A Look within LHCII: Differential Analysis of the Lhcb1−3 Complexes Building the Major Trimeric Antenna Complex of Higher-Plant Photosynthesis†

Stefano Caffarri,‡,§ Roberta Croce,|| Luigi Cattivelli,⊥ and Roberto Bassi*,‡,§

Dipartimento Scientifico e Tecnologico, Università di Verona, Strada Le Grazie 15, 37134 Verona, Italy, CNR-ITC Istituto di Biofisica, Sezione di Trento, via Sommarive 18, POVO (Tn), Italy, Université Aix-Marseille II, LGBP (Laboratoire de Génétique et Biophysique des Plantes), Faculté des Sciences de Luminy, Département de Biologie, Case 901-163, Avenue de Luminy, 13288 Marseille Cedex 09, France, and Istituto Sperimentale per la Cerealicoltura, Via S. Protaso 302, 29017 Fiorenzuola d'Arda (PC), Italy

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ABSTRACT: The major antenna complex of higher-plant photosynthesis, LHCII, is composed by the products of three genes, namely, Lhcb1-2-3. In this paper, the biochemical and spectroscopic properties of each of the three gene products were investigated. The three complexes were obtained by overexpression of the apoproteins in bacteria and refolding in vitro with purified pigments, thus allowing detection of differences in the structure/function of the pigment-binding gene products. The analyses showed that Lhcb1 and Lhcb2 complexes have similar pigment binding properties, although not identical, while Lhcb3 is clearly different with respect to both pigment binding and spectral properties and cannot produce homotrimers in vitro. Heterotrimers containing Lhcb3 together with Lhcb1 and/or -2 proteins were obtained upon assembly with Lhcb proteins purified from thylakoids. The major functional characteristics of Lhcb3 with respect to Lhcb1 and -2 consisted in (i) a red-shift of one specific chlorophyll *a* chromophore, strongly affecting the red-most region of the absorption spectrum and (ii) a different specificity for xanthophylls binding to sites L2 and N1. These properties make Lhcb3 a relative sink for excitation energy in isolated heterotrimers with Lhcb1 + Lhcb2, and potentially, a preferential site of regulation of the antenna function in excess light conditions.

LHCII, the major light-harvesting complex of higher plants, is the most abundant membrane protein on earth and the first chlorophyll-binding protein complex identified (*1*, *2*). A model for its three-dimensional structure has been proposed at near-atomic resolution (*3*) and refined by mutation analysis yielding identification of the chromophores bound to multiple binding sites (*4*−*9*). Thus, each LHCII polypeptide is thought to tightly bind seven Chl[1] (chlorophyll) *a*, five Chl *b*, and three xanthophyll molecules, while one additional Chl *a* and one xanthophyll chromophore are loosely bound to peripheral sites (*10*, *11*). Very recently, the structure of LHCII trimers has been resolved at 2.72 Å allowing obtaining information on the localization and orientation of all chromophores. Two additional Chl molecules were detected at the periphery of the complex, and also the neoxanthin and the violaxanthin binding sites were resolved (*12*). Our knowledge of LHCII has been inferred from Lhcb1, which served as a basis for interpretation of structural data on LHCII (*3*) and for mutation analysis (*4*, *7*, *8*). Nevertheless, the genes encoding LHCII components are highly redundant in nuclear genome of higher plants and green algae (*13*−*16*), and they can be clustered in three groups: Lhcb1, -2, and -3 (*13*). Differences in the biochemical and/or spectroscopic properties between these three LHCII components have not been described due to the difficulty in purifying each of them to homogeneity, although evidence for differential roles of individual complexes has been reported. Differently from Lhcb1−2, Lhcb3 cannot be phosphorylated during state 1−state 2 transitions (*17*) nor is it transferred from grana to stroma membranes (*18*). LHCII has been involved in several functions including light harvesting for PSII (photosystem II) (*2*) and PSI (*18*, *19*), regulation of excitation energy balance between PSI and PSII (*20*), and dissipation of excitation energy in excess (*21*), suggesting that Lhcb1−3 genes may have different functions. In this paper, we have analyzed the biochemical and spectroscopic properties of Lhcb1, Lhcb2, and Lhcb3. The genes were overexpressed in bacteria, and holoproteins were obtained by refolding the complexes in vitro with purified pigments to clarify the properties of individual gene products.

EXPERIMENTAL PROCEDURES

Native LHCII Purification. Thylakoid membranes of *Zea mays* plants were prepared and solubilized as described in

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* To whom correspondence should be addressed. Tel.: +390458027916; fax: +390458027929, e-mail: bassi@sci.univr.it.

‡ Università di Verona.

§ Université Aix-Marseille II.

|| CNR-ITC Istituto di Biofisica.

⊥ Istituto Sperimentale per la Cerealicoltura.

[1] Abbreviations: AA, amino acid; Car, carotenoid; Chl, chlorophyll; α(*β*)-DM, α(*β*)-D-dodecylmaltoside; CP, chlorophyll protein; His, histidine; Lhc, light-harvesting complex; Lute, lutein; LD, linear dichroism; LT, low temperature; Neo, neoxanthin; PG, L-α-phosphatidic acid dipalmitoil C16:0); PSII, photosystem II; RT, room temperature; Viola, violaxanthin; WT, wild type; Zea, zeaxanthin.

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refs *10* and *17*. IEF was performed following the protocol reported by Dainese et al. (*22*).

Lhcb1−3 cDNA Isolation. Lhcb1, Lhcb2, and Lhcb3 expressed genes of LHCII proteins were isolated from barley (cultivar Nure) by screening a cDNA library from leaves with a maize Lhcb1 probe (*23*) and by analyzing EST from the same library (*24*).

Protein Overexpression in Escherichia coli and Reconstitution in Vitro. The Lhcb1−3 genes (TC87157, TC87159, and TC87588 at the TIGR Barley EST database; http://www.tigr.org/tdb/tgi/hvgi/) coding for the mature proteins were cloned in the pQE50 expression vector using the *Bam*HI and *Hin*dIII sites created by PCR. The same sequences were also cloned in a pQE50 vector modified after the *Hin*dIII site to code a six histidine tail at the C-term of the protein.

Complex reconstitution was performed following the procedure described in ref *25* or modifying the procedure for the Ni^{2+} column (*26*). A ratio of 420 μg of apoprotein to 240 μg of chlorophyll (Chls *a*/*b* ratio 2.3 or 2.9 for the procedure in batch or in column, respectively) and 90 μg of carotenoids was used. The trimerization was induced by adding 0.1 mg/mL final of PG (L-α-phosphatidic acid dipalmitoil C16:0) in the solutions for the complex reconstitution (*27*).

Pigment Analysis. The pigment complement of the holoprotein was analyzed by fitting of the acetone extract with the spectra of the individual pigments (*28*) and by HPLC analysis (*29*).

Gel Electrophoresis. SDS 6M urea PAGE was performed with the Tris-sulfate buffer system as previously reported (*30*).

Spectroscopy. The absorption spectra were recorded by SLM-Aminco DK2000 spectrophotometer in 10 mM HEPES pH 7.5, 20% (v/v) glycerol (70% for LT), and 0.06% α-DM. The fluorescence emission spectra were measured on a Jasco FP-777 fluorimeter and corrected for the instrumental response. The bandwidths were 3 nm in excitation and 3 nm in emission. The samples were in 10 mM HEPES pH 7.5 and 0.03% α-DM (and 70% glycerol for LT measurements), and the Chl concentration was 0.1 μg/mL (RT) and 1 μg/mL (LT). The CD spectra were measured at 10 °C on a Jasco 600 spectropolarimeter. The samples were in the same solution described for the absorption. The LD spectra were recorded upon sample orientation of the particles by the polyacrylamide squeezing technique according to ref *31*.

IEF. Recombinant Lhcb3 complexes (600 μg in Chls) were mixed with monomeric antennas (800 μg in Chls) from barley thylakoids fractionated on a sucrose gradient and then purified by flat-bed preparative IEF as previously described (*22*). Green bands eluted from the gel were then fractionated by ultracentrifugation in a 0.1−1 M sucrose gradient containing 0.06% β-DM (20 h at 280 000*g* at 4 °C) yielding three bands: free pigments, monomeric antennas, and LHCII trimers.

Pigment Stoichiometry. For stoichiometric (pigment/protein ratio) determination, the protein concentration was determined by the ninhydrin method (*32*).

RESULTS

To determine the characteristics of Lhcb1, Lhcb2, and Lhcb3 complexes, thylakoids were solubilized in α-DM (α-D-dodecylmaltoside) and fractionated by sucrose gradient ultracentrifugation. The fraction containing monomeric Lhcb1−3 complexes was harvested and loaded on a preparative IEF (*22*). After fractionation of the IEF, the green bands were loaded on sucrose gradients to remove carrier ampholytes, and the polypeptide composition of each band was analyzed by SDS−PAGE. The more acid fraction with pI of 3.8−3.9 was particularly enriched in only one polypeptide, Lhcb3, while all the others showed the presence of different mixtures of the three proteins, thus not allowing the purification of Lhcb1 and Lhcb2 complexes (see Supporting Information).

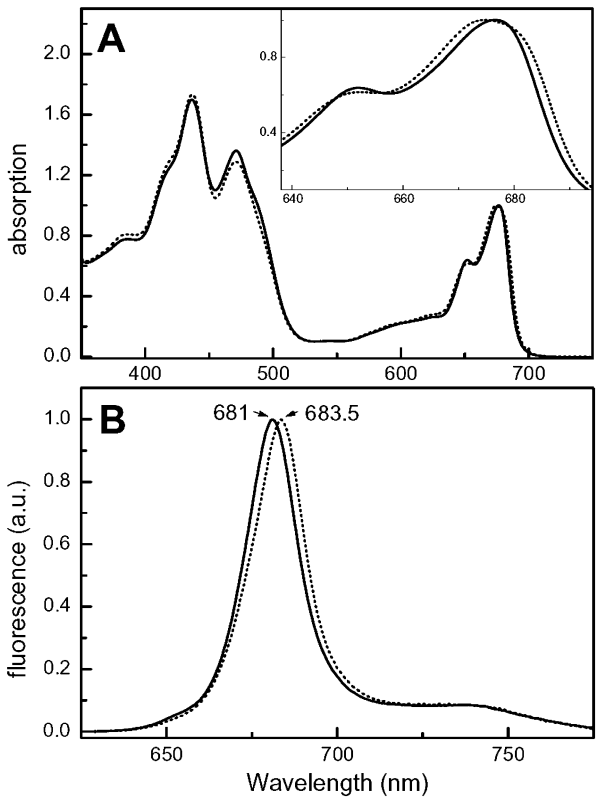

FIGURE 1: Absorption and fluorescence spectra of native Lhcb1−3 complexes. (A) Absorption spectra at RT of native Lhcb1-2 mixed complexes (solid) and native Lhcb3 (dot) are shown after normalization to the maximum in the Q_y region. Inset: RT absorption spectra of the same samples in the red region. (B) Fluorescence spectra of native Lhcb1-2 mixed complexes (solid) and native Lhcb3 (dot) complexes are shown after normalization to the maximum.

The fraction containing Lhcb3 was further analyzed by biochemical and spectroscopic methods. The Chl *a*/*b* ratio of this complex was 1.76, and the Chl/Car ratio was 4.35. Pigment analysis showed lower neoxanthin content with respect to complexes containing Lhcb1 and 2 from IEF (*33*). Pigment to protein stoichiometry indicated that IEF purified Lhcb3 bound 11 Chls (seven Chls *a* and four Chls *b*) versus 12 of the Lhcb1−2 isolated by IEF (*33*). The absorption and emission spectra of native Lhcb3 and the mixed Lhcb1−2 proteins are presented in Figure 1. The Lhcb3 complex showed a stronger absorption form at 682.4 and a red-shifted fluorescence emission maximum at 683.5 nm with respect to the fractions containing Lhcb1−2 polypeptides.

The major difficulty in purifying Lhcb1 and Lhcb2 complexes consists of their high sequence homology, which yields to proteins that are expected to be very similar in all the properties used in classical purification methods, namely, MW and pI. To overcome this problem, we used a different

FIGURE 2: Sequence alignment of the 1, 2, and 3 mature proteins. Different features are indicated: in yellow the AA different from the Lhcb1 sequence; violet rectangle, phosphorylation site; brown rectangles, trimerization sites; orange rectangles, putative carotenoid binding sites; purple rectangles, α-helices (the first three are transmembrane helices); green rectangles, Chl binding sites; and blue rectangles, residues involved in the stabilizing ionic pairs (indicated by dashed lines). The AA of Lhcb3 underlined in red are, between those ones different from the consensus sequence for Lhcb1, the most conserved in Lhcb3 sequences from different plant species.

approach consisting of the in vitro reconstitution of the complexes from apoproteins overexpressed in bacteria. Lhcb1−3 genes were cloned from a c-DNA library of barley (*24*) using a Lhcb1 probe. Sequencing of 48 clones among the many positive found by screening with a Lhcb1 probe allowed identification of 16 different Lhcb1−3 sequences encoding one each of Lhcb2 and Lhcb3 proteins and 14 different Lhcb1 proteins.

Lhcb1−3 apoproteins were overexpressed in *E. coli* and reconstituted in vitro with pigments to yield Chl/xanthophyll binding complexes. A preliminary experiment of in vitro reconstitution using four Lhcb1 representative apoproteins yielded holoproteins with very similar properties as judged from absorption spectra and pigment composition. We therefore chose one Lhcb1 sequence (see Experimental Procedures) for detailed analysis and comparison with Lhcb2 and Lhcb3. The alignment for the deduced mature proteins is presented in Figure 2. All the polypeptides were able to refold in vitro yielding stable monomeric complexes. The pigment content of the reconstituted products was analyzed, and the results are reported in Table 1. The differences in pigment binding between the three complexes were rather small with respect to both the Chls and Car (carotenoid) content.

Reconstituted Lhcb3 coordinates 12 Chls versus 11 of the native complex purified by IEF. This difference suggests that purification conditions lead to partial pigment loss from Lhcb3. To check this hypothesis, recombinant Lhcb3 was prepared by refolding in column (*26*, *34*) and submitted to IEF. The resulting Lhcb3 complex showed reduced neoxanthin content and 11 Chls per polypeptide, thus implying

Table 1: Pigment Composition of Recombinant Lhcb1−3 Complexes[a]

	Lhcb1	Lhcb2	Lhcb3
Chl *a*/Chl *b*	1.49	1.36	1.44
Chl *a* per mol	7.18	6.91	7.09
Chl *b* per mol	4.82	5.09	4.91
neo	0.94	0.99	0.89
viola	0.18	0.15	0.18
lute	1.82	1.69	1.77
Chl/car	4.09	4.23	4.23
Chl tot	12	12	12
car tot	2.93	2.84	2.83

[a] The pigment content of Lhcb1−3 proteins reconstituted in vitro with purified pigments from spinach is given. The values are normalized to the total Chl content per monomer. The error is less than 2%.

that purification by IEF lead to differential pigment loss in Lhcb3 with respect to Lhcb1 and 2 and suggesting that recombinant Lhcb3 was more intact than the IEF purified complex. On this basis, in the following all the analyses have been performed on the reconstituted complexes omitting the IEF step.

Absorption Spectra. The absorption spectra at 77 K of reconstituted Lhcb1, Lhcb2, and Lhcb3 are reported in Figure 3A along with their second derivative analysis (Figure 3B).

The spectra of Lhcb1 and Lhcb2 were very similar as observed from the second derivative analysis where contributions at 649.2, 661, and 670.4 nm were detected in both cases. The main negative signal was at 678 nm for Lhcb1, 677.2 nm for Lhcb2, and 678.8 nm for Lhcb3. Components at 650 and 670 nm could also be observed in the second derivative spectrum of Lhcb3.

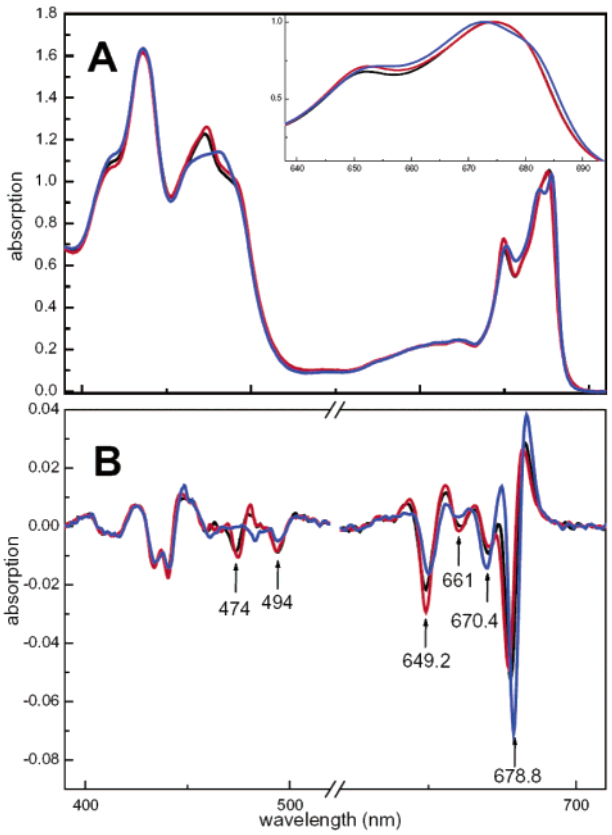

FIGURE 3: Absorption spectra of recombinant Lhcb1-3 complexes. (A) Absorption spectra at 77 K normalized to the Chl concentration. Inset: RT absorption spectra of the same samples in the Q_y region. (B) Second derivative analysis of the absorption spectra at LT of Lhcb1-3. The structureless 510/620 nm region was omitted. In each panel, Lhcb1 is indicated in black, Lhcb2 in red, and Lhcb3 in blue.

In the blue region of the spectrum, again Lhcb1 and Lhcb2 showed common features with contributions in the second derivative at 473−474 nm (attributed to the Chl *b* Soret band) and 494 nm (the red-most S0 > S2 transition sublevel of carotenoids). In the case of Lhcb3, the lack of the 473 nm contribution is possibly related to the appearance of a new negative signal at 483 nm, which can also be attributed to a Chl *b* Soret band, while the carotenoid band was detected at 494.4 nm.

For a more detailed analysis of the Q_y spectral region of the three complexes, the spectra at RT were fitted with Chl *a* and Chl *b* spectral forms in protein environment (*35*) upon normalization to the protein concentration (*36*). The fittings are reported in Figure 4.

The absorption spectra of the three complexes were described using three Chl *b* and five Chl *a* forms. The spectra of the three complexes could be satisfactorily fitted by using the same absorption forms, although the relative amplitude of each was different. On the basis of previous studies, the components peaking at 641, 650, and 655.4 nm were attributed to Chl *b* (*28*, *37*) and the remaining five to Chl *a* spectral forms. It is worth noting that the Chl *a*/*b* ratio that can be calculated from the spectral analysis is identical to the value obtained by pigment analysis. In Figure 4D, the amplitudes of the bands in the three complexes were compared. The description of Lhcb1 and Lhcb2 is almost identical, the only differences reflecting a small change in the Chl *a*/*b* ratio. In the case of Lhcb3, reduced amplitude of the 650 nm band is balanced by an increase at 655 nm, corresponding approximately to 1.0 Chl *b* molecules per polypeptide. In the Chl *a* absorption region, reduced 679 nm absorption is compensated by an increase of the red-most band, the amplitude corresponding to 0.6 Chl *a* molecules.

CD Spectra. The CD spectra of the three complexes showed common features (Figure 5), although the spectrum of Lhcb3 in the Q_y region was shifted 1 nm to the red as compared to the spectra of Lhcb1 and Lhcb2 and showed reduced amplitude of the contribution at 669 nm (+). Small changes can also be observed in the blue region where Lhcb3 showed increased amplitude of the signal at 472 (−) and 430 nm (+), possibly indicating changes in a Chl *a*−Chl *b* interaction.

The spectra of Lhcb2 and Lhcb1 are identical but for a small difference in the amplitude of 651 nm (−) and 670 nm (+) signals, both having higher amplitude in Lhcb2 as compared to Lhcb1.

Fluorescence. The fluorescence emission spectra at RT and 77 K of the three complexes upon excitation at 440 nm are presented in Figure 6.

The spectra of Lhcb1 and Lhcb2 overlap, while in the case of Lhcb3, a red shift of 1.5−2 nm is observed thus leading to a 683.5 nm maximum. At low temperature, the emission spectra of Lhcb1 and 2 were again slightly blue shifted with respect to Lhcb3 peaking at 680 nm. The most relevant feature was the effect of the temperature on the bandwidth: the three spectra had the same (20 nm) FWHM at room temperature. Spectral narrowing at 77 K down to 12 nm was observed in Lhcb1 and -2, while the effect was much stronger in Lhcb3 (9 nm). We have to emphasize that all these characteristics were also present in the native Lhcb3 purified by IEF (not shown).

To calculate the energy transfer efficiency of the three complexes, the fluorescence excitation spectra were measured and then compared to the absorption in the blue region upon fitting with the spectra of the pigments in protein environment (*38*). The excitation spectra of Lhcb1, Lhcb2, and Lhcb3 were deconvoluted into pigment components and the contribution of individual pigments as compared to those obtained from deconvolution of absorption spectra (not shown). The efficiency of energy transfer to Chl *a* was then determined from the ratio of the amplitudes in absorption versus fluorescence excitation (data not shown): in all cases, Chl *b* efficiency was 95%, and xanthophylls efficiency was 80%. These results are similar to those previously obtained with *Z. mays* Lhcb1 (*38*).

LD Spectra. The LD (linear dichroism) spectra of the three complexes recorded at 77 K are presented in Figure 7, where they are normalized at the carotenoid red-most peak. The maxima in the Q_y region were at 678.4, 677.4, and 679.2 nm for, respectively, Lhcb1, Lhcb2, and Lhcb3. The peak red-shift in Lhcb3 possibly indicates that the red-most 683 nm absorption form, increased in Lhcb3, has a positive LD contribution.

Again, the spectra of Lhcb1 and Lhcb2 showed the same components, although a small variation in the amplitude of individual bands could be observed. In the case of Lhcb3, differences were detected in the Chl *b* absorption regions, consistently with the absorption spectrum. The signals at 650 (+) and 658 (−) strongly decreased their amplitude as

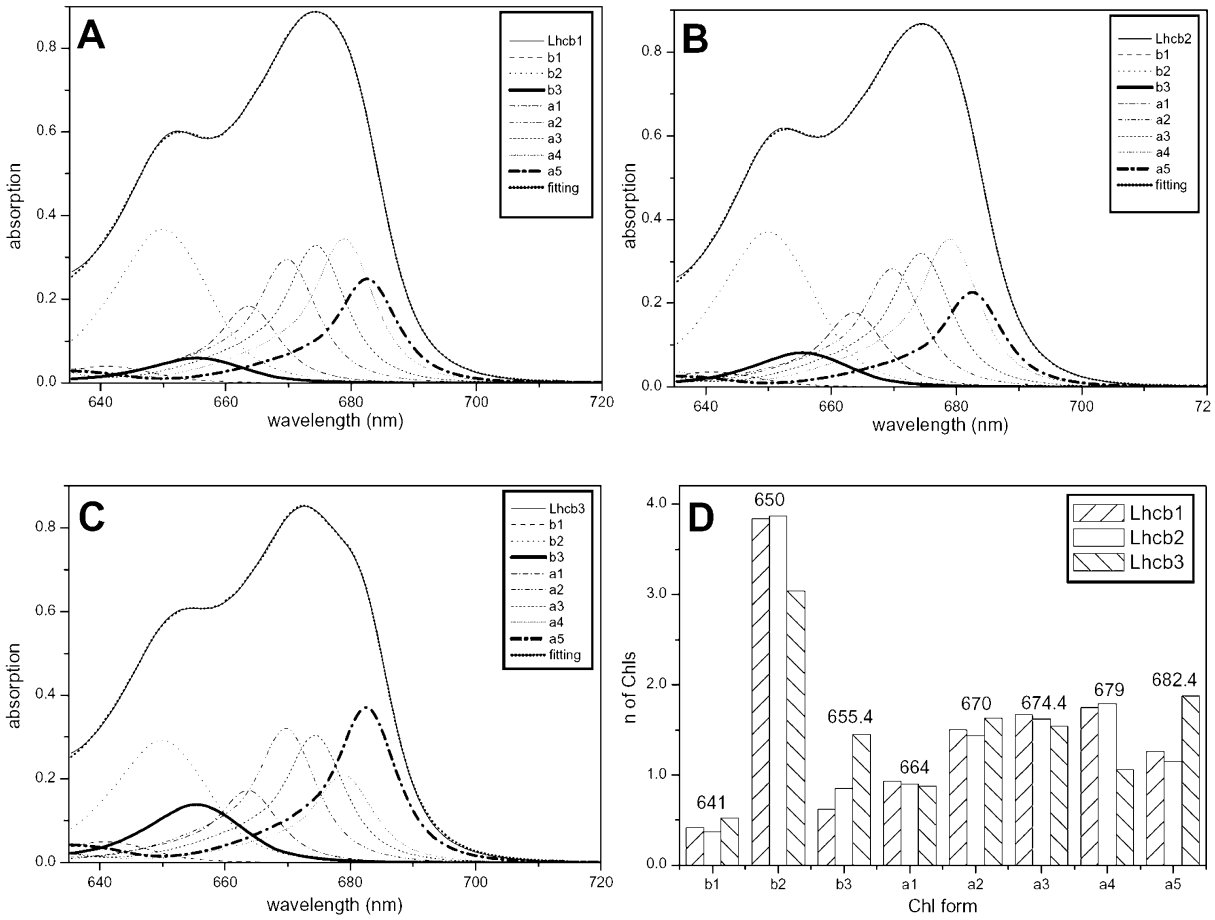

FIGURE 4: Decomposition of Lhcb1−3 absorption spectra. Fitting of Lhcb1 (A), Lhcb2 (B), and Lhcb3 (C) absorption spectra in the Q_y region was performed using absorption forms for Chl *a* and *b* in protein environment. In panel D, the number of Chl molecules absorbing at each wavelength is given.

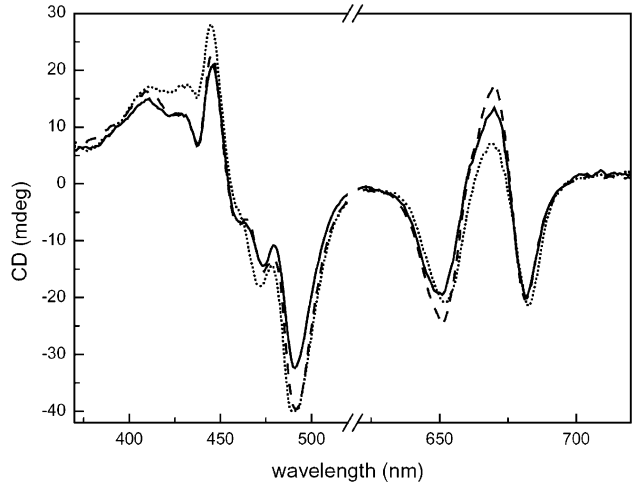

FIGURE 5: Circular dichroism spectra of Lhcb1−3 complexes at RT. The CD spectra normalized to the protein concentration are shown. Lhcb1 (solid), Lhcb2 (dash), and Lhcb3 (short dot).

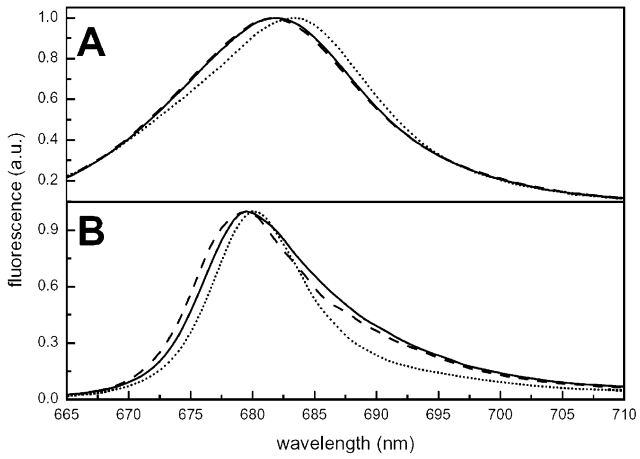

FIGURE 6: Fluorescence emission spectra of Lhcb1−3 complexes. The fluorescence emission spectra at RT (A) and 77 K (B) of Lhcb1 (solid), Lhcb2 (dash), and Lhcb3 (short dot) complexes are shown after normalization to the maximum.

compared to the spectra of the other two complexes. Differences in Chl *b* organization were also suggested by the analysis of the Soret absorption region: in Lhcb3, the signal at 470−475 nm (which characterizes both Lhcb1 and Lhcb2) disappears, while a new positive signal at 483 nm dominates this region of the spectrum.

Oligomerization of Lhcb3. When isolated from thylakoid membranes, LHCII is mainly organized into heterotrimers, although a small fraction is found in monomeric form. Mononomer−trimer interconversion has been shown to be affected by reversible phosphorylation of Lhc proteins (*39*), by carotenoid composition (*40*), and upon excess light stress (*41*). The ability of different Lhcb1−3 gene products to form trimers was assessed by reconstitution in the presence of the lipid PG (*27*), and the aggregation state was probed by sucrose gradient ultracentrifugation. It clearly appeared that Lhcb1 and Lhcb2 readily formed homotrimers in vitro, while Lhcb3 did not. Native Lhcb3 purified from thylakoids

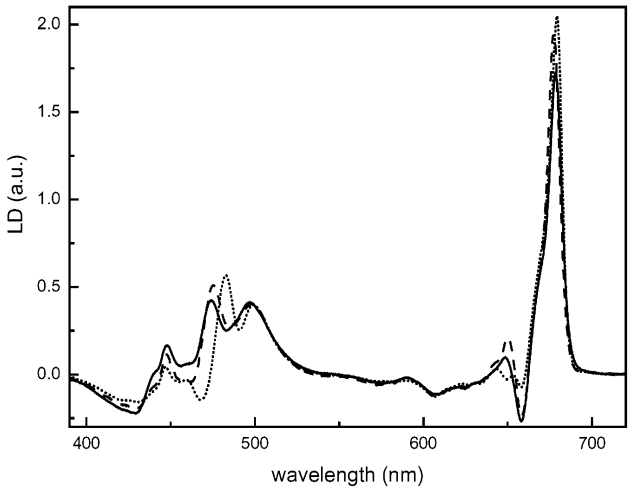

FIGURE 7: Linear dichroism spectra of Lhcb1−3 complexes at 77 K. The LD spectra of Lhcb1 (solid), Lhcb2 (dash), and Lhcb3 (short dot) complexes are shown after normalization at the carotenoid red most peak.

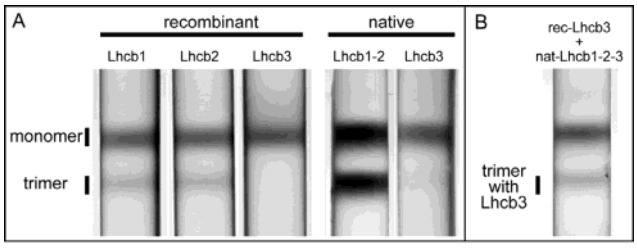

FIGURE 8: Oligomerization of Lhcb1−3 complexes. (A) Left: Lhcb1 and -2 recombinant complexes form homotrimers upon addition of the PG lipid, while Lhcb3 does not; right: similar results are obtained with the native complexes (the band of trimeric LHCII visible in the Lhcb3 native sample is due to little contamination from Lhcb1−2 complexes). (B) Recombinant Lhcb3 can instead form trimers with native Lhcb1−2 purified from solubilized thylakoids.

behaves the same (Figure 8A). It should be noted that the faint band visible at the trimer position in the tube loaded with the native Lhcb3 preparation is due to a low level contamination by Lhcb1−2 as verified by SDS−PAGE analysis (not shown). Since Lhcb3 has been found to form heterotrimers with Lhcb1 and 2 in vivo (*22*, *39*), the trimerization procedure was performed by using a mixture of recombinant Lhcb1 + -2 + -3 proteins where Lhcb3 carried a His-tag, while Lhcb1 and 2 did not. Lhcb3 containing complexes were recovered by binding to a Ni^{2+} column and analyzed by sucrose gradient ultracentrifugation. This procedure yielded Lhcb3 monomers. In contrast, when the same experiment was performed with either tagged Lhcb1 or -2, trimeric complexes were obtained that did not contain Lhcb3 but only Lhcb1 or -2. These results (not shown) suggest that a factor, not present in the in vitro reconstituted complexes, is essential for Lhcb3 trimerization. To test this hypothesis, recombinant tagged Lhcb3 was exposed to monomeric Lhcb1 + -2 + -3 isolated from barley thylakoids, then run on a nondenaturing IEF. The fractions containing recombinant Lhcb3 (separated from the other LHCII due to their higher pI owing to the His tail) were further fractionated on a sucrose gradient (Figure 8B). Analyses of the green bands obtained by spectroscopy and SDS−PAGE showed that, in these conditions, Lhcb3 was found in the trimeric

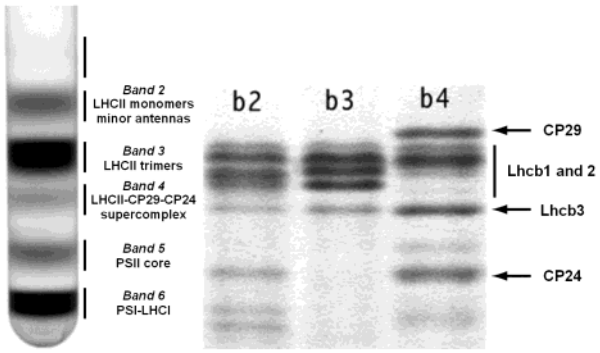

FIGURE 9: Localization of Lhcb3 in the PSII supercomplex. Lhcb3 is found in an oligomeric form in the band 3 of a sucrose gradient fractionation of solubilized thylakoid, and it is particularly enriched in band 4, a supercomplex containing LHCII-CP29-CP24. This result suggests a localization of Lhcb3 within the PSII at the interface between the peripheral LHCII trimers and the minor antenna proteins structure and moreover suggests the possibility that Lhcb3 needs a specific Lhcb1-2 partner for oligomerization.

band, implying that the hypothetic factor was contained into the fractions purified from thylakoids while not present in the recombinant protein preparation.

Distribution of Lhcb3 in the PSII−LHCII Supercomplex. The structure of PSII−LHCII supercomplexes has been reconstructed from electron microscopy and cross-linking experiments (*42*−*44*). Two PSII core complexes each binding 36 Chl *a* molecules are surrounded by monomeric CP26, CP29, and CP24 and by multiple trimeric LHCII. Tightly bound trimers, S, are connected to CP26; M trimers form a complex with CP29 and CP24 (*39*); and L trimers are arranged peripherally (*42*). The trimeric M LHCII forms a stable complex with one monomer each of CP24 and CP29. This complex was purified as previously reported (*39*) and analyzed for its polypeptide composition together with others LHCII containing bands from a sucrose gradient fractionation of maize thylakoids (Figure 9). Maize was used in this experiments instead of barley because in this species, a better separation between individual Lhcb1−3 proteins can be obtained by SDS−PAGE (*16*), but similar results were also obtained in barley. Densitometric analysis of the Coomassie stained SDS−PAGE bands shows that Lhcb3 constitutes one-third of the total LHCII proteins in this super complex, thus suggesting that the trimer is composed of heterotrimers containing one copy of Lhcb3 and two Lhcb1−2 copies according to the identification of maize LHCII polypeptides (*16*). Determination of Lhcb3 distribution in the sucrose gradient shows that Lhcb3 in M trimers is 20% of the total amount in thylakoids, while the ratio of (Lhcb1 + -2)/ Lhcb3 is about 10. This implies that Lhcb3 is a component of a portion of LHCII trimers present in the membranes, but LHCII trimers without Lhcb3 must also exist.

Affinity of Xanthophylls Binding Sites. Recombinant Lhcb1 has three tightly bound xanthophyll molecules, respectively, in sites called L1, L2, and N1 (*6*). Although the composition of Lhcb1−3 proteins was rather similar, the loss of carotenoids upon acidic treatment imposed by IEF suggested that the characteristics of the xanthophyll binding sites might be distinct. To clarify this point, we reconstituted Lhcb2 and -3 proteins with Chl *a*, Chl *b*, and combinations of individual xanthophyll species, as previously described for Lhcb1 (*6*), with particular attention to the binding of Viola (violaxanthin)

Table 2: Reconstitution of Lhcb1−3 Complexes with Different Carotenoids[a]

	1.L	2.L	3.L	1.V	2.V	3.V	2.LZ	2.LVZ	3.LV	3.LZ	3.LVZ
neo								0.1			0.1
viola				2.0	2.1	2.5	0.1	0.5	0.6	0.1	0.5
lute	2.0	2.0	2.0				1.3	1.2	1.8	1.4	1.2
zea							0.7	0.9	0.0	0.8	0.8
car tot	2.0	2.0	2.0	2.0	2.1	2.5	2.0	2.7	2.4	2.2	2.7

[a] Carotenoid composition per monomer of Lhcb1−3 complexes reconstituted with combinations of individual xanthophyll species. The number indicates the protein (1: Lhcb1; 2: Lhcb2; and 3: Lhcb3), and the letter represents the carotenoid composition used for each reconstitution (L: lutein; V: violaxanthin; and Z: zeaxanthin). The presence of neoxanthin in some samples is due to low contamination of the purified violaxanthin. The error is less than 5%.

and Zea (zeaxanthin), whose relative amounts change in thylakoids during excess light stress (Table 2). Lhcb1−3 bound two xanthophylls per polypeptide when Lute (lutein) was the only available xanthophyll species, thus suggesting that only sites L1 and L2 were occupied as previously assessed in Lhcb1 (*6*). When complexes were reconstituted with Viola, only Lhcb3 bound 2.5 xanthophylls versus 2 of Lhcb1 and Lhcb2. The higher binding capacity of Lhcb3 for violaxanthin was confirmed by its reconstitution with Lute + Viola yielding 2.6 xanthophylls, thus suggesting that site N1 could also bind violaxanthin in Lhcb3. In the presence of both violaxanthin and zeaxanthin, the latter xanthophyll competed mainly with lutein and only slightly with violaxanthin, suggesting that it is bound to site L2 rather than to site N1. Lutein was bound in at least one molecule per polypeptide even in the presence of excess Viola and Zea, thus suggesting that it was preferentially bound to site L1 in all Lhcb1−3 proteins.

DISCUSSION

Lhc multigene protein family includes many members with functions in light harvesting, photoprotection, and stress response (*13*). The first Lhc complex, LHCII, was discovered in 1966, while the presence of many other components was long overlooked due to difficulties of purification of these highly homologous membrane proteins that share the property of binding Chl *a*, Chl *b*, and several xanthophyll species. Many years of work were needed to identify the presence of individual Lhc gene products and to obtain their biochemical and functional characterization, but the physiological role of each is still the object of intense debate. In some cases, the characterization of individual pigment−protein complexes has been possible only upon expression of genes in bacteria and in vitro reconstitution of holoproteins since dissociation of heterooligomers often yields into partial denaturation of pigment−protein complexes (*37*, *45*). In the case of the major trimeric complex LHCII, to define the properties of each complex is particularly difficult because Lhcb1−3 gene products are found as heterotrimers, making their purification difficult in the native state. Isolation of individual Lhcb1−3 polypeptides has been possible only following denaturation (*16*, *46*); therefore, the characteristics of pigment−proteins are still unknown. Moreover, trimers into different location within PSII−LHCII supercomplex exhibit distinct composition, thus suggesting that individual gene products play different roles in PSII architecture and

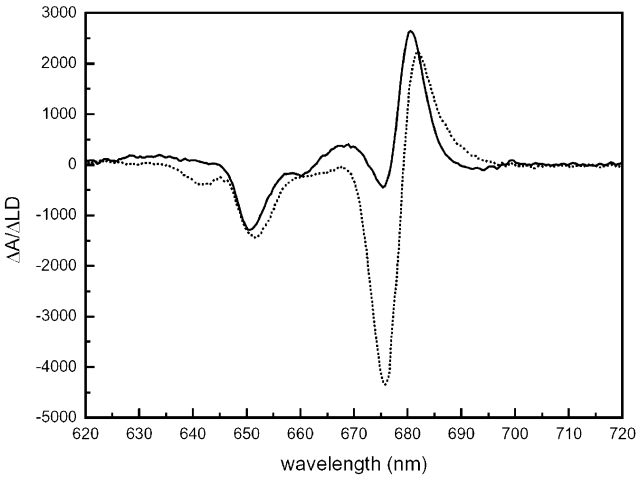

FIGURE 10: Lhcb1 and -2 absorption and LD difference spectra. Comparison between the Lhcb1 minus Lhcb2 absorption difference spectrum at 77 K (solid) and the LD difference spectrum (short dot) at the same temperature.

function. In this paper, as a step toward the identification of the function for the individual Lhcb proteins, we have isolated Lhcb1−3 genes from barley and obtained the biochemical and biophysical characterization of the recombinant pigment−protein complexes.

Sequence alignment allowed the comparison of the genes and proteins encoded (Figure 2). The similarity among the different polypeptides is high: the AA (amino acid) identity is of 82% between Lhcb1 and Lhcb2, 78% between Lhcb1 and Lhcb3, and 79% between Lhcb2 and Lhcb3 (homology 89, 84, and 86%, respectively). The transmembrane helix regions are the most conserved with all residues coordinating Chls fully conserved (in green), suggesting similar pigment organization in the proteins. However, the few substitutions close to the binding sites might modulate the pigment environment and therefore the spectroscopic properties of each Lhcb complex.

Lhcb1 versus Lhcb2. The data collected indicate that these two complexes have common biochemical and spectroscopic characteristics. The main difference observed is the lower Chl *a*/*b* ratio of Lhcb2 as compared to Lhcb1, which is possibly related to different affinity in the two complexes for Chl *a* and Chl *b* of one or more binding sites. In Figure 10, the Lhcb1 minus Lhcb2 absorption difference spectrum at 77 K is compared with the LD difference spectrum at the same temperature.

The absorption contribution at 680 nm in Lhcb1 seems to be substituted in Lhcb2 by a Chl *b* absorption at 650 nm. The same components are also observed in the LD difference spectrum. The spectra also suggest a similar orientation of the pigments giving rise to these two absorptions, thus supporting the view that they are the result of a different Chl *b* versus Chl *a* affinity of the same site. Surprisingly, the LD difference spectrum is dominated by a new negative contribution at 676 nm, which corresponds to a rather small absorption signal, suggesting that a Chl *a*, peaking at 676 nm, has a different orientation in Lhcb2 as compared to Lhcb1. Identification of the Chl binding site hosting the involved chromophore can be attempted on the basis of the absorption wavelength map reported by Remelli et al. (*4*) allowing for a 2 nm red-shift due to the RT (room temperature) versus LT (low temperature) measurements. On

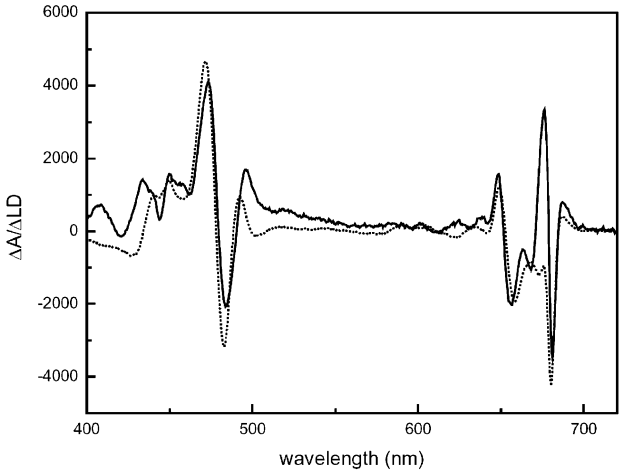

FIGURE 11: Lhcb1 and -3 absorption and LD difference spectra. Comparison between the Lhcb1 minus Lhcb3 absorption difference spectrum at 77 K (solid) and the LD difference spectrum (short dot) at the same temperature.

this basis, candidate sites are the Chl A6 and Chl A1/B1 cluster. The sequence around Chl A6 binding site (*3*) is highly conserved while, in the helix C−helix A loop, a sequence divergence is detected (Figure 2). Since the Chl A1/B1 cluster is located close to the stromal surface of the thylakoid membrane, the divergent sequences might be important in forming the binding pocket(s) for Chl A1 and/or B1. This is consistent with the finding that random mutations in this loop region severely affect Chl binding (*47*) and with the presence of a divergent sequence in Lhcb3, which shows the same spectral difference with respect to Lhcb1.

Lhcb1 versus Lhcb3. The pigment complements of Lhcb1 and Lhcb3 are similar with respect to both Chl *a*/*b* ratio and carotenoid composition. However, the spectroscopic analysis reveals that at least two Chl spectral forms are red-shifted in Lhcb3: (i) the absorption band of one Chl *b* molecule was shifted from 650 to 655 nm and (ii) the absorption of a Chl *a* was shifted from 678−9 to 682−683. In Figure 11, the comparison of the absorption and the LD difference spectra at low temperature is shown.

A clear shift in the Q_y absorption band of a Chl *b* molecule is observed from 650 to 655 nm. A related effect is also detectable in the Soret region, where the changes observed can be interpreted as the shift from 473 to 483 nm of the B_x band of the Chl *b*. The high similarity between the LD and the absorption difference spectra suggests that the orientation of this Chl *b* molecule is the same in the two complexes.

In the Chl *a* region, Lhcb3 shows an increased absorption at 681 nm (683 at RT) associated to a positive LD contribution. This low-energy band substitutes a Chl *a* absorption at 676 nm (679 nm at RT). In this case, a change in the orientation is observed, as shown by the opposite sign of this band in the absorption and LD difference spectra (Figure 11). From the comparison of the fluorescence spectra, it is clear that this form is responsible for the 2 nm red-shift of the fluorescence emission peak of Lhcb3.

At 77 K, the difference in the fluorescence spectra between Lhcb1 and Lhcb3 is best evident, the latter having a narrower spectrum. The difference indicates that Lhcb3 lacks emission at 690 nm. This emission can be related to an absorption band at around 686 nm, which is visible in Lhcb1 in the difference absorption spectra at LT. It was previously reported that in LHCII trimers a small absorption, accounting for less than 1% of the intensity, exists between 685 and 700 nm (*48*), and this result was confirmed by hole-burning spectra (*49*). Whereas several interpretations of this red band were proposed (*50*), it was suggested that this form is not intrinsic to all LHCII complexes. The analysis of the recombinant Lhcb proteins suggests that the presence of Lhcb3, which does not have this low-energy absorption, can modulate its amplitude in the native trimers.

By comparing the sequence of the three proteins (Figure 2), we note that the main differences are observed in the lumenal part of the transmembrane helix B and in helix C. The model of LHCII structure shows that three Chls are located in this region: A6, A7, and B6. Mutation analysis revealed that these sites accommodate half of the total Chl *b* complement of the protein (i.e., 2.5 molecules of Chl *b* and 0.5 of Chl *a*, with the Chl *b* molecule absorbing at 650−652 nm and the Chl *a* at 678−679 nm (RT) (*4*)). On this basis, we suggest that the changes in Chl absorption observed in Lhcb3, as compared to Lhcb1, are due to a different organization of the chromophores in the domain including sites A6, A7, and B6. Consistent with this hypothesis is the observation that Lhcb3, when submitted to low pH during IEF, undergoes partial denaturation with loss of Chl *b* and neoxanthin. Similar modifications can be induced in Lhcb1 by mutations at the Chl binding sites in this protein domain (*4*). On this basis, we conclude that the region near the C helix in Lhcb3 is perturbed, thus yielding to a weaker Chl *b* binding with respect to Lhcb1.

Xanthophyll Cycle and Site N1. Low pH dependent exchange of xanthophylls in Lhc proteins has been shown to be involved in the xanthophyll cycle: in high light conditions, violaxanthin is deepoxidized to zeaxanthin, which is then bound to site L2 (*51*), thus leading to a conformational change and dissipation of excess energy through fluorescence quenching (*52*). The previous results show that in Lhcb3, low pH affects xanthophyll binding to site N1. Low luminal pH induced exchange in site L2 has been described for site L2 (*51*). The present results suggest that the same effect may be present in site N1. Reconstitution with violaxanthin and zeaxanthin shows that both sites L2 and N1 can be occupied by xanthophyll cycle carotenoids with zeaxanthin binding to site L2 and violaxanthin to site N1. It is thus possible that during high light stress, Lhcb3 may undergo exchange of lutein versus zeaxanthin in site L2, while site N1 loses its neoxanthin ligand thus easing the conversion between conformations with different lifetimes. This is in agreement with data on recombinant protein showing that the presence of neoxanthin in site N1 stabilizes the long lifetime conformation (*53*). Experiments are in progress to understand if such a simultaneous carotenoid exchange might favor conformational change to a dissipative state (*52*).

Oligomerization and Localization of Lhcb1−3 Gene Products in Thylakoid Domains. The N-terminal region of the mature peptides shows the highest level of amino acid substitutions within the Lhcb1−3 subfamily. Many reports have shown that the N-terminal region is important for the regulation of LHCII activity and localization: threonine in position 3 is in fact involved in reversible phosphorylation, promoting the migration of LHCII from grana to stroma lamellae (state 1−state 2 transition) (*20*). The phosphoryla-

tion site is missing in Lhcb3, having a shorter N-terminus and known not to be phosphorylated (*17*). We suggest that the relative level of expression of Lhcb3 with respect to Lhcb1 and -2 gene products may control the capacity for state transitions.

Mutation analysis has shown that residues within the N-terminal domain (W16, Y17, and R21 of the mature peptide) are needed for trimerization of LHCII complexes (*54*). Although these residues are conserved in all Lhcb1−3 deduced sequences, their presence is not sufficient as shown by the impossibility to obtain homotrimers of Lhcb3 both in vivo and in vitro, while Lhcb1 and -2 can form trimers. This property of Lhcb3, which can only form heterotrimers, suggests that heterotrimers including Lhcb3 might have a special function in PSII supercomplexes. The special characteristics provided by Lhcb3 to LHCII trimers may possibly be related to the two major differences of this gene product with respect to Lhcb1 and -2, namely, (i) a peculiar Chl organization characterized by the enrichment of the 682 nm spectral form and the absence of the low amplitude absorption tail at wavelength >690 nm and (ii) the different xanthophyll binding characteristics of site N1. While we cannot suggest a possible functional implication of the former Lhcb3 property, the latter can be understood in the light of the recent finding that the xanthophyll cycle constitutes a signal transduction pathway for excess light stress (*10*, *55*).

Heterotrimerization of Lhcb3 has not been obtained by using recombinant Lhcb1 and/or -2 partners, while it was possible by using a monomeric Lhcb1−3 fraction from thylakoids. This result may have two explanations: (i) an unknown factor is present in the thylakoid extract, and it is needed for Lhcb3 interaction with Lhcb1−2 subunits, for instance, a lipid species different from those needed for trimerisation of Lhcb1 and -2 (*27*); alternatively, (ii) formation of Lhcb3 containing heterotrimers might require specific Lhcb1−2 partners, which were present in the thylakoid extract but did not correspond to the clone we have used for in vitro reconstitution in our experiments.

CONCLUSIONS

In this paper, we showed that the different Lhcb1−3 pigment−proteins have distinct biochemical and spectroscopic properties. In particular, Lhcb2 pigment−protein shows distinct spectroscopic properties with respect to Lhcb1 consisting of the reorientation of a Chl *a* chromophore and changes in affinity of a Chl binding site. Lhcb3 in addition to the spectroscopic features of Lhcb2 has an increased absorption component at 682−683 nm, which causes a red-shift in the fluorescence emission. Lhcb3 can only form heterotrimers and is enriched in the LHCII trimers, which are in contact with CP29 and CP24 within the PSII supercomplex. These properties are consistent with a role of Lhcb3 as a relative sink for excitation energy in heterotrimers with Lhcb1 + Lhcb2. Lhcb3 also exhibits a peculiar response of absorption and fluorescence spectra to temperature, possibly due to the lack of weak excitonic bands at wavelengths >690 nm and a different affinity for neoxanthin binding to site N1. The relevance of these features for the function of LHCII needs to be elucidated, and it will be the object of further experiments to verify the hypothesis that Lhcb3 may be a preferential site of regulation of the antenna function in excess light conditions.

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SUPPORTING INFORMATION AVAILABLE

Purification of the native Lhcb3, showing the IEF fractionation of the LHCII complexes; the sucrose gradient purification of the IEF eluted fractions; and the SDS−PAGE of each sucrose gradient green band. This material is available free of charge via the Internet at http://pubs.acs.org.

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