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The Major Antenna Complex of Photosystem II Has a Xanthophyll Binding Site Not Involved in Light Harvesting*

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We have characterized a xanthophyll binding site, called V1, in the major light harvesting complex of photosystem II, distinct from the three tightly binding sites previously described as L1, L2, and N1. Xanthophyll binding to the V1 site can be preserved upon solubilization of the chloroplast membranes with the mild detergent dodecyl-α-D-maltoside, while an IEF purification step completely removes the ligand. Surprisingly, spectroscopic analysis showed that when bound in this site, xanthophylls are unable to transfer absorbed light energy to chlorophyll *a*. Pigments bound to sites L1, L2, and N1, in contrast, readily transfer energy to chlorophyll *a*. This result suggests that this binding site is not directly involved in light harvesting function. When violaxanthin, which in normal conditions is the main carotenoid in this site, is depleted by the de-epoxidation in strong light, the site binds other xanthophyll species, including newly synthesized zeaxanthin, which does not induce detectable changes in the properties of the complex. It is proposed that this xanthophyll binding site represents a reservoir of readily available violaxanthin for the operation of the xanthophyll cycle in excess light conditions.

Light energy for higher plant photosynthesis is harvested by pigments including chlorophyll (Chl)[1] *a*, Chl *b*, and carotenoids bound to pigment binding proteins embedded into the thylakoid membrane. Each photosystem is made of two moieties: the core complex, containing Chl *a* and β-carotene bound to plastid-encoded polypeptides, and the light harvesting system, made up of nuclear encoded proteins of the Lhc family which, besides Chl *a*, also bind Chl *b* and the three xanthophylls lutein, violaxanthin, and neoxanthin. LHCII is by far the major antenna complex, since it binds about 50% of total chlorophyll. It is composed of heterotrimeric complexes of the Lhcb1, -2, and -3 gene products (1). Electron crystallography and mutation analysis showed that each Lhcb1 subunit contains five Chl *a* and four Chl *b* binding sites, while three additional sites can bind either Chl *a* or Chl *b*. Chlorophyll ligands are amino acid side chains belonging to three trans-membrane α-helices or to neighbor Chl (2, 3) through coordination of the Mg^{2+} atom at the center of each porphyrin. Within the pigment-protein complex are also located two carotenoid binding sites, called L1 and L2, cross-bracing helices A and B. These sites are occupied by lutein (L1) and by lutein (80%) and violaxanthin (20%) (L2) (4, 5). A third carotenoid binding site (N1), highly specific for neoxanthin, was localized in the C helix domain (6). Xanthophylls in sites L1, L2, and N1, are tightly bound to the complex even in very harsh conditions of purification. In addition, violaxanthin can be bound to LHCII when isolated by mild detergent treatment, while the interaction does not survive further purification steps, such as IEF, suggesting loose binding (7, 8).

Besides their role in light harvesting, xanthophylls are also involved in photoprotection by quenching ^{3}Chl* (4, 9), by preventing lipid peroxidation (10) and by dissipating ^{1}Chl* in excess with respect to the capacity of the electron transport chain (11, 12). Excess energy dissipation is catalyzed by the nonphotochemical quenching (NPQ) mechanism, whose full expression depends on the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin (VAZ) by violaxanthin de-epoxidase, which is activated by low luminal pH (13). Although it is generally accepted that NPQ is developed in the Lhc antenna system, the identity of the actual protein subunit catalyzing the energy dissipation reaction is still a matter of debate. The minor Lhc proteins CP29, CP26, and CP24 have been proposed as candidates for a primary role in NPQ (14) on the basis of several lines of evidence: (i) VAZ pigments were found to be tightly bound to CP29, CP26, and CP24 (15, 16); (ii) CP29 and CP26 undergo fluorescence quenching when zeaxanthin is bound (17, 18); and (iii) protonable sites were found to be exposed to the luminal side of CP26 and CP29, which could be covalently modified by the NPQ inhibitor dicyclohexylcarbodiimide (19, 20). Alternative sites for NPQ have been proposed to be the Lhc-like protein PsbS and LHCII. PsbS minus mutants showed a strong decrease in NPQ (21). Due to the binding of LHCII to multiple sites within PSII supramolecular complexes (22–24), violaxanthin loosely bound to LHCII may constitute the larger pool of this pigment in the thylakoid membrane and possibly the most readily available one for the xanthophyll cycle. It was suggested that the de-epoxidation state of the loosely bound VAZ could induce aggregation quenching by eliciting a conformational change in LHCII (7).

In this work, we report on the characteristics of the fourth xanthophyll binding site of LHCII, with the aim of understanding its function in the PSII light harvesting system. We found that violaxanthin is loosely bound to LHCII in a specific site, hereafter called the V1 site. Surprisingly, when bound to site

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[1] The abbreviations used are: Chl, chlorophyll; α-DM and β-DM, *n*-dodecyl-α-D-maltoside and *n*-dodecyl-β-D-maltoside; CP29, CP26, CP24, chlorophyll protein 29, 26, and 24 kDa, respectively; IEF, isoelectric focusing; Lhc, light harvesting complex; LHCII, light-harvesting complex of photosystem II; NPQ, nonphotochemical (energy) quenching; PSI and PSII, photosystem I and II, respectively; VAZ, violaxanthin-antheraxanthin-zeaxanthin; HPLC, high pressure liquid chromatography; μE, microeinstein(s); FPLC, fast protein liquid chromatography; LD, linear dichroism.

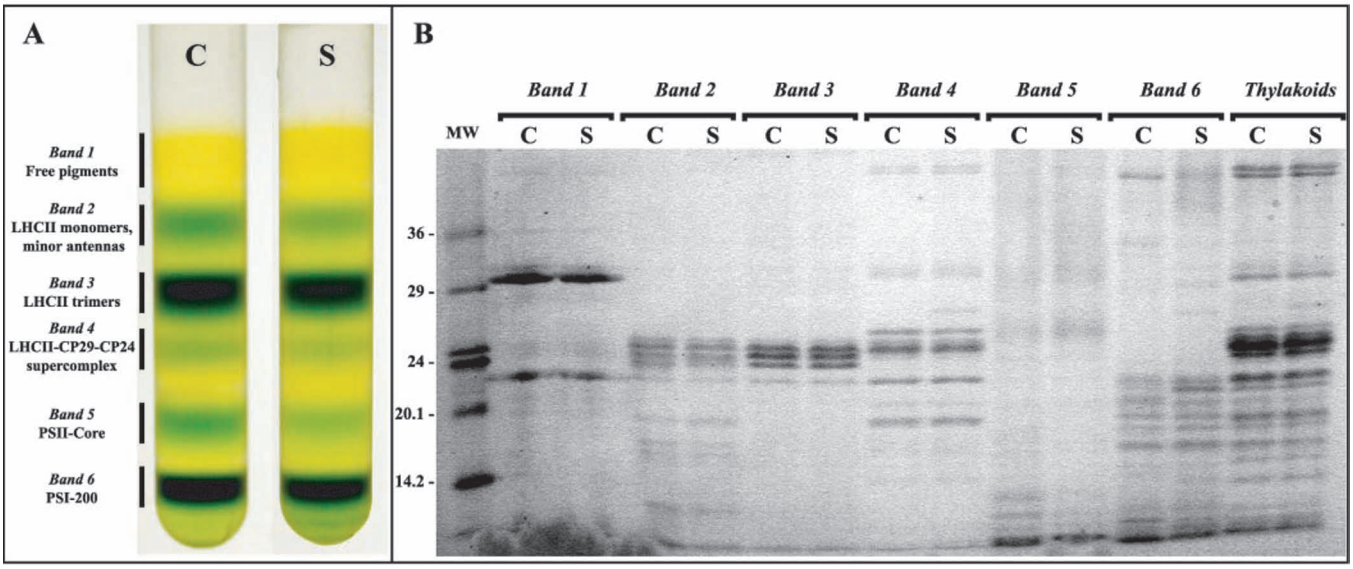

FIG. 1. **Sucrose density gradient profiles of solubilized thylakoids and gel electrophoresis of the gradient fractions.** *A*, thylakoids from control (*C*) and stressed (*S*) plants were solubilized in 0.6% α-DM and loaded on a sucrose gradient. For each tube, the six fractions indicated were harvested. *B*, SDS/6 M urea/Tris-sulfate PAGE of the six bands (*1–6*) from gradients and thylakoids from control and stressed plants.

V1, violaxanthin is not active in light harvesting. Also, its de-epoxidation to zeaxanthin does not produce fluorescence quenching or any detectable spectral differences which could suggest conformational changes. Possible functions of the V1 site are discussed.

EXPERIMENTAL PROCEDURES

Plant Material and Treatments—Zea mays (cv. Dekalb DK300) plants were grown after germination until an height of 10–15 cm at 23 °C at low light intensities (~80 μE, 14 h light/10 h dark). One set of plants was light-stressed at about 1000 μE m^{-2} s^{-1} for 2 h 30 min at 4 °C while control plants were maintained at growth conditions.

Thylakoid Preparation, Solubilization, and Sample Preparation—Thylakoid were isolated immediately after the light stress as described (25). Membranes corresponding to 500 μg of Chl were washed with 5 mM EDTA and then solubilized in 1 ml of 0.6% dodecyl-α-D-maltoside (α-DM), 10 mM Hepes, pH 7.5, by vortexing for 1 min. The solubilized samples were centrifuged at 15,000 × *g* for 10 min to eliminate unsolubilized material and then fractionated by ultracentrifugation in a 0.1–1 M sucrose gradient containing 0.06% α-DM and 10 mM Hepes, pH 7.5 (22 h at 280,000 × *g* at 4 °C). The green bands of the sucrose gradient were harvested with a syringe.

Trimeric LHCII, isolated from control plants, was further purified by flatbed preparative IEF as previously described (26). Green bands eluted from the gel were then fractionated by ultracentrifugation in a 15–40% glycerol gradient containing 0.06% β-DM (6 h at 480,000 × *g* at 4 °C), yielding three bands: free pigments, LHCII monomers, and LHCII trimers.

Pigment Analysis—The pigment composition of the complexes was analyzed by fitting the absorption spectrum of the acetone extract with the spectra of individual pigments (27) and by HPLC analysis (28).

Gel Electrophoresis—SDS-6 M urea PAGE was performed with the Tris-sulfate buffer system as previously reported (29).

Deconvolution of Absorption and Excitation Spectra—Deconvolution of the Soret region of the spectra of native complexes into individual pigment components was performed as previously reported (30).

Spectroscopy—Absorption spectra were obtained using a SLM-Aminco DW-2000 spectrophotometer at room temperature. Samples were in 10 mM Hepes, pH 7.5, 0.06% DM, 20% glycerol at 10 μg/ml Chl concentration. Fluorescence spectra were recorded at room temperature using a Jasco FP-777 spectrofluorimeter. Bandwidth was 3 nm for both excitation and emission. The Chl concentration was 0.1 μg/ml. The LD spectra were recorded upon sample orientation of the particles by the polyacrylamide squeezing technique according to Ref. 31.

Chlorophyll fluorescence quenching experiments were carried out as described in Ref. 32 but using a Chl concentration of 0.1 μg/ml.

Photobleaching—The samples were diluted to an absorbance of 0.6 at the maximum in the Q_y region. The protein was than illuminated with white light (4500 μE m^{-2} s^{-1}) from a halogen lamp. After each time interval, the cuvette was removed from the light source, and the absorption spectrum was recorded in the range 600–750 nm.

RESULTS

In order to check conditions for maintaining the highest xanthophyll binding level in LHCII complex, we solubilized thylakoid membranes with a series of alkyl-sugar detergents including dodecyl-β-D-maltoside, dodecyl-α-D-maltoside, octyl-β-D-glucoside, decyl-β-D-glucoside, and nonyl-β-D-glucoside and fractionated the solubilized material by sucrose gradient ultracentrifugation including each detergent slightly above its critical micellar concentration in the gradient. In each case, a green band was obtained at about 0.3 M sucrose upon ultracentrifugation for 22 h at 40,000 × *g* in an SW40 Beckman rotor, which contained pure LHCII as judged from SDS-PAGE, absorption spectra, and HPLC analysis. In each case, the Chl *a*/*b* ratio was close to 1.5, and the Chl/neoxanthin and Chl/lutein ratios were about 13 and 6, respectively. The Chl/violaxanthin ratio ranged between 50 and 18, indicating that each detergent had a different capacity of removing violaxanthin from LHCII. The best results were obtained with dodecyl-α-D-maltoside solubilization, which yielded the highest violaxanthin content. This detergent was thus utilized for purification of LHCII through this study.

Fig. 1*A* (*left*) shows a sucrose gradient loaded with solubilized thylakoids from control plants following ultracentrifugation. Six bands were obtained, the highest one being *yellow*, while bands 2–6 were green or *dark green*. SDS-PAGE analysis (Fig. 1*B*) showed that upper band only contained polypeptides of 33- and 23-kDa apparent molecular mass that were recognized by immunoblotting with OEE antisera, thus suggesting that these uncolored polypeptides co-migrated in the gradient with free carotenoids in detergent micelles. Carotenoids in this band accounted for 6% of the total carotenoids recovered from the bands in the sucrose gradient. HPLC analysis revealed traces of Chl accounting for less than 1% of total chlorophyll in the gradient (see Table I). According to previous results with dodecyl-β-D-maltoside (22), band 2 contained the minor Lhc proteins CP29, CP26, and CP24 as well as LHCII polypeptides, indicating that a small fraction of the LHCII trimeric complex dissociated into products co-migrating with monomeric minor Lhcs. Band 3 contained trimeric LHCII, and its properties will be described in detail below. Band 4 was the fainter band and

TABLE I
Percentage of pigments in the six bands of the sucrose gradient

The data are normalized considering 100 the sum of the Chls in the six bands. C, control; S, stressed; 1, free pigment; 2, Lhc monomers; 3, LHCII trimers; 4, "band four"; 5, PSII-core; 6, PSI-200; N, neoxanthin; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; L, lutein; b-C, β-carotene; Car tot, carotenoid total.

Sample	N	V	A	Z	L	b-C	Chl *b*	Chl *a*	Car tot	VAZ
	%	%	%	%	%	%	%	%	%	%
C.1	0.09	0.62	0.01	0.00	0.58	0.25	0.06	0.59	1.55	0.62
C.2	0.92	1.05	0.04	0.00	1.79	0.10	4.49	9.56	3.91	1.09
C.3	2.52	1.84	0.03	0.00	5.51	0.00	13.28	20.26	9.90	1.87
C.4	0.19	0.23	0.02	0.00	0.46	0.06	1.15	2.02	0.96	0.25
C.5	0.00	0.16	0.00	0.00	0.23	1.21	0.15	8.71	1.61	0.16
C.6	0.08	1.36	0.27	0.00	1.80	5.08	3.15	36.57	8.58	1.63
S.1	0.07	0.15	0.14	0.47	0.72	0.28	0.07	0.72	1.83	0.76
S.2	0.91	0.69	0.15	0.38	1.75	0.11	4.48	9.74	4.00	1.23
S.3	2.45	0.53	0.67	0.35	5.58	0.00	12.75	19.51	9.57	1.55
S.4	0.21	0.15	0.04	0.04	0.50	0.08	1.25	2.29	1.02	0.23
S.5	0.00	0.03	0.05	0.05	0.21	1.12	0.15	7.44	1.47	0.13
S.6	0.07	1.03	0.42	0.25	1.80	5.59	3.22	38.37	9.15	1.69

contained the supramolecular antenna complex composed of CP29-CP24-LHCII trimer previously described (33). Band 5 contained the PSII core complex binding Chl *a* and β-carotene, while band 6 contained the PSI-LHCI complex as suggested by its polypeptide composition and by the Q_y absorption peak red-shifted to 680 nm. SDS-PAGE analysis showed that LHCII polypeptides were only present in bands 4, 3, and 2 when in their supramolecular, trimeric, and monomeric forms, respectively.

Trimeric LHCII from Control Plants—The characteristics of LHCII from band 3 are as follows. The Chl *a*/*b* ratio was 1.53, the Chl/carotenoid ratio was 3.4, the Chl/lutein ratio was 6.1, the Chl/neoxanthin ratio was 13.3, and the Chl/violaxanthin ratio was 18.2. Recent work showed that each LHCII polypeptide tightly binds 12 Chl molecules and xanthophylls, namely 1.8 lutein, 0.2 violaxanthin, and 1.0 neoxanthin (3, 4) per polypeptide, thus yielding a Chl/carotenoid ratio of 4.0, Chl/lutein of 6.7, Chl/neoxanthin of 12, and Chl/violaxanthin of 60. It is evident that violaxanthin and lutein content in LHCII from sucrose band 3 is strongly increased with respect to both recombinant LHCII (4) and highly purified LHCII (15). Assuming 12 Chl per polypeptide (2, 22) a xanthophyll content of 3.5 molecules per polypeptide is obtained. However, this figure it is not fully convincing, since it implies a content in neoxanthin lower than expected (0.9 *versus* 1.0 mol per polypeptide). By assuming for LHCII trimer from sucrose gradient a neoxanthin content as in recombinant and highly purified LHCII, a pigment complement of 13 Chl, 2.1 lutein, 1.0 neoxanthin, and 0.7 violaxanthin per polypeptide is obtained, implying that loosely bound xanthophylls account for 0.5 violaxanthin and 0.3 lutein molecules per LHCII polypeptide (Table II).

LHCII from Light-stressed Plants—In conditions of excess light with respect to the capacity of the electron transport chain, xanthophyll cycle operates, thus de-epoxidizing violaxanthin to antheraxanthin and zeaxanthin. In order to verify if de-epoxidation affects xanthophyll distribution, we have analyzed thylakoids obtained from plants upon activation of xanthophyll cycle by treating plants for 2.5 h at 4 °C with strong light (1000 $\mu E\ m^{-2}\ s^{-1}$). Fig. 1*A* (*right*) shows the separation obtained with de-epoxidized membranes (($Z + 1/2A$)/VAZ = 40%, where *Z* represents zeaxanthin and *A* is antheraxanthin). The distribution of chlorophyll among sucrose gradient bands did not show significant differences with respect to the separation from control plants, and also the polypeptide composition was not affected (Fig. 1*B*). However, xanthophyll composition of bands 1–3 was significantly modified.

In the following, we will indicate as "LHCII-S" the trimeric complex purified from stressed plants and as "LHCII-C" the one purified from control plants. The pigment composition of the fractions 1–6 from control "C" and stressed "S" plants are reported in Table I. The violaxanthin content of LHCII-S was (5.8% of total carotenoid), strongly decreased with respect to LHCII-C (18.5%). This loss is compensated by an increase of lutein (from 55.5 to 58.1%) and the appearance of small amounts of zeaxanthin (3.6%) and antheraxanthin (7%) (Table I). It is worth noting that the amount of VAZ in the two samples was different. While in the LHCII-C 9.2 mol of VAZ were found per 100 Chl *a*, in the case of LHCII-S, this value dropped to 8.1. The amount of zeaxanthin plus antheraxanthin bound to LHCII-S was as low as 0.4 mol per mol of polypeptide. The VAZ content in thylakoid membranes, normalized to the total chlorophyll content, from S and C plants, was essentially the same, indicating that, although a significant part of the violaxanthin bound to LHCII-C complexes has been converted to zeaxanthin under stress conditions, LHCII-S is not totally efficient in binding the zeaxanthin produced.

TABLE II
Pigment to protein stoichiometry determination of LHCII-C

Pigment content per monomer (mon) of LHCII purified from control plants by sucrose gradient (LHCII-C) normalized at 12 and 13 Chls compared with the pigment composition of recombinant LHCII (LHCII-rec) and highly purified LHCII (LHCII-IEF) is shown. Car, carotenoid; L, lutein; N, neoxanthin; V, violaxanthin.

	Car/mon	L/mon	N/mon	V/mon	Chl/mon
LHCII-C (12 Chl)	3.5	2.0	0.9	<0.7	12
LHCII-rec and LHCII-IEF	3.0	1.8	1.0	0.2	12
LHCII-C (13 Chl)	3.8	2.1	1.0	>0.7	13

The absorption spectra of LHCII trimers from stressed and control plants are shown in Fig. 2 after normalization at the Q_y absorption peak. No differences were detected in the red region of the spectra. In the Soret region (400 and 520 nm), including the contribution of carotenoids, the two spectra are slightly different as shown by the LHCII-C/LHCII-S difference spectrum (see *inset* in Fig. 2). The absorption of control sample is higher in the 350–480-nm range, while LHCII-S shows increased absorption in the 480–550-nm red range consistently with the higher amount of red-shifted carotenoids lutein, antheraxanthin, and zeaxanthin (4) in the latter complex.

Removal of Loosely Bound Pigments from LHCII—LHCII-C trimers (fraction C3) were subjected to flatbed IEF followed by ultracentrifugation in sucrose gradient, and the collected green fractions containing LHCII were merged together in order to

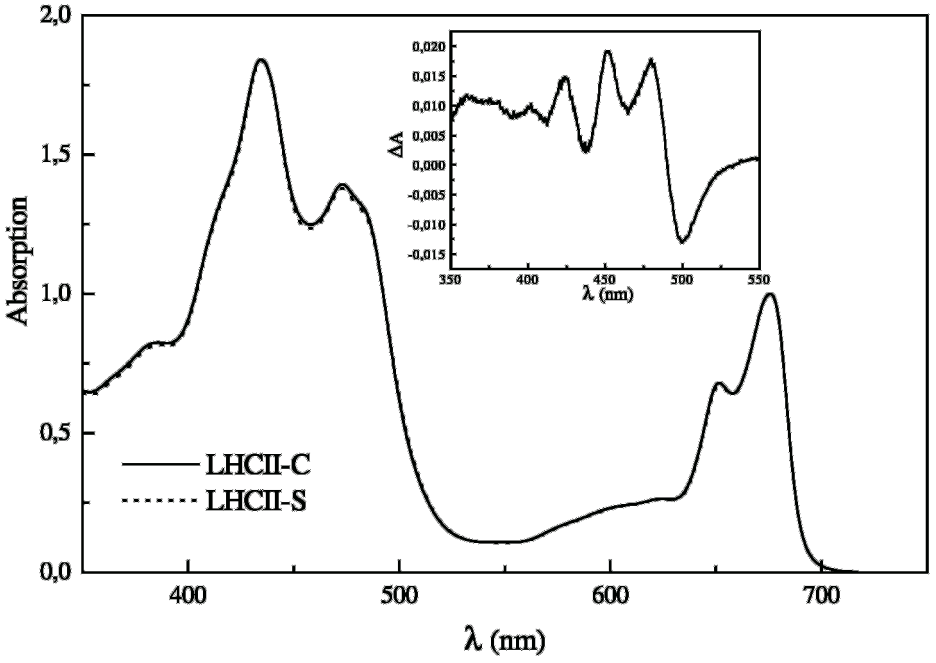

FIG. 2. **Absorption spectra at room temperature of LHCII prepared from control and stressed plants.** LHCII-C (*solid line*) and LHCII-S (*dashed line*) absorption spectra were normalized to the Chl content. *Inset*, the difference spectrum in the Soret region.

avoid introducing differences in the polypeptides composition with respect to the complex in C3. This treatment was shown to be effective in removing loosely bound pigments (7, 8, 15). LHCII-C-IEF trimeric complex thus obtained had biochemical and spectroscopic properties identical to those previously reported (14). Thus, 12 Chl (seven Chl *a* and five Chl *b*) and three xanthophylls (1.8 lutein, 1.0 neoxanthin, and 0.2 violaxanthin) were bound per LHCII polypeptide (Table III). These values are also fully consistent with the composition of monomeric LHCII as studied by mutation analysis of recombinant LHCII (3, 4). Thus, the IEF treatment removed 0.5 molecules of violaxanthin, 0.3 of lutein, and one of Chl *a* from the LHCII-C complex purified by sucrose gradient ultracentrifugation. When the IEF procedure was applied to LHCII-S, a LHCII-S-IEF complex was obtained, which was indistinguishable from LHCII-C-IEF with respect to its pigment composition and absorption spectra. In this case, lutein (0.4 molecules) and zeaxanthin-antheraxanthin (0.4 molecules) were removed, showing that the pigment content of internal L1, L2, and N1 sites was not affected by the operation of the xanthophyll cycle (Table III). On the other hand, these results also show that, although the loose binding is preferential for violaxanthin, it can also bind lutein and, upon de-epoxidation, antheraxanthin and zeaxanthin. Neoxanthin content did not show any significant change, suggesting that this xanthophyll is not involved in the loose binding to LHCII.

The absorption spectra of the LHCII-C and LHCII-C-IEF are shown in Fig. 3 after normalization to Chl content (34). The difference spectrum shows peaks at 431, 453, 485, and 665 nm. The 431- and 665-nm signals can readily be attributed to Chl *a*. The 453- and 485-nm signals are tentatively attributed to removed xanthophylls, mainly violaxanthin, as suggested by the pigment analysis. This indicates a shift of the loosely bound violaxanthin by 12 nm as compared with 80% acetone (473 nm for the red-most S_0-$S2_{0-0}$ transition). The difference spectrum is wider than the violaxanthin spectrum in the 490–510-nm region, accounting for the loss of lutein. It has been previously observed that the absorption spectrum of the violaxanthin in the two internal sites shows a shift of 19 nm as compared with the absorption in 80% acetone (4). This corresponds to an environment with refractive index of 1.55. The shift by 12 nm, observed for the loosely bound violaxanthin, indicates a refractive index of 1.5. Since violaxanthin red-most absorption, in α-DM micelles, peaks at 477 nm (corresponding to a refractive index of 1.43), loose binding to LHCII provides an environment with a refractive index intermediate between internal sites and detergent micelles, possibly indicating a peripheral location. In order to test the possibility that unspecific binding to LHCII could yield a similar shift, we used recombinant LHCII reconstituted *in vitro* with violaxanthin; when purified by sucrose gradient ultracentrifugation, this complex still retains unspecifically bound violaxanthin, which is removed by a further purification step by FPLC (35). The difference absorption spectra before and after FPLC showed that unspecifically bound violaxanthin absorbs at 477 nm.

Orientation of Loosely Bound Violaxanthin—To gain additional information on the binding of peripheral violaxanthin, we proceeded to the determination of the orientation of the violaxanthin transition moment, lying along the polyene chain, with respect to the normal to the plane of the thylakoid membrane according to the procedure previously described for the N1 site of LHCII (6). LD measurements were performed at 100 K on LHCII-C and LHCII-C-IEF complexes oriented by the polyacrylamide squeezing technique. The spectra are reported in Fig. 4. Normalization was performed in the 505–640-nm spectral region, where the difference absorption spectrum had zero amplitude. The IEF treatment produced changes in the Chl Q_y region at around 645–670 nm corresponding to the differences observed in absorption (taking in to account the blue shift induced by temperature). The major change was due to the loss of a Chl *a* with 664-nm absorption and a positive LD signal. Very small differences were also observed at 675 and 680 nm and a slightly larger one at 648 nm, possibly indicating that the treatment introduced small changes in the orientation of some Chl within the complex. However, the shape and amplitude of the major peak of bulk Chl a were essentially unaffected by the treatment. In the 500–530-nm range, the lutein signal dominates the LD spectrum, as shown in Fig. 4 by comparison with the lutein absorption spectrum.

The difference (LHCII-C-IEF minus LHCII-C) LD shows a major positive signal in the Soret region peaking at 455 nm and other signals of lower amplitude at 477 and 492 nm. The 455-nm peak is attributed to the S_0-$S_{2,1}$ transition of loosely bound violaxanthin on the basis of its absorption at 485 nm

TABLE III

Pigment composition of LHCII complexes purified with different methods

The values are normalized to the number of Chls per monomer. tot, total; N, neoxanthin; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; L, lutein; Car, carotenoid; C, control; S, stressed; IEF, control after IEF. The measurements have been repeated at least six times with two different methods. The maximum error is 0.02.

Sample	Pigments								
	Chl *a*/*b*	Chl tot	N	V	A	Z	L	Car tot	Chl/car
LHCII-C	1.53	13	0.98	0.71	0.01		2.13	3.84	3.39
LHCII-S	1.53	13	0.99	0.21	0.27	0.14	2.25	3.86	3.37
LHCII-C-IEF	1.42	12	0.99	0.22			1.80	3.0	4.00
LHCII-S-IEF	1.41	12	1.00	0.21			1.80	3.0	4.00

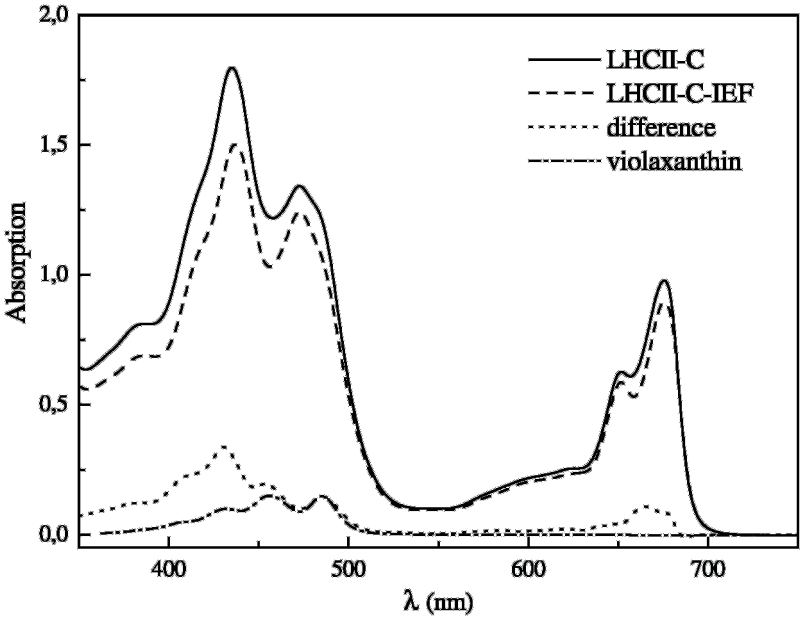

FIG. 3. **Comparison of the absorption spectra of LHCII-C and LHCII-C-IEF.** Absorption spectra of LHCII-C (*solid line*) and LHCII-C-IEF (*dashed line*) were normalized to Chl content. The *dotted line* represents the difference spectrum (LHCII-C minus LHCII-C-IEF). The spectrum of violaxanthin shift of 12 nm with respect to the absorption in 80% acetone is also shown (*dashed and dotted line*).

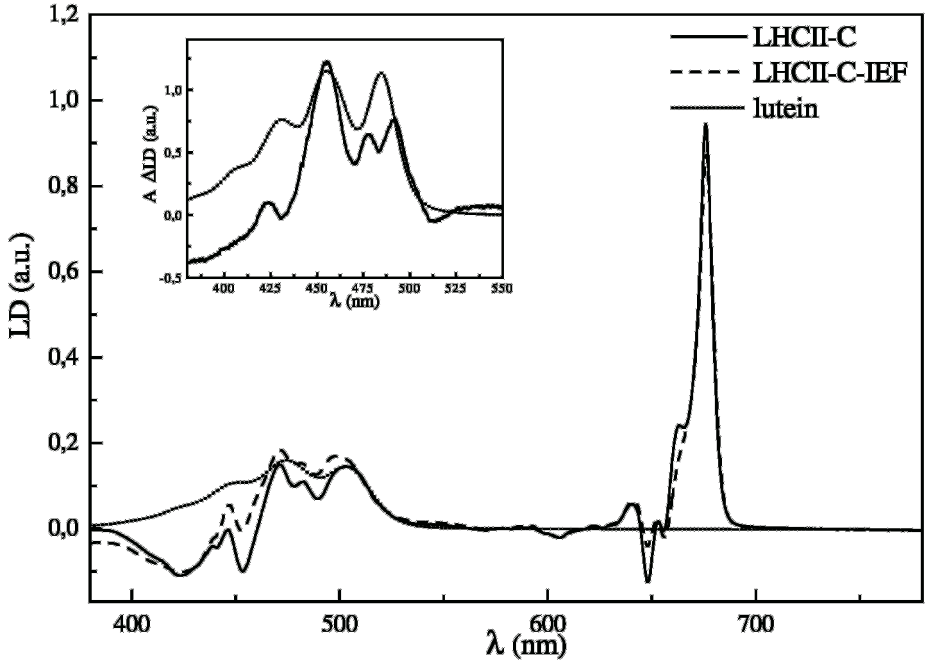

FIG. 4. **LD spectra at 100 K of LHCII-C and LHCII-C-IEF.** LHCII-C (*solid line*) and LHCII-C-IEF (*dashed line*) are shown. The spectrum of lutein, used as an internal standard, is shown as a *dotted line*. *Inset*, the difference spectrum between LHCII-C-IEF and LHCII-C (*solid line*) together with the absorption spectrum of violaxanthin (*dotted line*).

(Fig. 3) and of the energy gap of 28 nm between $S_{2,0}$ and $S_{2,1}$ vibrational states of violaxanthin in acetone. The fact that the third peak ($S_{0,0}$-$S_{2,0}$ transition) does not appear in LD can be due to other changes in the complex, such as the loss of lutein (positive signal at 490 nm, +12 nm shift with respect to the absorption of lutein in acetone) and/or a change in the orientation on one Chl *b* molecule as suggested by the analysis of the Q_y region. We used the signal from the tightly bound two lutein molecules as an internal standard to calculate the orientation of the loosely bound pigment (6), since their orientation with respect to the normal to the membrane in which the LHCII molecule is inserted is known from structural data (2). The LD signal associated to violaxanthin is negative, indicating that for the carotenoid in the fourth site, the transition moment forms

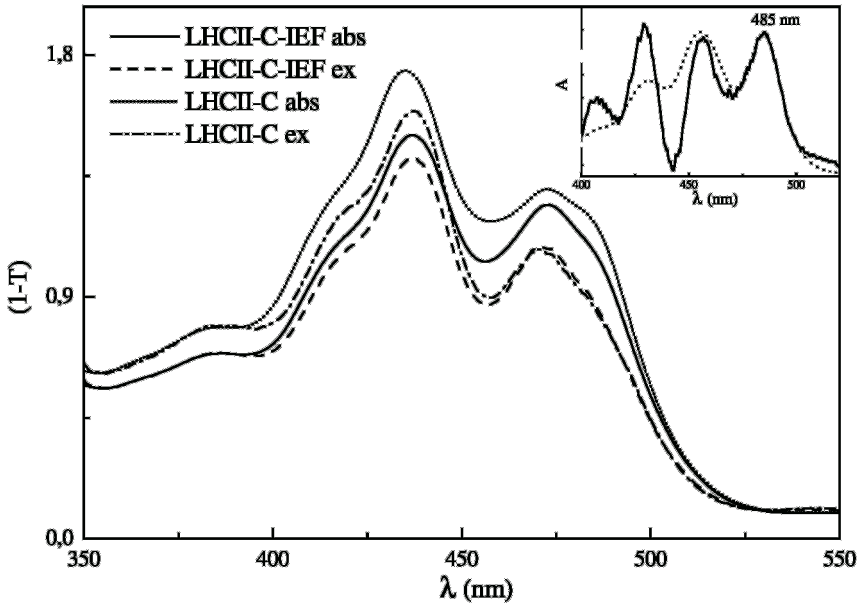

FIG. 5. **Comparison of absorption and excitation spectra of LHCII-C and LHCII-C-IEF.** Absorption (*dotted line*) and excitation spectra (*dotted and dashed line*) of LHCII-C and absorption (*solid*) and excitation (*dashed*) spectra of LHCII-C-IEF. See "Results" for details about normalization. *Inset*, difference spectrum (*solid*) between the nontransferring pigment component in the two samples ((absorption − excitation)$_{\text{LHCII-C}}$ − (absorption − excitation)$_{\text{LHCII-C-IEF}}$) is shown together with the spectrum of violaxanthin (*dotted*) shifted by 12 nm with respect to the absorption in 80% acetone.

a ϕ angle of less than 54.6° with the normal to the membrane plane. Calculation of amplitude ratio, considering stoichiometry of 1.8 lutein in the two central sites *versus* 0.8 violaxanthin/lutein in the fourth site, indicates a value of 53 ± 2° for the orientation of this carotenoid with respect to the normal to the membrane plane (see also Ref. 6 for a detailed description of the calculus).

This ϕ value is slightly smaller than observed for xanthophylls in the L1, L2, and N1 sites. Identical results have been obtained analyzing the two trimers purified with the same procedure form barley (data not shown).

Energy Transfer from Loosely Bound Violaxanthin to Chlorophyll a—In order to evaluate the ability of violaxanthin in transferring energy to Chl *a* emitters, the absorption and the fluorescence excitation spectra of the LHCII-C (four xanthophylls) and LHCII-C-IEF (three xanthophylls) differing for the occupation of the peripheral binding site, have been compared following normalization. First, the two absorption spectra were normalized to their Chl content (13 *versus* 12 Chls). Second, the fluorescence excitation spectra were normalized to the absorption spectra assuming 100% efficiency for Chl *a* to Chl *a* energy transfer, based on emission spectra that show thermal equilibration in the samples here analyzed (data not shown). In practice, the excitation spectrum for each sample was normalized to its absorption spectrum in the region between 350 and 390 nm, where the signal is almost entirely due to Chl *a* absorption (Fig. 5). It clearly appears that, while absorption spectra of the two complexes differ in the wavelength range of xanthophyll absorption, fluorescence excitation spectra are very similar, only differing in the amplitude of the Chl *a* signal. This suggests that while the Chl *a* molecule removed by the IEF treatment is active in energy transfer, xanthophylls in the fourth site are not. By subtracting the absorption minus fluorescence excitation difference spectra of LHCII-C-IEF from that of LHCII-C, the spectrum of a carotenoid is obtained, having the red-most peak at 485 nm (*inset* in Fig. 5). This clearly indicates that violaxanthin at 485 nm is not active in energy transfer to Chl *a*. For a more quantitative analysis, spectra were analyzed in terms of the energy level and amplitude of the spectral components in the Soret region (30). The results of the fitting are reported in Table IV. Two Chl *a*, three Chl *b*, two lutein, one neoxanthin, and one violaxanthin absorption form were needed in order to obtain the best fit of absorption spectra. With respect to previous results with recombinant monomeric LHCII, a small spectral population of lutein with the red-most peak ($S_{2,0}$ level) at 510 nm (7% of the total carotenoid absorption) was needed for the best fit. This lutein spectral form was present in both LHCII trimers independently from the occupancy of the fourth xanthophyll binding site. In the absorption spectrum of LHCII-C, one violaxanthin absorption form peaking at 485 nm was needed, in agreement with the results of the absorption difference spectrum, while in LHCII-C-IEF the violaxanthin component was of much smaller amplitude and shifted to the red at 492 nm. By comparing the results of the spectral deconvolution of absorption spectra *versus* excitation spectra, it was possible to evaluate the transfer efficiency for the individual pigments. The results are reported in Table IV. Despite an error that can account for 5–10% of the transfer efficiency values, somehow higher in the LHCII-C sample where many pigments are present, the results clearly indicate that loosely bound violaxanthin was unable to transfer energy to Chl *a*.

TABLE IV
Energy transfer efficiency for individual pigment in LHCII-C and LHCII-C-IEF

Transfer efficiency of Chl *a* is assumed to be 100%. The error is assumed to be 5–10%. N, neoxanthin; V, violaxanthin; L, lutein.

Sample	Energy transfer efficiency to Chl *a*				
	Chl *a*	Chl *b*	N	V	L
	%	%	%	%	%
LHCII-C	100	92.6	80	0	88
LHCII-C-IEF	100	89	73	ND	88

It is worth mentioning that the excitation spectrum from LHCII-S is identical to the spectrum of LHCII-C (data not shown), thus suggesting that also zeaxanthin and antheraxanthin in the fourth site are not able to transfer energy.

Effect of De-epoxidation on Resistance to Photobleaching and Fluorescence Yield—Besides their role in light harvesting, xanthophylls have been involved in photoprotection by quenching ^{3}Chl*, thus preventing formation of harmful $^1O_2$* and quenching ^{1}Chl* during NPQ. We therefore proceeded to verify if changes in the loosely bound xanthophyll pigment pool influence the photoprotection properties of LHCII. In a first experiment, we have evaluated the kinetics of photobleaching obtained by illuminating the complexes in the presence of oxygen

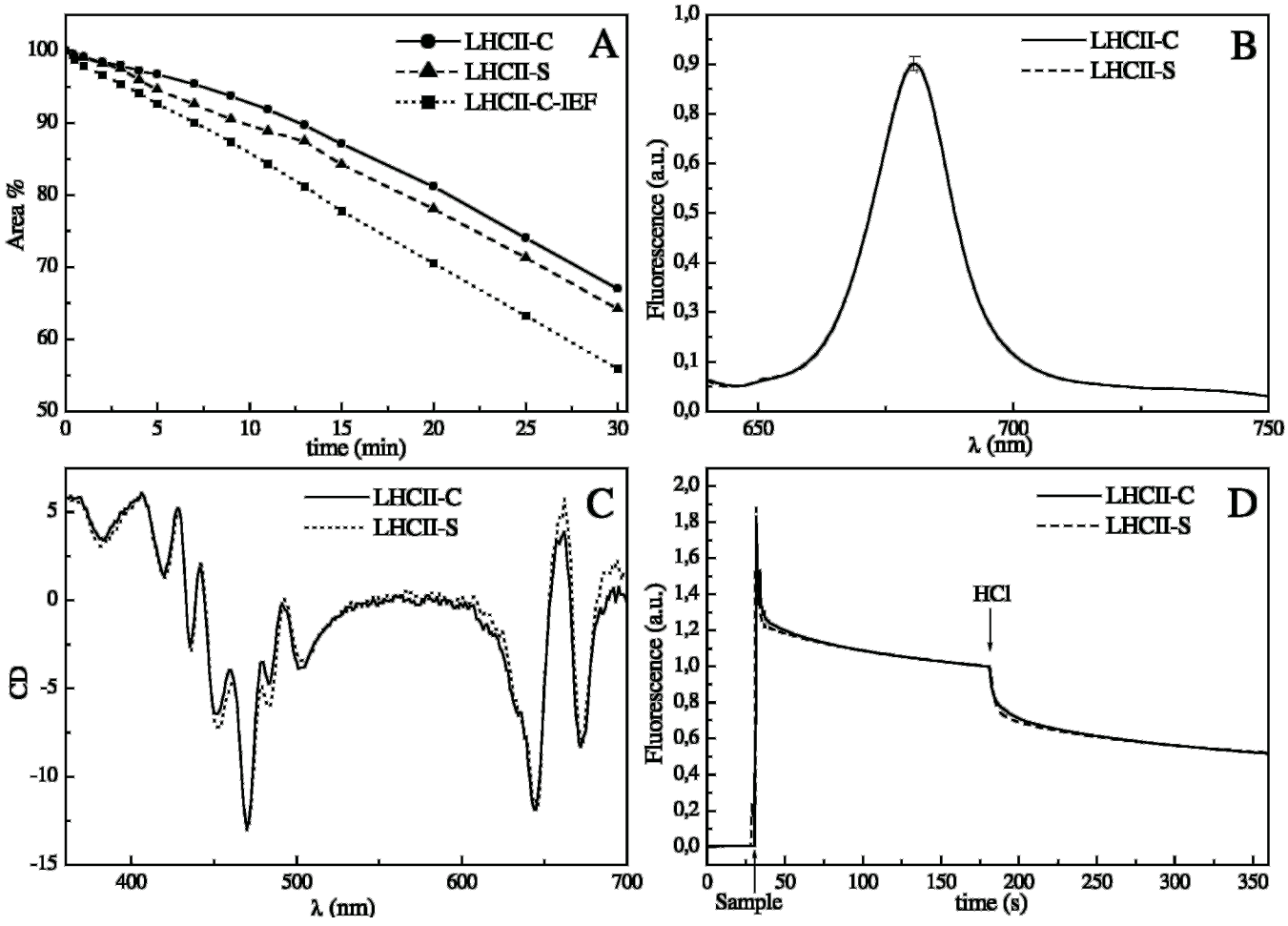

FIG. 6. **Effect of de-epoxidation on LHCII trimers.** *A*, decrease of the chlorophyll absorption due to photobleaching. The area decrease in the red absorption region (600–750 nm) is reported as a function of the time interval of high light treatment. The points represent experimental data for different samples. ●, LHCII-C; ▲, LHCII-S; ■, LHCII-C-IEF. *B*, comparison of fluorescence quantum yield of LHCII-C (*solid line*) and LHCII-S (*dotted line*) upon excitation at 440 nm. The data presented are the average of six independent measurements; the *error bar* is shown. *C*, circular dichroism spectra of LHCII-C (*solid line*) and LHCII-S (*dotted line*); the spectra are normalized to the Chl content. *D*, fluorescence quenching in LHCII-C (*solid line*) and LHCII-S (*dotted line*) upon dilution into 6 μM DM. The samples were excited at 440 nm, and the fluorescence emission was detected at 681 nm. The time of the addition of the sample and HCl are reported in the *figure*. The pH after acidification was 5.5.

(4). Fig. 6*A* shows that LHCII-C and LHCII-S are very resistant to photobleaching, since exposure to strong light (4500 μE m^{-2} s^{-1}) for 30 min only yielded less than 35% bleaching of Chl absorption. The two kinetics were identical within the experimental error. It is worth noticing that LHCII-C-IEF, in which the external xanthophyll has been removed, is bleached with a higher rate with respect to the samples with a full xanthophyll complement.

We then proceeded to verify if binding of zeaxanthin *versus* violaxanthin in the external site could induce a quenching of Chl fluorescence of the LHCII complex through a conformational change according to the model recently proposed (7, 36). The relative fluorescence yield was measured upon careful normalization of the samples at the excitation wavelength (625 nm). Fig. 6*B* shows that the averaged fluorescence emission spectra from six independent measurements are essentially identical in both shape and amplitude.

One of the most sensitive techniques for detection of the mutual organization of chromophores is circular dichroism. In Fig. 6*C* the CD spectra of the LHCII-C and LHCII-S upon normalization to Chl absorption at 674 nm are shown to be identical, thus suggesting that if a conformational change is produced by the substitution of part of loosely bound violaxanthin by zeaxanthin, it does not yield any change in the mutual organization of pigments in the complex.

An alternative method of evaluating NPQ-related fluorescence quenching in isolated Lhc proteins is the comparison of the fluorescence during low pH-induced aggregation of the pigment-protein at low detergent concentration (37). The results of such an experiment are shown in Fig. 6*D* and indicate that LHCII-C and LHCII-S are identical in their aggregation-induced fluorescence behavior.

DISCUSSION

In this study, we have characterized the biochemical and functional properties of xanthophylls loosely bound to the major light harvesting complex of higher plant photosystem II. Solubilization screening with a range of mild alkyl-sugar detergents decreased the fraction of the xanthophylls in the yellow band migrating on the top of sucrose gradients following ultracentrifugation of solubilized thylakoids and increased the fraction bound to green bands 2–6 containing chlorophyll-binding proteins. About 70% of LHCII was contained in sucrose band 3, thus making this preparation largely representative of this complex in the thylakoid membranes. The distribution of pigments between the pool tightly bound into internal sites, and the loosely bound pool was determined by comparing the LHCII complex from sucrose gradient with the one obtained by removing the loosely bound pool by IEF followed by gradient ultracentrifugation, thus obtaining a LHCII complex only re-

taining tightly bound pigments (7, 8). The pigment composition and stoichiometry of IEF purified LHCII complex from maize was consistent with previous work (14, 15, 22) and also identical to that of the recombinant complex obtained by overexpression in bacteria of the Lhcb1 gene product followed by *in vitro* reconstitution (3, 4): each LHCII polypeptide thus binds 12 Chl, with a Chl *a*/*b* ratio of 1.4–1.45, and three xanthophylls in the ratio of 1.8 lutein:1.0 neoxanthin:0.2 violaxanthin. Since neoxanthin is very tightly bound to LHCII with 1.0 molecule per complex, this pigment can be used for normalization of the remaining pigments in the complex. On this basis, LHCII-C binds 13 Chl $a + b$, 1.0 neoxanthin, 2.1 lutein, and 0.7 violaxanthin. The same conclusion can be reached by considering the difference in the Chl *a*/*b* ratio between the two complexes; this value is higher in the LHCII-C compared with LHCII-C-IEF, strongly suggesting loss of Chl *a* during IEF. This makes the other possibility, namely the increase of Chl *b* after IEF, very unlikely. We conclude that mildly solubilized LHCII binds close to one (0.8) xanthophyll and one Chl *a* molecule per polypeptide in a loosely bound form. This is consistently obtained by computing the difference between the composition of the LHCII-C *versus* LHCII-C-IEF and by directly analyzing the pigment removed by IEF and recovered in the subsequent sucrose gradient as free pigment band. Although we tried solubilization with very low detergent concentrations, it was never possible to obtain more than about 1.0 xanthophyll per LHCII polypeptide in the loose pool, thus suggesting that a specific site, rather than multiple nonspecific sites, is present in LHCII. These results are consistent with a previous report on LHCII from spinach PSII membranes (7).

Specificity of the Loose Xanthophyll Binding to LHCII—Both violaxanthin and lutein are involved in loose binding to LHCII rather than only violaxanthin as previously reported (7, 8). Upon de-epoxidation, significant amounts of zeaxanthin and antheraxanthin were found to be bound to LHCII-S. Nevertheless, the total amount of bound carotenoids per polypeptide did not significantly change, while removal of the loosely bound pool by IEF from the control and stressed LHCII showed that the tightly bound pool did not change. Therefore, we conclude that the specificity of loose binding is low and that any xanthophyll but neoxanthin can participate to this pool. On the basis of the availability of the individual carotenoids in the different conditions (calculated from the amount of individual xanthophylls in the free pigment fraction), it is possible to propose that the affinity of this site for the xanthophylls is as follows: antheraxanthin > violaxanthin > lutein > zeaxanthin.

The above data suggest that the loosely bound xanthophylls may occupy a specific site in the LHCII complex. Two observations support this hypothesis: refractive index of the binding site and the orientation of violaxanthin.

Refractive Index of the Binding Site—Xanthophyll absorption is strongly affected by the environment. Using the absorption shift, it is possible to calculate the refractive index of the protein domain where a particular xanthophyll is located (38). The red-most, S_0-$S_{2,0}$, transition of violaxanthin peaks at 472.8 in 80% acetone, while it is red-shifted by 19 nm when in the L1/L2 sites of LHCII (4) and by 12 nm in the loose binding site and by 4 nm in detergent micelles. We conclude that loose violaxanthin is bound to a site with refraction index of 1.5, intermediate between L1/L2 sites and detergent micelles or unspecifically bound violaxanthin. It is worth noting that lutein belonging to the loose pool undergoes the same 12-nm absorption shift, suggesting that it is bound to the same site as violaxanthin.

Orientation of Violaxanthin—Comparison between the LD spectra of LHCII binding either three or four xanthophylls allowed determination of the angle that the violaxanthin transition moment, lying along the polyene chain, forms with respect to the normal to the membrane plane. The ϕ value thus obtained is $53 \pm 2°$, suggesting a specific orientation despite the loose binding and an orientation of the loosely bound violaxanthin similar to that of tightly bound lutein ($\phi = 56$ and 59°) and neoxanthin ($\phi = 57°$). Since $\phi = 24°$ was reported for violaxanthin in lipid bilayers (39), it appears that violaxanthin orientation is strongly affected by its loose but specific binding to LHCII. Our determination of violaxanthin ϕ value strongly differs from the previous report of 71° (40), implying planar orientation of this pigment with respect to the plane of the thylakoid membrane. We attribute this discrepancy to the attribution to violaxanthin of a small absorption component at 510 nm (41). However, recent Raman analysis suggested the 510-nm signal to a lutein population distorted by the interaction within trimeric LHCII (42). This is also our finding. In order to fit the Soret region of the absorption spectrum of trimeric LHCII (but not monomers), a lutein component with a strong shift (33 nm with respect to the absorption in 80% acetone, corresponding to 510 nm) is needed. Since this absorption form persists after removal of xanthophylls by IEF, it clearly does not originate from loosely bound violaxanthin.

From the above observations, namely the stoichiometry of approximately 1.0 with respect to LHCII polypeptides, a distinct refractive index as determined from the 12-nm red shift of both lutein and violaxanthin and the specific orientation with respect to the plane normal to the membrane, different from that formed by free violaxanthin, we conclude that a fourth specific binding site, hereafter called V1, is present in LHCII complex. It was previously suggested that the occupancy of this site varied in dependence to the light intensity during growth (7, 8). On the basis of the present data, it is possible to suggest that the relative occupancy of the V1 site by violaxanthin *versus* lutein rather than the occupancy level may change. However, we cannot exclude differences due to the plant species here used (*Z. mays*) *versus Spinacia oleracea* and *Vinca major* used in previous studies.

LHCII Complex Binds 13 Chlorophylls—Previous stoichiometric determinations of Chl binding to LHCII were performed on the highly purified complex (22) or the recombinant complex (3, 4) lacking the loosely bound xanthophyll pool. These determinations yielded a value of 12 Chl $a + b$ per monomeric complex, consistent with structural data (2). The present data clearly show that mildly purified LHCII complex binds an additional Chl *a* molecule. This Chl *a* molecule absorbs at 664 nm. Since Chl *a* ligands deeply buried in the LHCII structure have energy levels of their Q_y transitions red-shifted up to 681 nm (3, 43), we suggest a surface-exposed site in agreement with this pigment being readily extractable from the complex. Nevertheless, fluorescence spectra show that the 13th Chl is fully functional in energy transfer.

Function of Xanthophylls Bound to V1 Site—Spectroscopic analysis clearly shows that xanthophylls bound to V1 site are not able to transfer the absorbed energy to Chl *a*. This effect is irrespective of the xanthophyll species in both epoxidized (violaxanthin, lutein) and de-epoxidized state (antheraxanthin, zeaxanthin). This is rather surprising, since xanthophylls bound to the previously characterized binding sites are active in energy transfer to Chl *a* although with different efficiencies (27, 44, 45). Lack of energy transfer cannot be imputed to the overlap integral between violaxanthin and Chl *a*/*b* chromophores, in fact this is probably better upon blue shift of violaxanthin in V1 with respect to what is observed for the tightly bound xanthophylls in L1, L2, and N1 sites. We rather

suggest that lack of energy transfer is due to the distance between the carotenoid and the Chls as also suggested by the fact that no changes in the absorption spectra of Chl have been detected following substitution of V1 pigments.

Functions of carotenoids alternative to light harvesting include photoprotection by either quenching of ^{3}Chl* in order to prevent $^1O_2$ or singlet energy dissipation by NPQ. The removal of xanthophyll in the fourth site significantly increases the rate of photo-oxidation. Our measurement does not distinguish between a photoprotection by direct quenching of Chl *a* triplets or by scavenging of singlet oxygen. The former effect seems unlikely, since it has been shown above that this xanthophyll is unable to transfer energy to Chl *a*; a scavenging effect on reactive oxygen species eventually formed by Chl triplets escaping carotenoid quenching is more likely. Occupancy of the V1 site by either epoxidized or de-epoxidized xanthophylls did not affect resistance to photobleaching of LHCII, thus showing that the exchange of violaxanthin for zeaxanthin in this site upon high light exposure does not yield an increased photoprotection, suggesting that this is not the specific function of the violaxanthin *versus* zeaxanthin exchange in the V1 site. We then proceeded to test whether the characteristics of LHCII from control *versus* light-stressed plants could fit with the expectations from the different models that have been proposed for NPQ. We observed, in agreement with previous work with spinach (7), that newly formed zeaxanthin and antheraxanthin remain in part bound to LHCII-S specifically in the V1 site, since these pigments can be removed by IEF. Nevertheless, the level of xanthophyll cycle pigments in site V1 is higher in LHCII from control plants (0.5 mol per monomer) than in the complex from light-stressed plants (0.4 mol per monomer), lutein complementing for the reduced VAZ level. The origin of this lutein complement may well be a new synthesis as previously reported in *Vinca* upon high light stress (8). Consistently, we found in thylakoid membranes a slightly increased (10–15%) lutein content on a Chl basis upon high light stress. A first model for NPQ was proposed by Frank *et al.* (46) based on the lower S1 energy level of zeaxanthin with respect to violaxanthin as calculated by the energy gap law. The Chl *a* Q_y transition being in between the S1 value of epoxidized and de-epoxidized xanthophyll, it was concluded that while violaxanthin could act as energy supplier for Chl *a*, zeaxanthin was a quencher. Recent direct measurement of S1 (47) showed, however, that violaxanthin and zeaxanthin transitions are in fact isoenergetic. Alternatively, it was proposed that quenching in LHCII could be elicited by conformational changes induced by binding of zeaxanthin to the external (V1) site of LHCII (7, 48). Since both antheraxanthin and zeaxanthin were shown to be active in NPQ (49) the comparison between the LHCII-C, binding violaxanthin, and LHCII-S, binding antheraxanthin and zeaxanthin, should allow verification of this model. Determination of the relative fluorescence yields in these two complexes showed, however, that binding of zeaxanthin plus antheraxanthin to the V1 site did not induce any quenching in LHCII. Also, CD spectra, previously shown to be effective in detecting conformational changes in LHC proteins (50), were identical, suggesting that the LHCII complex did not undergo conformational changes upon binding of zeaxanthin plus antheraxanthin. It was proposed that low pH-induced aggregation quenching of Lhc proteins mimics a similar phenomenon induced in the thylakoid membranes (37). According to this procedure, no differences were detected between LHCII-C and LHCII-S.

We have shown that xanthophylls in the V1 site neither are active in light harvesting nor affect the photoprotection properties of LHCII, at least *in vitro*. Therefore, what is their function? The result of de-epoxidation is a redistribution of xanthophylls among Lhc subunits with an increase of antheraxanthin and zeaxanthin free in the lipid membrane or tightly bound to CP29, CP26, and CP24 (7, 8, 15, 16), while LHCII increases its binding of lutein *versus* VAZ. We tentatively suggest that the LHCII V1 site acts as a source of readily available violaxanthin for the de-epoxidation. De-epoxidation products play a dual role: (i) they are released in the lipid membrane to play a role as stabilizer and antioxidant (10), and (ii) they act as signal transducers by binding to minor Lhc proteins and induce quenching by conformational change (51).[2]

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