The N-terminal domain of the light-harvesting chlorophyll *a/b*-binding protein complex (LHCII) is essential for its acclimative proteolysis

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Received 11 November 1999

Edited by Richard Cogdell

Abstract Variations in the amount of the light-harvesting chlorophyll *a/b*-binding protein complex (LHCII) is essential for regulation of the uptake of light into photosystem II. An endogenous proteolytic system was found to be involved in the degradation of LHCII in response to elevated light intensities and the proteolysis was shown to be under tight regulation [Yang, D.-H. et al. (1998) Plant Physiol. 118, 827–834]. In this study, the substrate specificity and recognition site towards the protease were examined using reconstituted wild-type and mutant recombinant LHCII. The results show that the LHCII apoprotein and the monomeric form of the holoprotein are targeted for proteolysis while the trimeric form is not. The N-terminal domain of LHCII was found to be essential for recognition by the regulatory protease and the involvement of the N-end rule pathway is discussed.

Key words: Light-harvesting complex II; Proteolysis; Acclimative protease; N-terminal domain; Recognition site; Recombinant light-harvesting complex II

1. Introduction

The steady-state levels of chloroplast proteins, like all cellular proteins, are balanced by coordination between their synthesis and degradation. Regulatory proteolysis has been found to play a number of essential roles in determining the overall function of an organelle [1]. Endogenous protein degradation is a complicated and often a highly specific biological process, which shows a high degree of regulation. It may require a multitude of proteolytic systems combined with other enzymatic reactions [1,2]. Moreover, regulatory proteolysis often requires not only an active protease, but also a signal within the substrate protein itself [3].

We have recently identified a novel endogenous proteolytic activity involved in the acclimative degradation of the major light-harvesting complex (LHCII), when the light-harvesting antenna of photosystem II (PSII) is reduced in response to elevated light intensities [4,5]. The protease involved is extrinsically bound to the outer surface of the stroma-exposed thylakoid regions and is of serine or cysteine type. The proteolytic activity was found to be strictly ATP-dependent. It was further demonstrated that initiation of the acclimative proteolysis of LHCII requires regulation at both the enzyme and the substrate levels [5]. It was found that the non-phosphorylated form of LHCII is the substrate for the protease while the phospho-LHCII is protected from proteolysis [5]. It is unclear, however, whether the native trimeric form of LHCII can be the direct target for the protease or whether it has to be dissociated into monomers to be recognized by the protease and whether this is influenced by the phosphorylation/dephosphorylation state. Moreover, the identity of the protease and the location of the substrate recognition site for the regulatory protease remain unknown.

Monomeric LHCII can be reconstituted in vitro from the purified apoprotein, overexpressed in *Escherichia coli*, and pigments purified from chloroplast membranes, in the presence of detergents [6]. The reconstituted LHCII monomers can be assembled into the trimeric form in vitro in the presence of lipid [7]. The resulting monomeric and trimeric complexes closely resemble the native LHCII monomers and trimers in their stability, electrophoretic behavior, spectroscopic properties and pigment composition [6,7]. Moreover, mutant LHCII protein with a truncated N-terminal can successfully be reconstituted into mutant monomeric form of LHCII [8,9]. This opens up the experimental possibility to investigate the substrate specificity and the mechanism of substrate recognition by the LHCII proteolytic system. In this work, we report that the LHCII apoprotein degrades faster than the holoprotein. The monomeric form of LHCII is specifically recognized for proteolysis whereas the LHCII trimer is resistant to the proteolytic digestion. The N-terminal domain of the protein was found to be essential for the recognition of the protein by the acclimative protease system, indicating a degradation via the N-end rule pathway [10,11].

2. Materials and methods

2.1. Preparation of thylakoid membranes and protease-enriched salt wash supernatant

Spinach (*Spinacia oleracea*) plants were grown for 4 weeks at a photon flux density (PFD) of 30 μmol/m^2/s (low light). The light/dark cycle was 10 h/14 h. The mature low light grown spinach plants were transferred to a PFD of 600 μmol/m^2/s (high light) for 2.5 days including the dark night period. Thylakoids were isolated from the high light acclimating plants and washed three times in incubation medium containing 0.5 M NaCl as described before [5]. The protease-enriched wash supernatant was concentrated by centrifugation on centricons, dialyzed overnight at 4°C against incubation medium

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Abbreviations: chl, chlorophyll; ECL, enhanced chemiluminescence; LHCII, light-harvesting chlorophyll *a/b* protein complex of photosystem II; LM, lauryl maltoside; OG, octyl glucoside; PFD, photon flux density; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

PII: S0014-5793(00)01107-8

(pH 7.5) and used for assays of proteolytic activity. The protein concentration in the supernatant was determined according to Bradford [12].

2.2. Preparation of native LHCII trimer, monomer and apoprotein

LHCII trimers were isolated from spinach according to Burke et al. [13]. LHCII monomers were generated by incubation of the trimeric complex with 3 μg phospholipase A_2/ml from bee venom (Sigma) in a medium containing 0.6% (w/v) octyl glucoside (OG) with a chlorophyll (chl) concentration of 1 mg/ml according to Nußberger et al. [14]. After incubation, the samples were dialyzed against incubation buffer containing 0.1% (w/v) lauryl maltoside (LM) and then loaded onto a 12.5–25% sucrose gradient and centrifuged at 300 000 × *g* for 18 h at 4°C [7]. The monomer band was removed and concentrated. For preparation of LHCII apoprotein, the purified LHCII trimer was washed three times with 80% (v/v) acetone. The white pellet after centrifugation was solubilized in 0.1% (w/v) sodium dodecyl sulfate (SDS) and dialyzed against 1% (w/v) OG overnight.

2.3. Reconstitution and trimerization of LHCII monomer from overexpressed LHCII protein

The pea (*Pisum sativum*) *lhcb1*2* clone derived from the AB 80 gene [15] was constructed stepwise by replacement of the 5′-proximal region of the reading frame in XLHCP-2 with complementary synthetic oligonucleotides as described before [6,16]. The N-terminal deletion mutants ΔN-11 and ΔN-58 were constructed by exonucleolytic digestion and religation detailed by Paulsen and Hobe [8]. The full-length Lhcb1 protein and N-terminally truncated mutants were overexpressed in *E. coli*, purified and reconstituted with pigments to monomeric complexes according to Paulsen et al. [17]. Standard reconstitution assays (100–500 μl) contained 0.4 mg/ml LHCP, 1.0 mg/ml chl (chl *a*:*b* = 1) and 0.15 mg/ml xanthophyll in reconstitution buffer (2% LDS, 100 mM Tris–HCl, pH 9.0, 12.5% sucrose, 5 mM ε-aminocaproic acid, 1 mM benzamidine). Trimerization of reconstituted monomeric LHCII was carried out by adding dipalmitoylphosphatidylglycerol (200 μg/ml) to the reconstitution mixture followed by sonication (5–15 min) [9]. LHCII trimer was separated on 12.5–25% sucrose density gradient containing 0.1% LM as described by Hobe et al. [9].

2.4. Proteolysis of LHCII by the acclimative protease

Proteolytic activity was assayed by adding either the native or recombinant LHCII trimer, monomer or apoprotein to the protease-enriched wash supernatant at a ratio of 1:50 (w/w) of LHCII to the proteins in the wash supernatant in the presence of 0.5 mM ATP and 0.05% (w/v) OG or LM. The reaction mixture was incubated at 30°C in darkness. Aliquots were taken at different time intervals and frozen in liquid nitrogen. The loss of LHCII was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) [18] and immunoblotting using antibody against LHCII [5] and visualized by the alkaline phosphatase-conjugated enhanced chemiluminescence (ECL) method (Zymed). The amount of LHCII was quantified by laser densitometry scanning.

3. Results

3.1. Proteolysis of different forms of native LHCII and overexpressed LHCII protein after reconstitution

Our previous studies have shown that the acclimative proteolytic system for LHCII was extrinsically located at the outer surface of the stroma-exposed thylakoid regions and could be removed by washing of the isolated membranes using a high-salt medium [4]. The proteolytic activity could be reconstituted by readdition of the desalted wash supernatant. This active supernatant was therefore used as an experimental tool to study the substrate specificity of the endogenous LHCII protease.

The proteolytic activity in the protease-enriched salt wash supernatant was assayed with different types of LHCII samples. A low concentration of detergent (0.05% OG or LM) was included in the experimental system to avoid protein aggregation during the incubation period. Such detergent conditions have proven not to be inhibitory to the proteolytic activity (not shown). The apoprotein of LHCII was degraded almost to completion after 4 h incubation with the protease extract (Fig. 1). This is consistent with earlier observations [19,20] that LHCII is rapidly degraded in the thylakoid membrane during biogenesis unless there are stabilizing pigments present.

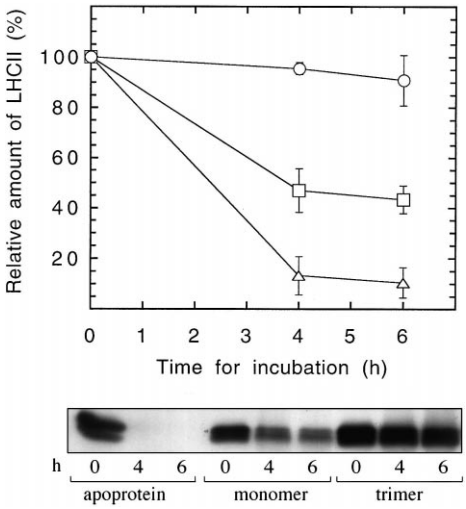

Fig. 1. Degradation of native spinach LHCII trimer, monomer and apoprotein by the acclimative protease. Proteolytic activity against the trimeric form (circles), monomeric form (squares) and apoprotein (triangles) of LHCII was assayed using a high-salt wash supernatant of thylakoids obtained from spinach leaves acclimated to high light for 2.5 days. After incubation in the presence of 0.5 mM ATP at the time indicated, samples were taken and loaded onto SDS–PAGE with a gradient of 12–22.5% (0.5 μg LHCII protein per well). The LHCII protein was detected by immunoblotting, visualized by alkaline phosphatase-conjugated ECL and quantified by laser densitometry scanning. Representative immunoblots of LHCII are shown in the lower panel. The amount of LHCII protein before incubation with the proteolytic fraction was taken as 100%. The results are the means ± S.E.M. of four independent experiments.

Furthermore, the two oligomeric forms of native LHCII, monomer and trimer, were found to behave differently when subjected to the acclimative protease. The LHCII monomer was degraded up to 50% after 4 h proteolytic treatment, whereas the trimer remained stable within a period of 6 h incubation (Fig. 1).

As a second approach, overexpressed full-length apo-

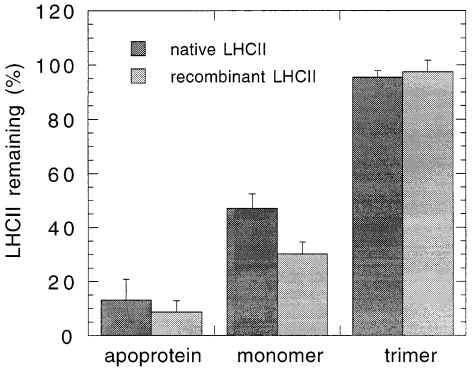

Fig. 2. Proteolysis of recombinant LHCII protein and its pigment-reconstituted monomer and trimer after 4 h incubation with the proteolytic supernatant. Samples were analyzed as shown in Fig. 1. The results are the means ± S.E.M. of four independent experiments.

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                    10        20        30        40        50        60        70
                    |         |         |         |         |    ↓    |         |
Lhcb1   MRKSATTKKVASSGSPWYGPDRVKYLGPFSGESPSYLTGEFPGDYGWDTAGLSADPETFSKNRELEVIHSR~
ΔN-11           MR----------------------------------------------------------~
ΔN-58                                                                 MSSL-----~
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Fig. 3. N-terminal amino acid sequences of the pea Lhcb1 protein and its truncated derivatives. Numbering of amino acids begins one residue downstream from the leading methionine [30]. Dashed lines indicate amino acid identity with LHCII and conserved potential phosphorylation sites are underlined [16]. The start of the first transmembrane helix is marked by the arrow [31].

LHCII protein and its pigment-reconstituted monomer and trimer were applied as substrate for the acclimative protease at the same experimental conditions as for the native LHCII isolated biochemically from spinach. The results show that the overexpressed and reconstituted LHCII monomer and trimer closely resembled the native monomeric and trimeric form of LHCII in their response towards the added protease (Fig. 2).

3.2. Proteolysis of N-terminally truncated LHCII proteins

To study the possibility that the N-terminal domain of LHCII is involved in substrate recognition by the protease, the full-length recombinant LHCII protein and two N-terminally truncated derivatives, one with the first 11 amino acid residues missing (ΔN-11) and the other with the first 58 amino acid residues deleted (ΔN-58) (Fig. 3), were reconstituted with pigments to monomeric complexes. The resulting monomers were incubated with the salt wash supernatant enriched in the LHCII protease activity at the same detergent conditions as for the native LHCII. It was found that complexes of the two N-terminally truncated mutant proteins form aggregates at such conditions (not shown) because of the reduction of positively charged amino acid residues at the N-terminal region. To compromise between conditions required for maintenance of the reconstituted mutant monomers in solution and for keeping the protease active, 0.1% OG (w/v) was used as a final detergent concentration in the incubation system for the two mutant LHCII species.

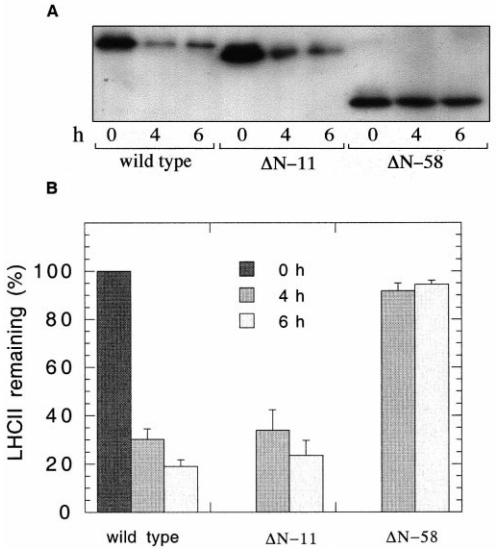

Fig. 4. Proteolysis of reconstituted monomers of recombinant LHCII protein and N-terminally truncated derivatives. Samples were analyzed as described in Fig. 1. The remaining of LHCII protein (wild-type) and its derivatives was detected by immunoblotting followed by alkaline phosphatase-conjugated ECL (A) and quantified by laser densitometry scanning (B). The amount of wild-type and mutant proteins before incubation with the proteolytic fraction was taken as 100% for each sample. The results are means ± S.E.M. of five independent experiments.

Deletion of N-terminal amino acids of LHCII protein results in a lower apparent molecular weight of the mutant species as detected by immunoblotting using antibody raised against LHCII (Fig. 4A). The mutant ΔN-11 shows the same degradation rates as the wild-type LHCII protein. Notably however, LHCII becomes stable towards the acclimative protease when 58 amino acid residues are deleted from the N-terminus (ΔN-58) (Fig. 4). This result strongly suggests that the N-terminal domain of LHCII between amino acids 12 and 58 is essential for the recognition by the acclimative proteolytic system to allow degradation to occur.

4. Discussion

The long term acclimation of the PSII antennae to fluctuations in the light intensity is an important factor for the photoprotection of the photosynthetic apparatus. The removal of LHCII in response to increased irradiance is a complicated process involving not only a specific proteolytic cleavage but also changes in the oligomeric state of the complex, protein phosphorylation and lateral migration of LHCII from the appressed to non-appressed thylakoids [4,5]. Our present results show that several of these steps are directly or indirectly controlled via the N-terminal region of LHCII.

The regulatory protease only recognizes the monomeric and not the trimeric form of LHCII. From a physiological point of view, this appears logical. Upon acclimative removal, LHCII trimers dissociate from PSII and migrate to the stroma-exposed thylakoid regions where the complex is in the monomeric form and where the protease is located [4,21,22]. It has previously been shown that the N-terminal domain of LHCII is important for trimerization [14,23] involving structural elements between amino acids 16 and 21 [9]. It is believed that there is a close interaction between the N-termini of the three interacting LHCII monomers and this interaction renders these domains inaccessible toward externally added proteases such as trypsin or chymotrypsin. Our present work demonstrates that this is also the case for the endogenous regulatory LHCII protease.

Furthermore, the fact that a mutant form of LHCII lacking the N-terminal domain loses its ability to undergo proteolysis reveals that this domain contains a recognition site for the proteolytic system and that the protease then cleaves in a different domain further downstream of the LHCII protein. Such a recognition site, functioning as a signal for LHCII degradation, may relate to the N-end rule [10]. The N-end rule pathway emphasizes the importance of the N-terminal domain for the stability of a protein and is primarily focused on the very N-terminal amino acid residue [10]. More recently, it is suggested that an N-terminal domain, not located at the very proximal N-terminus but involving an internal sequence motif, may function as a degradation signal as

well [11]. In the case of the LHCII protein, the potential degradation signal should be located between amino acids 12 and 58 at its N-terminal domain. In eukaryotes, the N-end rule is believed to be part of the ubiquitin/proteasome system [24,25]. However, most experimental evidence speaks against the presence of this proteolytic system in plant chloroplasts [2,26]. It is suggested that the general function of the proteasome in chloroplasts is rather carried out by a Clp AP protein [27], an enzyme homologous to a bacterial ATP-dependent protease that is involved in the bacterial N-end rule pathway [11]. However, the membrane-associated acclimative proteolytic system is not likely to correspond to the Clp AP protein [5]. Further studies on identification of the LHCII protease system and its relationship to other LHCII-degrading proteases [28] are therefore still required.

Our finding that the N-terminus is important for the degradation of LHCII also has significance for our understanding on how the proteolytic system can distinguish between the phosphorylated and unphosphorylated forms of the protein. It is known that the LHCII phosphorylation sites are at Thr-5 or Thr-6 of the N-terminal segment [29]. Recent work on LHCII phosphorylation has shown that more than one residue along the length of the N-terminal domain of LHCII is susceptible to phosphorylation, even within the truncated proteins [16]. There are still five conserved potential phosphorylation sites between amino acids 12 and 58 (Fig. 3) [16]. Modification of these N-terminal residues by a phosphate group could shield the recognition site for the proteolytic system, thereby hindering proteolysis of LHCII.

Acknowledgements: This work was supported by the Swedish Natural Science Research Council, Deutsche Forschungsgemeinschaft (Pa 324/3-1) and Fonds der Chemischen Industrie. We thank Drs. Chunhong Yang, Monika Meyer, Stephan Hobe and Ms. Christine Huschenbett, Kirstin Kosemund, Henriette Wolf and Birgit Schreiner for assistance in the reconstitution experiments and valuable discussions.

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