of proteolytic activity. This enzyme induction will occur only after the plants are exposed for a prolonged period to a high light intensity, as shown by the observed lag phase in the physiological response (Fig. 1).

Such a simple mechanistic connection between short- and long-term acclimation of the PSII antenna would be consistent with the fact that both responses are targeted mainly to LHCII-25 (Larsson et al., 1987a, 1987b). However, it should be stressed that a role for protein phosphorylation during LHCII degradation can only be indirect, because it...
brings the protein substrate and enzyme together in the thylakoid membrane system. We found that phosphorylated LHCII is a poor substrate for the degradation compared with the unphosphorylated form (Fig. 4). LHCII degradation, therefore, may be more similar to D1-protein degradation during photoinhibitory conditions, in which the phosphorylated form of the photodamaged protein is protected from proteolysis and no degradation occurs in the absence of dephosphorylation (Koivuniemi et al., 1995; Ebbert and Godde, 1996; Rintamäki et al., 1996).

It should also be considered that high light intensities can lead to kinase inactivation (Schuster et al., 1986; Horton and Hague, 1988; Walters and Horton, 1991), which could complicate a potential physiological role of protein phosphorylation during long-term acclimation to high light. However, at the rather moderate nonphotoinhibitory irradiance applied in our present acclimation studies (600 µE m⁻² s⁻¹), no reduction in the kinase activity took place (not shown). Kinase inactivation readily occurred at higher light intensities (>1000 µE m⁻² s⁻¹), in accordance with previous studies (Schuster et al., 1986; Horton and Hague, 1988; Walters and Horton, 1991).

Degradation of LHCII should potentially release chlorophyll molecules that would be highly toxic to the photosynthetic apparatus if they were not rapidly degraded or quenched by some other mechanism. ELIPs may be involved in the binding of liberated chlorophyll molecules, thereby providing quenching of any toxic effects during LHCII degradation. This suggestion would be in accordance with the function proposed for ELIPs as “transient pigment-binding proteins” (Adamska, 1997) and with the experimental finding that ELIPs bind both chlorophyll and lutein (I. Adamska, unpublished data). ELIPs in spinach leaves were recently shown to have unusually high expression under conditions of LHCII degradation (Lindahl et al., 1997).

In our efforts to identify the LHCII protease, we have at least ruled out that it could be related to the chloroplast Clp or FtsH proteases. On the other hand, it has previously been shown that a detergent-activated proteolytic activity against LHCII is present in isolated bean thylakoids (Anastassiou and Argyroudi-Akoyunoglu, 1995). A comparison of the proteases responsible for acclimative proteolysis of LHCII and the detergent-induced degradation reveals similarities as well as discrepancies. Both enzymes are of the Ser type and probably contain sulfhydryl groups essential for the catalytic activity. The detergent-induced proteolytic activity of LHCII is, in contrast to the acclimative degradation of LHCII, independent of ATP. The relationship between the two proteases is still uncertain, because additional information concerning the localization and the nature of the association with the thylakoid membrane of the detergent-activated protease is lacking.

Recently, a proteolytic activity involved in low-light-induced degradation of ELIPs, which are homologous to the Cab proteins, has been characterized in vivo and in vitro and the protease has been partially purified (Adamska et al., 1996). This ELIP protease was also found to be of the Ser type and extrinsically located at the stroma-exposed thylakoid surfaces. However, the fact that its activity does not require ATP argues against an involvement of this protease in the acclimative degradation of LHCII. The identity of the LHCII protease, therefore, remains unknown, and its identification and characterization provide a central challenge for gaining further knowledge of the light acclimation of PSII and its light-harvesting antenna.

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LITERATURE CITED


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