ELSEVIER

Biochimica et Biophysica Acta 1556 (2002) 53–64

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Proteolytic activity against the light-harvesting complex and the D1/D2 core proteins of Photosystem II in close association to the light-harvesting complex II trimer

John H. Georgakopoulos[a,*], Anna Sokolenko[b], Michael Arkas[a], Georgia Sofou[a], Reinhold G. Herrmann[b], Joan H. Argyroudi-Akoyunoglou[a]

[a] *Institute of Biology, NCSR "Demokritos", Aghia Paraskevi, Attiki, Athens, Greece*
[b] *Institute of Botany, LMU, Menzingerstr. 67, D-80638 Munich, Germany*

Received 7 January 2002; received in revised form 16 July 2002; accepted 5 August 2002

Abstract

Light-harvesting complex II (LHCII) prepared from isolated thylakoids of either broken or intact chloroplasts by three independent methods, exhibits proteolytic activity against LHCII. This activity is readily detectable upon incubation of these preparations at 37 °C (without addition of any chemicals or prior pre-treatment), and can be monitored either by the LHCII immunostain reduction on Western blots or by the Coomassie blue stain reduction in substrate-containing "activity gels". Upon SDS–sucrose density gradient ultracentrifugation of SDS-solubilized thylakoids, a method which succeeds in the separation of the pigment–protein complexes in their trimeric and monomeric forms, the protease activity copurifies with the LHCII trimer, its monomer exhibiting no activity. This LHCII trimer, apart from being "self-digested", also degrades the Photosystem II (PSII) core proteins (D1, D2) when added to an isolated PSII core protein preparation containing the D1/D2 heterodimer. Under our experimental conditions, 50% of LHCII or the D1, D2 proteins are degraded by the LHCII–protease complex within 30 min at 37 °C and specific degradation products are observed. The protease is light-inducible during chloroplast biogenesis, stable in low concentrations of SDS, activated by Mg^{2+}, and inhibited by Zn^{2+}, Cd^{2+}, EDTA and *p*-hydroxy-mercury benzoate (pOHMB), suggesting that it may belong to the cysteine family of proteases. Upon electrophoresis of the LHCII trimer on substrate-containing "activity gels" or normal Laemmli gels, the protease is released from the complex and runs in the upper part of the gel, above the LHCII trimer. A polypeptide of 140 kDa that exhibits proteolytic activity against LHCII, D1 and D2 has been identified as the protease. We believe that this membrane-bound protease is closely associated to the LHCII complex in vivo, as an LHCII–protease complex, its function being the regulation of the PSII unit assembly and/or adaptation.

Keywords: Light-harvesting complex protease; D1/D2 protease; LHCII trimer–protease complex; Photosystem II; Protease; Thylakoid membrane

1. Introduction

The supramolecular Photosystem II (PSII) complex embedded in the thylakoid membrane of higher plant chloroplasts consists of more than 20 polypeptides, chlorophylls (Chls), carotenoids and other chromophores [1–3]. It is organized in a way to capture, transfer, and convert light energy. Among the complex biochemical pathways that are engaged in the assembly and stability of the PSII complex, proteolysis has been lately under intensive investigation. Proteolytic action against polypeptides of the PSII complex is thought to be involved in a number of processes such as the degradation of misled or damaged proteins, the constant protein turnover processes and the adaptation of the plant to different light regimes. Proteolytic activity against PSII proteins has been detected in all stages of development starting from the etiolated seedling to the mature plant. Despite obvious progress made in the study of proteolytic phenomena in the thylakoid system, the proteases directed against the main proteins of PSII [such as the antenna complexes light-harvesting complex II

* Corresponding author. Tel.: +30-1-6511-577; fax: +30-1-6511-767.
E-mail address: ingeorg@bio.demokritos.gr (J.H. Georgakopoulos).

0005-2728/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0005-2728(02)00306-7

(LHCII) and the PSII core protein D2] have not yet been identified. On the other hand, the physiological substrates for a number of recently isolated chloroplast proteases remain still unknown [4–7].

Recent work has shown that thylakoids isolated from *Phaseolus vulgaris* plants possess proteolytic activity directed against the LHCII, which is developmentally regulated and seems to originate in a peripherally bound protease [8–10]. A similar proteolytic activity directed against LHCII has been detected during high light acclimation of spinach leaves, ascribed also to a protease peripherally bound to thylakoids [11,12]. A 38-kDa protease isolated from the degreened *y-1* strain of *Chlamydomonas reinhardtii* has been shown to degrade LHCII [13]; a 68-kDa polypeptide, considered to be part of a light-inducible 270-kDa protease (SppA), isolated lately from thylakoids of *Arabidopsis thaliana* [14], has been suggested to be involved in the degradation of PSII antenna complexes. Two proteases that can degrade the photodamaged PSII core protein D1 upon its light-induced turnover have been isolated recently. The DegP2 protease [15], peripherally attached at the stromal face of the thylakoid membrane, is responsible for the primary cleavage of the protein, leading to a 23-kDa primary fragment, and the FtsH protease [16], an integral membrane metallopeptidase, is probably involved in further cleavage of this fragment. The action of these proteases directed against D1, however, can only be observed on photoinhibited or heat-stressed thylakoids. This suggests that they may not be involved in the degradation that takes place under physiological conditions, since high rates of D1 degradation have also been observed in plants under low to moderate in vivo light conditions [17], in thylakoids at low light [18], in the dark at 0 °C without any stress pretreatment, as in the case of a mutant of *Synechocystis* sp. *PCC 6803* [19], and even under conditions at which the thylakoid kinase is activated [20,21]. These observations raise the question as to whether various proteases may be involved in D1 turnover under different conditions. Recently, for example, a stromal protease (of about 15 kDa) has been found to degrade the cross-linked products of the D1 protein, which are generated by photoinhibition of the PSII complex [22].

Here we report the isolation of a protease from bean thylakoids that degrades isolated LHCII protein, but also the D1 and D2 proteins in the isolated PSII core complex. No pretreatment or preconditioning of the substrates nor addition of any chemicals is required for degradation. The rate of degradation is high (t 1/2 = 0.5 h), and at the early stages of degradation, specific fragments, similar to those identified in earlier studies [8,23,24], can be observed. The proteolytic activity copurifies with the LHCII trimer, and can be separated from the "LHCII trimer–protease complex" by SDS-PAGE as a 140-kDa band in the Coomassie-stained gel. We believe that this protease is closely associated to the LHCII complex in vivo, its function being the regulation of the PSII unit assembly and/or adaptation.

2. Materials and methods

2.1. Plants and handling

P. vulgaris plants, red kidney var., were grown in the dark in a Conviron plant growth chamber at 22 °C and 80% humidity. For routine protease preparations, 6-day etiolated plants were transferred to continuous light (3000 lx; incandescent and fluorescent lamps) for 7 days.

For studies during greening, cotyledons were harvested from 6- to 9-day-old etiolated plants, one cotyledon was removed, and the leaves, attached to the remaining cotyledon, were placed in covered petri dishes on moist filter paper and either exposed to continuous light, or to intermittent light (ImL, in cycles of 2 min light every 98 min dark). Leaves were then withdrawn for thylakoid isolation. In case of ImL exposure, all manipulations were under a green safe light, not permitting PChl(ide) photoreduction.

2.2. Isolation of intact chloroplasts

Intact bean chloroplasts were isolated on linear Percoll gradients as follows: 40 g fresh weight leaves was homogenized in 80 ml ice-cold grinding buffer (0.4 M sorbitol–50 mM Tricine–NaOH, pH 7.8–10 mM NaCl) by three bursts of 3 s each at low speed in an ice-cold Waring blender. After filtration of the homogenate through two layers of nylon gauze (40 and 75 μm pore size), the chloroplast pellet, recovered after centrifugation at 1000 × g for 1 min, was resuspended in 4 ml grinding buffer and loaded onto a linear Percoll gradient in grinding buffer. The gradient was made by mixing 45 ml Percoll with 3 g sorbitol, adjusting the pH to 7.9, and adding grinding buffer to 50 ml. On 30 ml linear Percoll gradients [0–100% (v/v) in grinding buffer], 2 ml chloroplast suspension was loaded. The gradients were centrifuged at 8500 × g for 15 min in a HB-4 swinging bucket rotor (Sorvall centrifuge), and intact chloroplasts, separated as a clear lower zone, were removed. Intactness of the chloroplasts was verified by light microscopy. The chloroplasts were diluted with an equal volume of grinding buffer, repelleted at 2000 × g for 5 min, and lysed in ice-cold 50 mM Tricine–NaOH, pH 7.3. Thylakoids were separated from stroma by centrifugation at 10,000 × g for 10 min. The recovery of Chl from 40 g fresh weight bean leaves was approximately 500 μg. Thylakoids were also isolated as before [25] from broken chloroplasts.

2.3. Isolation of envelopes from intact chloroplasts

Intact chloroplasts were lysed in 10 mM Tricine–NaOH, pH 7.6, 4 mM $MgCl_2$ and layered onto a discontinuous sucrose density gradient [26]. After centrifugation for 1 h at 20,000 rpm (SW 27:1 rotor), envelopes were separated as a yellow band at the interface of 0.93 and 1.2 M sucrose. This band was collected, diluted with 10 mM Tricine–NaOH, pH 7.6, and centrifuged at 25,000 rpm for 1 h; the resulting

membrane pellet was suspended in 10 mM Tricine–NaOH, pH 7.6, at a protein concentration of 1.5 mg/ml and stored at −30 °C.

2.4. LHCII isolation

LHCII was separated from SDS-solubilized thylakoids by ultracentrifugation on sucrose gradients [5–22% (w/v) sucrose] in 0.05 M Tris–borate (pH 9.5) containing Triton X-100/deoxycholate/SDS [0.2% (v/v)/0.2% (w/v)/0.1% (w/v), respectively] [27]. In this case, thylakoids separated from intact or broken chloroplasts, washed twice with 50 mM Tricine–NaOH, pH 7.3, were diluted to 1500 μg Chl/ml. An equal volume of SDS-containing 0.3 M Tris–HCl buffer [10% glycerol, 1% (w/v) in SDS], pH 8.6, was added, and 0.3–0.5 ml solubilized material was layered onto ice-cold sucrose gradients and centrifuged at 170,000 × *g* for 17 h at 4 °C. The gradient was fractionated thereafter, the Chl and proteolytic activity of each fraction was determined, and oligomeric and monomeric LHCII were removed from the gradient. LHCII was also isolated according to Argyroudi-Akoyunoglou [28] by differential Mg^{2+} precipitation from SDS-solubilized thylakoids, and according to Burke et al. [29] by sucrose density ultracentrifugation of Triton X-100-solubilized thylakoids.

2.5. Assessment of proteolytic activity

Proteolysis was assessed (a) by the extent of LHCII, D1 or D2 immunostain reduction on Western blots, following incubation of samples at 37 °C [8,9], and (b) by analysis of samples (gradient fractions, or LHCII preparations) on gelatin substrate-containing ‘‘activity gels’’ [6]. In (a), samples of various LHCII preparations were incubated at 37 °C. Incubation mixtures, containing 0.1–0.2 μg LHCII protein/μl in 40 mM Tris–HCl, pH 8.6, 0.2% (v/v) Triton X-100 (assay buffer), were incubated at 0 or 37 °C. Aliquots of defined protein concentration were removed at various time intervals, added to an equal volume of SDS-solubilization sample buffer [0.1 M Tris–HCl, pH 7.6–4% (w/v) SDS–0.6 M sucrose, 8% (v/v) mercaptoethanol], immediately frozen in liquid N_2, and kept in the freezer thereafter. SDS-PAGE was performed on mini-gels (Hoeffer SE 250, USA) prepared according to Hoober et al. [30]. Aliquots containing 1 μg total protein were loaded per slot, which is in the linear range of LHCII immunostain [9]. After electrophoresis, proteins were transferred to nitrocellulose membranes and the LHCII present was immunodetected according to Towbin et al. [31] and Blake et al. [32]. The reduction in LHCII immunostain (reflecting LHCII degradation), monitored by scanning in a Biometra Scan-Pack II densitometer, was used as a measure for proteolytic activity. In (b), gelatin-containing ‘‘activity gels’’ (mini-gels) were used [6]. In this case, Laemmli gels [33], 10% (w/v) in polyacrylamide, were supplemented with gelatin (2 mg/ml). Samples of LHCII preparations, without addition of SDS-containing sample buffer, were loaded onto the gels. Following electrophoresis, the gels were incubated at 37 °C in assay buffer overnight, and stained by Coomassie brilliant blue thereafter. Upon destaining, the transparent non-Coomassie-stainable protease zones were scanned in a Biometra Scanpack II densitometer.

2.6. Separation of the protease from the LHCII trimer by SDS-PAGE

For the isolation of the protease, the LHCII trimer–protease complex zone, after its separation from SDS-solubilized thylakoids by SDS–sucrose density gradient ultracentrifugation, was used [27]. Concentrated thylakoid samples with 2200 μg Chl/ml were solubilized with an equal volume of SDS-solubilization buffer and loaded onto the gradient. The LHCII trimer–protease zone, as obtained from a gradient tube (150 μl, 180 μg Chl/ml, Chl *a*/*b* ratio = 1.5) was lyophilized and solubilized in 25 μl sample buffer (containing no sucrose) at 1 mg Chl per milliliter. Forty microliters of such samples was pooled, and incubated at 37 °C for 30 min. Thereafter, the protease was separated from the LHCII trimer by SDS-PAGE in the cold, at 10 mA for 3 h on a 10% (w/v) PA Laemmli gel, containing no EDTA and urea. Following electrophoresis, the gel was separated in three parts. One part was dissected from the top to the bottom (2-mm-wide gel slices) and the protease activity in each slice was assessed by reelectrophoresis on gelatin-containing ‘‘activity gels’’. The second part was stained by Coomassie brilliant blue G-250 for protein detection. From the third part, the gel slice corresponding to the activity zone was incubated at 4 °C in a minimal volume (150 μl) of a 0.025 mM Tris–0.003 M borate buffer, pH 9.5, containing 12.5% (w/v) sucrose, 0.05% (w/v) SDS, 0.2% (v/v) Triton X-100 and 0.2% (w/v) deoxycholate, and the extract was tested for proteolytic activity against LHCII, D1 and D2. The protease separation from the LHCII trimer–protease complex has been always successful. We report here representative results of at least six trials. The test for proteolytic activity of the 140-kDa extract has been repeated three times for each protein used as substrate.

2.7. Miscellaneous methods

Chl was determined spectrophotometrically according to Mackinney [34] and protein according to Lowry et al. [35]. A ratio of 130 μg Chl/mg protein was determined in the isolated LHCII preparations.

For delipidation of the LHCII monomer, following its isolation from the sucrose gradient, 80% (v/v) acetone was added and the sample was left in an ice bath for a few hours. The protein pellet was washed once more with 90% (v/v) acetone, recovered by centrifugation and dried under a stream of N_2.

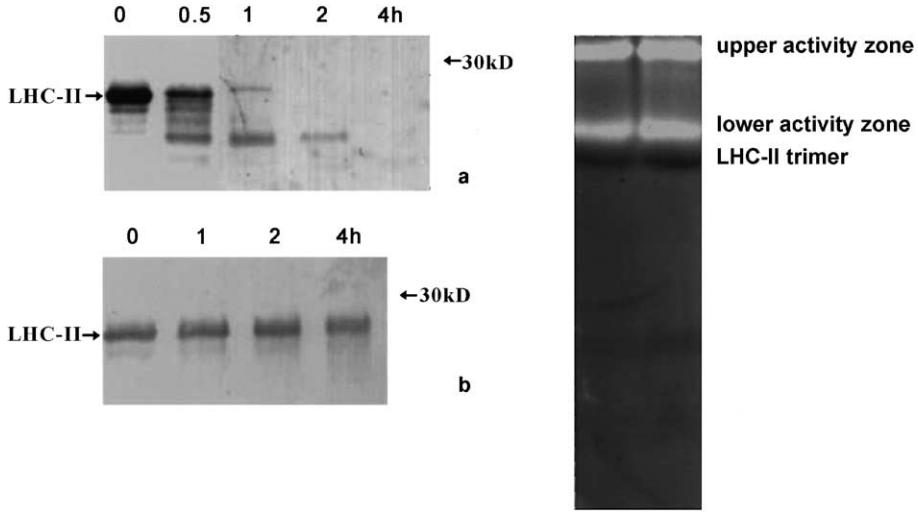

Fig. 1. Detection of proteolytic activity in the LHCII trimer on Western blots (left) or on gelatin-containing "activity gels" (right). Left: Self-degradation of LHCII preparations during incubation at 37 °C, as monitored by the reduction of immunostain on Western blots. (a and b) Trimer and monomer, respectively, isolated by SDS–sucrose density gradient ultracentrifugation [27]. Incubation mixtures contained 0.2 μg LHCII protein/μl assay buffer. Incubation at 37 °C was for 0 (0), 30 min (0.5), 1 h (1), 2 h (2), 4 h (4). Right: LHCII trimer (6 μg Chl), without addition of SDS-containing sample buffer was loaded onto a gelatin-containing "activity gel" and electrophoresed. After electrophoresis, the gel was incubated at 37 °C in assay buffer overnight, and Coomassie stained thereafter. The activity zones were visualized after destaining.

Grana and stroma lamellae were separated by differential centrifugation from French press-disrupted chloroplasts which were suspended in 0.1 M sorbitol–30 mM Tricine–NaOH, pH 7.8–5 mM Mg^{2+}–10 mM NaCl [36].

3. Results

3.1. Proteolysis of LHCII

During our studies, we invariably observed that, irrespective of the preparation method used, LHCII preparations were self-digested to a higher or lesser extent, when incubated at 37 °C. This suggested that some kind of a proteolytic system was present in close association to the LHCII. In an attempt to isolate the protease responsible for this degradation, we first tried to find out which of the various preparations used exhibited highest proteolytic activity. Three independent methods for LHCII isolation were employed, namely (a) the salting-out method affected by Mg^{2+}, which succeeds in fractional precipitation of thylakoid proteins from SDS-solubilized thylakoids [28], (b) the sucrose density gradient ultracentrifugation method, which succeeds in the separation of LHCII from Triton X-100-solubilized thylakoids [29], and (c) the SDS–sucrose density gradient ultracentrifugation method which succeeds

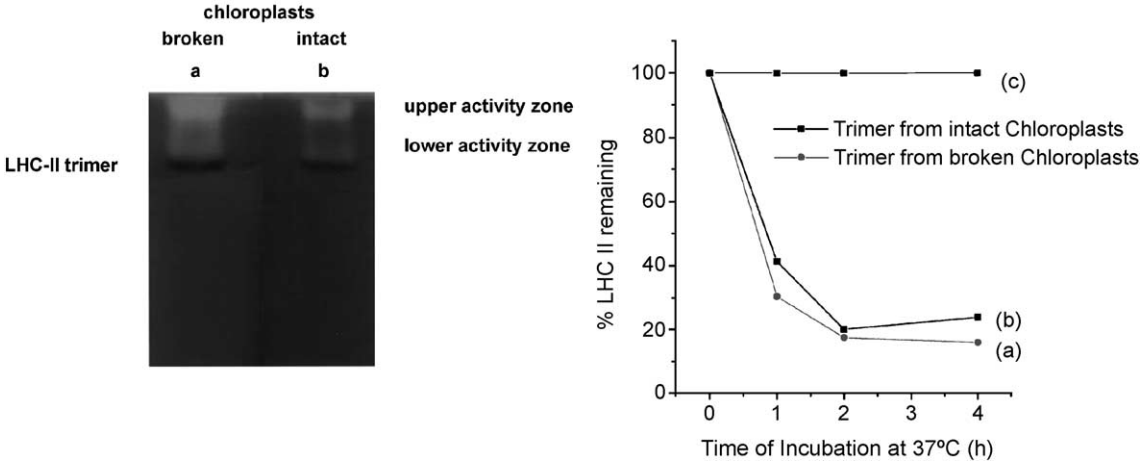

Fig. 2. Proteolytic activity in LHCII trimer preparations, as detected by SDS-PAGE on gelatin-containing "activity gels" (left), or by the LHCII immunostain reduction in samples incubated at 37 °C (right). LHCII trimer was isolated by SDS–sucrose density gradient ultracentrifugation from SDS-solubilized thylakoids of broken (a) or intact chloroplasts (b). Thirty microliters of LHCII trimer zone (5 μg Chl; Chl *a*/Chl *b* = 1.5) was loaded without addition of SDS sample buffer onto the activity gel (left) or incubated at 37 °C (0.2 μg protein/μl in assay buffer) (right). (c) LHCII trimer preheated for 3 min at 95 °C.

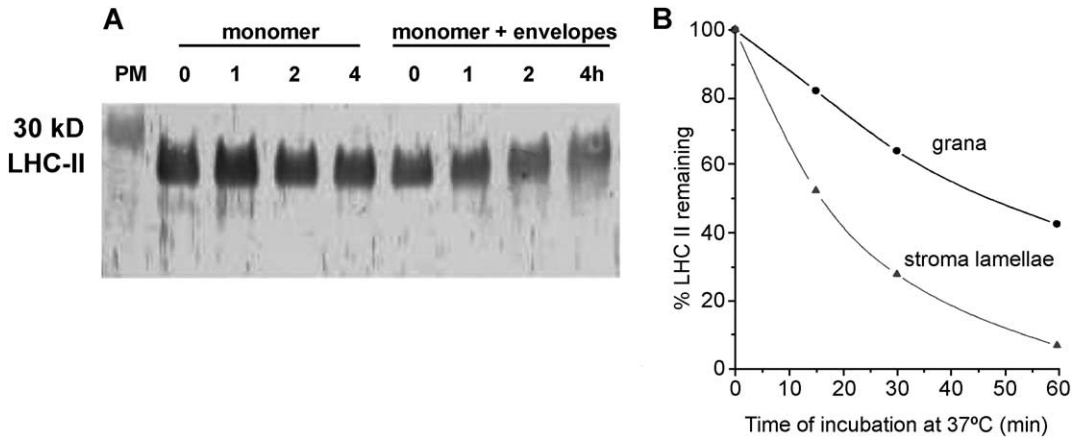

Fig. 3. Detection of protease activity against LHCII in envelope membranes (A), grana and stroma lamellae (B), as monitored by LHCII immunostain reduction on Western blots. (A) LHCII monomer (0.1 μg protein/μl assay buffer) without additions, or LHCII monomer (0.1 μg protein/μl assay buffer) with envelope membranes (0.1 μg protein/μl assay buffer) were incubated at 37 °C for 0, 1, 2 and 4 h. PM: prestained markers. For immunodetection, 1 μg protein was loaded per slot. (B) Stroma and grana thylakoids were lyophilized and suspended in assay buffer to a concentration of 0.05 mg Chl/ml. Thereafter, they were incubated at 37 °C. For immunodetection, 0.125 μg Chl (grana) or 0.25 μg Chl (stroma lamellae) was loaded per slot.

in the separation of all pigment–protein complexes from SDS-solubilized thylakoids, and, in addition, can distinguish between LHCII trimeric and monomeric forms [27]. In all cases, the LHCII preparations exhibited proteolytic activity, but the trimer form separated by SDS–sucrose density gradient ultracentrifugation [27] exhibited the highest. Representative results are shown in Fig. 1 (left). This figure shows Western blots, immunostained with anti-LHCII, of LHCII trimer (a) and monomer (b), isolated by SDS–sucrose density gradient ultracentrifugation [27] and incubated at 37 °C for several hours. Self-degradation of LHCII is evident in the case of the LHCII trimer (a), but not in the case of the LHCII monomer (b) which remains stable at least for 4 hours of incubation.

The proteolytic activity in the LHCII trimer isolated by the SDS–sucrose density gradient ultracentrifugation can also be monitored by electrophoresis of the LHCII trimer on gelatin-containing "activity gels" (see Fig. 1, right). Two transparent activity zones are distinguished on such substrate-containing gels, called here "upper" and "lower" activity zones; the upper one is considered to be due to the strong binding of the protease to the gelatin substrate upon its entrance in the gel; the lower one, which runs just over the LHCII trimer (the strongly Coomassie-stainable band), may possibly be the protease dissociated from the gelatin binding during electrophoresis.

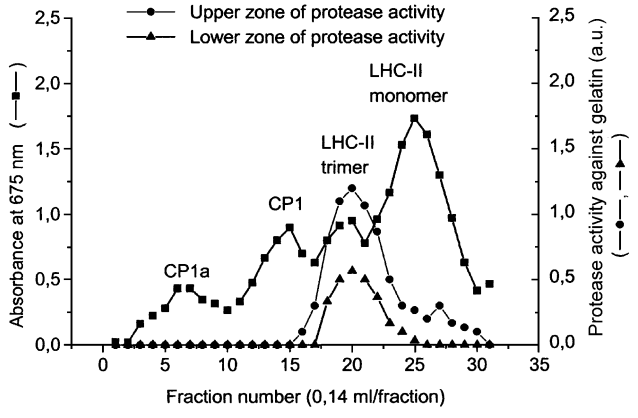

Fig. 4. Fractionation of pigment–protein complexes (as detected by the absorbance of Chl at 675 nm) and of proteolytic activity (as detected on gelatin-containing "activity gels") by SDS–sucrose density gradient ultracentrifugation of SDS-solubilized bean thylakoids.

3.2. Localization of the protease

To check whether this activity is indeed present in the chloroplast, rather than being due to an unspecific association of a high-affinity cellular protease to its substrate,

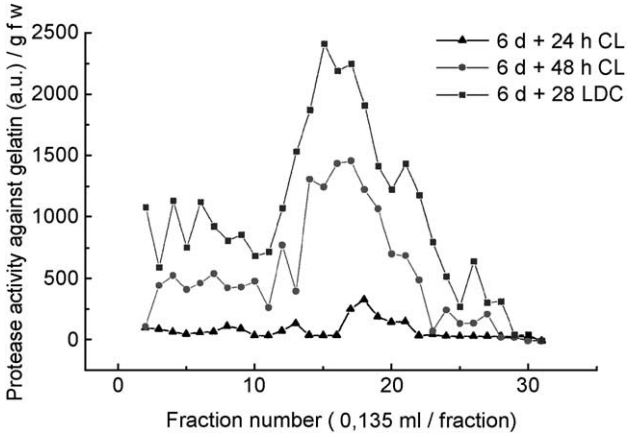

Fig. 5. Resolution of proteolytic activity by SDS–sucrose density gradient ultracentrifugation from thylakoids isolated during greening of etiolated bean plants. Thylakoids were isolated from leaves of 6-day etiolated plants, transferred either to continuous light (CL) for 24 or 48 h, or to intermittent light (28 light–dark cycles, LDC). Gradients were fractionated, and the protease activity in each fraction was assessed on gelatin-containing "activity" gels. Data are normalized on a gfw basis of leaves used for isolation of the thylakoids that were loaded onto each gradient.

LHCII trimer was isolated also from thylakoids of intact chloroplasts and its proteolytic activity tested. Fig. 2 (left) shows gelatin-containing "activity gels" in which LHCII trimer preparations isolated from thylakoids of broken (a) or intact (b) chloroplasts have been analysed. Fig. 2 (right) illustrates "self-degradation" of LHCII in these preparations during incubation at 37 °C, as monitored by the reduction of immunostain on Western blots. As shown, the LHCII trimer zone, isolated by ultracentrifugation of SDS-solubilized thylakoids from intact (a) or broken (b) chloroplasts, exhibits comparable proteolytic activity, as detected by both methods: the transparency of the non-Coomassie-stainable bands on the gelatin activity gel (left), or the reduction of the LHCII immunostain (right). For the same amount of Chl loaded on the activity gel, the ratio of proteolytic activity to the LHCII trimer stain is approximately equal in preparations isolated from intact or broken chloroplasts (vis. average values found 5.25 and 4.1, respectively). In addition, the similar kinetics of the LHCII immunostain reduction shown in Fig. 2 (right) suggest that the protease is indeed localized in the organelle. Fig. 2 right (c) also shows that no degradation is observed in LHCII trimer samples preheated for 3 min at 95 °C. The complete abolishment of degradation by this brief heat pre-treatment speaks in favour of an enzyme-mediated degradation.

In addition, when testing for proteolytic activity various subchloroplast membrane preparations (Fig. 3), very low activity against exogenously added LHCII monomer was found in the membrane fraction derived from the envelopes

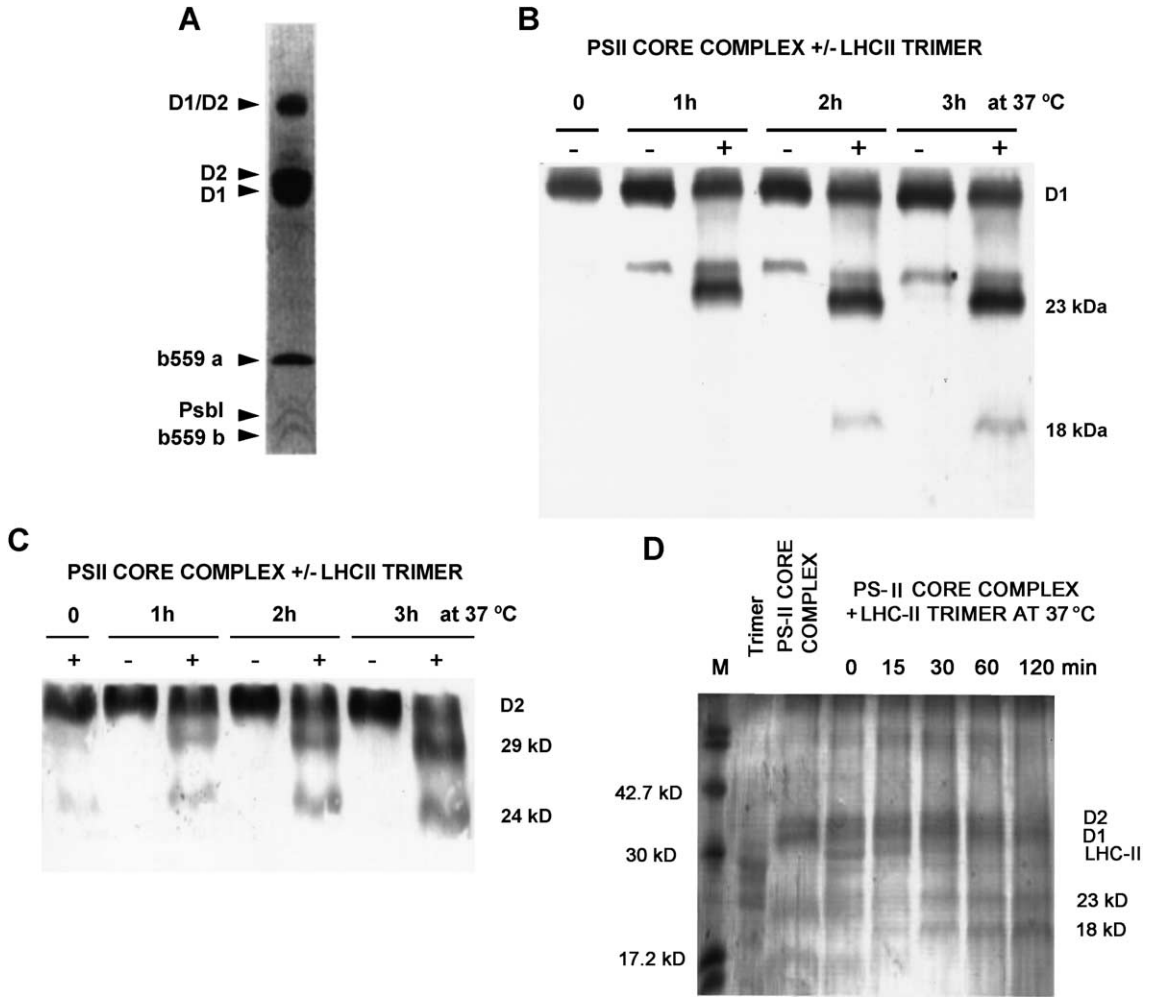

Fig. 6. Proteolysis of the D1 and D2 proteins in a D1/D2/cytb559 PSII core by the LHCII trimer–protease complex during incubation at 37 °C. (A) The Coomassie-stained PSII core protein preparation. (B) Proteolysis of D1, monitored by the D1-immunostain reduction on Western blots and the accumulation of the D1 protein degradation products (anti-D1 raised against the A–B segment of D1). (C) Proteolysis of D2, monitored by the D2-immunostain reduction and the appearance of the D2 protein degradation products [anti-D2 raised against peptide AFNPTQ (235–241 amino acids of spinach D2 sequence)]. (D) PSII core protein degradation as monitored by the degradation products visualized by silver staining. One hundred fifty micrograms PSII core protein in 1350 μl assay buffer was mixed with 150 μl of LHCII trimer zone (187 μg protein) and incubated at 37 °C. At various time intervals, aliquots were withdrawn, lyophilized, resuspended in sample buffer, analysed by SDS-PAGE and either immunodetected on Western blots (B,C) or silver stained (D). In (B) and (C), 7 μg PSII core protein was loaded per slot. In (D), 20 μg PSII core protein was loaded per slot. In (D), the LHCII trimer protein (20 μg, Trimer), and the PSII core protein (20 μg, PSII core protein) before their mixing are also shown. M: molecular weight markers.

(Fig. 3A). For a 50% degradation of LHCII monomer by the envelope fraction, at least 4 h was needed, while in the case of the thylakoid-derived LHCII trimer, 50% degradation is reached in half an hour. In addition, we could not detect any proteolytic activity in the envelope fraction against D1 or gelatin (as judged on an activity gel). These indicate that the protease activity found in thylakoids is not due to contamination from extra-chloroplastic material or envelope membranes. Fig. 3B further shows that in thylakoid fractions, tested for activity against their endogenous LHCII, stroma lamellae exhibit higher activity than grana, consistent with a previous report [8].

3.3. Comigration of the protease with the LHCII trimer band on SDS–sucrose gradients

The results presented in Fig. 1 suggested that the protease activity copurified with the LHCII trimer following ultracentrifugation of SDS-solubilized thylakoids on SDS–sucrose density gradients. Indeed, as shown in the fractionation pattern obtained from such a gradient (see Fig. 4), the protease activity as detected on a gelatin-containing "activity gel" comigrates with the LHCII trimer zone. In contrast, the proteolytic activity in the LHCII monomer fraction or in the CPIa and CPI (the PSI pigment–protein complexes) is almost undetectable. This can explain why "autolysis" of the monomer is lower than that of the trimer (Fig. 1b vs. a, respectively), and supports our finding that the protease directed against LHCII is mainly associated with the LHCII trimer complex.

Fig. 5 shows the resolution of proteolytic activity on SDS–sucrose density gradients following ultracentrifugation of thylakoids obtained during the very early stages of greening. The activity zone peaks at similar fractions, even in thylakoids deficient in LHCII (e.g. the sample obtained from ImL plants—6-day etiolated exposed to 28 light–dark cycles). Here, the area under the proteolytic activity peak is normalized to the fresh weight of leaves used for the isolation of the thylakoids loaded on the gradient. It becomes evident that the activity increases in parallel with leaf age, a finding reflecting the biogenesis of thylakoid membranes. Normalization of the activity to the amount of Chl loaded on each gradient shows a more or less constant ratio of activity units per Chl in samples exposed to continuous light (data not shown). This suggests that accumulation of the protease parallels the accumulation of LHCII. On the contrary, the activity per Chl in the ImL plant is many times higher; this suggests photoactivation of the protease.

3.4. Proteolysis of D1/D2

The proteolytic activity in the LHCII trimer zone has been found to be directed also against the D1 and D2 proteins of the D1/D2 heterodimer in a PSII core protein preparation, kindly offered by K. Satoh (Okayama, Japan). The polypeptide composition of this preparation, reproduced from Tomo and Satoh [37], is shown in Fig. 6A. Incubation of LHCII trimer with the isolated PSII core at 37 °C results in drastic degradation of the D1 and the D2 proteins, as monitored by immunodetection with anti-D1 or anti-D2, respectively (Fig. 6B and C, respectively). Two major degradation products of 23 and 18 kDa are detected with anti-D1; two major degradation products of 29 and 24 kDa are detected with anti-D2. All products accumulate with time of incubation at 37 °C of the PSII core with the LHCII trimer–protease complex; some of these can actually be detected by silver staining (Fig. 6D). The degradation products, which accumulate during incubation at 37 °C, are clearly of PSII core origin, and not of LHCII origin, since the degradation products of LHCII do not accumulate during incubation (Fig. 1). Such products have been

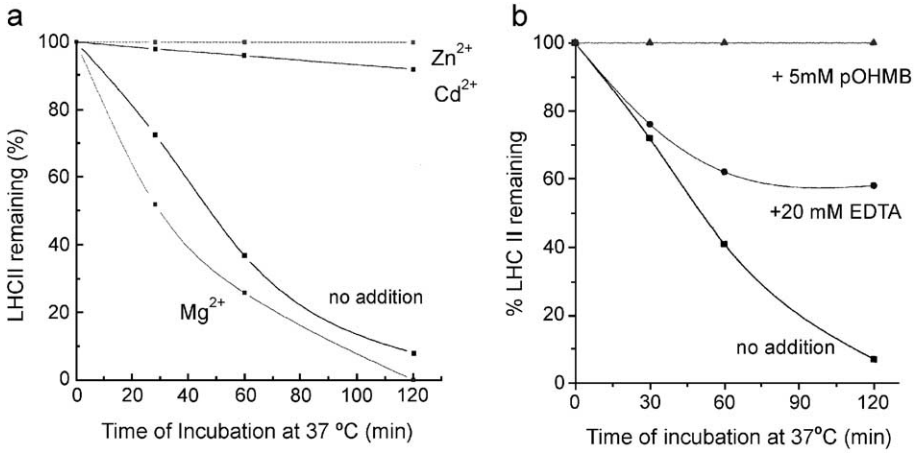

Fig. 7. Proteolytic activity against LHCII, in the LHCII trimer–protease complex, as affected by Mg^{2+}, Cd^{2+}, Zn^{2+} (left), and pOHMB and EDTA (right). Cation concentration was 1 mM. Degradation is monitored by the reduction of LHCII immunostain in assay mixtures containing 0.2 μg LHCII protein/μl assay buffer. All mixtures were first preincubated in an ice bath for 30 min.

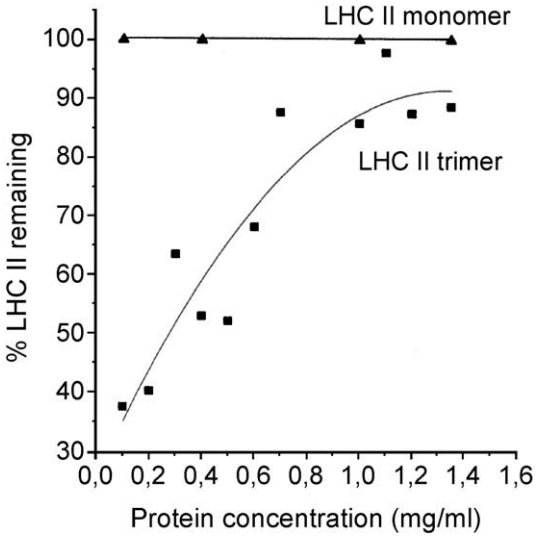

Fig. 8. Self-degradation in LHCII trimer and monomer preparations, as affected by their concentration during incubation in assay buffer at 37 °C for 30 min. Before and following incubation, samples were withdrawn and mixed with an equal volume of SDS sample buffer. For SDS-PAGE, 1 μg LHCII protein was loaded per lane. Degradation is monitored by the LHCII immunostain reduction on Western blots.

reported to accumulate in photoinhibited PSII reaction centers from pea [24]. In addition, these polypeptides have been earlier reported to accumulate upon degradation of the D1 and D2 proteins [19,24]. Thus, the protease activity associated to and directed against LHCII, appears also to degrade the PSII core proteins D1 and D2. This finding suggests that this protease may play a regulatory role exclusively in the assembly and/or adaptation of PSII [9,12,38].

3.5. Characteristics of the enzyme

Fig. 7 shows the activation and inhibition of LHC-II proteolysis in the LHCII trimer–protease complex by metal cations, *p*-hydroxy-mercury benzoate (pOHMB) and EDTA. As shown, Mg^{2+} is an activator, in contrast to Zn^{2+} and Cd^{2+} which inhibit the activity. Twenty millimolars EDTA or 5 mM pOHMB also inhibit the degradation of LHCII in this LHCII–protease complex (by 75% or 100%, respectively, in 30 min at 37 °C). Other proteolytic inhibitors as PMSF were found to have no significant effect on the enzyme (data not shown).

The question, therefore, arises of how the activity of this thylakoidal protease is controlled in the plant. It is more or less generally accepted that Chl stabilizes the LHCII apoprotein in thylakoids, rescuing it from protease attack. To assess whether delipidation of the LHCII monomer enhances its degradation by the LHCII trimer–protease complex, LHCII trimer was incubated at 37 °C with lipid containing or delipidized LHCII monomer. It was found that both monomer preparations were degraded by the LHCII trimer–protease complex at a more or less equal rate (data not shown), suggesting that at least under our experimental conditions during incubation [0.1% (w/v) SDS in the isolated trimer, 0.2% (v/v) Triton X-100 in the assay buffer], Chl and other lipids, expected to be bound on the lipid-containing LHCII, either cannot rescue the protein from degradation, or they have been already dissociated from the apoprotein via the SDS action.

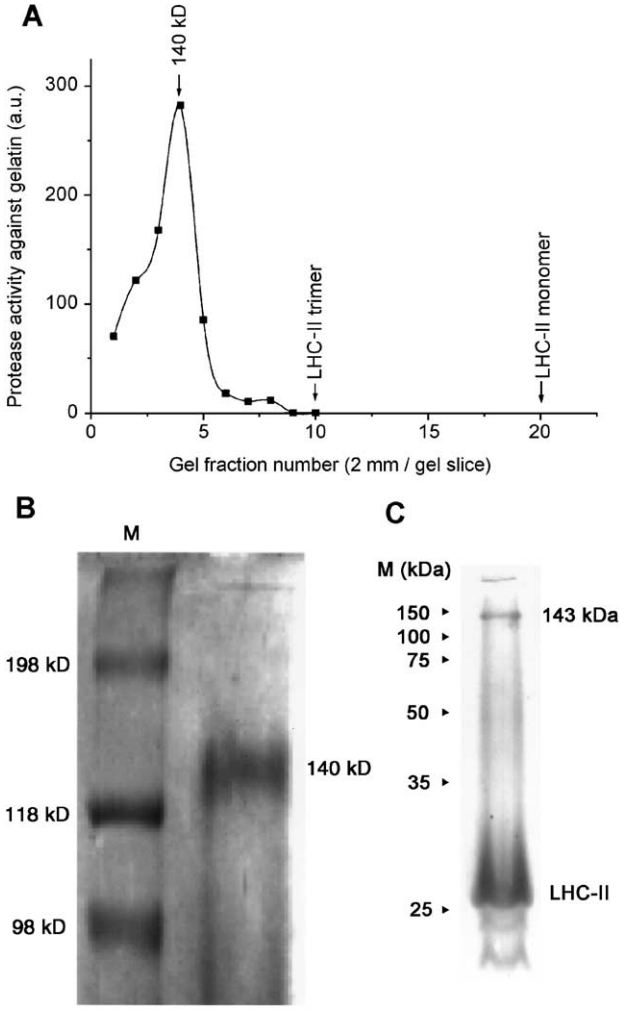

Fig. 9. Separation of protease activity from the LHCII trimer–protease complex by SDS-PAGE. The LHCII trimer–protease zone isolated from the sucrose density gradient following ultracentrifugation of SDS-solubilized thylakoids (150 μl, 180 μg Chl/ml, Chl *a*/Chl *b* ratio = 1.5) was lyophilized and solubilized in 25 μl sample buffer by incubation at 37 °C for 30 min. Forty microliters of such a preparation was loaded into a slot of a 10% (w/v) PA Laemmli mini-gel and electrophoresed for 3 h at 10 mA in the cold. Following SDS-PAGE, the gel was sliced/fractionated (2 mm/slice) and the slices reloaded (without extraction) on a gelatin-containing "activity" gel. After SDS-PAGE, and incubation of the activity gel at 37 °C overnight, the gel was Coomassie blue stained and the transparent protease activity zones were scanned. (A) Fractionation of the activity as monitored by the transparency on the gelatin activity gel. (B) A slot loaded with a preparation as in (A) was Coomassie blue stained; a 140-kDa protein corresponding to the proteolytic activity peak in (A) is detected. (C) The LHCII trimer zone (concentrations as above) was loaded into a slot of a 12.5% PA Laemmli mini-gel containing 6 M urea. Following electrophoresis, the gel was Coomassie blue stained.

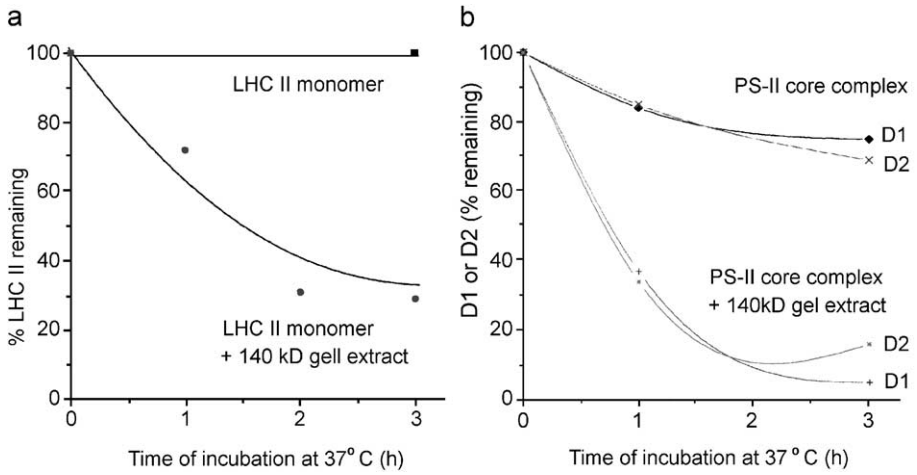

Fig. 10. Degradation of LHCII monomer (left) or of D1 and D2 proteins (right) by the extract of the 140-kDa band (in Fig. 9B). Extraction of the protease from the gel slice was by incubation and shaking of the slice overnight at 4 °C in 150 μl 12% (w/v) sucrose, 0.2% (v/v) Triton X-100, 0.2% (w/v) deoxycholate, 0.05% (w/v) SDS, 0.025 M Tris–0.003 M borate, pH 9.5. Left: Forty microliters of this extract, mixed with 60 μl LHCII monomer in assay buffer (0.1 μg LHCII protein/μl final concentration), was incubated at 37 °C; for immunoblot, 1 μg LHCII was loaded per slot. Right: Forty microliters of this extract, mixed with 260 μl PSII core proteins in assay buffer (0.1 μg PSII core proteins/μl final concentration), was incubated at 37 °C; for immunoblot, 7 μg PSII core proteins was loaded per slot. Anti-D1 was raised against the D–E loop of D1; anti-D2 was raised against peptide AFNPTQ (235–241 amino acids of spinach D2 sequence).

Self-degradation of LHCII in preparations of the trimer, as affected by the concentration of the trimer–protease complex incubated at 37 °C, is shown in Fig. 8. The extent of LHCII proteolysis is gradually reduced as the concentration of the LHCII trimer–protease complex is increased. Here, the Triton X-100 in the assay mixtures at increasing trimer–protease concentrations ranged from 0.186% (v/v) to 0, and the SDS from 0.007% to 0.1% (w/v), respectively. These concentration ranges do not affect the enzyme activity. Thus one may conclude that at high LHCII concentrations, as expected to prevail in thylakoids, the extent of proteolysis is drastically reduced. In addition, assuming that the protease concentration in the complex is very low, one can calculate that at a LHCII-trimer protein concentration of 0.2 μg/μl, 0.6 μg LHCII protein is degraded per microgram LHCII protein present in the LHCII–protease complex during a 30-min incubation at 37 °C, that is, 0.02 μg or about 1×10^{-6} μmol LHCII is degraded per minute.

3.6. Attempts to separate the protease from the LHCII trimer–protease complex

Fig. 9 illustrates the results of attempts to separate the protease from the LHCII trimer preparation by SDS-PAGE. The LHCII trimer–protease complex was lyophilized and dissolved in solubilization sample buffer to a final protein concentration of approximately 8 μg/μl. The mixture was incubated at 37 °C for 30 min to induce dissociation of the protease from its substrate; the proteolytic activity of the sample, as tested on a gelatin activity gel, was found to be reduced by about 50% by this treatment. Following electrophoresis on a 10% (w/v) polyacrylamide gel for 3 h, part of the gel was fractionated (sliced) and each slice tested for protease activity and another part was Coomassie stained for protein detection. As shown in Fig. 9A,B, only one band, that is resolved well above the LHCII trimer band, exhibited proteolytic activity. This band corresponds to a 140-kDa polypeptide (Fig. 9B), which runs as a 143-kDa polypeptide on 6 M urea-supplemented 12.5% PA gels (Fig. 9C). For the latter molecular weight estimation, recombinant protein molecular weight markers were used. The 140-kDa polypeptide band (Fig. 9B) was cut off, extracted from the gel and when tested for proteolytic activity, was found to be active against LHCII monomer and also against the D1 and D2 proteins of the PSII core (Fig. 10). During incubation at 37 °C, the substrate concentration (LHCII monomer or PSII core) was 0.1 μg/μl, and the ratio of substrate to protease was 4:1 (w/w).

4. Discussion

In this work we demonstrate, to our best knowledge, for the first time, the existence of a protease in the thylakoid membrane in close association with the antenna complex, capable of degrading the LHCII antenna but also the core proteins of PSII. The protease is bound peripherally at the stromal face of thylakoids, as already shown [9], it is directed against the mature LHCII already preaccumulated in thylakoids and accumulates in thylakoids during chloroplast development.

It is hard to extrapolate the significance of these in vitro results to the in vivo system; however, the close association of the protease to the LHCII trimer and the finding that it can also degrade drastically the D1 and D2 proteins in a PSII core preparation, suggest that it may function as a

regulatory factor in PSII unit stabilization/adaptation, but also in the vital D1 turnover, considered to take place in stroma lamellae, that occurs following the inevitable photoinactivation of all PSII units under normal non-stressed conditions.

In addition, the finding that stroma lamellae exhibit higher proteolytic activity against their endogenous LHCII than grana do, may further suggest that the activity of the protease may regulate the content of LHCII and the proportion of PSIIa vs. PSIIb centers in thylakoids; as known grana are rich in LHCII and PSIIa centers in contrast to stroma lamellae which are deficient in LHCII and contain PSIIb centers. The higher protease activity in stroma lamellae fraction may also reflect the fact that the protease is bound peripherally at the stromal face of the thylakoid membrane.

The specific fragments released during its action argue for a specific protease. In the case of LHCII degradation, these specific fragments are further degraded by the protease, but in the case of the D1 and D2 proteins, the degradation products accumulate with time. In the case of the D1 protein, the main degradation product of 23 kDa (Fig. 6B) is known to result from the primary cleavage in the stromal loop, between the transmembrane D and E helices, of the D1 protein [23]. Its accumulation suggests resistance to further degradation, and requirement of an additional protease for its complete digestion. A good candidate for the latter might be the FtsH metalloprotease [16], shown to degrade the 23-kDa primary cleavage product. In the case of the D2 protein, the main degradation products that one can see to accumulate under our experimental conditions are of 29 and 24 kDa (products already shown to originate in D2 [19,24]). The accumulation of these degradation products parallel to D1 and D2 immunostain reduction speaks for proteolysis of both proteins by this protease.

Our results on the inhibition of the enzyme (Fig. 7) suggest that the protease may belong to the cysteine class of proteases, a suggestion made also earlier for the protease involved in LHCII degradation during high light acclimation [11], as well as during biogenesis of chloroplasts [8]. In studies concerning the D1 degradation, it has been suggested that the putative protease belongs to the serine class of proteases. However, in our experiments, no significant inhibition using serine protease inhibitors such as PMSF was found.

The molecular weight of the protease, found to be in the order of 140 kDa (Fig. 9A), is relatively high. This may indicate that it is not a single polypeptide but an enzyme complex. However, even prolonged incubation (2 h at 37 °C) of the LHCII trimer–protease complex with different sample buffers (with high β-mercaptoethanol or dithiothreitol content) releases the same 140-kDa polypeptide. This suggests that even in case the protease is a complex composed of a number of proteins, these should be tightly associated.

The evidence obtained by Hoober and Eggink [39] shows that during chloroplast biogenesis, the nuclear-coded LHCII protein, to be imported into the chloroplast, in the absence of adequate amount of Chl, is degraded outside the chloroplast, in the cellular vacuoles, a site of proteolysis. Our work suggests that LHCII, once bound and accumulated in thylakoids as a Chl–protein complex, is degraded by a thylakoid-localized protease in the chloroplast.

Earlier work had suggested that the protease is free to act on LHCII when thylakoids are detergent solubilized [8,9,40], or incubated under conditions of pigment photooxidation (high light) [8]. The present work further shows that LHCII is degraded faster in the LHCII–protease complex when present in dilute solutions (see Fig. 8), conditions under which the trimer is expected to be less organized.

The generally accepted concept that LHCII accumulating in thylakoids is rescued from proteolytic attack via Chl–protein complex formation is now questioned. As found in this work, delipidized LHCII monomer is as good a substrate as the non-delipidized one, suggesting that Chl and other lipids may not confer stability to this protein, their presence being necessary mainly for anchoring the protein into the lipophilic thylakoid membrane. In such a case, Chl may play a role in LHCII stabilization only at the very early stages of LHCII import in the chloroplast [39]. The formation of the pigment–protein complex may be a prerequisite for rescuing the Lhcb gene translation products in the chloroplast envelope during import and not at later stages, when preaccumulated LHCII in chloroplast thylakoids is degraded. However, it is also possible that pigment and other lipids are not strongly bound to the isolated monomer protein under our experimental conditions so that delipidation does not add to further dissociation. This is supported by earlier results obtained in vitro but also in vivo. The former show that high proteolytic activity against LHCII is detected in bean thylakoids after their solubilization in detergent [8,9] or upon photooxidation of their pigments (non-solubilized thylakoids) [8]. The results in vivo show that thylakoids of leaves greened in continuous light display higher proteolytic activity when incubated in the presence of Chl biosynthesis inhibitors [41]. In thylakoids of plants, first exposed briefly to continuous light and then transferred to the dark—where further Chl synthesis is inhibited, but new PSU reaction centers continue to be formed—the preaccumulated LHCII is degraded [25,42]. The question, therefore, still remains whether the Chl-to-protein binding is the means for rescuing preaccumulated LHCII in thylakoids. Based on the results presented in Fig. 8, suggesting that degradation of LHCII is blocked at high LHCII trimer–protease complex concentrations, one may conclude that a means for LHCII rescuing in thylakoids may also be the attainment of high LHCII concentrations in thylakoids. As estimated, thylakoids of mature plants have a Chl concentration of 2 M (2 μmol/μl, i.e. 2000 μg/μl). Based on a ratio of 130 μg Chl/mg LHCII protein in the isolated trimer, thylakoids may

contain 15 mg LHCII protein/μl, a density high enough to inhibit the protease action completely.

Our work also shows that D1 and D2 are as good a substrate for this protease as LHCII. This activity is detected under non-photoinhibitory conditions, and the degradation of D1 is very rapid. This protease may therefore be part of the D1 turnover system. We have found earlier that degradation of D1/D2 is dependent on the phosphorylation state of the thylakoid [20,21]. We do not know whether phosphorylation of this protease may affect its activity, but it is worth looking for an experimental system that will help check it. The data showing that this protease is detectable even in etiolated tissues (Fig. 5) suggest that it may be responsible for the generation of the 23-kDa product of the D1 protein, observed in etiolated tissues where mature D1 is not detectable despite the presence of its psbA transcripts [43,44].

Recently, a DegP2 protease has been shown to perform the primary cleavage of the photodamaged D1 protein [15]. However, the DegP2 protease was found not to degrade LHCII, a finding which speaks against the possibility that it is the same protease as that isolated in the present study. Note that in this work we have concentrated on the LHCII, D1 and D2 degradation by the protease. Other proteins of the thylakoid membrane may be targets of this protease as well, a possibility to be tested in further work.

A similar proteolytic system directed against LHCII, activated under high light, isolated in the high salt wash of thylakoids, was shown recently to degrade LHCII trimer of spinach at a lower rate than its LHCII monomer, which was generated by incubation of the trimer with phospholipase [45]. In contrast, under our experimental conditions, trimer and monomer seem to be degraded at a more or less equal rate, by the LHCII protease copurified with the trimer. This indicates that the two proteases may not be identical.

As noted above, all LHCII preparations, irrespective of method of isolation used, exhibit “autolysis”. In addition, all mild efforts to separate the proteolytic activity from the LHCII trimer were unsuccessful; even after incubation of the LHCII trimer with 100 mM Na^+ and ultracentrifugation on an SDS-gradient containing 100 mM Na^+, only partial separation could be achieved. The only method that succeeded in separating the protease from the LHCII trimer was that of SDS-PAGE (see Fig. 9). All these results exclude the possibility that the association of the protease to the LHCII trimer is due to a simple copurification, and suggest that the protease may belong to the PSII supercomplex, functioning in situ during assembly and adaptation of the unit.

Acknowledgements

This work was supported by grants PENED-1998 (Greek Ministry of Development—General Secretariat of Research and Technology) and DFG So.448/2.

The antiserum raised against LHCII was a kind offer of Prof. Dr. K. Kloppstech (Hannover, Germany); the isolated PSII core protein preparation and the anti-D1, raised against the A–B loop, were kindly offered by Dr. K. Satoh (Okayama, Japan); the anti-D1, raised against the D–E loop, was a kind offer of Drs. I. Ohad (Jerusalem, Israel) and E.-M. Aro (Turku, Finland).

References

[1] A. Zouni, H.-T. Witt, J. Kern, P. Fromme, N. Krauß, W. Saenger, P. Orth, Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution, Nature 409 (2001) 739–743.
[2] F.-A. Wollman, L. Minai, R. Nechushtai, The biogenesis and assembly of photosynthetic proteins in thylakoid membranes, Biochim. Biophys. Acta 11411 (1999) 21–85.
[3] J. Nield, E.V. Orlova, E.P. Morris, B. Gowen, M. van Heel, J. Barber, 3D map of the plant photosystem II supercomplex obtained by cryoelectron microscopy and single particle analysis, Nat. Struct. Biol. 7 (2000) 44–47.
[4] Z. Adam, Protein stability and degradation in chloroplasts, Plant Mol. Biol. 32 (1996) 773–783.
[5] B. Andersson, E.-M. Aro, Proteolytic activities and proteases of plant chloroplasts, Physiol. Plant. 100 (1997) 780–793.
[6] A. Sokolenko, L. Altschmied, R.G. Herrmann, Sodium dodecyl sulphate-stable proteases in chloroplasts, Plant Physiol. 115 (1997) 827–832.
[7] I. Adamska, Light stress-activated proteases in the thylakoid lumen, in: J.H. Argyroudi-Akoyunoglou, H. Senger (Eds.), The Chloroplast: From Molecular Biology to Biotechnology, NATO ASI, vol. 3/64, Kluwer Academic, Netherlands, 1999, pp. 265–270.
[8] R. Anastassiou, J.H. Argyroudi-Akoyunoglou, Thylakoid-bound proteolytic activity vs LHCII apoprotein in bean, Photosynth. Res. 43 (1995) 241–250.
[9] L.-A. Tziveleka, J.H. Argyroudi-Akoyunoglou, Implications of a developmental-stage-dependent thylakoid-bound protease in the stabilization of the light-harvesting pigment–protein complex serving photosystem II during thylakoid biogenesis in Red Kidney Bean, Plant Physiol. 117 (1998) 961–970.
[10] L.-A. Tziveleka, J.H. Argyroudi-Akoyunoglou, Proteolytic mechanism in LHCII stabilization, in: J.H. Argyroudi-Akoyunoglou, H. Senger (Eds.), The Chloroplast: From Molecular Biology to Biotechnology, NATO ASI, vol. 3/64,Kluwer Academic, Netherlands, 1999, pp. 277–282.
[11] M. Lindahl, D.-H. Yang, B. Andersson, Regulatory proteolysis of the major light-harvesting chlorophyll *a*/*b* protein of photosystem II by light-induced membrane associated enzymic system, Eur. J. Biochem. 213 (1995) 503–509.
[12] D.-H. Yang, J. Webster, Z. Adam, M. Lindahl, B. Andersson, Induction of acclimative proteolysis of the light-harvesting chlorophyll *a*/*b*–protein of photosystem II in response to elevated light intensities, Plant Physiol. 118 (1998) 827–834.
[13] J.K. Hoober, M.J. Hughes, Purification and characterization of a membrane-bound protease from *Chlamydomonas reinhardtii*, Plant Physiol. 99 (1992) 932–937.
[14] M. Lensch, R.G. Herrmann, A. Sokolenko, Identification and characterization of SppA, a novel light-inducible chloroplast protease complex associated with thylakoid membranes, J. Biol. Chem. 276 (2001) 33645–33651.
[15] K. Haußühl, B. Andersson, I. Adamska, A chloroplast DegP2 protease performs the primary cleavage of the photodamaged D1 protein in plant photosystem II, EMBO J. 20 (2001) 713–722.
[16] M. Lindahl, C. Spetea, T. Hundal, A. Oppenheim, Z. Adam, B.

Andersson, The FtsH protease plays a role in the light-induced turnover of the photosystem II D1 protein, Plant Cell 12 (2000) 419–431.
[17] C. Sundby, S. McCaffery, J.M. Anderson, Turnover of the photosystem II D1 protein in higher plants under photoinhibitory and non-photoinhibitory irradiance, J. Biol. Chem. 268 (1993) 25476–25482.
[18] N. Keren, A. Berg, P.J.M. van Kan, H. Levanon, I. Ohad, Mechanism of photosystem II photoinactivation and D1 protein degradation at low light: the role of back electron flow, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 1579–1584.
[19] E. Kanervo, N. Murata, E.-M. Aro, Massive breakdown of the photosystem II polypeptides in a mutant of the cyanobacterium *Synechocystis* sp. PPC 6803, Photosynth. Res. 57 (1998) 81–91.
[20] J.H. Georgakopoulos, J.H. Argyroudi-Akoyunoglou, Implication of D1 degradation in phosphorylation-induced state transitions, Photosynth. Res. 53 (1997) 185–195.
[21] J.H. Georgakopoulos, J.H. Argyroudi-Akoyunoglou, Thylakoid protein phosphorylation is suppressed by "free radical scavengers". Correlation between PSII core protein degradation and thylakoid protein phosphorylation, Photosynth. Res. 58 (1998) 269–280.
[22] A. Ferjani, S. Abe, Y. Ishikawa, T. Henmi, Y. Tomokawa, Y. Nishi, N. Tamura, Y. Yamamoto, Characterization of the stromal protease(s) degrading the cross-linked products of the D1 protein generated by photoinhibition of photosystem II, Biochim. Biophys. Acta 1503 (2001) 385–395.
[23] B.M. Greenberg, V. Gaba, A.K. Matto, M. Edelman, Identification of a primary in vivo degradation product of the rapidly-turning-over 32 kd protein of photosystem II, EMBO J. 6 (1987) 2865–2869.
[24] C.A. Shipton, J. Barber, In vivo and in vitro photoinhibition reactions generate similar degradation fragments of D1 and D2 photosystem II reaction center proteins, Eur. J. Biochem. 220 (1994) 801–808.
[25] J.H. Argyroudi-Akoyunoglou, A. Akoyunoglou, K. Kalosakas, G. Akoyunoglou, Reorganization of the PSII unit in developing thylakoids of higher plants after transfer to darkness. Changes in Chl*b*, LHCII content and grana stacking, Plant Physiol. 70 (1982) 1242–1248.
[26] R. Douce, R.B. Holtz, A.A. Benson, Isolation and properties of the envelope of spinach chloroplasts, J. Biol. Chem. 248 (1973) 7215–7222.
[27] J.H. Argyroudi-Akoyunoglou, H. Thomou, Separation of thylakoid pigment–protein complexes by SDS-sucrose density gradient centrifugation, FEBS Lett. 135 (1981) 177–181.
[28] J.H. Argyroudi-Akoyunoglou, Isolation of the 25–23 Kdalton polypeptide(s) from SDS-solubilized thylakoids: their importance in the onset of the cation-induced control of spillover and grana formation, in: G. Akoyunoglou, J.H. Argyroudi-Akoyunoglou (Eds.), Chloroplast Development, Elsevier/North-Holland Biomedical Press, 1978, pp. 401–412.
[29] J.J. Burke, C.L. Ditto, C.J. Arntzen, Involvement of the light-harvesting complex in cation-regulation of excitation energy distribution in chloroplasts, Arch. Biochem. Biophys. 187 (1978) 252–263.
[30] J.K. Hoober, R.H. Millington, L.P. D'Angelo, Structural similarities between the major polypeptides of thylakoid membranes from *Chlamydomonas reinhardtii*, Arch. Biochem. Biophys. 202 (1980) 221–234.
[31] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 4350–4354.
[32] M.S. Blake, K.H. Johnston, G.J. Russel, E.C. Gotschlich, A rapid sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots, Anal. Biochem. 136 (1984) 175–180.
[33] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 277 (1970) 680–685.
[34] G. Mackinney, Absorption of light by chlorophyll solutions, J. Biol. Chem. 140 (1941) 315–322.
[35] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, Protein measurement with Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[36] J.H. Georgakopoulos, J.H. Argyroudi-Akoyunoglou, On the question of lateral migration of LHCII upon thylakoid protein phosphorylation in isolated pea chloroplasts: the stroma lamellar fraction separated from phosphorylated chloroplasts is not homogeneous, Biochim. Biophys. Acta 1188 (1994) 380–390.
[37] T. Tomo, K. Satoh, Nearest neighbour analysis of D1 and D2 subunits in the photosystem II reaction center using a bifunctional cross-linker, hexamethylene diisocyanate, FEBS Lett. 351 (1994) 27–30.
[38] G. Tzinas, J.H. Argyroudi-Akoyunoglou, G. Akoyunoglou, The effect of dark interval in intermittent light on thylakoid development: photosynthetic unit formation and light-harvesting protein accumulation, Photosynth. Res. 14 (1987) 241–258.
[39] K. Hoober, L. Eggink, Assembly of light-harvesting complex II and biogenesis of thylakoid membranes in chloroplasts, Photosynth. Res. 61 (1999) 197–215.
[40] T. Honda, H. Ito, Y. Tanaka, A. Tanaka, H. Tsuji, Proteolytic digestion of apoproteins of light-harvesting chlorophyll *a*/–protein complexes in barley leaves, in: J.H. Argyroudi-Akoyunoglou (Ed.), Regulation of Chloroplast Biogenesis, NATO ASI, vol. A226, Plenum, New York, 1991, pp. 337–341.
[41] R. Anastassiou, J.H. Argyroudi-Akoyunoglou, Thylakoid bound proteolytic activity vs exogenous azocoll substrate during chloroplast development in bean, J. Plant Physiol. 145 (1995) 24–30.
[42] G. Akoyunoglou, J.H. Argyroudi-Akoyunoglou, Posttranslational regulation of chloroplast differentiation, in: G. Akoyunoglou, H. Senger (Eds.), Regulation of Chloroplast Differentiation, A.R. Liss, NY, 1986, pp. 329–338.
[43] J.E. Mullet, P.G. Klein, R.R. Klein, Chlorophyll regulates accumulation of the plastid-encoded chlorophyll apoproteins CP 43 and D1 by increasing apoprotein stability, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 4038–4042.
[44] J.H. Georgakopoulos, A. Prombona, J.H. Argyroudi-Akoyunoglou, The abundance of Cab and psbA transcripts and of their products in greening etiolated leaves transferred to darkness, in: J.H. Argyroudi-Akoyunoglou, H. Senger (Eds.), The Chloroplast: From Molecular Biology to Biotechnology, Kluwer Academic Publishers, Netherlands, 1999, pp. 247–252.
[45] D.H. Yang, H. Paulsen, B. Andersson, The N-terminal domain of the light-harvesting chlorophyll *a*/*b* binding protein complex (LHCII) is essential for its acclimative proteolysis, FEBS Lett. 466 (2000) 385–388.