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Biochemical Characterization of Photosystem II Antenna Polypeptides in Grana and Stroma Membranes of Spinach[1]

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ABSTRACT

The photosystem (PS) II antenna system comprises several biochemically and spectroscopically distinct complexes, including light-harvesting complex II (LHCII), chlorophyll-protein complex (CP) 29, CP26, and CP24. LHCII, the most abundant of these, is both structurally and functionally diverse. The photosynthetic apparatus is laterally segregated within the thylakoid membrane into PSI-rich and PSII-rich domains, and the distribution of antenna complexes between these domains has implications for antenna function. We report a detailed analysis of the differences in the polypeptide composition of LHCII, CP29, and CP26 complexes associated with grana and stroma thylakoid fractions from spinach (*Spinacia oleracea* L.), making use of a very high-resolution denaturing gel system, coupled with immunoblots using monospecific antibodies to identify specific antenna components. We first show that the polypeptide composition of the PSII antenna system is more complex than previously thought. We resolved at least five type I LHCII apoproteins and two to three type II LHCII apoproteins. We also resolved at least two apoproteins each for CP29 and CP26. In state 1-adapted grana and stroma thylakoid membranes, the spectrum of LHCII apoproteins is surprisingly similar. However, in addition to overall quantitative differences, we saw subtle but reproducible qualitative differences in the spectrum of LHCII apoproteins in grana and stroma membrane domains, including two forms of the major type II apoprotein. The implications of these findings for models of PSII antenna function in spinach are discussed.

Most of the Chl in the thylakoid membrane of higher plants and green algae is bound by specialized antenna complexes associated with either PSI or PSII (5, 15, 33). The light-harvesting antenna system of PSII includes several distinct CP[3] complexes. The major component, LHCII (designated LHCIIb in Thornber's nomenclature; 27), accounts for approximately half of the total Chl as well as of the total protein in the membrane and has a Chl *a/b* of about 1.1 (21). The minor PSII antenna complexes CP29, CP26, and CP24 (LHCIIa, LHCIIc, and LHCIId in Thornber's nomenclature, 27; these are products of the genes Lhb4, Lhb5, and Lhb6, respectively, 19) each account for a few percent of the total Chl. The CP29 complex has a Chl *a/b* ratio of 2.2 to 2.5 (5, 27) and has an apoprotein several kD larger than those of the LHCII complex. CP26 also has a higher Chl *a/b* ratio than LHCII but generally comigrates with LHCII in both native and fully denaturing SDS-PAGE systems (4, 27). Coupled with the difficulty in separating CP29 from the LHCII apoproteins in some species (particularly in spinach [16, 17]), this has contributed to confusion in the literature as to the identities of these two complexes. This issue has recently been settled by the comparison of partial protein sequence data for CP29 and CP26 in barley (*Hordeum vulgare*) with a full-length gene sequence for CP29 (26), confirming that CP29 and CP26 are distinct complexes, both from each other and from LHCII.

In this paper we will use the terms CP26 and CP29 as defined in ref. 4. Little is known for certain about the physical arrangement of the PSII antenna system, but the isolation of O_2-evolving complexes lacking LHCII but still containing CP29 suggests that CP29 is more closely associated with the reaction center (9). The gel systems used in ref. 9 did not distinguish between CP29 and CP26; therefore, no conclusions could be drawn about the relationship of CP26 to the reaction center.

Although LHCII comprises a family of closely related polypeptides (11), it is both functionally and structurally the most diverse and complex antenna system of higher plant thylakoids. In addition to its role in gathering light energy to drive PSII, LHCII, which is found as a trimer in the membrane (21), is involved in the formation and regulation of thylakoid architecture (33) and in the regulation of energy distribution between the two photosystems (34). The LHCII apoproteins are encoded by multigene families (called Lhb1, Lhb2, and Lhb3 *cab* gene families; 19) in every species examined to date (11). For example, in tomato (*Lycopersicon esculentum*), in which almost the entire *cab* gene family has been molecularly characterized, LHCII is encoded by at least nine distinct genes (29). Genes for LHCII apoproteins have been divided into three types based on sequence similarities. Type I and II apoproteins (products of the Lhb1 and Lhb2 *cab* genes; 19) differ by a number of diagnostic amino acid residues, by the presence of introns in type II genes, and by the slightly smaller predicted size of type II LHCII apoproteins (11, 29). The type III family (products of Lhb3 *cab* genes; 19) is represented by only a few members and does not fit well into either type I or II classifications. The finding by Piechulla et al. (29) that type I and II genes are differentially expressed, both between the two classes and within members of a single

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[3] Abbreviations: CP, Chl-protein complex; LHCII, light-harvesting complex II; IEF, isoelectric focusing; CF, coupling factor.

class, strengthens the notion of functional differences between different gene products (18, 22, 23).

One approach to assessing the functional properties of LHCII is to determine the distribution of individual apoproteins in different membrane domains. The thylakoid membrane is laterally segregated into appressed grana regions and interconnecting, nonappressed stromal membranes. This structural differentiation is accompanied by substantial lateral segregation of thylakoid membrane components. Thus, most PSII, as well as the bulk of the main PSII light-harvesting antenna, LHCII, is located in the grana membranes. Most PSI and virtually all of the chloroplast-CF (CF1, CF0) are located in the stroma membranes (reviewed in refs. 1 and 33). In addition to the lateral segregation of PSI and PSII, there are also differences between the PSII centers found in grana versus those found in stroma, as well as between PSI centers found in the two domains. Thus, the PSII found in the grana regions, designated PSIIα, has a larger antenna size than does the stroma membrane-located PSIIβ (1, 25). Similarly, the fraction of PSI centers found in the grana membranes has a larger antenna size than the PSI found in the stroma membranes (3).

To our knowledge, only one complete *cab* gene sequence is available in spinach (*Spinacia oleracea*, 24), but partial N-terminal sequence data indicate that there are at least four distinct LHCII apoproteins in spinach thylakoids (6). However, most biochemical analysis to date is based on the assumption that there are two apoproteins with apparent molecular masses of approximately 27 and 25 kD (18, 20, 22, 23). The precise relationship between the multiple roles played by LHCII, the multiple polypeptides found in the membrane, and the complexity of the gene family that encodes the LHCII apoproteins is not clear. Our first objective in this study was to reexamine the polypeptide composition of LHCII, CP29, and CP26 in spinach using a high-resolution SDS-PAGE protocol, to show that the apoprotein families are at least as complex as seen in other species. This effort included classifying apoproteins based on their reactivity to monospecific antisera. The second main goal was to use these same techniques to reexamine the polypeptide composition of grana and stroma thylakoid fractions and to determine the lateral distribution of type I and II LHCII, CP29, and CP26 apoproteins.

MATERIALS AND METHODS

Thylakoid Membrane Preparation

Deveined spinach (*Spinacia oleracea*) leaves were homogenized in a chilled buffer containing 0.1 M Tricine (pH 7.8), 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, and the protease inhibitors benzamidine (5 mM) and ϵ-amino caproic acid (1 mM). The homogenate was filtered through four layers of Miracloth (Calbiochem, La Jolla, CA). Intact and semi-intact chloroplasts were pelleted at 5000*g* for 10 min at 4°C. Pellets were washed twice (10,000*g*, 10 min, 4°C) in popping buffer containing 10 mM Tricine (pH 7.8), 10 mM NaCl, 10 mM $MgCl_2$, and the protease inhibitors as described above. Washed pellets were resuspended in the same buffer containing 10% glycerol, frozen in liquid nitrogen, and stored at −80°C. The α and β fractions of grana and stroma were prepared according to the method of Andreasson et al. (3).

The BS and Y100 fractions were prepared according to the procedures reported in refs. 17 and 35. All of these fractions were a generous gift of Dr. Cecilia Sundby (University of Lund, Lund, Sweden). The Y100 fraction was stored frozen in a buffer containing 10 mM sodium phosphate (pH 7.4), 5 mM NaCl, 5 mM $MgCl_2$, and 100 mM sucrose. The remaining samples were stored in the same medium without $MgCl_2$. DMSO (5%) was added to samples before freezing. Pigment determinations were performed on a Perkin-Elmer 330 spectrophotometer, in dimethyl formamide, using the simultaneous equations of Porra et al. (30).

IEF

Fluid phase IEF of solubilized spinach thylakoids was carried out in a Rotofor Cell (Bio-Rad Instruments, Richmond, CA). The anode solution was 0.1 M phosphoric acid, and the cathode solution was 0.1 M sodium hydroxide. The final cell volume was 55 mL. Isolated spinach thylakoids containing 12 mg of Chl were washed twice (10,000*g*, 10 min, 4°C) in 2 mM Tris maleate (pH 7.0) plus protease inhibitors as described above. The washed pellet was solubilized on ice with stirring for 15 min in 25 mL of a solution containing 0.5% octyl glucoside, 0.5% decyl maltoside, 2 mM Tris maleate (pH 7.0), and 10% glycerol. This was then brought up to 55 mL with a solution containing 10% glycerol, 10% trehalose, and 1.5 mL of pH 4 to 6 ampholytes (Pharmacia LKB, Piscataway, NJ) and loaded into the precooled cell. The fractionation proceeded for 4 h at 12 W of constant power. The voltage reached a plateau at 3 h. The cell was fractionated, and after pH determinations had been made, fractions were dialyzed overnight at 4°C in the dark against 2 mM Tris maleate (pH 7.0). Dialyzed samples were frozen in liquid nitrogen and stored at −80°C before use.

Fully Denaturing SDS-PAGE

Fully denaturing SDS-PAGE was carried out using the ammediol (2-amino-2-methyl-1,3-propanediol) buffer system described by Bury (8) with the modifications described in ref. 2. For most analytical work, samples are electrophoresed on 0.75-mm thick, 30-cm long gels, containing 18% acrylamide at an acrylamide to bis-acrylamide ratio of 90:1. Because bromophenol blue electrophoreses well ahead of the Chl front in this gel system, we use malachite green as a dye marker. When all proteins below about 20 kD are to be run off the bottom of the gel, congo red, which electrophoreses with an apparent molecular mass of about 30 kD in this system, is used. The gel shown in Figure 1 is a 15-cm, 0.75-mm thick gel containing a linear 18 to 26% polyacrylamide gradient. Samples were solubilized for 15 min at 55°C in solubilization buffer containing 1× stacking gel buffer, 2% SDS, 2% β-mercaptoethanol, and 10% glycerol. Samples were loaded at 1 μg of Chl per lane. Gels were stained using the low background silver stain protocol described by Blum et al. (7).

For two-dimensional gels, 1.5-mm thick strips were excised from green gel lanes, incubated for 15 min at 55°C in

solubilization buffer containing 1× stacking gel buffer, 2% SDS, 2% β-mercaptoethanol, and 10% glycerol. Treated gel strips were loaded directly onto the stacking gel of a 1.5-mm minigel containing 18% polyacrylamide with an acrylamide to bis-acrylamide ratio of 60:1.

For immunoblots, proteins were transferred to polyvinylidine fluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 3% nonfat dry milk, decorated with primary antisera, stained with goat anti-rabbit horseradish peroxidase conjugate secondary antibodies, or with goat anti-mouse horseradish peroxidase conjugated secondary antibodies (Bio-Rad Laboratories, Richmond, CA), and visualized according to the Bio-Rad horseradish peroxidase protocol.

For video densitometry, negatives of silver-stained gels were digitized using a Star 1 cooled CCD video camera (Photometrics, Ltd., Tucson, AZ). These images were analyzed using the National Center for Supercomputing Applications (Champaign, IL) Image program on a Macintosh computer.

RESULTS

Separation of CP Complexes by Fluid Phase IEF

We first sought to determine whether the light-harvesting complexes of PSII in spinach, including CP29 and CP26, contained a larger number of polypeptides than had been resolved to date. To this end, we have partially purified LHCII, CP29, and CP26 by native fluid phase IEF. Our IEF system was designed to maintain most components as pigmented, multisubunit complexes, but because of the relatively harsher environment of IEF and the longer sample handling time required, it is not as gentle as low ionic strength native SDS-PAGE systems (2, 27). Whole, isolated spinach thylakoids were solubilized in a mixture of octyl glucoside and decyl maltoside and separated over an ampholyte range of pH 4 to 6. Chl *a/b* ratios for the various fractions are given in Table I, and polypeptide compositions of these fractions are shown in Figure 1. The conditions of the IEF separation dissociate most LHCII from the PSII reaction center, whereas the LHCI subunits remain attached to PSI.

Table I. *Pigment Composition of IEF Fractions*

pH	Chl *a/b* Ratio	Percent Total Chl
3.8	1.65	10
4.2	1.54	11
4.35	1.46	13
4.5	1.46	12
4.6	1.67	9
4.75	2.02	7
4.9	3.93	5
5.1	6.71	5
5.2	6.84	9
5.35	>10	9
5.5	>10	7
5.7	>10	2
5.9	>10	1
5.95	>10	<1
6.1	>10	<1
6.4	>10	<1

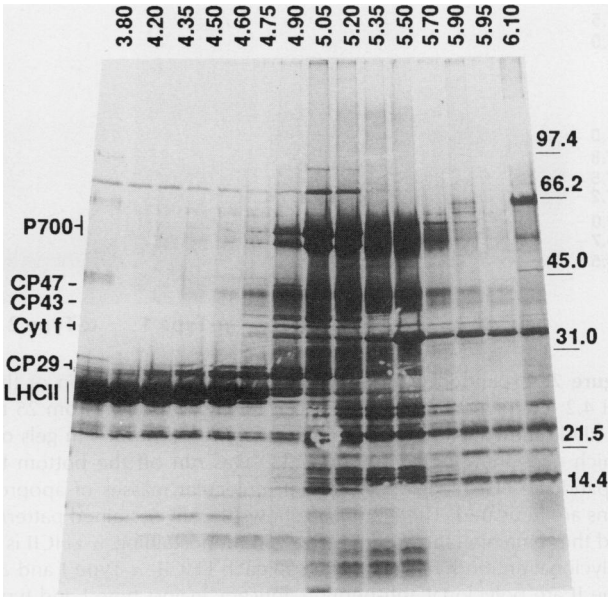

Figure 1. Separation of thylakoid components by preparative fluid phase IEF. Fractions have been separated by fully denaturing SDS-PAGE on a 15-cm gel and silver stained. The pH of each fraction is indicated at the top of the lane. Positions of molecular mass markers are indicated along the right side. Selected landmarks of the polypeptide pattern are indicated along the left side. P700, P700 apoproteins of PSI.

The first five fractions, from pH 3.8 to pH 4.6, have Chl *a/b* ratios of approximately 1.5 and, as shown in Figure 1, contain the bulk of the LHCII. Most of this LHCII material is in trimeric form, as assayed by native green gel electrophoresis of these fractions (data not shown). The bulk of the CP29 and CP26 apoproteins focus from pH 4.5 to 5.35. CP29 and CP26 comigrate with each other and not with the LHCII complexes. The CP26 apoproteins, approximately 27.5 kD, are obscured by the LHCII apoproteins (Fig. 3) and thus are not labeled in Figure 1. Most PSII and PSI, as judged by the CP47 and CP43 apoproteins of PSII and the P700 apoproteins of PSI, are in the region from pH 4.9 to 5.5. The Cyt complex focuses above pH 5.5.

Type I and II LHCII Apoproteins in Spinach Thylakoids

Our next goal was to classify the LHCII apoproteins resolved with the ammediol SDS-PAGE system based on reactivity with monospecific antisera. To this end, we probed immunoblots of high-resolution SDS-PAGE gels, using antisera specific for type I and type II LHCII sequences (32).

Figure 2 illustrates the polypeptide composition of the pH 4.2 IEF fraction, which is highly enriched for LHCII but also contains small amounts of CP29 and CP26 (Fig. 3). The figure shows just the LHCII/CP29 apoprotein region of 30-cm gels on which all polypeptides below about 20 kD have

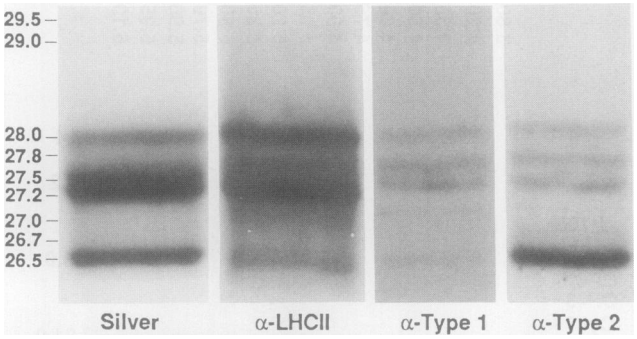

Figure 2. Expanded view of the polypeptide composition of the pH 4.2 IEF fraction shown in Figure 1. Only the region from 25 to 30 kD is shown. Samples were electrophoresed on 30-cm gels on which everything below about 20 kD was run off the bottom to expand the LHCII region. Apparent molecular masses of apoproteins are indicated. The first panel shows the silver-stained pattern, and the remaining three panels show immunostaining. α-LHCII is a polyclonal antibody raised against spinach LHCII. α-Type I and α-type II are polyclonal anti-peptide antisera against type I and type II LHCII sequences (T1-B1 and T1-B2, see text).

been run off the bottom of the gel to maximize resolution in the LHCII region. The first panel is the silver-stained pattern, and the other panels are immunoblots of similar gels. The nine polypeptides labeled in this figure have been assigned on the basis of the antibody binding described below and on published characterizations (4, 5, 12, 16).

The second panel shows immunoreactivity with an antiserum prepared against purified spinach LHCII (14). This antiserum reacts with the whole range of LHCII apoproteins of a variety of different plant species. All of the bands recognized by this antiserum are also recognized by one or more anti-peptide antisera prepared against LHCII sequences (32; see below). This antiserum labels all of the LHCII bands from 28.0 to 26.5 kD. The apoproteins of CP26 and CP29 are very weakly recognized by this antiserum (not shown). This finding is in agreement with other studies showing that CP29 and CP26, although distinct from LHCII, share some epitopes with LHCII (12).

The third and fourth panels show immunostaining with anti-peptide antisera prepared against the petunia type I LHCII sequence PDRVKYLGPFSGEAPS (antiserum T1-B1; 32) or against the petunia type II LHCII sequence EDRPKYLGPFSEQTPS (antiserum T2-B1; 32). Although these peptide sequences were selected to take maximum advantage of differences between type I and II LHCII *cab* gene sequences, the two peptides are about 50% identical. In particular, both share the sequences KYLGPFS. Thus, although these antisera successfully distinguish between type I and type II LHCII apoproteins (32), there is some cross-reactivity between the two types. Identification of type I and II apoproteins may be made by comparing the relative strength of reaction of the two antisera. The anti-type I antiserum reacts most strongly with the larger LHCII apoproteins from 28.0 to 27.0 kD and reacts more weakly with the 26.5-kD apoprotein. The anti-type II antiserum reacts most strongly with the 26.5-kD apoprotein. Also, the faint 26.7-kD band is much more strongly recognized by the anti-type II antiserum than by the anti-type I antiserum.

On the basis of these results, we assign polypeptides 28.0 through 27.0 kD as type I LHCII (excluding the CP26 polypeptides, see below) and the 26.7 and 26.5 kD polypeptides as type II LHCII. This is in agreement with Sigrist and Staehelin (32), who found that, in general, the type II apoproteins are less numerous and smaller than the type I apoproteins, and with protein sequence data (20), indicating that the faster migrating LHCII polypeptide is encoded by a type II gene. This is also consistent with molecular data showing that the type II LHCII *cab* genes are less numerous than are the type I (11, 29).

Apoproteins of CP29 and CP26 in Spinach Thylakoids

The apoproteins of CP29 are well separated from LHCII using the ammediol SDS-PAGE system, but the CP26 apoproteins are obscured by LHCII. Our next goal was to identify the apoproteins of CP29 and CP26. Figure 3 shows the LHCII/CP29 apoprotein composition of the IEF fractions from pH 3.8 to 4.75. Again, for simplicity, only the region from 25 to 30 kD is shown. Figure 3A is the silver-stained pattern. Figure 3B is a double immunostain with the anti-type II LHCII antibody described above (T2-B1) and the MLH2 monoclonal antibody directed against CP29 (10, 12). Note that in this double-stained blot, MLH2 reacts only with the 29.5-kD CP29 apoprotein. The other reactions visible on the blot are from the anti-type II antiserum. Figure 3C shows staining with a monoclonal antibody directed against CP26 (PS2CF-02-FC8-H10-F12; 31). In the original description of this monoclonal antibody, the antigen was identified as

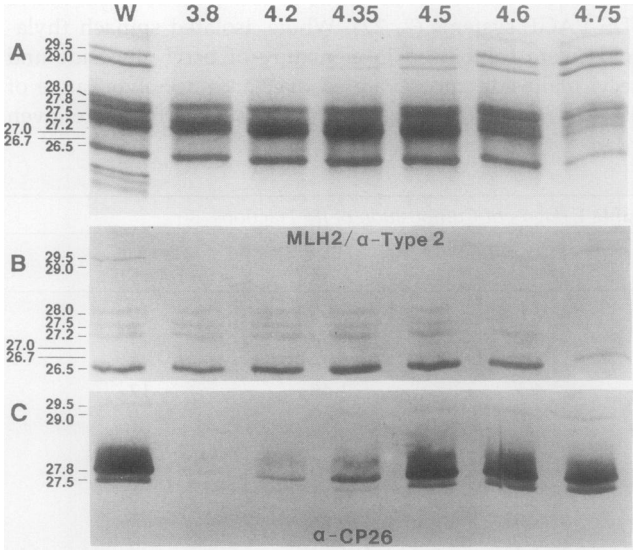

Figure 3. Polypeptide compositions and immunostaining patterns of LHCII, CP29, and CP26 complexes separated by fluid phase IEF. The pH of each fraction is indicated. A, Silver stain; B, double immunostain with the α-type II antiserum used in Figure 2 and MLH2, a monoclonal directed against CP29. C, Anti-CP26 monoclonal antibody, PS2CF-02-FC8-H10-F12.

CP29. This band corresponds to the "type I CP29" described by Pichersky et al. (28) and corresponds to the CP26 previously described by Bassi et al. (4). We are, therefore, using the original CP26 nomenclature of ref. 4 to denote this band.

In the silver-stained pattern, as well as in the anti-type II LHCII staining in Figure 3B, it is apparent that the concentration of the LHCII apoproteins decreases rapidly as one moves from the pH 4.5 to the pH 4.75 fraction. The CP29 and CP26 apoproteins follow an opposite pattern, increasing in intensity in the less acidic fractions. The anti-CP26 monoclonal antibody recognizes two bands at 27.8 and 27.5 kD (and possibly a third, higher band). These two bands would ordinarily be obscured by the LHCII apoproteins, but because the concentration of the CP26 apoproteins increases as the concentration of the LHCII apoproteins decreases in the less acidic fractions, these bands are clearly visible even in the silver-stained pattern. The 27.5-kD band is a doublet, the top half of which is a CP26 apoprotein. The MLH2 monoclonal antibody reacts only with the upper of the two CP29 apoproteins. We are assigning both the 29.5- and 29.0-kD bands as CP29 apoproteins based on (a) the exact comigration of these two bands in a variety of different fractionation techniques, (b) the fact that both are faintly recognized by antisera directed against CP26 or LHCII (Fig. 3C, and data not shown), and (c) the fact that both of these polypeptides are present in preparations of the CP29 green band using a gel electrophoresed according to the method of Dunahay and Staehelin (13, not shown). The original characterization of the MLH2 antiserum indicated that the epitope is located near the N terminus of the molecule (10). The fact that MLH2 reacts only with the upper CP29 apoprotein suggests that the lower 29.0-kD band is a truncated form of the 29.5-kD apoprotein.

Polypeptide Compositions of Grana and Stroma Thylakoid Fractions

The second major goal of this study was to reexamine the polypeptide composition of grana and stroma thylakoid fractions in spinach. Polypeptide compositions of whole spinach thylakoids (Chl a/b ratio = 3.2) and appressed and nonappressed membrane fractions from two different nondetergent separation procedures are shown in Figure 4. The fractions β and α were prepared as described in ref. 3. The β nonappressed fraction has a Chl a/b ratio of 4.8, and the α appressed membrane fraction has a Chl a/b ratio of 2.4. The second set (Y100/BS) is described in ref. 17 (Y100) and in ref. 35 (BS). The Y100 nonappressed membrane fraction has a Chl a/b ratio of 5.1. The BS appressed membrane fraction, with a Chl a/b ratio of 1.9, is prepared by further purification of the α fraction. Because the bulk of the Chl b in the membrane is located in the grana, it is clear from the Chl a/b ratios that Y100/BS is a more extreme separation of appressed and nonappressed thylakoids than is β/α. This is also apparent from the polypeptide compositions of these fractions.

The CF is the only thylakoid complex that is exclusively located in the nonappressed membranes, and therefore, it is a good indicator of the degree of separation (Fig. 4). The CF1 α and β subunits at approximately 55 kD are substantially enriched in the Y100 and β-stroma membrane fractions but

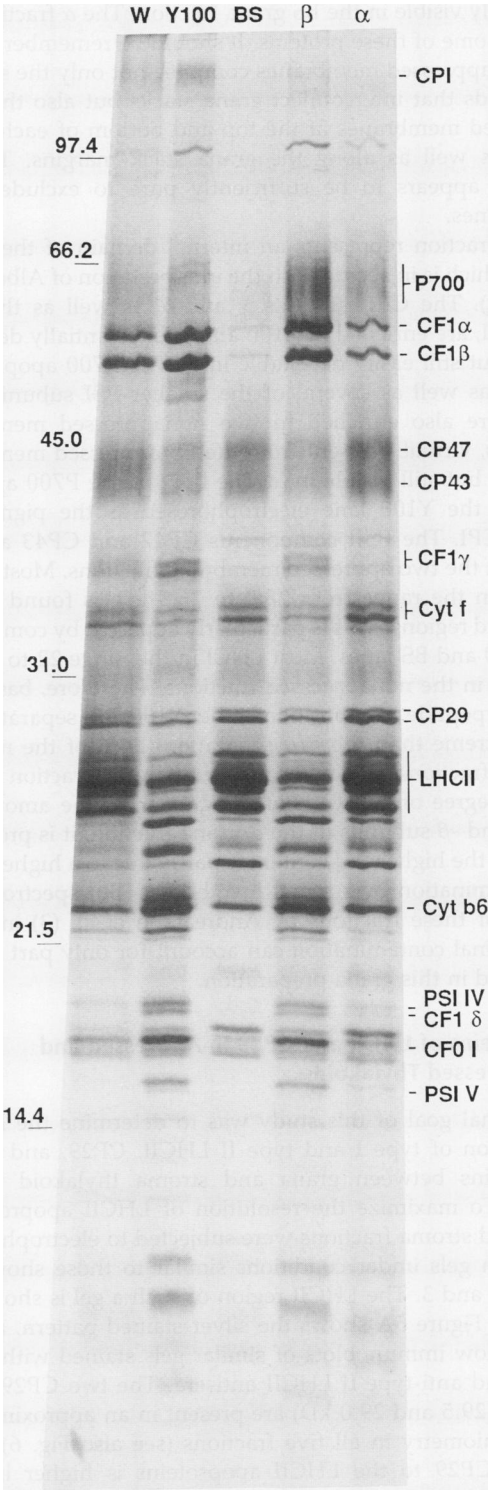

Figure 4. Polypeptide compositions of appressed (BS and α) and nonappressed (Y100 and β) thylakoid fractions separated on a 30-cm gel. Positions of soluble molecular mass markers, and of principal thylakoid membrane components, are marked. CF1, α, and β subunits of the chloroplast CF.

are barely visible in the BS grana fraction. The α fraction still retains some of these proteins. It should be remembered that the nonappressed membranes comprise not only the stromal thylakoids that interconnect grana stacks but also the nonappressed membranes at the top and bottom of each grana stack, as well as along the grana stack margins. The BS fraction appears to be sufficiently pure to exclude these membranes.

This fraction represents an internal domain of the grana stack, which is in accord with the interpretation of Albertsson et al. (1). The CF1 subunits γ and δ, as well as the CF0 subunit I, are enriched in Y100 and β, substantially depleted in BS, but still easily detectable in α. The P700 apoproteins of PSI, as well as several of the smaller PSI subunits and LHCI, are also enriched in two nonappressed membrane fractions, virtually absent from the BS-appressed membrane fraction, but still visible in α. The bulk of the P700 apoproteins in the Y100 lane electrophoresed as the pigmented dimer, CPI. The PSII components CP47 and CP43 are enriched in the two appressed membrane fractions. Most of the LHCII, in the range from 28.0 to 26.5 kD, is found in the appressed regions. This is particularly apparent by comparing the Y100 and BS lanes. Most LHCI in the range 22 to 25 kD is found in the nonappressed fractions. Therefore, based on the polypeptide compositions, the Y100/BS separation is more extreme than the β/α separation. Both of the nonappressed fractions are quite pure, but the α fraction has a higher degree of PSI content. Judging from the amount of CF1-α and -β subunits in the α-grana fraction, it is probably true that the higher PSI content is partly due to a higher level of contamination by stromal membranes. But spectroscopic studies of these fractions by Andreasson et al. (3) indicate that stromal contamination can account for only part of the PSI found in this grana preparation.

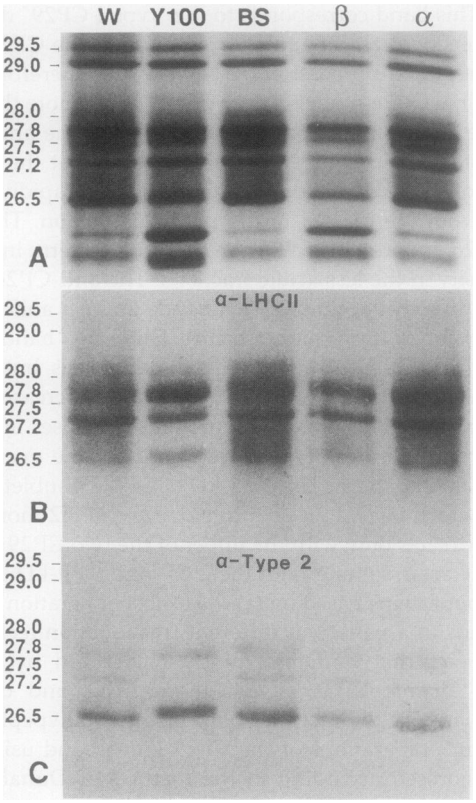

Figure 5. Expanded view of LHCII, CP29, and CP26 apoproteins in whole thylakoid and grana and stroma fractions electrophoresed on a 30-cm gel on which everything below about 20 kD has been run off the bottom. Apparent molecular masses are indicated. A, Silver stain; B, anti-LHCII; C, anti-type II LHCII.

Apoproteins of LHCII and CP29 in Appressed and Nonappressed Thylakoids

The final goal of this study was to determine the lateral distribution of type I and type II LHCII, CP29, and CP26 apoproteins between grana and stroma thylakoid membranes. To maximize the resolution of LHCII apoproteins, grana and stroma fractions were subjected to electrophoresis on 30-cm gels under conditions similar to those shown in Figures 2 and 3. The LHCII region of such a gel is shown in Figure 5. Figure 5A shows the silver-stained pattern, and B and C show immunoblots of similar gels stained with anti-LHCII and anti-type II LHCII antisera. The two CP29 apoproteins (29.5 and 29.0 kD) are present in an approximately 1:2 stoichiometry in all five fractions (see also Fig. 6). The ratio of CP29 to the LHCII apoproteins is higher in the stroma than in the grana fractions. The faint bands at 26.7 and 27.0 kD seen in Figure 2 electrophoresed as diffuse bands on these gels and hence are not visible and are not marked in Figure 5. The CP26 apoproteins at 27.5 and 27.8 kD are obscured by the LHCII apoproteins. In the Y100 and β-stroma fraction, the 26.5-kD LHCII band (see Fig. 5) migrates more slowly than it does in the BS and α-grana fractions. This effect, although subtle at this protein loading, has been observed on several different gels. Furthermore, on very lightly loaded gels, the 26.5-kD band resolves as a doublet, indicating that two type II apoproteins are contained in this band.

The immunoblots shown in Figure 5 demonstrate that, although the silver-stained patterns for all of these fractions are quite similar, there are subtle differences in the LHCII apoprotein populations in stacked and unstacked membranes. As shown in Figure 2, the anti-type II LHCII antiserum reacts most strongly with the 26.5-kD apoprotein, with weaker reactions to the larger, type I apoproteins. The differences in the migration of the 26.5-kD apoprotein are again apparent in the anti-type II LHCII blot. This suggests that there are two forms of the major type II LHCII apoprotein, one enriched in stroma and one enriched in grana thylakoids. These together would give rise to a slightly broader band when whole thylakoid fractions are electrophoresed or to the doublet seen on very lightly loaded gels. Note also the differences in staining intensity of the 27.2-kD band in the various fractions. In the silver-stained and anti-LHCII panels, this band appears to be approximately equally represented in all fractions, perhaps even a little enriched in the stroma fractions. Yet, in the anti-type II LHCII blot, this band is almost undetectable in the stroma fractions but is still easily detectable in the grana fractions. Therefore, although there are no differences in migration, the differences in reactivity

of the type II antiserum indicate the presence of two different components in this band. Thus, there is a more "type II-like" component enriched in grana membranes and a more "type I-like" component enriched in stroma thylakoids.

To determine whether there are changes in the stoichiometries of the LHCII apoproteins in appressed and nonappressed membranes, images of the whole thylakoid, Y100 and BS lanes of the silver-stained gel in Figure 5A, were digitized and used to produce the densitometric scans in Figure 6. This technique provides an approximate quantitation, relying on the assumption that, because all of the LHC apoproteins have very similar polypeptide compositions (11), they will react to equal extents with the silver stain reagent. At the minimum, this procedure allows for the comparison of polypeptide stoichiometries between these different fractions. The distinct peaks at 29.5 and 29.0 kD are the CP29 apoproteins. The 27.8-kD apoprotein of CP26 is visible in all fractions, but the 27.5-kD apoprotein is obscured by the LHCII apoproteins. One or both of the peaks at 25.0 and 24.5 kD, which are substantially enriched in the Y100 nonappressed membrane fraction, are LHCI subunits (Figs. 4 and 5). The ratio of the 29.5- and 29.0-kD CP29 apoproteins to the 27.8-kD CP26 apoprotein is remarkably constant in all three lanes. But the ratio of CP29 and CP26 to the LHCII apoproteins is much higher in the Y100 stroma membrane sample than in either the whole membrane or the BS-appressed membrane samples. This is consistent with the smaller antenna size of PSII in the nonappressed regions (1, 25). The relative stoichiometries of the individual LHCII apoproteins remain quite constant in all three fractions.

Figure 7 shows a series of immunoblots of the LHCII/CP29 section of 15-cm gels on which the grana (BS, α) and stroma (Y100, β) fractions have been separated. This provides a somewhat compressed view of the pattern obtainable with 30-cm gels. As in the previous figure, this series of blots shows that the differences between LHCII apoproteins in the grana and stroma fractions are, in general, quantitative rather than qualitative. Figure 7A shows immunostaining with anti-LHCII antiserum. At this loading there is no detectable cross-reaction to the CP29 apoproteins. At least five of the LHCII apoproteins in the range from 26.5 to 28.0 kD are resolved.

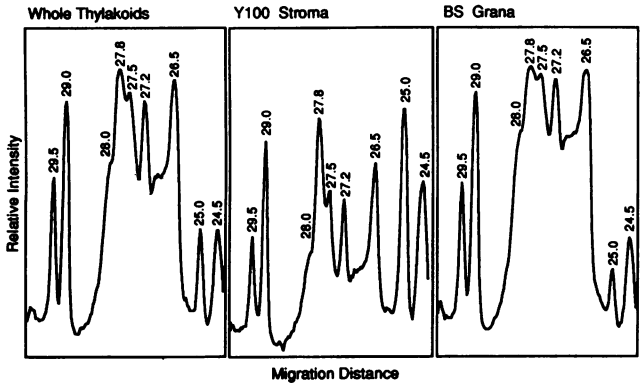

Figure 6. Densitometric scans of the silver-stained whole membrane, Y100, and BS lanes from Figure 5A. Apparent molecular masses are indicated at the top of each peak.

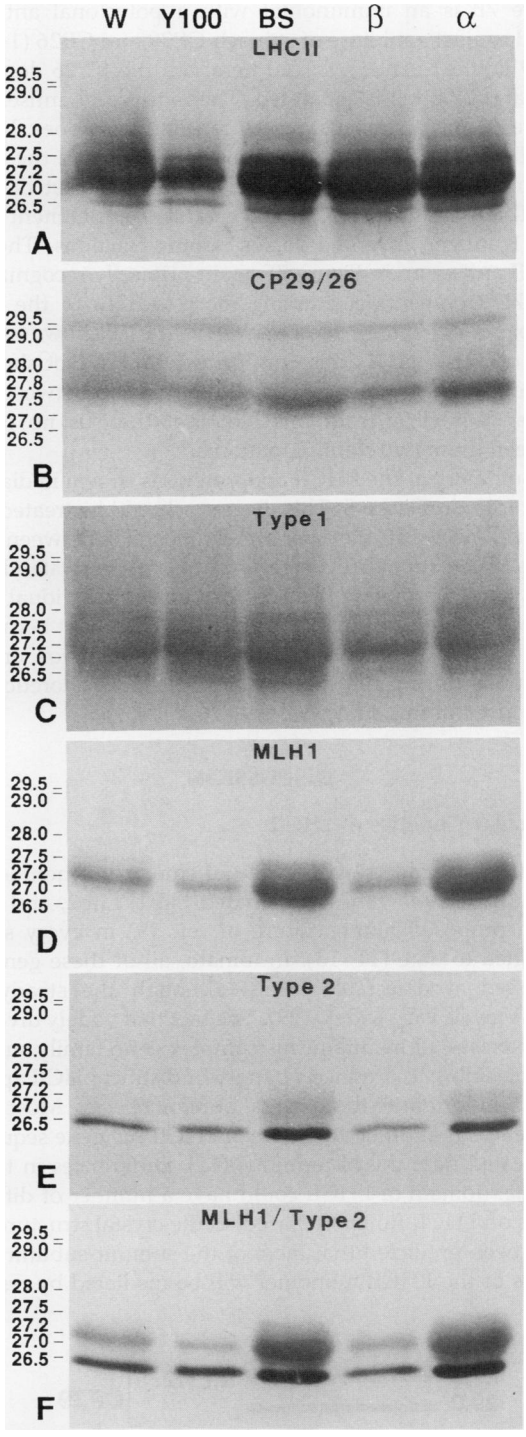

Figure 7. Immunoblots of LHCII and CP29 polypeptides in whole membranes and the two grana/stroma separations separated on 15-cm gels. Antisera are indicated on each panel. The anti-CP29/26 polyclonal antiserum (B) was raised against a mixture of spinach CP29 and CP26. F, A double stain using the MLH1 monoclonal antibody and the anti-type II LHCII antiserum.

Figure 7B is an immunoblot with a polyclonal antiserum raised against a mixture of spinach CP29 and CP26 (14). The CP29 doublet is not resolved here, but the CP26 doublet at 27.5 and 27.8 kD is separated. The anti-type I antiserum in Figure 7C has a similar range of reactivities as does the anti-LHCII. Figure 7D shows immunoreaction with the MHL1 monoclonal antibody, which recognizes a subset of type I LHCII apoproteins (10). There is a large enrichment of the MLH1 antigen in grana versus stroma fractions. The anti-type II antiserum in Figure 7E again primarily recognizes the 26.5-kD polypeptide, crossing more weakly to the type I polypeptides from 27.0 to 28.0 kD. Figure 7F shows a second stain of anti-type II antiserum on the MLH1 blot shown in Figure 7D, illustrating the spatial relationship between these bands. It is clear from this blot that there is no overlap between these two staining patterns.

A summary of the LHCII apoproteins is shown in diagrammatic form in Figure 8. This diagram should be treated as an idealized view of the spatial relationships between these bands. Even though every effort was made to standardize conditions, the precise relative positions of individual bands may vary slightly from experiment to experiment because of small variations in polyacrylamide polymerization or exact conditions during the high-resolution electrophoretic separation used in this study.

DISCUSSION

Molecular Genetics of LHCII

LHCII is encoded by a family of similar but nonidentical genes (LHCII *cab* genes, designated Lhb1, Lhb2, and Lhb3, in the proposed nomenclature of ref. 19) in every species examined to date (11, 15). In tomato, all of these genes are expressed at detectable levels, although the steady-state mRNA levels vary widely (29). The fact that widely divergent plant species all maintain this complex gene family suggests that the ability to produce a battery of distinct LHCII apoproteins is important to the survival of plant species. Most of the differences that do occur between LHCII *cab* gene sequences are located near the N terminus (11). Differences in the N-terminal domain of LHCII could have a number of different effects on LHCII function. Based on the crystal structure (21), it has been predicted that most of the subunit-subunit interactions of the LHCII monomer will be mediated by residues in the N-terminal domain. Thus, alterations near the N terminus may affect trimer formation or association of LHCII with the PSII reaction center. Furthermore, the LHCII phosphorylation site is located very near the N terminus of the molecule (6). Studies of the kinetics of phosphorylation of synthetic peptides indicate that basic residues on either side of the phosphorylation site are important for the kinetics of the reaction (reviewed in ref. 6).

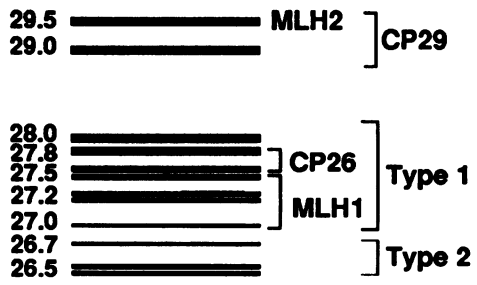

Figure 8. Summary diagram of LHCII, CP29, and CP26 apoproteins. Apparent molecular masses and antibody recognitions are indicated.

Relatively little molecular information exists for the spinach *cab* gene family. To our knowledge, only one LHCII *cab* gene sequence from spinach has been published to date (24). The application of Edman degradation to obtain protein sequence has not been successful because, as is the case in many other species, the spinach LHCII apoproteins are blocked by N-terminal acetylation (6). However, the phosphorylation sites of four distinct spinach LHCII peptides have been sequenced using a different approach. Proteolytic fragments of phosphorylated LHCII were separated by metal ion affinity chromatography and HPLC, resulting in four distinct fragments. These were then sequenced by fast atom bombardment spectroscopy (6). Three of these peptides appeared to be type I LHCII and the remaining peptide was type II. Our data indicate the presence of at least five type I LHCII apoproteins and at least two type II apoproteins.

Pichersky and coworkers (28) isolated a tomato gene encoding a 26-kD mature light-harvesting polypeptide that is clearly distinct from the 29-kD apoprotein of CP29 (4, 5, 17). They designated this gene "type I CP29." This nomenclature is based on green gel and fully denaturing SDS-PAGE conditions that did not distinguish between CP29 and CP26 and led to the conclusion that both the 26- and 29-kD polypeptides were components of the same complex (see, for example, refs. 9 and 15). Improvements in native SDS-PAGE (4, 5, 27, 33) have shown that CP29 and CP26 are distinct complexes with distinct properties. We believe, in concurrence with Morishige and Thornber (26), that the Pichersky sequence is a CP26 polypeptide, as defined by Bassi's group (4) rather than a CP29 polypeptide. This view is supported by the fact that the partial protein sequence for CP29 from both spinach (16) and barley (26), as well as a complete gene sequence for barley CP29 (26), shows very little homology to the tomato sequence. We believe that these sequences are much too distantly related to be considered type I and type II CP29. The biochemical, spectroscopic, and immunological differences already described between CP29 and CP26 (4, 5, 12, 27) also support the view that these are distinct complexes. Thus, we recommend that the original nomenclature used by the authors who first described the CP26 complex (4) should be used.

Partial protein sequence data obtained by Jansson et al. (20) indicates that the 25-kD polypeptide (resolved as at least two bands in this study) is encoded by a type II *cab* gene, based on the presence of several diagnostic amino acid residues. By comparison with other species, these authors inferred that the 27-kD polypeptide (resolved as five bands in this study) is encoded by a type I *cab* gene. We have confirmed this using monospecific antibodies directed against synthetic peptides derived from type I and type II *cab* gene sequences (32). At this point, it is not clear how much of the LHCII polypeptide heterogeneity we observe is the result of

expression of multiple LHCII *cab* genes and how much is the result of differential processing or other posttranslational modification of a smaller number of gene products. We can rule out differential phosphorylation as a source of the polypeptide heterogeneity, because we can observe no differences in the LHCII polypeptide pattern between phosphorylated and dephosphorylated thylakoids (K.D. Allen and L.A. Staehelin, unpublished data).

Models of the Spinach LHC

Initial biochemical characterizations of spinach LHCII resolved two polypeptides with apparent molecular masses of 27 and 25 kD (9, 18, 20, 22, 23). The larger of these two is more abundant in thylakoids, with estimates of the 27/25 molar ratio based on densitometric scans of Coomassie or silver-stained gels of 4:1 (22) or 3:1 (18). This has provided the basis for a simple and very useful model for the functional aspects of spinach LHCII. The 25-kD type II polypeptide is much more rapidly phosphorylated during the state transition than the 27-kD polypeptide (23, and refs. therein). But, although the initial rate of phosphorylation of the 25-kD polypeptide is typically 3 times higher than that for the 27-kD form, the final extent of phosphorylation is quite similar for the two (18).

The differential phosphorylation rates of the 25- and 27-kD polypeptides are paralleled by a faster rate of migration of the 25-kD polypeptide from grana to stroma membranes during a state 1 to state 2 transition (23). This would suggest that LHCII may be divided into two populations. The inner LHCII is more tightly associated with the PSII reaction center, is less highly phosphorylated, and does not migrate during the state transition. The peripheral LHCII is more loosely associated with the reaction center, is more highly phosphorylated, and migrates preferentially during the state transition. Furthermore, the inner population should be enriched for the 27-kD polypeptide, whereas the peripheral should contain a higher proportion of the 25-kD polypeptide. A second layer of complexity is imposed on this pattern by the finding of Islam (18) that LHCII phosphorylation is always substoichiometric in spinach and that there is an approximately equal distribution of incorporated phosphate between grana and stroma membranes. These results indicate that only a fraction of either the 25- or 27-kD polypeptide pools are phosphorylated and that a substantial proportion of the phosphorylated LHCII does not migrate during the state transition. Thus, the LHCII pool must be divided into at least three subpopulations: phosphorylated mobile, phosphorylated nonmobile, and nonphosphorylated nonmobile (18).

Our results are in general agreement with many aspects of this model, but other aspects will clearly need to be expanded or reevaluated to account for the increased complexity of the LHCII population reported here and on our finding that differences in the population of LHCII apoproteins between state 1-adapted grana and stroma thylakoid domains are surprisingly subtle. We find that the type II "25-kD" polypeptide actually consists of at least two and possibly three polypeptides. The "27-kD" type I band contains at least five LHCII apoproteins, in addition to the two CP26 apoproteins. Furthermore, CP29 is a doublet at 29.5 and 29.0 kD. This increased complexity clearly has implications for any discussion of the relative stoichiometries of type I and type II polypeptides in spinach thylakoids. The nature of this problem becomes apparent by comparing, for example, the densitometric trace in our Figure 6 with those in figure 1 of ref. 22 or in figure 1 of ref. 18. It should further be noted that, even in Figure 6, the densitometry only shows a fraction of the heterogeneity of the LHCII region. Thus, the peak at 27.8 kD is primarily a CP26 apoprotein, but there is also a comigrating type I LHCII polypeptide in this region. The heterogeneity of the 27.2- and 26.5-kD bands demonstrated in Figure 5 is also not reflected in this densitometric trace.

Our data are not consistent with a model in which, in state 1-adapted thylakoids, the grana-associated LHCII is enriched for the smaller, type II LHCII apoproteins, whereas the stroma preferentially contains the larger, type I apoproteins. These data do not, however, impinge on the well-established observation that during the state transition the type II polypeptides are preferentially phosphorylated and preferentially migrate from grana to stroma membranes (18, 22, 23).

In summary, we found that the antenna complexes of PSII in spinach, including LHCII, CP29, and CP26, contain a greater number of polypeptides than reported previously. We saw only subtle differences in the LHCII apoproteins found in grana and stroma fractions from state 1-adapted thylakoids. We did, however, see a higher ratio of the CP29 and CP26 apoproteins to the LHCII apoproteins in stromal fractions versus grana fractions. This is consistent with the idea that CP29 and CP26 are part of a basic PSII unit (PSIIγ) with a constant stoichiometry to the reaction center core (25) and with the observation that most LHCII is concentrated in the grana membranes (1, 33).

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