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Light-harvesting chlorophyll *a*/*b* proteins (LHCII) populations in phosphorylated membranes

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The properties of the light-harvesting chlorophyll *a*/*b*-protein complex of Photosystem II (LHCII) have been analyzed, in thylakoids and PS II membrane after phosphorylation. Using a newly developed fractionation method, by flat-bed electrofocusing in granulated gel, seven Chl *a*/*b* proteins have been separated from thylakoids. Their phosphorylation level and polypeptide composition have been evaluated. PhosphoLHCII represent only 30% of the total Chl *a*/*b* proteins in thylakoids and the 'mobile' fraction binds 20% of the total LHCII chlorophyll. Tightly bound LHCII differs from the mobile fraction for the presence of a 26 kDa polypeptide characterized by the absence of the N-terminal LHCII proteolytic fragment which is phosphorylated during state transition.

Introduction

When chloroplasts are exposed to light preferentially absorbed by Photosystem II (PS II), a low fluorescence state (State II) is induced as a consequence of a redistribution of excitation energy between the photosystems. Under these conditions the change in antenna size, which is the basis for more energy being funnelled to PS I, avoids imbalance in electron flow [1,2].

The activation of thylakoid bound kinase(s) [3] by reduced plastoquinone is the mechanism underlying state transitions [4]. The phosphorylation of a 9 kDa PS II polypeptide has been associated to the inhibition of PS II photochemical activity [5], while the PS II antenna size is modulated by the phosphorylation of LHCII polypeptides. The addition of negatively charged phosphate groups to the LHCII complex is proposed to induce its dissociation from PS II, and to favour its migration out of the appressed regions of the grana into the stroma lamellae [6]. In this membrane compartment, LHCII is thought to transfer excitonic energy to PS I centres [7,8]. However, recent results on the distribution of LHCII after phosphorylation show that only a fraction of the PS II antenna system is transferred to the stroma membranes [7,9].

Accordingly, although LHCII polypeptides appear to be heavily phosphorylated, roughly half of the total phosphate incorporated into LHCII is found associated with the grana fraction [6,10,11]. These findings suggest that the different components of the PS II antenna system may play different roles in the molecular mechanism by which the

Abbreviations: *pI*, isoelectric point; IEF, isoelectrofocusing; LHCII/I, light-harvesting chlorophyll *a*/*b* protein complex of Photosystem II/I; PS II/I, Photosystem II/I; RC, reaction centre; Tricine, *N*-2-hydroxy-1,1-bis(hydroxymethyl) ethylglycine; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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'mobile' population of LHCII is induced to dissociate from the PS II-LHCII matrix. We have recently described the isolation and characterization of the 'mobile' LHCII from maize stroma membranes after light-induced transition to State II and have shown that the polypeptide composition of this LHCII population is different from that of the population tightly bound to PS II [7].

In this paper we describe the application of a newly developed method for the separation of native chlorophyll-proteins to the study of the different LHCII populations generated after phosphorylation. Up to seven chlorophyll *a/b*-binding complexes have been resolved by this method with distinct polypeptide composition. One of these, corresponding to the 'mobile' antenna obtained from stroma membranes, is found to disappear from grana membranes after phosphorylation.

Those remaining attached to PS II in the grana moiety include a heavily phosphorylated complex besides other LHCII chlorophyll-protein complexes which do not incorporate phosphate groups.

Materials and Methods

Preparation of thylakoid membranes. *Zea mays* L. seedlings were grown for 2–3 weeks in a growth chamber at 28/22°C day/night at a light intensity of 8000 lux. Thylakoids from mesophyll chloroplasts were prepared as previously described [12]. PS II membranes were obtained according to the method of Berthold et al. [13] using the modifications described by Dunahay et al. [14].

Phosphorylation of thylakoids. Seedlings leaves were dark-adapted for 1 h at room temperature to ensure dephosphorylation of thylakoid chlorophyll-proteins. Thylakoids were then isolated according to Farchaus et al. [3] and resuspended, at 200 μg chlorophyll/ml, in 100 mM sucrose/10 mM NaCl/5 mM $MgCl_2$/50 mM Hepes-NaOH (pH 7.5). 5 mM NaF was added to inhibit phosphatase activity. 30 μCi/ml of [^{32}P]ATP was added to a final ATP concentration of 1 mM. The suspension was illuminated in a flat glass cuvet 100 × 80 × 3 mm to ensure even light exposure. After illumination, thylakoids were pelleted by centrifugation and resuspended in 5 mM Tricine (pH 8.0)/1 mM EDTA/5 mM NaF or in 25 mM Hepes (pH 7.6)/5 mM $MgCl_2$/10 mM NaCl/5 mM NaF for the preparation of PS II membranes.

Sucrose gradient ultracentrifugation. PS II membranes or thylakoids were resuspended at 1 mg Chl/ml in 5 mM Tricine (pH 8.0)/1 mM EDTA/2 mM NaF and solubilized for 5 min at 0°C in the presence of 2% (w/v) *n*-octyl β-D-glucopyranoside (Sigma) and then centrifuged at 15000 × *g* for 15 min. Samples (1 ml) were loaded onto a 0.1 to 1 M sucrose gradient containing 1% octylglucoside and run at 39000 rpm in an SW41 rotor (Beckman) at 4°C for 15 h.

Isoelectrophocusing technique. The technique of Radola [15,16] was used. A bed (18 ml) of 5% Ultrodex (LKB), 2% Ampholine carrier ampholites (pH 3.5–5.0), 1% glycine, 1% octylglucoside was prepared and made on a 4 mm layer using a special tray 21 cm long. The sample (0.8 mg Chl) was applied and focused at a constant power of 3 W, for 14 h, at 4°C. The green bands were then eluted from small plastic columns with a small volume of 25 mM Hepes (pH 7.6)/1% octylglucoside and tested for pH.

Gel electrophoresis. Samples (5–15 μg Chl) were solubilized with 2% SDS/10 mM Tris-sulfate (pH 9.0) and run in a gel containing 6 M urea as previously described [12]. Gels were dried and exposed to autoradiography with a Kodak X-Omat ARS X-ray film.

Immunological techniques. For immunoblot assays, the LHCII oligomeric complex was obtained by preparative electrophoresis [17]. Apoproteins were resolved by urea-SDS-PAGE as described above and transferred to a nitrocellulose filter (Millipore). The filters were then assayed with the monoclonal antibody as in Ref. 18.

Miscellaneous. Absorption spectra of the chlorophyll-proteins were recorded using a Perkin-Elmer Lambda-5 spectrophotometer. Chlorophyll concentration and *a/b* ratio were determined in 80% acetone according to Arnon [19]. Proteolysis was carried at 20°C for 30 min. Trypsin (Boehringer) was used at 5 μg/ml concentration.

Results

Although the ATP-dependent fluorescence quenching during light-induced state transition was complete after 5 min, the phosphorylated

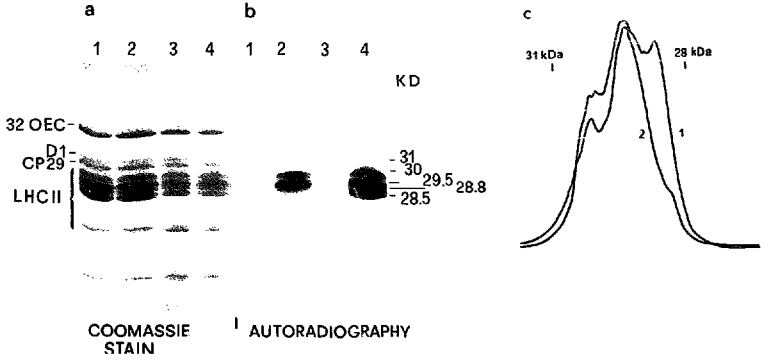

Fig. 1. SDS-PAGE (a) and autoradiography (b) of ^{32}P-labeled and control PS II and thylakoid membranes. (1) Control PS II membranes; (2) phosphorylated PS II membranes; (3) control thylakoids; (4) phosphorylated thylakoids. 10 μg of chlorophyll were loaded in each lane. (c) Densitometric scan of the autoradiography: (1) thylakoids; (2) PS II membranes.

thylakoid membranes used throughout this study were subjected to the high light conditions for 15 min. After this exposure, the phosphorylation of the protein-chlorophyll complexes was found to be almost complete. From this material, PS II membranes were prepared following the procedure of Berthold et al. [13] with the modifications described in Dunhaway et al. [14]. As control samples we used thylakoid membranes obtained from dark-adapted plants which had been subjected to the same light conditions in the absence of ATP. Thylakoids and PS II membranes were then examined by high resolution denaturating SDS-PAGE and autoradiography (Fig. 1). In the 27–30 kDa region, where the polypeptides of LHCII are located, no differences were detected in the pattern of phosphorylated and control samples, both in thylakoids and PS II membranes, as far as could be judged by densitometry of the Coomassie-stained gels. Nevertheless, liquid scintillation counting showed a significant difference between the samples. Thus the radioactivity associated with the grana membranes per unit chlorophyll was found to be about 40% of that found in thylakoids. The same result was obtained when PS II membranes were prepared by exposing the thylakoids to Triton X-100 for shorter times during fractionation (5 and 10 min vs. 20 min in the standard procedure) (Table I). Milder solubilizations gave PS II particles slightly contaminated by stroma components, namely CF1 and PS I polypeptides (Fig. 2), but excluded the possibility that PS II particles prepared from light-adapted membranes might lose some of their chlorophyll-protein complexes.

Examined by electron microscopy after negative staining with uranyl acetate, the PS II membranes, from phosphorylated and control

TABLE I

CHARACTERISTICS OF ^{32}P-LABELED THYLAKOIDS AND PS II MEMBRANES OBTAINED BY DIFFERENT SOLUBILIZATION TIMES

The quoted values for specific labeling are normalized to the chlorophyll content.

	% Chl	Chl *a*/*b* ratio	Specific ^{32}P label
Thylakoids	100	3.47	100
Ps II membranes 5 min	50	2.3	36
PS II membranes 10 min	35	2.2	38
PS II membranes 20 min	32	1.85	40

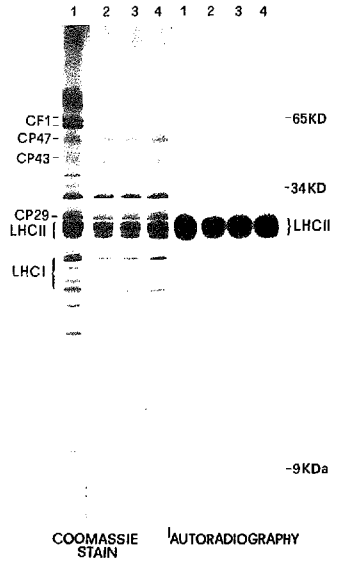

Fig. 2. SDS-PAGE and short-exposure autoradiography of thylakoids and PS II membranes obtained by different times of solubilization with Triton X-100: (1) thylakoids; (2) PS II membranes treated for 5 min; (3) PS II membranes treated for 10 min; (4) PS II membranes treated for 20 min.

thylakoids, appeared as flat membrane sheets with an average diameter of 0.5 –0.8 μm. These values are not very different from that of grana stacks in intact maize mesophyll chloroplasts.

Fig. 1b reports the autoradiography of PS II and thylakoid membranes. It clearly shows that the difference in ^{32}P labeling between the two samples is due mainly to polypeptides belonging to LHCII. Moreover, the densitometric scan of autoradiography in the LHCII range (Fig. 1c) shows that the decrease of labeling in PS II membranes is not evenly distributed among the LHCII polypeptides, but rather is concentrated on the bands of 28.5 and 30 kDa. This finding suggests that some of the LHCII phosphoproteins are preferentially removed from grana membranes during phosphorylation. Fig. 3 shows that minor phosphoproteins of apparent mass 65, 34, 17, 14, 10 and 9 kDa are also detected with a longer exposure. These phosphoproteins, with the exception of the 34 and the 9 kDa bands, were removed during the preparation of PS II membranes.

The fractionation pattern obtained by sucrose gradient centrifugation is depicted in Fig. 4. Four green bands were resolved from phosphorylated thylakoids and three from PS II membranes. In both cases the upper band with low chlorophyll *a/b* ratio contains light-harvesting complexes, while the other bands with high chlorophyll *a/b* ratio contain reaction-centre complexes. This is shown in Fig. 4b by the polypeptide composition of the green fractions [17,20]. Lane 1 of panel b shows the SDS-PAGE gel of the upper green band from thylakoid membranes. This fraction contains the polypeptides of the LHC complexes and some

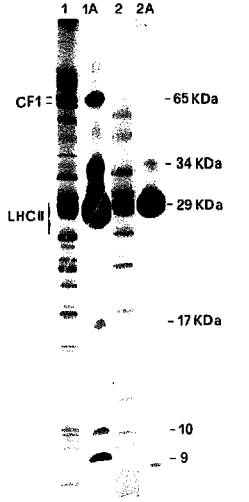

Fig. 3. SDS-PAGE and long-exposure autoradiography of thylakoids (1) and PS II membranes (2).

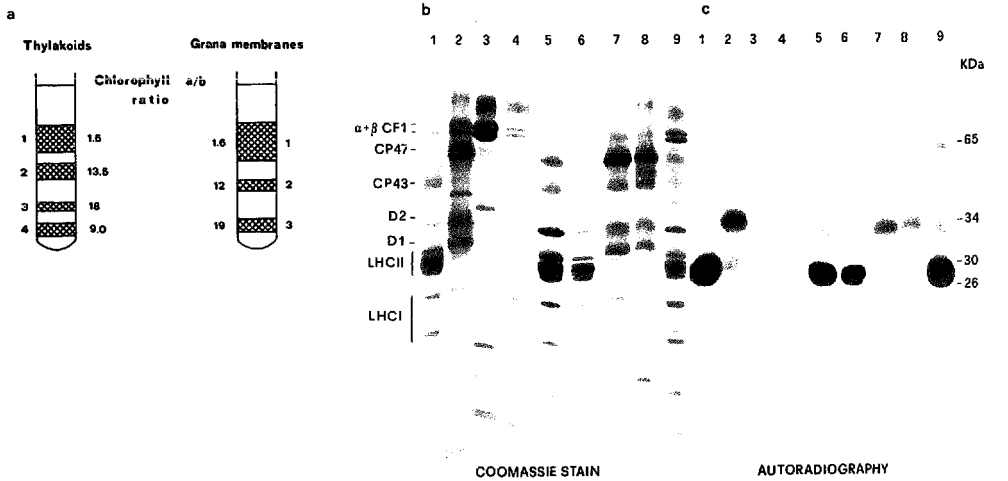

Fig. 4. (a) Scheme of the separation pattern of PS II and thylakoid membranes obtained by solubilization and sucrose gradient ultracentrifugation. (b) Polypeptide composition and (c) autoradiography of the green bands of panel (a). lane 1, band 1 from thylakoids; lane 2, thylakoid band 2; lane 3, thylakoid band 3; lane 4, band 4; lane 5, PS II membranes; lane 6, band 1 from PS II membranes; lane 7, band 2 from PS II; lane 8, band 3 from PS II; lane 9, thylakoid membranes. The upper Coomassie band in lanes 2, 7 and 8 is thought to derive from the aggregation of D1 and D2 polypeptides of PS II, since it is ^{32}P labeled and is recognized by a polyclonal antibody against PS II reaction centre (not shown).

polypeptides of the OEC. The autoradiography reported in Fig. 4c (lane 1) shows that, besides LHCII, the 65 kDa minor phosphoprotein is also present in this fraction. The second green band (lane 2) contains PS II RC and some contamination of coupling factor. This PS II fraction appears partially depleted of the 43 kDa subunit, which is enriched in band 1, but it contains all the ^{32}P associated with the 34 and 9 kDa phosphoproteins (the latter polypeptide is not visible in the gel of fig. 4). The third and fourth bands contain PS I RC fractions including LHCI. No phosphorylated bands are detected in these fractions.

With the exception of the 65 kDa phosphoprotein, the band 1 from PS II membranes (lane 6) is identical to the corresponding fraction from thylakoids. The other two green bands both derive from PS II RC but are characterized by different composition. Band 2 is depleted of the 43 kDa component and other minor polypeptides, whereas the OEC polypeptides of 32 and 23 kDa are present (lane 7). Band 3 contains the 43 kDa polypeptide but the OEC components are removed together with the 9 kDa phosphoprotein (lane 8).

The upper bands from the sucrose gradient, containing the chlorophyll *a/b*-proteins from both samples (thylakoids and PS II membranes), were further fractionated by nondenaturating IEF. Seven chlorophyll *a/b*-proteins were resolved as green bands from the thylakoids and six from the PS II membranes. The IEF pattern of PS II membranes is shown in Fig. 5a as an example. The relevant characteristics of the chlorophyll-protein complexes so obtained are summarized in Table II and their polypeptide composition and autoradiography are shown in Fig. 6.

All these complexes differ in their polypeptide composition. All of them, except the less acidic one (p*I* = 4.62), contain polypeptides in the 26–30 kDa range. A singie polypeptide of 31 kDa, belonging to the latter complex, can be easily identified as the CP29 by its chlorophyll *a/b* ratio (2.8) and characteristic absorption spectrum (λ_{max} = 677 nm) [17]. The other chlorophyll *a/b* complexes show similar chlorophyll *a/b* ratios in the range 1.15–1.35 with different red maxima (see Table II). The polypeptide with the lowest ap-

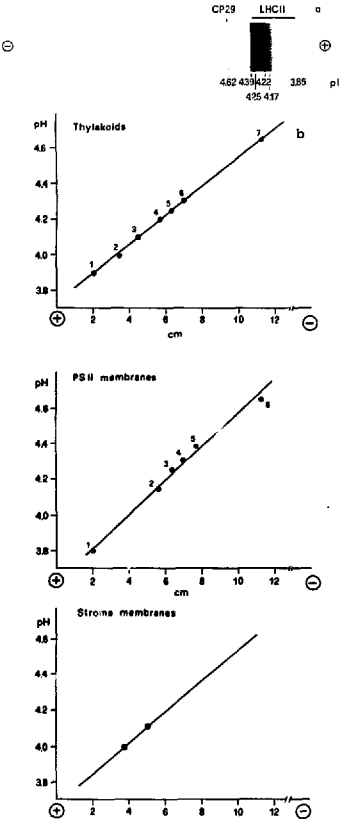

Fig. 5. (a) IEF pattern of LHCII isolated from ^{32}P labelled PS II membranes. Numbers in the bottom line refer to isoelectric points of the green bands. (b) Schematic drawing of the IEF pattern from phosphorylated thylakoids, PS II membranes and stroma membranes. The solid lines represent the pH gradient in the gel. The points represent the positions of the green bands in the IEF gels. Numbers are given which will be referred to in the text and in the next Fig. 6.

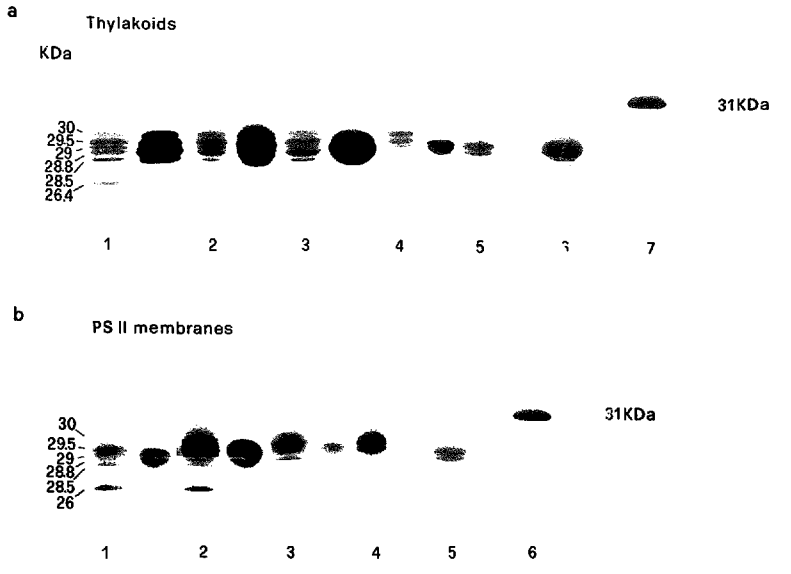

Fig. 6. SDS-PAGE and autoradiography of the Chl *a/b* proteins obtained by IEF from ^{32}P-labeled thylakoids and PS II membranes. Numbers at the bottom correspond to those in Fig. 5b and mark the Coomassie-stained patterns. Autoradiography is shown on the right of each numbered pattern.

parent mass (26 kDa) is present only in the IEF band 1 together with five other polypeptides (28.5, 28.8, 29, 29.5, 30 kDa). The other bands contain different combinations of the polypeptides in the 28.5–30 kDa range. Five polypeptides are clearly resolved in bands 2 and 3, while a lower number of peptides, in different proportions, are contained in bands 4, 5 and 6. The autoradiography shows that bands 1, 2 and 3 are the most heavily phosphorylated, band 4 and 5 carry little ^{32}P, while bands 6 and 7 are not labelled at all. Taking together the data of Table II and Fig. 6, we may calculate that the amount of chlorophyll associated with the highly phosphorylated bands represents no more than 30% of the total *a/b* chlorophyll carried by the chlorophyll *a/b*-proteins of light-harvesting complex.

Panel b of Fig. 5 shows the comparison between the IEF pattern of thylakoids and PS II membranes. Two heavily phosphorylated bands (p*I* 4.0 and 4.1) are missing in PS II membranes, while four rather then three bands are resolved in the 4.17–4.39 pH range. Most of the ^{32}P label is found associated with bands 1 and 2, those which contain the 26 kDa apoprotein. These bands together represent 11% of the chlorophyll. These data suggest that the two missing bands, characterized by p*I* = 4.0 and 4.1, largely correspond to the 'mobile' LHCII.

To check this possibility we have isolated stroma

membranes from State-II-adapted leaves and fractionated by sucrose gradient ultracentrifugation as previously described [7]. When the fraction corresponding to LHCII was analyzed by IEF, two bands were resolved, with p*I* of 4.00 and 4.12, which contained five polypeptides in the 28.5–30 kDa range whereas the 26 kDa apoprotein was lacking. It is worth noting that no significant differences were observed in the IEF pattern from thylakoids and PS II membranes when they were not subjected to phosphorylation.

As outlined above, a well-resolved 26 kDa polypeptide is found only in the LHCII subpopulation which is strongly phosphorylated and yet remains associated to the PS II reaction centres during state transition.

The 26 kDa polypeptide is present only in phosphoLHCII tightly bound to PS II in grana membranes. A distinctive characteristic of this polypeptide is found in its property of being resistant to trypsinization. When the isolated LHCII complex was treated with 5 μg/ml trypsin for 30 min and then separated in SDS-PAGE, the results shown in Fig. 7 were obtained. It clearly appears

TABLE II

CHARACTERISTICS OF THE Chl *a*/*b* PROTEINS SEPARATED BY FLAT-BED IEF IN THE PRESENCE OF 1% OCTYLGLUCOSIDE

Band	p*I*	chl *a*/*b*	Red max (nm)	% Chl	% ^{32}P labeling
Thylakoids					
1	3.92	1.27	674.0	9	70
2	4.00	1.29	673.5	6.7	100
3	4.10	1.17	673.0	14.4	84
4	4.2	1.22	675.0	27	10
5	4.23	1.24	673.5	21.5	4
6	4.32	1.34	676.0	13	1
7	4.62	2.8	677.0	9.3	–
PS II membranes					
1	3.85	1.25	674.0	6.0	18
2	4.17	1.22	675.0	5.0	100
3	4.22	1.24	673.0	15.0	8
4	4.25	1.21	674.0	25.1	2
5	4.39	1.30	676.0	36.9	–
6	4.62	2.8	677.0	12.0	–
Stroma membranes					
1	4.00	1.3	670.0	45.0	– [a]
2	4.12	1.15	671.0	65.0	– [a]

[a] Not determined.

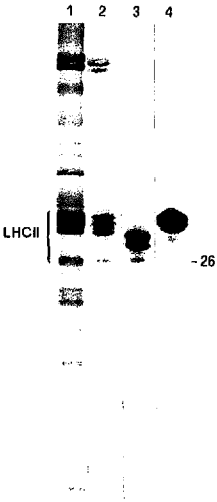

Fig. 7. SDS-PAGE (lanes 1–3) and Western blot (4) of LHCII complex. (1) Thylakoids; (2) LHCII untreated; (3) LHCII treated with trypsin as described in Materials and Methods; (4) monoclonal antibody reaction to LHCII: the nitrocellulose filter was assayed with the MLH 1 monoclonal directed to the N-terminal fragment of LHCII.

that all polypeptides are cleaved by the proteolytic enzyme except for the 26 kDa one. Western blotting with a monoclonal antibody (also shown in Fig. 7), directed to the 2 kDa N-terminal proteolytic fragment, which is known to be present in the major LHCII polypeptides [20,21], does not recognize the 26 kDa apoprotein.

Discussion

The phosphorylation of LHCII polypeptides by a membrane-bound protein kinase(s), regulated by the redox state of the plastoquinone pool, and its dephosphorylation by intrinsic phosphatase has been proposed to be the mechanism for the energy redistribution between PS I and PS II [4,23]. Further studies have shown the existence of different

LHCII components with regard to their ability to migrate from grana to stroma and to the extent of phosphorylation they are subjected to during state transition.

Spinach LHCII has been resolved into two polypeptides of 25 and 27 kDa in the stoichiometric ratio 1 : 3. The 25 kDa component is reported to be preferentially contained in the 'mobile' LHCII population and to be more heavily phosphorylated [24]. The ATP-induced fluorescence quenching was correlated to the phosphorylation kinetics of the 25 kDa polypeptide rather than to the ^{32}P incorporation into the whole LHCII fraction [9,11].

More detailed knowledge is available for the maize PSII antenna system, which has been resolved into four Chl *a/b*-proteins having polypeptides in the 20–31 kDa range [17]. These have been indicated as LHCII** (with an apparent mass in the native green gels of 64 kDa) and CP29, CP26, and CP24, which are present at lower concentration. The latter three minor chlorophyll-proteins are not phosphorylated, and all of the ^{32}P label is incorporated into the major (LHCII**) complex. The previous study therefore implies that both 'mobile' and 'tightly bound' phospho-LHCII subpopulations belong to the major LHCII** complex.

The results of Fig. 1a–c show that a specific mobile LHCII has been removed from grana following phosphorylation, while a tightly bound LHCII remains there, representing approx. 40% of the ^{32}P label incorporated in the antenna system. On the other hand, no detectable difference in the relative amount of the Coomassie-stained bands is found in the light-adapted PS II membranes with respect to control thylakoids. The 60% reduction of the ^{32}P labeling in PS II membranes, after ATP induced migration, seems therefore in contradiction with the report of Hawort et al. [25] that 80–90% of LHCII is phosphorylated. Our results rather suggest that phosphoLHCII is a small fraction of total LHCII and that a large pool of unphosphorylated proteins of LHCII is included in the tightly bound subpopulation. These results are in agreement with a recent report of substoichiometric ^{32}P labeling of LHCII [26].

The analysis by isoelectrophocusing of LHCII fractions, obtained from sucrose gradient fractionation, confirms this view. Thus, under our experimental conditions, where a degree of phosphorylation close to saturation is induced [9,26], phosphochlorophyll *a/b*-proteins represent only 30% of the chlorophyll associated with LHCII, including both 'mobile' and 'non-mobile' antenna (see Table II). The application of IEF to the mobile LHCII population resolve two chlorophyll proteins with slightly different isoelectric points. These were found to correspond to bands 2 and 3 from IEF of LHCII prepared from phosphorylated thylakoids. These two complexes show essentially the same polypeptide composition but slightly different degree of phosphorylation. The identification of these complexes as 'mobile' LHCII is confirmed by their absence in phosphorylated PS II membranes, while they are the only LHCII complexes present is isolated stroma membranes from State-II-adapted leaves (Fig. 5b).

Tightly bound phosphoLHCII is characterized by the presence of a 26 kDa component besides the five polypeptides which are present in the mobile LHCII and represent approx. 11% of the total LHCII chlorophyll. A more complete characterization of the chlorophyll proteins obtained by IEF will be the subject of a forthcoming paper. We will focuse here on the 26 kDa polypeptide whose presence is the more obvious difference between 'tightly bound' and 'mobile' populations. This polypeptide is not phosphorylated and is not recognized by a monoclonal antibody directed to the N-terminal fragment that is cleaved by trypsin [22]. Its mobility is not changed by trypsinization that cleaves the other LHCII polypeptides in the 28.5–30 kDa range (Fig. 7). We propose that the deletion of this phosphorylatable, trypsin-cleavable, N-terminal fragment is a major characteristic of the 26 kDa polypeptide. Whether this characteristic is correlated to its property of staying tightly bound to RC in the grana region is still unclear, since other phosphorylatable apoproteins are present in the same complex.

From isoelectrophocusing, the considerable complexity of the polypeptide composition of LHCII is clearly revealed. Six polypeptides can be resolved from a single IEF band and Coomassie bands from different IEF fractions, with the same apparent mass, could well correspond to different polypeptides. Moreover, the recent report that in-

complete phosphorylation of LHCII apoproteins is due to intrinsic characteristics of different LHCII populations rather than to a modulation of the kinase activity [26] suggests that phosphorylated and unphosphorylated polypeptides differ in their amino-acid sequence.

Although higher plant LHCII has been resolved into a few (mainly two) polypeptides [24,26,28], molecular genetic data show that LHCII is much more complex in petunia, lemna and tomato [27,29–31]. In *Zea mays*, twelve distinct cDNA clones have been obtained [32] and differential expression, depending on the cell type, has been demonstrated [32,33]. The results reported here strongly suggest that one of the possible reasons for the expression of several different LHCII genes may lie in the slightly different role they play in the phosphorylation-dependent state transition. Another reason could be the possibility of differential expression in response to changes in development or environmental conditions [34].

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