

# The Three Isoforms of the Light-harvesting Complex II

SPECTROSCOPIC FEATURES, TRIMER FORMATION, AND FUNCTIONAL ROLES\*

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**The major light-harvesting complex (LHC-II) of higher plants plays a crucial role in capturing light energy for photosynthesis and in regulating the flow of energy within the photosynthetic apparatus. Native LHC-II isolated from plant tissue consists of three isoforms, Lhcb1, Lhcb2, and Lhcb3, which form homo- and heterotrimers. All three isoforms are highly conserved among different species, suggesting distinct functional roles. We produced the three LHC-II isoforms by heterologous expression of the polypeptide in *Escherichia coli* and *in vitro* refolding with purified pigments. Although Lhcb1 and Lhcb2 are very similar in polypeptide sequence and pigment content, Lhcb3 is clearly different because it lacks an N-terminal phosphorylation site and has a higher chlorophyll *a/b* ratio, suggesting the absence of one chlorophyll *b*. Low temperature absorption and fluorescence emission spectra of the pure isoforms revealed small but significant differences in pigment organization. The oligomeric state of the pure isoforms and of their permutations was investigated by native gel electrophoresis, sucrose density gradient centrifugation, and SDS-PAGE. Lhcb1 and Lhcb2 formed trimeric complexes by themselves and with one another, but Lhcb3 was able to do so only in combination with one or both of the other isoforms. We conclude that the main role of Lhcb1 and Lhcb2 is in the adaptation of photosynthesis to different light regimes. The most likely role of Lhcb3 is as an intermediary in light energy transfer from the main Lhcb1/Lhcb2 antenna to the photosystem II core.**

The light-harvesting pigment protein complexes (LHCs)<sup>1</sup> in the thylakoid membranes of chloroplasts are efficient collectors of solar energy. Unlike the reaction center complexes in which chlorophyll (Chl) *a* is the only green pigment, the LHCs contain defined amounts of both Chl *a* and Chl *b*, and are therefore often referred to as Chl *a/b* binding complexes (1, 2). LHC-II, the most abundant Chl *a/b* complex, alone accounts for roughly one-third of the total membrane protein in plant thylakoids (2).

It is mainly associated with photosystem II (PS II) forming the PS II-LHC-II supercomplex in which LHC-II surrounds the

reaction center. The main role of LHC-II in this assembly is the absorption and transfer of solar energy to the reaction center through the so-called minor LHCs (Lhcb4, Lhcb5, and Lhcb6) (3), which are similar to LHC-II in pigment content and gene sequence. In addition, LHC-II plays an important role in balancing the transfer of excitation energy to PS I and II. Under conditions in which an excess of light energy is transmitted to PS II, a redox-controlled kinase phosphorylates LHC-II at a threonine near the N terminus. The outer LHC-II complexes then detach from the PS II-LHC-II supercomplex, thereby reducing the amount of excitation energy directed to PS II (4). In this way, plant photosynthesis is able to adapt optimally to varying light conditions on a short timescale.

The structure and function of LHC-II have been studied extensively. It is well known that LHC-II is a trimer in detergent solution (5) and in two-dimensional crystals (6) and most likely also in the thylakoid membrane. Electron image processing of PS II-LHC-II supercomplexes revealed a large variety of assemblies (7–9). At least three kinds of LHC-II binding sites were found, suggesting that LHC-II is playing an important role in the organization of these supercomplexes.

The excitation energy transfer within the LHC-II complex occurs on a timescale of femtoseconds (10–13) and is dependent on the type, orientation, and exact position of the bound pigments. High performance liquid chromatography (HPLC) analysis revealed that each LHC-II monomer binds 7–9 Chl *a*, 5–7 Chl *b*, two luteins, and one neoxanthine, plus small, nonstoichiometric amounts of violaxanthine (5, 14, 15). An atomic model based on cryoelectron microscopy of two-dimensional crystals (16) showed the arrangement of 12 Chl *a* and *b* and two luteins in the protein scaffold. Recently, the positions of the two remaining Chl and of neoxanthine and violaxanthine were determined in an x-ray structure at 2.72 Å resolution (17). The structure also revealed the identities of the Chl as Chl *a* or Chl *b*, and the majority of LHC-II binds therefore eight Chl *a*, six Chl *b*, two luteins, one neoxanthine, and one violaxanthine.

LHC-II isolated from thylakoid membranes is a mixture of several closely related gene products. The LHC-II genes are divided into three classes, referred to as Lhcb1, Lhcb2, and Lhcb3. Typically, all three are found in multiple copies in the genomes of higher plants (18, 19). In *Arabidopsis thaliana*, for example, there are five and four copies of Lhcb1 and Lhcb2, respectively, but only one copy of Lhcb3 (20). Each gene encodes an apoprotein of about 25 kDa. The protein products of these gene classes are referred to as LHC-II isoforms and can be correlated directly to the corresponding genes (21). The most abundant Lhcb1 isoform accounts for roughly 70% of the total LHC-II protein, whereas Lhcb2 and Lhcb3 account for ~20 and ~10%, respectively (18, 22). The Lhcb1–3 isoforms are similar enough to form homo- and heterotrimers in various combinations. At least three combinations, namely Lhcb1 homotrimers,

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<sup>1</sup> The abbreviations used are: LHC, light-harvesting complex; Chl, chlorophyll; DDM, dodecyl maltoside; HPLC, high performance liquid chromatography; PS, photosystem.

Lhcb1/Lhcb2 heterotrimers, and Lhcb1/Lhcb2/Lhcb3 heterotrimers, were identified by nondenaturing isoelectric focusing in *A. thaliana* (22). The situation in other plants is almost certainly similar.

LHC-II isoforms are highly conserved among different species. Their polypeptide sequences have undergone little change in several hundred million years of evolution (20). It can therefore be expected that they have distinct functional roles. Indeed, a spatial distribution of different isoforms around the PS II reaction center has been suggested by biochemical investigations of the PS II-LHC-II supercomplex. The LHC-II population on the PS II periphery was found to be enriched in Lhcb2 (23, 24), whereas Lhcb3 was more tightly attached and thus closer to the minor LHC next to the PS II core (25, 26).

Plants grown in low light contain higher levels of Lhcb2 (24, 27), indicating a role of this isoform in long term light acclimation. *In vitro* phosphorylation studies with a mixture of LHC-II isolated from plant thylakoids showed that one fraction was phosphorylated three times more rapidly than the bulk of the complex (28). Similar studies indicated that the rapidly phosphorylated form is enriched in Lhcb2 (29), consistent with an involvement of this isoform in the adaptation to different light regimes.

So far, studies of LHC-II have been carried out either with inherently heterogeneous LHC-II preparations isolated from plants or with recombinant Lhcb1 produced *in vitro* from its components. A detailed understanding of the functional implications of LHC-II heterogeneity, however, depends on the availability of pure LHC-II isoforms in the amounts necessary for spectroscopic and biochemical investigations. We have produced all three isoforms by *in vitro* folding of functional complexes from bacterially expressed polypeptide, pigments, and lipids and characterized them biochemically and spectroscopically. Our results indicate a high degree of similarity between Lhcb1 and Lhcb2, whereas Lhcb3 is clearly different with respect to pigment organization, trimer formation, and spectroscopic properties.

#### EXPERIMENTAL PROCEDURES

**Genetic Material and Cloning**—Apoproteins were expressed with a C-terminal His<sub>6</sub> tag in *Escherichia coli*. Expression levels were similar for the three isoforms, and typically 1 liter of bacterial culture yielded about 100 mg of polypeptide. For expression of the Lhcb1 polypeptide we used the clone C3.2h, which carries the gene *lhcb1*\*2 of pea (30, 31) in a pDS 12 plasmid. cDNAs carrying the *lhcb2*\*1 and Lhcb3 genes from *Arabidopsis* were provided by the Arabidopsis Biological Resource Center (Ohio State University).

For expression of Lhcb2, plasmids from the clone C.32h were amplified by PCR with the His<sub>6</sub> tag but without the *lhcb1* gene. The amplified *lhcb2* gene was inserted into this vector by an introduced SphI cleavage site and transformed to XL1-blue *E. coli* competent cells. Clones were sequenced and tested for expression. The Lhcb2 apoprotein was expressed in XL1-blue *E. coli* cells.

The *lhcb3* gene did not express under the control of the T5 promoter and was produced using the pET expression system controlled by the stronger T7 promoter. NdeI and XhoI cleavage sites were used to insert the *lhcb3* gene in a pET-20b(+) vector. To create the XhoI site the additional sequence CTCGAG was added to the 3'-end of *lhcb3* resulting in additional Leu and Glu residues at the C terminus of the expressed polypeptide. After transformation the construct was checked by sequencing. The Lhcb3 apoprotein was expressed in BL21(DE3) pLys *E. coli* cells.

**Sequence Comparison**—Sequence comparisons were performed with the program ClustalW (1.81) on the EMBL Web site (www-db.embl-heidelberg.de).

**Refolding and Protein Isolation**—All three different LHC-II polypeptides formed inclusion bodies during expression and were purified as described previously (32). Pigments were isolated from spinach by acetone extraction followed by dioxane precipitation of the carotenoids. LHC-II monomers were refolded by the detergent exchange method (33). 0.6 mg of apoprotein, 0.5 mg of Chl (*a/b* = 3.0), and 0.15 mg of carotenoids were used for each refolding experiment.

Trimers of refolded LHC-II monomers formed on a nickel chelating column as described in Ref. 31. The eluted green fraction was loaded onto partly denaturing gels (see below) or sucrose density gradients (5–35% sucrose, 0.1% *n*-dodecyl  $\beta$ ,D-maltoside (DDM), 25 mM Tris-HCl, pH 7.5). Gradients were centrifuged at 40,000 rpm and 4 °C. For further analysis, monomers and trimers were removed from the gradient and prepared as described below.

**Gel Electrophoresis**—Monomeric and trimeric complexes were separated under partly denaturing conditions on 15% acrylamide gels without SDS at 4 °C in the dark. Samples were loaded in 10% glycerol without SDS. The SDS concentration in the running buffer was 0.1%.

To investigate the isoform content of trimers, the complexes were run on SDS-PAGE (34). Trimers were first purified on sucrose density gradients and concentrated using Centricon ultrafiltration devices with a 50 kDa cutoff. Equal amounts of complexes were purified further on partly denaturing gels. Gel slices containing LHC-II trimers were excised and transferred to an Eppendorf tube. Protein was extracted by shaking overnight in 30  $\mu$ l of SDS loading buffer. Complexes were denatured by heating to 95 °C for 5 min prior to loading on the gels. The same procedure was applied to monomeric Lhcb3 complexes. As a further control a fraction at the position of the trimer band was removed from the Lhcb3 gradient and treated the same way.

**HPLC**—Pigments were extracted from sucrose gradient fractions by adding *sec*-butyl alcohol and NaCl up to a concentration of 33% and 1 M, respectively. Samples were centrifuged for 1 min in a tabletop centrifuge and the green supernatant diluted to a concentration of 80% acetone prior to injection onto the column (Chromolith SpeedROD; RP-18e; Merck). Pigments were separated by applying a gradient of acetone/water buffered with 0.2 mM Tris-HCl to pH 7.0. The pure pigments were quantified by comparing the integrated 440 nm peak with a calibration curve of purified standards.

**Spectroscopy**—Chl concentrations were determined photometrically by the method of Porra *et al.* (35). For circular dichroism (CD) and 77 K absorption spectroscopy, sucrose density gradient purified monomers and trimers were concentrated for 3 h at 100,000 rpm in a tabletop ultracentrifuge. Clear supernatant was removed and the remaining sample adjusted with 20% sucrose, 0.1% DDM, 25 mM Tris-HCl, pH 7.5, to an  $A_{437}$  of 1.5. Fluorescence emission measurements were performed on samples directly from sucrose density gradients diluted with 20% sucrose, 0.1% DDM, 25 mM Tris-HCl, pH 7.5.

CD spectra were recorded with a path length of 1 cm and a slit width of 0.2 nm. The measuring cell was cooled with water to 4 °C.

77 K absorption spectra were recorded with a path length of 1 cm and a slit width of 0.2 mm. Prior to freezing in liquid nitrogen, glycerol and DDM were added to final concentrations of 60 and 0.05%, respectively. A base line recorded with 20% sucrose, 0.05% DDM, 25 mM Tris-HCl, pH 7.5, 60% glycerol was subtracted from the spectra. Spectra were normalized to  $A_{437}$ . To minimize noise, five measurements of the spectral region between 600 and 750 nm were averaged prior to calculation of the fourth derivative.

77 K fluorescence emission spectra were recorded with an excitation of 469 nm (slit width 2 nm) where Chl *a* absorption is negligible. Emission spectra were recorded with a spectral resolution of 0.5 nm.

#### RESULTS

**Sequence Comparisons**—A comparison of the polypeptide sequence of the three LHC-II isoforms from *Arabidopsis* with Lhcb1 from pea is shown in Fig. 1. The residues are numbered as in Lhcb1 from pea for ease of comparison with former studies.

The sequences of Lhcb1 from *Arabidopsis* and pea are very similar, with 91.8% identity and only 2.6% of nonconservative substitutions, mostly within the first 12 residues near the N-terminal phosphorylation site. This is a much higher level of sequence identity than between two isoforms of the same species, as demonstrated by Lhcb1 and Lhcb2 from *Arabidopsis*, which shows only 66.8% identical residues and 14.2% nonconservative substitutions. When all three isoforms of *Arabidopsis* are compared, the homology is even lower, with 56.6% identical residues and 24.6% nonconservative substitutions. Major differences occur at the N and C termini and the loop connecting helices C and A.

Even though there is considerable sequence variation between the isoforms, most residues with known functions are

FIG. 1. Alignment of primary sequences of Lhcb1-3 from *A. thaliana* and Lhcb1 from pea (*Pisum sativum*). Conserved Chl ligands are green. Residues involved in trimer formation are dark blue. Main phosphorylation sites are red,  $\alpha$ -helical regions are shaded light blue according to Kühnbrandt *et al.* (16).

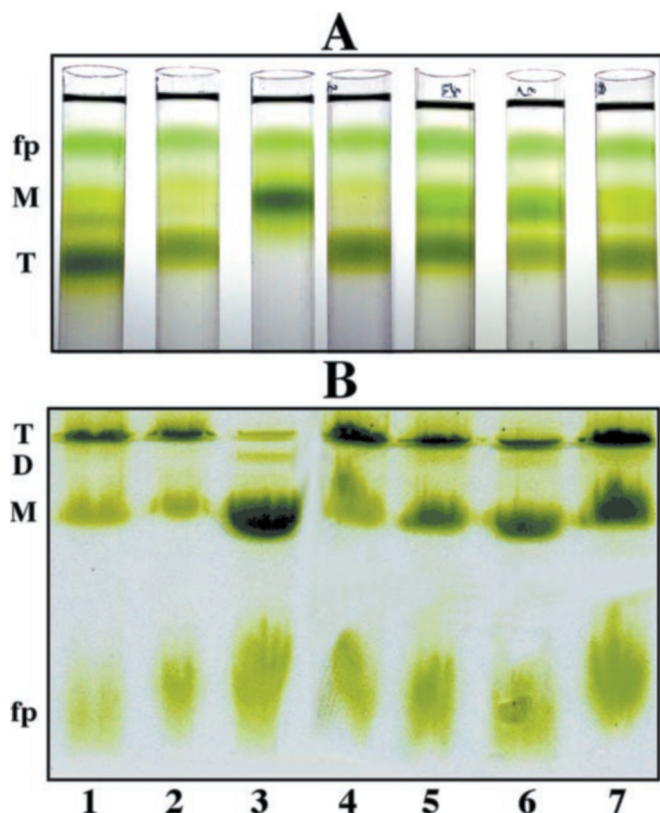
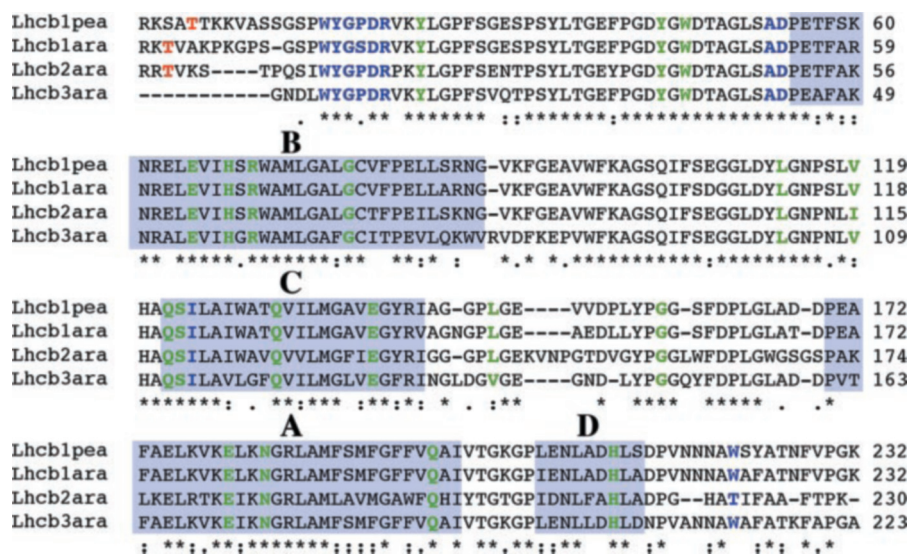


FIG. 2. Oligomeric state of LHC-II isoforms. Monomers of each isoform were refolded by the detergent exchange method. Pure monomeric isoforms, 1:1 mixtures of two isoforms, or 1:1:1 mixtures of all three isoforms were bound via the cloned His<sub>6</sub> tag to a nickel-chelating column. Protein was eluted, and oligomeric states were investigated by sucrose density gradient centrifugation (A) or partly denaturing gel electrophoresis (B). Slightly different positions of the bands on the gradient reflect variations in sucrose concentration. Lane 1, Lhcb1; lane 2, Lhcb2; lane 3, Lhcb3; lane 4, Lhcb1/Lhcb2; lane 5, Lhcb1/Lhcb3; lane 6, Lhcb2/Lhcb3; lane 7, Lhcb1/Lhcb2/Lhcb3; T, trimer; D, dimer; M, monomer; fp, free pigment.

conserved. This includes most of the side chains involved in Chl binding. The only two exceptions are valine 119, which is changed to an isoleucine in Lhcb2, and the change of leucine 148 to valine in Lhcb3. Both substitutions are conservative, and because the respective Chl are bound via the main chain carbonyl, no effect on Chl content is expected. Another con-

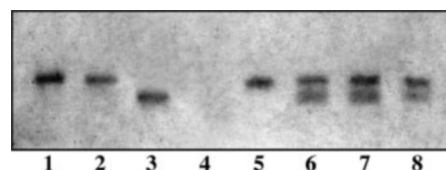


FIG. 3. Isoform composition of LHC-II trimers. Pure monomeric isoforms, 1:1 mixtures, and 1:1:1 mixtures of different isoforms were trimerized. Trimers were purified by sucrose density gradient centrifugation. Residual monomers were removed by a subsequent partly denaturing gel electrophoresis. Bands were cut out of the gel, and protein was extracted by overnight incubation in SDS sample buffer prior to separation by denaturing SDS-PAGE. Because pure Lhcb3 did not form trimers, the monomer was used instead. As a control a fraction from the Lhcb3 gradient at the level of the trimer was treated the same way. Lanes 1, Lhcb1; lanes 2, Lhcb2; lanes 3, Lhcb3; lanes 4, control; lanes 5, Lhcb1/Lhcb2; lanes 6, Lhcb1/Lhcb3; lanes 7, Lhcb2/Lhcb3; lanes 8, Lhcb1/Lhcb2/Lhcb3.

served motif is the presumed phosphatidylglycerol binding site at position 16 (WYGPDR) required for trimer formation (36, 37). The only change of this motif was found in *Arabidopsis* Lhcb1 where proline 19 is changed to a serine. The other sequence motifs thought to have a role in trimer formation Ala<sup>53</sup>-Asp<sup>54</sup> (38) and Ile<sup>124</sup>-Trp<sup>222</sup> (38, 39) are similarly conserved, with the single exception of threonine 222 in Lhcb2. The main N-terminal phosphorylation site (threonine 5) is present in both Lhcb1 and Lhcb2. The N terminus of Lhcb3 is 10 residues shorter and lacks the phosphorylation site, implying that this isoform is unable to participate in the redox-controlled regulation of energy distribution.

**Refolding and Trimer Formation**—All three isoforms were refolded into pigment-protein complexes by the addition of the photosynthetic pigments and exchanging the harsh detergent lauryl dodecyl sulfate against octyl glucoside, a mild, nonionic detergent (33). The resulting Chl *a/b* ratio of the refolded complex depends to some extent on the Chl *a/b* ratio used for refolding (40). We chose an initial *a/b* ratio of 3, which results in complexes that are virtually identical to native LHC-II in pigment composition.

Partly denaturing gel electrophoresis, which separates free pigment from the folded complex and monomers from trimers, showed single green monomer bands for the pure refolded isoforms. The absence of bands of higher molecular mass indicated that none of the isoforms produced trimers spontaneously under these conditions. Yields were estimated by the Chl content of monomer bands and were very similar for all three isoforms.

TABLE I  
Pigment stoichiometry of LHC-II isoforms as determined by HPLC analysis of monomers and trimers derived from sucrose density gradient centrifugation

Numbers refer to molecules/pLHC-II monomer, assuming two luteins/ppolypeptide. Averages  $\pm$  S.D. of four independent refolding experiments are presented. M, monomer; T, trimer; Nx, neoxanthin; Vx, violaxanthin; Lu, lutein.

	Nx	Vx	Lu	Chl <i>b</i>	Chl <i>a</i>	Chl <i>a/b</i>
Lhcb1 M	0.86 $\pm$ 0.05	0.11 $\pm$ 0.02	2	7.06 $\pm$ 0.36	7.82 $\pm$ 0.31	1.11
Lhcb2 M	0.80 $\pm$ 0.09	0.14 $\pm$ 0.02	2	6.90 $\pm$ 0.36	8.01 $\pm$ 0.08	1.16
Lhcb3 M	0.72 $\pm$ 0.05	0.14 $\pm$ 0.01	2	6.13 $\pm$ 0.28	8.32 $\pm$ 0.26	1.36
Lhcb1 T	0.86 $\pm$ 0.02	0.07 $\pm$ 0.01	2	7.72 $\pm$ 0.15	8.49 $\pm$ 0.24	1.10
Lhcb2 T	0.80 $\pm$ 0.08	0.07 $\pm$ 0.01	2	7.58 $\pm$ 0.28	8.48 $\pm$ 0.28	1.12

An effective method to induce trimer formation of Lhcb1 *in vitro* is the slow elution of the monomers from a nickel-nitrilotriacetic acid column in the presence of the lipid phosphatidylglycerol (31). We used this method to determine whether Lhcb2 and Lhcb3 form trimers under the same conditions as Lhcb1. Eluted samples were again analyzed by partly denaturing gel electrophoresis (Fig. 2). The relative amounts of monomers and trimers obtained were very similar for Lhcb1 and Lhcb2. In both cases, the trimer was the predominant form, whereas only a faint trimer band was found for Lhcb3 under the same conditions. The Lhcb3 trimer band was observed only when the protein was loaded onto the gel within 30 min after elution from the column, indicating that the Lhcb3 homotrimer is unstable. Unlike Lhcb1 and Lhcb2 in  $\sim$  50% of the experiments Lhcb3 also showed a faint dimer band, possibly because of nonspecific aggregation, but most of the protein remained in the monomeric state.

Monomers and trimers of the isoforms were prepared by sucrose density gradient centrifugation for biochemical and biophysical studies. Typical sucrose density gradients are shown in Fig. 2. Lhcb1 and Lhcb2 produced reproducible monomer and trimer bands on the gradient. For Lhcb3 only a strong monomer band was found, indicating that the small amount of Lhcb3 homotrimer seen on the gels was unstable on the timescale of a sucrose density gradient centrifugation.

Plants contain not only LHC-II homotrimers but heterotrimers as well. To investigate the *in vitro* formation of heterotrimers, all permutations of monomeric isoforms were applied in equal amounts to a nickel-nitrilotriacetic acid column. The amounts of monomers and trimers obtained in each case were compared on partly denaturing gels and sucrose density gradients (Fig. 2). A mixture of Lhcb1 and Lhcb2 gave a trimer band of comparable intensity to trimers of pure Lhcb1 or Lhcb2. All permutations containing Lhcb3 (Lhcb1/Lhcb3, Lhcb2/Lhcb3, Lhcb1/Lhcb2/Lhcb3) also showed a dominant trimer band but a higher amount of monomers than those that did not contain this isoform. This demonstrates that Lhcb3 has a stronger tendency to remain monomeric even in the presence of the other isoforms.

The polypeptide composition of the trimers was studied by SDS-PAGE (Fig. 3). Trimers derived from pure isoforms, 1:1 mixtures of two isoforms, and a 1:1:1 mixture of all three isoforms were purified by sucrose density gradient centrifugation. Any residual monomers were removed by partly denaturing gel electrophoresis. Trimers or, in the case of Lhcb3, monomers, were investigated by SDS-PAGE. Because of their nearly identical molecular mass, the Lhcb1 and Lhcb2 polypeptides migrated as a single band. Lhcb3 migrated at lower molecular mass and was therefore easily distinguished from the other two isoforms. All trimers from mixtures containing Lhcb3 (Lhcb1/Lhcb3, Lhcb2/Lhcb3, Lhcb1/Lhcb2/Lhcb3) showed a clear band of the Lhcb3 polypeptide. Lhcb3, although unable to form stable trimers by itself, is therefore able to form oligomers with both Lhcb1 and Lhcb2. In all mixtures the intensity of the Lhcb3 band was significantly lower than that of Lhcb1 and

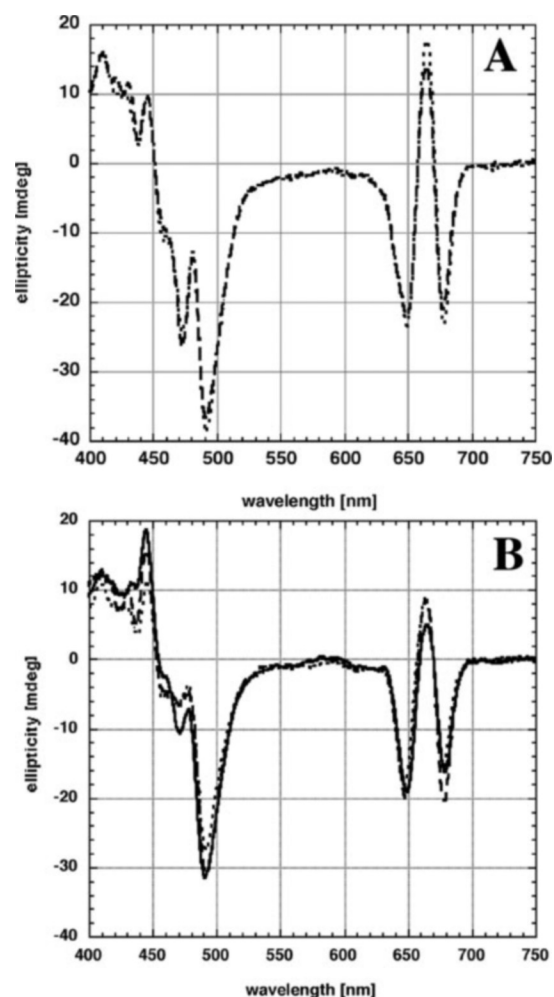


FIG. 4. CD spectra at 4 °C of trimeric (A) and monomeric (B) LHC-II isoforms. Monomers and trimers were purified by sucrose density centrifugation and concentrated by ultracentrifugation. Dotted line, Lhcb1; dashed line, Lhcb2; solid line, Lhcb3.

Lhcb2, probably because Lhcb1 and Lhcb2 have strong tendencies to form homotrimers, so there is less material available for heterotrimers with Lhcb3.

**HPLC**—LHC-II isolated from plant thylakoids contains Chl *a*, Chl *b*, and the three carotenoids lutein, neoxanthine, and violaxanthine. The pigment content of the three LHC-II isoforms was determined by HPLC of refolded monomers and trimers separated on sucrose density gradients. Because there are two copies of lutein/LHC-II monomer (2) we used this carotenoid as an internal standard to quantify the number of pigment molecules bound/polypeptide. Results of the HPLC analysis of monomers and homotrimers from four independent folding experiments are presented in Table I.

Overall, the pigment content of the three isoforms was close to that found by previous HPLC analyses of native LHC-II and

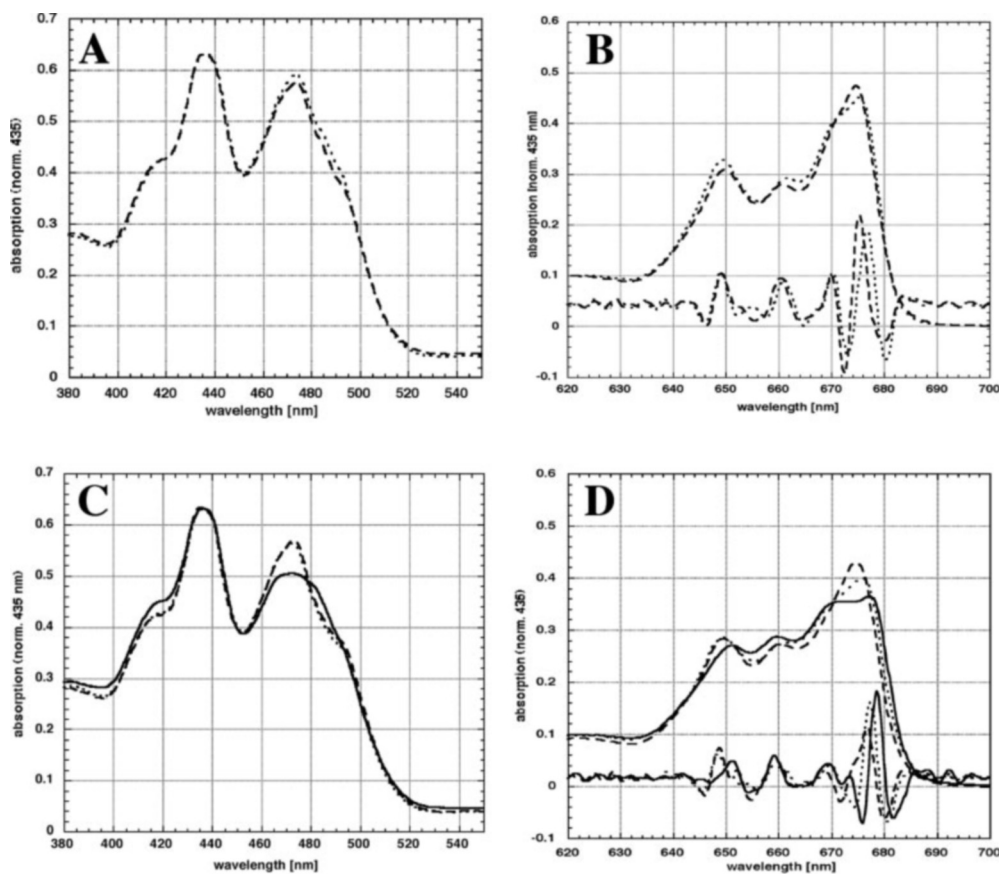


FIG. 5. Absorption spectra at 77 K of trimeric (A and B) and monomeric (C and D) LHC-II isoforms. Samples were isolated from sucrose density gradients and concentrated by ultracentrifugation. For better comparison only the blue (A and C) and red regions (B and D) of the visible spectrum are presented. Spectra were normalized at 437 nm. To resolve the spectral substructure, the fourth derivative was calculated from the red part of the spectrum. Dotted line, Lhcb1; dashed line, Lhcb2; solid line, Lhcb3.

refolded Lhcb1 (5, 14, 15) (3–4 carotenoids and 12–16 Chl/monomer) and is, within experimental error, in good agreement with the recent x-ray structure (17).

The Chl *a/b* ratio of refolded Lhcb1 and Lhcb2 trimers was 1.10 and 1.12, respectively. The observed number of Chl was 16, rather than 14–15 in the monomers, probably because of small amounts of nonspecifically attached Chl *a* and Chl *b* that were not removed on the sucrose gradient. Although the pigment content of Lhcb1 and Lhcb2 monomers was roughly the same, that of Lhcb3 was different. The neoxanthine content of that isoform was slightly lower but remained within the standard deviation of the experiments. Its Chl *a/b* ratio of 1.36 was significantly higher, mostly because of a lower Chl *b* content corresponding to about one Chl *b* less than in Lhcb1 and Lhcb2 monomers.

**CD Spectroscopy**—The CD spectrum of LHC-II in the visible wavelength range is a sensitive monitor of intramolecular pigment-pigment interactions. In addition, it distinguishes between monomeric and trimeric LHC-II. The CD spectrum of trimeric LHC-II is characterized by a strong negative peak around 472 nm, which is absent in the monomer (41). CD spectra of the refolded monomeric and trimeric LHC-II isoforms are shown in Fig. 4. They are similar to one another and to the CD spectra of native LHC-II (41), confirming that all complexes were folded correctly. The spectra of Lhcb1 and Lhcb2 are almost identical, and the trimers show the expected peak at 472 nm. The CD signal of Lhcb3 is very similar to that of monomeric Lhcb1 and Lhcb2. The most prominent difference of Lhcb3 is a peak at 470 nm. Although close to the 472 nm trimer peak seen in the other two isoforms, this peak must be the result of differences in pigment content or pigment inter-

actions because we have shown above that Lhcb3 does not form stable homotrimers under our conditions.

**77 K Absorption Spectroscopy**—Even though the absorption spectra of the three isoforms appeared similar at room temperature, some clear spectral differences emerged at 77 K. Fig. 5 shows the low temperature absorption spectra of monomeric and trimeric LHC-II isoforms. Initial comparison showed only small differences between Lhcb1 and Lhcb2. The fourth derivative, however, indicated a shift of 1–1.5 nm toward shorter wavelength in the Chl *a* region of Lhcb2 compared with Lhcb1. This shift was the same for monomers and trimers.

Again, the largest differences were found for Lhcb3. In the spectral region around 470 nm, dominated by Chl *b* and carotenoids, Lhcb3 absorbs significantly less than Lhcb1 or Lhcb2. Because HPLC analysis showed virtually no differences in the carotenoid content, the lower absorption in this region confirms the lower Chl *b* content. Consistent with this, the red part of the Lhcb3 spectrum, where carotenoids do not absorb, indicates lower Chl *b* absorption around 650 nm. In the Chl *a* region, several sub-bands of Lhcb3 have different relative heights compared with Lhcb1. In contrast to the Chl *a* bands in Lhcb2, which were blue-shifted with respect to Lhcb1, this part of the Lhcb3 spectrum is shifted ~1.5 nm to the red. At the red end of the spectrum, the three LHC-II isoforms thus absorb light of different energy, with Lhcb2 absorbing at shorter and Lhcb3 at longer wavelength than Lhcb1.

**77 K Fluorescence Emission Spectroscopy**—The intramolecular energy transfer from Chl *b* to Chl *a* in the recombinant pure LHC-II isoforms was monitored by fluorescence emission spectroscopy with an excitation wavelength of 469 nm where Chl *a* absorption is negligible (Fig. 6). All three isoforms

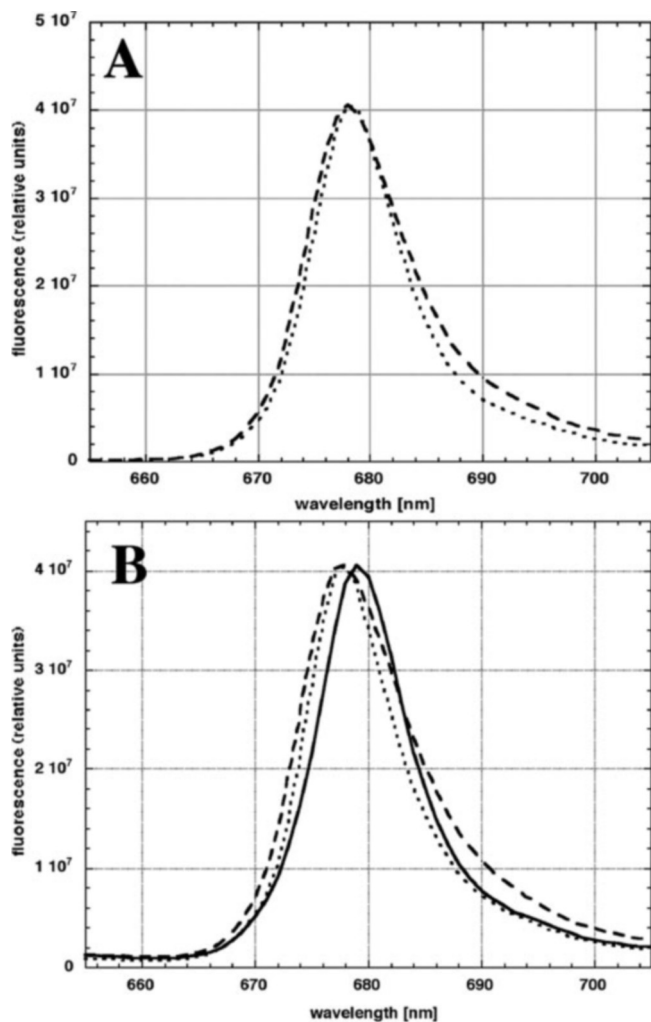


FIG. 6. 77 K fluorescence emission spectra of monomeric and trimeric LHC-II isoforms derived from sucrose density gradients. The samples were excited at 469 nm where Chl *a* absorption is negligible. A, trimeric complexes; B, monomeric complexes Lhcb1 (dotted line), Lhcb2 (dashed line), and Lhcb3 (solid line).

showed efficient energy transfer without Chl *b* emission, confirming that they are fully intact. For Lhcb1 the emission maximum was at 678 nm with a full width at half-maximum of 9.5 and 9 nm, respectively, for both monomers and trimers.

The emission peak of Lhcb2 monomers and trimers was at the same wavelength as for Lhcb1, but the full width at half-maximum was wider by 2 nm. This increase in fluorescence emission width could be caused by heterogeneity in pigment binding introduced by the refolding procedure. However, because no Chl *b* emission was detected and the same increase was observed in monomers and trimers, we believe it is a true characteristic of the Lhcb2 isoform.

Lhcb3 monomers again differed most clearly from the other two isoforms, with an emission peak shifted to the red by 1.5–679.5 nm. The full width at half-maximum of 9.5 nm was the same as for Lhcb1 monomers.

#### DISCUSSION

**Recombinant LHC-II Isoforms**—Pure, intact isoforms of LHC-II have not yet been prepared from plant material, and this has prevented a thorough understanding of the functional role of LHC-II heterogeneity. We cloned and expressed the Lhcb2 and Lhcb3 isoforms from *A. thaliana* and compared their folding, oligomer formation, and spectroscopic features with the well characterized Lhcb1 gene product from pea. Sequences

of Lhcb1 from pea and *Arabidopsis* are 91.8% identical, and the corresponding protein complexes are therefore expected to be very similar. In comparison, the sequence identity of Lhcb1 and Lhcb2 from *Arabidopsis* is only 66.8%. This shows that the same isoforms in different species are more similar to each other than the different isoforms within one species, in agreement with previous comparisons of LHC-II genes, which has led to the classification into three groups (18, 19). Minor sequence differences between species are therefore unlikely to affect the common characteristics of LHC-II isoforms. Accordingly, we show that Lhcb1 from pea and Lhcb2 from *Arabidopsis* are almost indistinguishable in their biochemical and spectroscopic properties, whereas Lhcb3 from *Arabidopsis* is clearly different from Lhcb2 of the same species.

It is well established that *in vitro* refolding yields LHC-II essentially identical to the native complex (40–42). By this approach, we were able to refold the three expressed LHC-II isoforms into monomeric pigment-protein complexes with comparable efficiency. The pigment stoichiometry of the refolded complexes is an important indicator of the quality of recombinant LHC-II. For all three isoforms the pigment stoichiometry was close to that of native LHC-II (5). The fluorescence emission spectrum is another indicator of the native-like state of refolded LHC-II. The emission spectra of all three isoforms show a complete transfer of excitation energy from Chl *b* to Chl *a* without residual Chl *b* emission. This demonstrates that they are in a fully functional state, with all pigment molecules in the correct position and orientation.

**Pigment Organization of LHC-II Isoforms**—The type and number of bound pigments and their organization in the pigment-protein complex are of particular importance for the function of LHC-II. Both parameters are reflected in the spectral properties of the complex. Because LHC-II isoforms are highly homologous and all pigment-binding residues are conserved, the overall biochemical and spectroscopic properties of the complexes are not expected to vary much. Indeed all three isoforms showed the features typical for native LHC-II. They were binding similar numbers of pigments and had comparable CD, absorption, and fluorescence spectra, indicative of a similar pigment organization. Nevertheless, there are significant differences, not only in the number of bound pigments but also in their fluorescence and absorption properties. The most significant spectral differences were found in the Chl *a* absorption, most likely reflecting different local protein environments of particular Chl *a* molecules. The absorption band around 680 nm is of special interest because it has been shown that Chl *a*2 (Chl  $\alpha$ 612 in the x-ray structure (17)) absorbs in this region (43). This Chl can be eliminated from the complex by removing the coordinating side chain (14). The resulting shift of the fluorescence emission peak suggested that Chl *a*2 is the final emitter of excitation energy in LHC-II (14). Interestingly, Lhcb3 shows comparable differences in the fluorescence emission spectra. These results demonstrate that, despite their similar pigment organization, LHC-II isoforms, and particularly Lhcb3, absorb and emit light at slightly different wavelengths.

A striking difference between Lhcb3 and the other two isoforms is the peak at 470 nm in the CD spectrum of the monomer, which is close to the 472 nm peak observed in native LHC-II, and in Lhcb1 and Lhcb2 trimers. This peak is thought to arise from the pairwise interaction of Chl in different monomers (41). The wavelength suggests that a Chl *b* is involved. Likely candidates are Chl *b*601 and Chl *b*609 because these are the only Chl with strong excitonic coupling between monomers within the trimer (17). Because Lhcb3 does not form homotrimers, the negative CD signal at 470 nm might reflect the loss

of one of these Chl. This is in agreement with the lower absorption of Lhcb3 around 470 nm and the loss of a Chl *b* molecule indicated by the HPLC analysis. Therefore the 470 nm CD signal probably reflects the absence of Chl *b*601 or Chl *b*609 in Lhcb3.

**Trimer Formation**—In the thylakoid membrane, LHC-II is believed to function mainly as a trimer. Native LHC-II trimers are not homogeneous but consist of homo- and heterotrimers in different combinations of the various LHC-II isoforms. The strict sequence conservation over a long evolutionary distance indicates that the different trimers are likely to perform specific functions in light harvesting, energy transfer, and regulation of photosynthesis. The refolded monomeric LHC-II isoforms are a good starting point for investigating trimers of all monomer combinations and their possible functional roles. Our results show that Lhcb1, Lhcb2, and mixtures of both form trimers with equal efficiency. SDS-PAGE does not distinguish between Lhcb1 and Lhcb2, and therefore we do not have direct experimental proof of Lhcb1/Lhcb2 heterotrimers. However, both Lhcb1 and Lhcb2 form heterotrimers with Lhcb3, to which they are much less similar. It is therefore safe to assume that Lhcb1/Lhcb2 heterotrimers will form under the same conditions. A Lhcb2 homotrimer has so far not been found in plant thylakoids. The high degree of similarity between Lhcb1 and Lhcb2 and the ease with which Lhcb2 homotrimers form *in vitro* suggest that they occur *in vivo* as well. Plants grown under optimal high light conditions express significantly more Lhcb1 than Lhcb2. Assuming a stochastic distribution of these two isoforms in native LHC-II trimers, this would lead to a majority of Lhcb1 homotrimers, some Lhcb1/Lhcb2 heterotrimers, and a small number of Lhcb2 homotrimers, which may explain why the latter have not been found *in vivo*.

By contrast, Lhcb3 did not form stable homotrimers *in vitro*, even though all sites that are thought to be involved in trimer formation are conserved in its sequence. However, we show here that Lhcb1/Lhcb3 and Lhcb2/Lhcb3 heterotrimers do form *in vitro* and that they are of stability comparable to that of the Lhcb1 and Lhcb2 homotrimers because they do not dissociate during density gradient centrifugation and subsequent partly denaturing gel electrophoresis. We have no direct evidence for a Lhcb1/Lhcb2/Lhcb3 heterotrimer, but because Lhcb1 and Lhcb2 were indistinguishable in their trimer formation, it seems certain that these heterotrimers do form as well.

Our experiments thus suggest strongly that LHC-II trimers exist in all possible combinations of the three isoforms, with the only exception of an Lhcb3 homotrimer. This implies that the function of Lhcb3 is connected to the heterotrimeric state of LHC-II.

**A Physiological Role for LHC-II Heterogeneity**—The Chl *a/b*-containing LHC are highly conserved throughout the plant kingdom, as expected from their vital role in photosynthesis and photoprotection. Normally, Lhcb1 is the most abundant isoform. In *Arabidopsis* a ratio of about 6:2:1 was found for Lhcb1, Lhcb2, and Lhcb3 (22). However, this ratio varies depending on growth conditions. In particular, the expression of Lhcb2 can increase by a factor of 2–3 under limited light conditions (27). A stochastic distribution of Lhcb1 and Lhcb2 in LHC-II trimers would then result in a higher number of Lhcb2 homo- and heterotrimers under low light. Because Lhcb2 is enriched in the more peripheral, “outer” LHC-II subpopulation (23, 24), this would result in a larger peripheral antenna and could provide a mechanism to adapt photosynthesis to low light intensities. Because this mechanism is based on the expression of Lhcb2, it is mostly relevant on a timescale of hours or days. A faster light adaptation of photosynthesis is triggered by phosphorylation of the outer LHC-II subpopulation. Both

Lhcb1 and Lhcb2 have a phosphorylation site, but there is evidence that Lhcb2 is phosphorylated three times more rapidly (28, 29). It has been proposed that only Lhcb1/Lhcb2 heterotrimers can associate with PS I and that Lhcb2 varies from Lhcb1 in its energetic properties to allow energy transfer to PS I. A putative additional Chl *a* molecule was thought to be involved in this process (27). This is in conflict with our measurements, which did not indicate a difference in pigment content of Lhcb1 and Lhcb2. In addition, the small spectroscopic differences we observed are inconsistent with a preferential energy transfer from Lhcb2 to PS I. Our results suggest that the main differences between Lhcb1 and Lhcb2 are the different localization in the light-harvesting antennae and the faster phosphorylation kinetics of Lhcb2. The main role of Lhcb2 therefore seems to be in the adaptation of photosynthesis to different light conditions, both on a short and a long timescale.

Lhcb3 is less abundant than either Lhcb1 or Lhcb2, and little is known about its role in the organization of PS II. Its smaller size is mainly the result of the lack of a N-terminal phosphorylation site. Trimers containing Lhcb3 are therefore less likely to be phosphorylated and detach from PS II, suggesting that Lhcb3 does not belong to the mobile outer LHC-II, but rather to the “inner” LHC-II, which remains associated with PS II in different light regimes. This would agree with the reduced fitness of field-grown plants lacking Lhcb1 and Lhcb2 but not Lhcb3 (44). Based on this line of evidence we propose that of the three LHC-II isoforms Lhcb3 is the closest to the PS II reaction center and is not involved in light adaptation of photosynthesis. We show that Lhcb3 differs significantly in its biochemical and spectral properties. Absorption spectra of Lhcb3 indicate a clear red shift of the longer wavelength Chl *a* molecules and the emitted light is clearly red-shifted as well. Because Lhcb3 is unlikely to occur as an isolated monomer in the membrane, we propose that its main function is in the adaptation of LHC-II to specific positions in the light-harvesting antennae and that Lhcb3-containing LHC-II trimers function as intermediaries in excitation energy transfer from the main Lhcb1/Lhcb2 antenna to the PS II core.

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