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Dynamics of Chromophore Binding to Lhc Proteins *in Vivo* and *in Vitro* during Operation of the Xanthophyll Cycle*

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Three plant xanthophylls are components of the xanthophyll cycle in which, upon exposure of leaves to high light, the enzyme violaxanthin de-epoxidase (VDE) transforms violaxanthin into zeaxanthin via the intermediate antheraxanthin. Previous work (1) showed that xanthophylls are bound to Lhc proteins and that substitution of violaxanthin with zeaxanthin induces conformational changes and fluorescence quenching by thermal dissipation. We have analyzed the efficiency of different Lhc proteins to exchange violaxanthin with zeaxanthin both *in vivo* and *in vitro*. Light stress of *Zea mays* leaves activates VDE, and the newly formed zeaxanthin is found primarily in CP26 and CP24, whereas other Lhc proteins show a lower exchange capacity. The de-epoxidation system has been reconstituted *in vitro* by using recombinant Lhc proteins, recombinant VDE, and monogalactosyl diacylglycerol (MGDG) to determine the intrinsic capacity for violaxanthin-to-zeaxanthin exchange of individual Lhc gene products. Again, CP26 was the most efficient in xanthophyll exchange. Biochemical and spectroscopic analysis of individual Lhc proteins after de-epoxidation *in vitro* showed that xanthophyll exchange occurs at the L2-binding site. Xanthophyll exchange depends on low pH, implying that access to the binding site is controlled by a conformational change via lumenal pH. These findings suggest that the xanthophyll cycle participates in a signal transduction system acting in the modulation of light harvesting *versus* thermal dissipation in the antenna system of higher plants.

Supramolecular complexes of the thylakoid membrane called photosystems catalyze higher plant photosynthesis. Each photosystem is composed of a core moiety containing electron transport components and binding Chl[1] *a* and β-carotene (2, 3) and by an antenna moiety containing, as light harvesting pigments, Chl *a*, Chl *b*, and a number of xanthophylls, bound to proteins belonging to the Lhc family (4). When light intensity exceeds the capacity for electron transport from water to $NADP^+$, excess energy can be diverted to molecular oxygen with the formation of reactive species harmful for the chloroplast, thus leading to photoinhibition of photosystems (5). In these conditions photoprotection mechanisms are activated leading to the thermal dissipation of excess chlorophyll singlet states (6). At the same time, the pigment composition of thylakoid membranes is modified by the operation of the xanthophyll cycle, consisting of the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin by the lumenal enzyme VDE, which binds to thylakoids upon activation by low lumenal pH. During operation of the xanthophyll cycle, violaxanthin bound to a low affinity site of LHCII (7) is released into the membrane lipids where it is de-epoxidized. Newly synthesized zeaxanthin has been reported to act freely in the membrane together with tocopherol in the scavenging of reactive oxygen species (8). Moreover, zeaxanthin can be exchanged for violaxanthin in high affinity binding sites of Lhc proteins where it induces a conformational change leading to increased thermal dissipation (1, 9, 10). Knowledge of the xanthophyll exchange in different Lhc proteins is limited, and the understanding of the mechanisms is very low. In this study we analyzed the extent of the xanthophyll exchange in the different Lhc proteins *in vivo* upon activation of the xanthophyll cycle by strong illumination. We compared these results with those obtained *in vitro* by using a reconstituted system composed of recombinant Lhc proteins and the recombinant VDE enzyme. The extent of zeaxanthin binding to Lhc proteins strongly differed among members of the Lhc protein family. The results obtained by the simple *in vitro* system closely reproduce those obtained *in vivo*, thus suggesting that differences in protein structure are the major determinants for the regulation of xanthophyll exchange. Biochemical and spectroscopic analysis of Lhc proteins upon *in vitro* de-epoxidation showed that xanthophyll exchange occurs specifically at the L2-binding site. This site was previously shown (10) to act as an allosteric regulator of thermal dissipation activity in Lhc proteins by controlling the transition between two conformations of Lhc proteins (1). These data suggest that the xanthophyll cycle is part of a signal transduction system acting in the modulation of light harvesting *versus* thermal dissipation in the photosystems of higher plants.

EXPERIMENTAL PROCEDURES

Plant Material and Treatments—Z. mays (cv. Dekalb DK300) plants were grown for 2 weeks at 23 °C at low light intensities (~80 μE, 14 h light/10 h dark). One set of plants was light-stressed at ~1000 μE m^{-2} s^{-1} for 30 min at 20 °C, whereas control plants were maintained at growth conditions. After treatment leaves were rapidly harvested, cooled in ice, and chloroplast membranes were isolated as previously reported (11). Thylakoids were solubilized with 1% DM and fractionated by flatbed preparative isoelectric focusing as previously described (12). Fractions from IEF were further fractionated by sucrose gradient ultracentrifugation to eliminate co-migrating pigments. Free pigment formed a yellow band in the upper part of the gradient, whereas Lhc proteins formed multiple green bands migrating at higher sucrose den-

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[1] The abbreviations used are: Chl, chlorophyll; β-DM: *n*-dodecyl-β-D-maltoside; CP, chlorophyll protein; HPLC, high performance liquid chromatography; Lhc, light harvesting protein; Lhca, light harvesting complex of PSI; Lhcb, light harvesting complex of PSII; LHCII, major light harvesting complex of PSII; PS, photosystem; VDE, violaxanthin de-epoxidase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MGDG, monogalactosyl diacylglycerol; NPQ, excitation energy.

sities. The green fractions from each tube were pooled for further analysis.

Pigment Analysis—The pigment content was determined by HPLC (13) and fitting of the acetone extract with the spectra of the individual pigments (14).

Gel Electrophoresis—SDS-PAGE was performed with the Tris-Tricine buffer system as previously reported (15).

Expression of Recombinant VDE—The construct QAV expressing VDE was a kind gift of Prof. Yamamoto (16). For the VDE expression, *Escherichia coli* cultures (SG13009 strain) (17) with a 600-nm absorbance of 0.6 were induced with 1 mM IPTG for 3 h and purified on a Ni^{2+} affinity column. The protein was denatured in 6 M guanidine-HCl, 20 mM HEPES, pH 8, 0.2 M NaCl and then refolded with a slow dilution of the denaturant with the renaturation buffer (10% glycerol, 0.25% *n*-octyl-β-D-glucoside, 20 mM HEPES, pH 7.5, 100 mM NaCl) (16).

Isolation of Overexpressed Lhc Apoproteins from Bacteria—Lhc were expressed and isolated from *E. coli* following a protocol as previously described (11, 18).

Reconstitution and Purification of Lhc-pigment Complexes—Lhca1 and Lhca4 from *Arabidopsis thaliana*, Lhcb4 (CP29) and Lhcb5 (CP26) from *Z. mays*, and Lhcb1, Lhcb2, and Lhcb3 from *Hordeum vulgare* were reconstituted as described (19) with the following modifications. The reconstitution mixture contained 420 μg of Lhc apoprotein and 240 μg of chlorophyll *a* plus *b*. The Chl *a*/*b* ratio in the pigment mixture varied from 2.3 to 4.5 as optimized for the different Lhc proteins: Lhcb1–3, 2.3; CP26, 3.0; CP29, 4.5; and Lhca1/4, 4.0. Xanthophyll content was 90 μg of violaxanthin for Lhcb1, Lhcb2, and Lhcb3 and 60 μg for CP26, CP29, and Lhca1, 4.

Spectroscopy—The absorption spectra at room temperature were recorded by a SLM-Aminco DK2000 spectrophotometer and a 0.4-nm step was used. The CD spectra were measured at 10 °C on a Jasco 600 spectropolarimeter. Samples were in 10 mM HEPES, pH 7.5, 20% glycerol, and 0.06% β-DM.

Deconvolution of Spectra into Absorption Forms—Absorption spectra were analyzed in terms of the contribution of individual pigments by using the absorption spectra of pigments in Lhc proteins as previously reported (20).

De-epoxidation Reaction in Vitro—Lhc proteins (3 μg of chlorophyll) were mixed with 60 μg/ml monogalactosyl diacylglycerol (MGDG) and added to the reaction mixture containing 250 mM citrate buffer, pH 5.1, and 0.02% β-DM and 1.5×10^{-3} units of the VDE enzyme preparation. The de-epoxidation was performed at 28 °C for 30′ and started by adding 30 mM of ascorbate as described in Ref. 21. The reaction was stopped by the addition of 250 μl of Tris-HCl 3 M, pH 8.45. Following the reaction, proteins were concentrated in Centricon tubes (10 kDa cut-off) and purified from free pigments by ultracentrifugation in 15–40% glycerol gradient containing 0.06% β-DM and 10 mM HEPES-KOH, pH 7.5.

RESULTS

De-epoxidation in Vivo—Maize plants were exposed to high light intensity to induce de-epoxidation. Thylakoids from light stressed plants were isolated and fractionated into different Lhc complexes by preparative IEF (12). Fig. 1 shows the polypeptide composition of the different fractions as determined by SDS-PAGE. We obtained 12 different fractions ranging from a pI of 3.9–6.5. Although IEF did not allow purification of individual pigment-binding proteins, the distribution of each Lhc polypeptide among different fractions was determined by immunoblotting with specific antibodies. The reactions obtained with α-CP24, α-CP26, α-CP29, α-LHCI, and α-LHCII antibodies are also indicated in Fig. 1. The pigment composition of individual fractions was determined by HPLC analysis upon separation of free pigments from pigment-protein complexes by sucrose gradient ultracentrifugation (12). The violaxanthin and zeaxanthin levels in each fraction are shown in Fig. 2.

Fractions 1–4 contain only LHCII and have a low level of zeaxanthin (0.4–0.6 mol/100 mol of Chl *a*). It is interesting to observe that fractions enriched in Lhcb3 (fraction 1) have the highest level of zeaxanthin. These data suggest that Lhcb3 can bind zeaxanthin more efficiently than other LHCII components, although to a low level.

The fractions with the highest zeaxanthin content (0.9–1.3 mol/100 mol Chl *a*) were those with pI ranging from 4.2 to 4.5

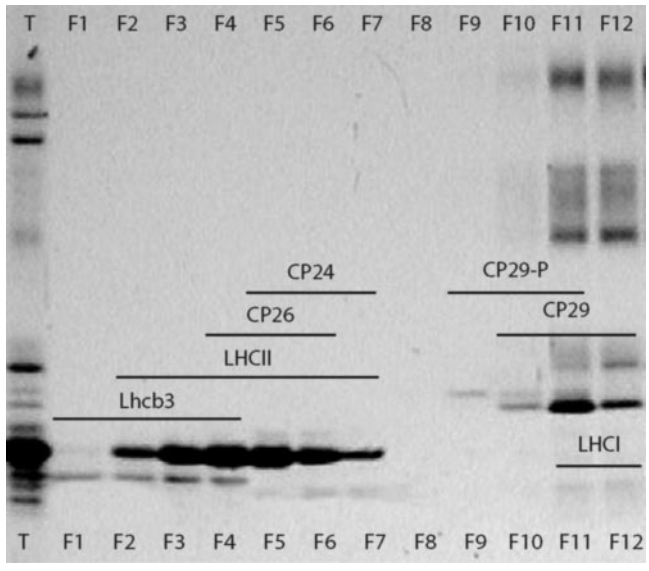

FIG. 1. **SDS-PAGE of fractions obtained from the IEF separation of *Z. mays* thylakoids.** *Lanes* are as follows: *T*, thylakoids; *F1-F12*, fractions 1–12. The *black lines* indicate the polypeptide presence as detected by reactions with specific antibodies.

and numbered from 5 to 7. These fractions contained LHCII, CP26, and CP24. Because fractions 1–4 contained only LHCII and showed a very low zeaxanthin content, we conclude that zeaxanthin is mostly bound to CP26 and CP24. Their enhanced zeaxanthin level is even more significant if we consider that LHCII is the most abundant component in these fractions. In fact, densitometric analysis of SDS-PAGE showed that CP26 and CP24 content in these fractions is in the range of 5–10% of the total protein.

Fraction 8 did not contain any polypeptide, and even immunoblotting with α-Lhc proteins could not detect any specific reaction, suggesting no Lhc proteins were present in this fraction. CP29 in its phosphorylated form is the only protein present in fraction 9 (22, 23). Zeaxanthin is present in this fraction at the level of 0.45 mol/100 mol of Chl *a*, similar to fractions 1–4 containing LHCII.

The remaining fractions (10–12) contained CP29 in its non-phosphorylated form and the PSI-LHCI complex. The level of zeaxanthin in these fractions was low (0.2 mol/100 mol of Chl *a*).

To obtain a better estimation of the zeaxanthin content of LHCI, we have purified the PSI-LHCI complex from CP29 by sucrose gradient. The zeaxanthin content of this preparation was 0.2 mol/100 mol of Chl *a*. Due to the presence of LHCI together with PSI core (see Fig. 1), which binds high amounts of Chl *a*, the actual content of zeaxanthin in LHCI proteins is probably underestimated.

Reconstituted in Vitro System to Examine Exchange of Violaxanthin for Zeaxanthin—Although the determination of the zeaxanthin content of individual Lhc proteins upon de-epoxidation *in vivo* is physiologically relevant, little information can be obtained on the biochemical factors controlling xanthophyll exchange. In fact, zeaxanthin is exchanged for violaxanthin, whose content in individual Lhc proteins ranges from 0.2 mol/polypeptide in Lhcb1 to 1.2 mol/polypeptide in Lhca1. Moreover, the accessibility of zeaxanthin-binding sites to newly formed zeaxanthin can be different depending on the aggregation state of individual Lhc proteins in the thylakoid supramolecular assemblies. To determine the intrinsic capacity of individual Lhc proteins to exchange violaxanthin for zeaxanthin, we have used a simplified reconstituted system (21) in which de-epoxidation is performed *in vitro* using a recombinant VDE

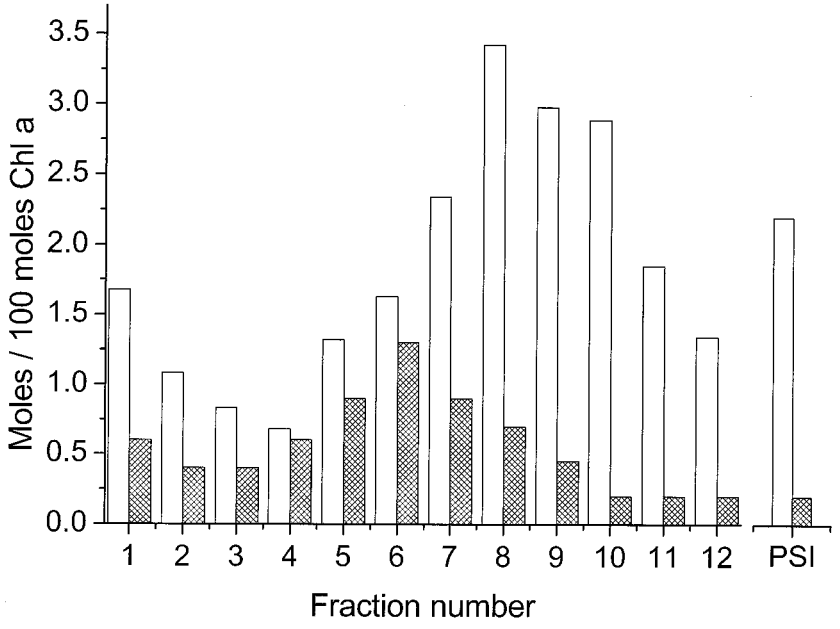

FIG. 2. **Violaxanthin and zeaxanthin content of fractions from IEF separation.** Violaxanthin (*empty bars*) and zeaxanthin (*filled bars*) content in different fractions is expressed in mol/100 mol of Chl *a*.

enzyme (16) expressed in *E. coli* and purified by affinity chromatography. As substrate we used recombinant Lhc proteins reconstituted *in vitro* from the apoprotein expressed in bacteria and purified pigments. To overcome the problem of a different content of violaxanthin and evaluate their specific exchange capacity, we have reconstituted the different Lhc proteins with violaxanthin as the only xanthophyll (24–26), thus obtaining recombinant proteins with a comparable xanthophyll composition.

Expression of Recombinant VDE in E. coli—Violaxanthin de-epoxidase from *A. thaliana* (16) was expressed in *E. coli* and purified by affinity chromatography. To increase the specific activity, this preparation was subjected to a denaturation/renaturation cycle by first treating with 6 M guanidine-HCl and, upon binding to a Ni^{2+} column, slowly diluting the guanidine-HCl with renaturation buffer. VDE obtained by this procedure showed 12 times higher activity (350 nmol of violaxanthin de-epoxidized min^{-1} mg $protein^{-1}$) with respect to the protein purified in the native state, suggesting that an inefficient folding had occurred in the bacterial host.

Reconstitution of Different Lhc Proteins with Violaxanthin—Seven different Lhc polypeptides were expressed in *E. coli* and reconstituted with violaxanthin as the only carotenoid: Lhcb1, Lhcb2, Lhcb3, Lhcb4 (CP29), Lhcb5 (CP26), Lhca1, and Lhca4. The pigment complement of different polypeptides, as obtained by HPLC and fitting of acetone extracts, is summarized in Table I. The xanthophyll content of different Lhc proteins ranged between 2 and 3 per polypeptide. The lower value was obtained in the case of CP29 (1.9 mol/mol of polypeptide), whereas Lhca1 yielded a value near 3 (2.8 mol/polypeptide). Lhcb3 protein bound ~2.4 violaxanthin/polypeptide. This latter value is clearly different from previous results obtained with Lhcb1 from *Z. mays* showing a value of 2.0 (24). The reason for this difference must be ascribed to the different gene product and strongly suggests that the affinity of individual binding sites for different xanthophyll species can vary between individual Lhcb gene products.

We then analyzed recombinant proteins reconstituted with violaxanthin as the only xanthophyll to assess whether the modification in xanthophyll composition did actually modify Lhc protein conformation. To this aim we compared the absorption and CD spectra of Lhc proteins reconstituted with the whole set of xanthophylls to those of the same complexes reconstituted with violaxanthin only. Differences were detected in the Soret range due to the direct absorption of xanthophylls; however, in the Qy range the absorption and CD spectra of Lhc proteins with violaxanthin only were essentially identical to those of the corresponding control Lhc protein (see CP26 and Lhca1, Fig. 3, *A–D*). The Qy absorption and CD spectra are an excellent probe of protein conformation because Chl absorption is modulated by each binding site to distinct energy levels and responds to conformational changes (23, 27). The observation that only very minor changes could be detected in this spectral region clearly shows that Lhc proteins reconstituted with violaxanthin only are representative of their control forms also binding lutein and neoxanthin. This is consistent with previous work with Lhcb1 and CP26 (24, 25).

TABLE I
Pigment composition of Lhc complexes reconstituted with violaxanthin

Pigment composition of Lhcb1, Lhcb2, Lhcb3, CP26, CP29, Lhca1, and Lhca4 reconstituted *in vitro* with violaxanthin as the unique carotenoid. All values are indicated as moles per polypeptide. The number of chlorophylls used for normalization was in Refs. 35, 41, 42, and 51. For Lhcb2 the same value of Lhcb1 was used.

	Lhcb1	Lhcb2	Lhcb3	CP26	CP29	Lhca1	Lhca4
Chl *a*/Chl *b*	1.5	1.4	1.9	2.3	2.9	4.2	2.6
Chl *a*	7.1	7.0	7.2	6.2	5.9	8.1	7.2
Chl *b*	4.9	5.0	3.8	2.8	2.1	1.9	2.8
Violaxanthin	2.5	2.1	2.7	2.2	1.9	2.8	2.2
Chl tot	12	12	11	9	8	10	10

De-epoxidation in Vitro—A reconstituted *in vitro* system for Lhc xanthophyll de-epoxidation was accomplished by mixing recombinant Lhc proteins with recombinant VDE plus MGDG and ascorbate, previously shown to be essential for VDE activity (28). Preliminary experiments were run at different temperatures and pH values by using the activity assay previously reported (29) with purified violaxanthin as a substrate rather than Lhc proteins. The de-epoxidation activity was strongly dependent on temperature with a 5-fold increase between 20 and 28 °C and also a sharp pH optimum at 5.2. The assay conditions were therefore set at 28 °C and pH 5.2 for 30 min when using Lhc-bound violaxanthin as a substrate with 60 μg/ml MGDG and 30 mM ascorbate as co-factors. These assay conditions were successful for all Lhc proteins eccept CP24, which was denatured by prolonged incubation at 28 °C, consistent with a previous report (30) of low stability of this

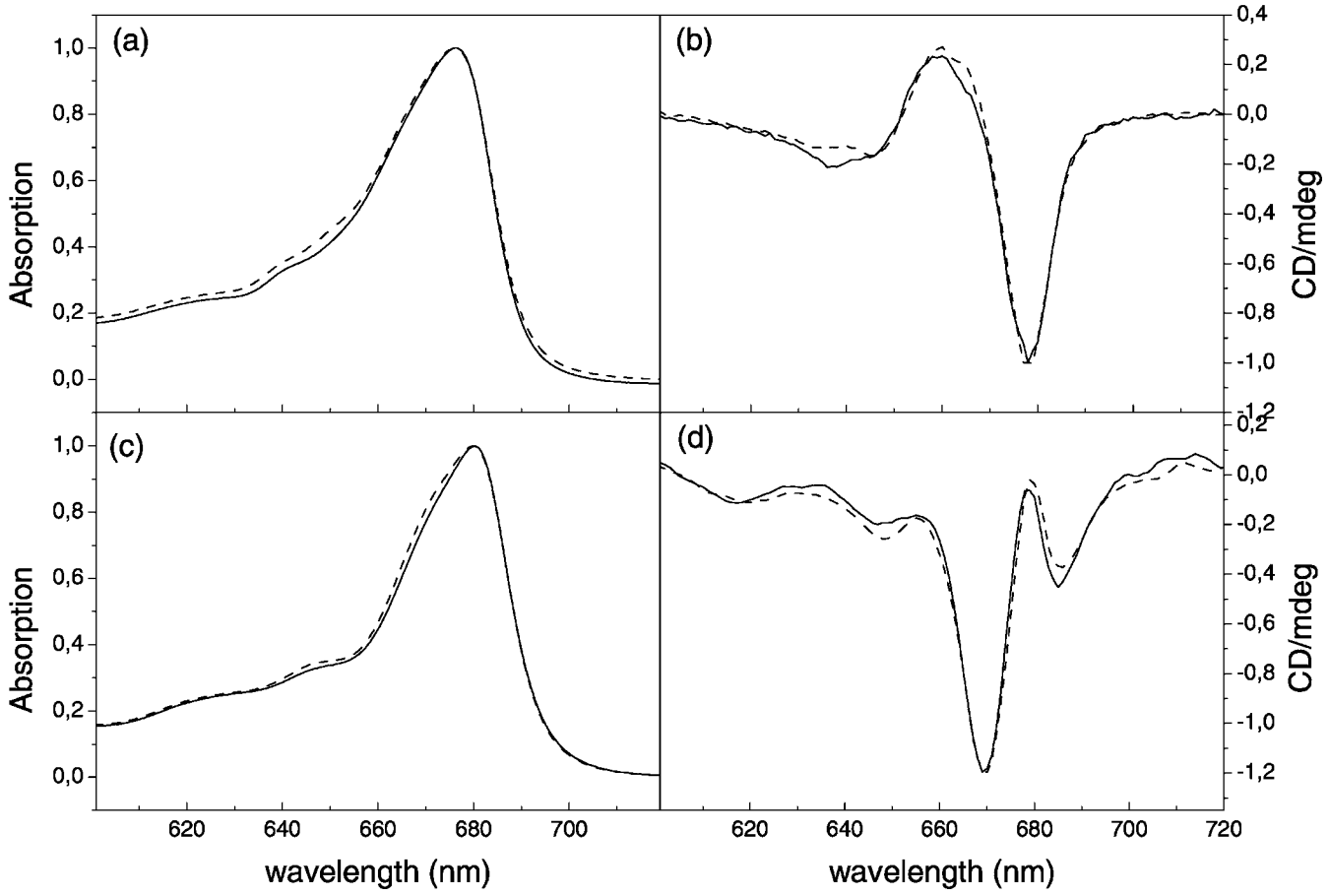

FIG. 3. **Spectral comparison of Lhc reconstituted with only violaxanthin and with a control carotenoid content.** Absorption and CD spectra of CP26 (*a* and *b*, respectively) and Lhca1 (*c* and *d*, respectively), control (*solid line*), and reconstituted with violaxanthin as the unique xanthophyll (*dashed line*) are shown.

pigment-protein complex both recombinant or purified from thylakoids.

Following incubation, Lhc complexes were re-purified by a glycerol gradient to separate pigments freed during the incubation from pigment-protein complexes. The pigment content of the two fractions was analyzed by HPLC and fitted to acetone extracts. In all cases, the free pigment fractions, obtained as a yellow-green band on the upper region of the gradient, showed the highest level of de-epoxidated xanthophylls, in particular antheraxanthin, implying the favored substrate for de-epoxidation was the freed xanthophyll in MGDG rather than the protein-bound form. Only traces of antheraxanthin were found to be protein-bound, suggesting that the affinity of the protein-binding sites was higher for either violaxanthin or zeaxanthin than antheraxanthin. The results in Fig. 3 show that all proteins are able to exchange violaxanthin with zeaxanthin, although the amplitude of the effect was very different depending on the gene product.

Among Lhc complexes, CP26 showed the highest level of zeaxanthin after 30′ of de-epoxidation (7.7 mol/100 mol of Chl *a*) as compared with the rest of the Lhcb proteins. In fact, CP29, Lhcb1, Lhcb2, and Lhcb3 had reduced levels of zeaxanthin after incubation under the same conditions (0.8–2.2 mol zeaxanthin/100 mol of Chl *a*). Longer periods of incubation led to a decrease in differences between the individual Lhc proteins when the reaction approached saturation (90′-120′). An interesting result was obtained with Lhca1 and Lhca4 proteins, which showed a violaxanthin-to-zeaxanthin exchange efficiency comparable with CP26 (4.1 and 6.0 mol zeaxanthin/100 mol of Chl *a*, respectively).

The control samples were incubated in the same conditions but without the enzyme. In Fig. 4 we show the CP26 results because it was the most efficient protein in violaxanthin exchange. After 30′ incubation at 28 °C, pH 5.2, without VDE, the complex was purified by gradient ultracentrifugation. Even in the absence of the enzyme, the content in violaxanthin was reduced, although to a lower extent with respect to the sample incubated with VDE (6.3 mol/100 mol of Chl *a* *versus* 8.7 mol/100 mol of Chl *a*). If zeaxanthin was added in excess (1 μg/ml) to the mixture at the beginning of the incubation, the total xanthophyll content did not decrease; however, part of the violaxanthin was substituted by zeaxanthin. These data indicate that xanthophyll exchange at low pH does not depend on the presence of the VDE enzyme but only on the xanthophylls present in the reaction mixture. Similar results were obtained with Lhca4. In this case when zeaxanthin was supplied in the reaction mixture in the absence of VDE enzyme, it was bound by the protein to a level of 18 mol/100 mol of Chl *a*.

In a second experiment, Lhca4 was incubated with an excess of zeaxanthin at neutral pH (7.5). In this case, the exchange level was ~3 times lower than at pH 5.2 (5.5 *versus* 18 mol of zeaxanthin bound/100 mol of Chl *a*). Consistently, the level of violaxanthin that remained bound to the Lhca4 protein after incubation with zeaxanthin was 16 and 27 mol/100 mol of Chl *a*, at pH 5.2 and pH 7.5, respectively.

A further observation on the effect of the incubation of Lhc proteins was that the Chl *a*/*b* ratio decreased upon incubation in the reaction medium (Table II). Either a loss of chlorophyll *a* or the gain of chlorophyll *b* can explain this effect. The latter hypothesis seems unlikely in this *in vitro* system in which

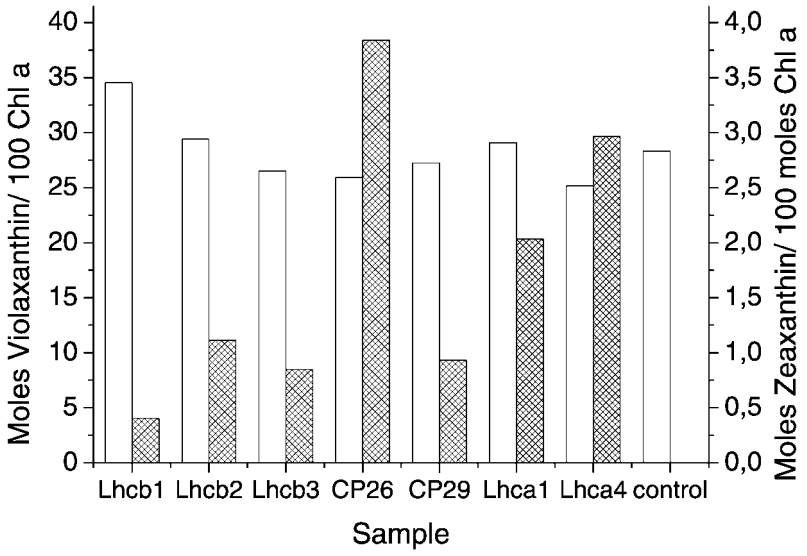

FIG. 4. **Violaxanthin and zeaxanthin content of different Lhc complexes after the de-epoxidation *in vitro*.** Violaxanthin (*empty bars*) and zeaxanthin (*filled bars*) content in different Lhc proteins is expressed in mol/100 mol of Chl *a*.

TABLE II

Pigment composition of Lhc complexes after de-epoxidation in vitro

Values are normalized following the hypothesis of one Chl is lost during the reaction. Difference values are obtained from normalized pigment binding data of Lhc complex before (Lhc-Vx, Table 1) and after the reaction (Lhc-Depox). All values are indicated as moles per polypeptide.

	Lhc complexes after de-epoxidation							
	Lhcb1	Lhcb2	Lhcb3	CP26	CP29	Lhca1	Lhca4	Control
Chl *a*/Chl *b*	1.3	1.3	1.9	1.7	2.5	4.2	2.0	1.8
Chl *a*	6.2	6.3	6.5	5.0	5.0	7.3	6.0	5.2
Chl *b*	4.8	4.7	3.5	3.0	2.0	1.7	3.0	2.8
Violaxanthin	2.1	1.8	1.7	1.3	1.4	2.1	1.5	1.5
Zeaxanthin	0.0	0.1	0.1	0.4	0.1	0.3	0.4	0.0
Chl tot	11	11	10	8	7	9	9	8
	Difference: (Lhc-Vx) − (Lhc-Depox)							
Chl *a*	0.9	0.7	0.7	1.2	0.9	0.8	1.2	1.1
Chl *b*	0.1	0.3	0.3	−0.2	0.1	0.2	−0.2	−0.1
Violaxanthin	0.4	0.2	1.0	0.9	0.5	0.7	0.7	0.7

excess Chl *b* is not available, and the alternative hypothesis of a loss of Chl *a* is also supported by an increase of the Chl *a*/*b* ratio in the free pigment fraction after the de-epoxidation (not shown). Because the Chl *a*/*b* ratio did not change upon incubation at pH 7.5, we suggest that the low pH treatment was necessary not only for xanthophyll exchange but also for the loss of Chl *a*. When normalized to the Chl-to-protein stoichiometry of the individual Lhc proteins, the amplitude of Chl *a* release corresponded to one mol/mol of polypeptide (1.0 ± 0.1 Chl *a*/Lhc polypeptide, Table II).

DISCUSSION

In this work we analyzed the de-epoxidation of violaxanthin to zeaxanthin and its binding to Lhc proteins *in vivo* and *in vitro*. The integration between these two approaches should allow both to identify the relative level of involvement of the different Lhc proteins into the xanthophyll cycle mechanism and to determine to what extent this is determined by the structure of individual gene products *versus* other factors such as the accessibility of Lhc to the enzyme. The first approach consisted of the exposure of maize seedlings to excess light, a condition that activates VDE and induces accumulation of zeaxanthin in the thylakoid membrane (31, 32). The treatment was for 30′ to obtain sufficient levels of de-epoxidation. Moreover, 30 min of illumination with saturating light allows the saturation of excitation energy (NPQ) (not shown). Shorter treatments yielded essentially the same results, although the extent of de-epoxidation and the level of accumulation in individual Lhc proteins was lower. Nevertheless, the distribution of zeaxanthin was essentially the same as detected following illumination for 30′. De-epoxidation *in vitro* was also carried out for 30′. Previous work (21) showed that saturation of zeaxanthin incorporation into Lhcb1 protein is attained at ~70 min of reaction at 28 °C. From preliminary experiments, we chose 30′ incubation to efficiently detect the differences between individual Lhc proteins and at the same time obtain significant levels of de-epoxidation.

The major physiological mechanism in which the xanthophyll cycle has been involved so far is the thermal dissipation of NPQ, which is thought to be devoted mainly to protection of PSII from photoinhibition (8).

The Minor Antenna Complexes—Data from de-epoxidation *in vivo* showed that zeaxanthin binds mainly to CP26 and CP24. This is confirmed also by *in vitro* experiments where CP26 is the Lhc protein that shows the highest rate of violaxanthin exchange with zeaxanthin. In the case of CP24, we only have data from *in vivo* experiments because recombinant CP24 showed to be unstable at assay conditions in agreement with previous reports (30) with both the native and recombinant proteins. Nevertheless, the finding of high zeaxanthin in fraction 7 of the IEF separation, where there is no CP26 (as detected by specific antibodies), suggests that CP24 can exchange violaxanthin with zeaxanthin at a similar rate to CP26. It is possible that longer treatments may induce even higher zeaxanthin content in CP24 due to the fact that in CP24 neoxanthin is absent and is substituted by corresponding amounts of violaxanthin (30, 33). It is worth noting that selective depletion of CP26 (34) in transgenic tobacco led to the alteration of the xanthophyll cycle and inhibition of energy dissipation under stress conditions.

CP29 was well separated by the IEF procedure. In fraction 9 we only find phosphorylated CP29, which binds little zeaxanthin. It has been previously shown that phosphorylation does not affect the pigment binding properties of CP29 (23). Data from *in vivo* and *in vitro* experiments consistently show that CP29 has a low capacity for zeaxanthin binding, similar to Lhcb2 and Lhcb3, at least in the present experimental conditions. This result is somewhat surprising because of the high similarity between CP29 and CP26. These two proteins bind a similarly low number of chlorophylls (35), show a similar distribution of xanthophylls among binding sites with lutein in L1 and violaxanthin/neoxanthin in L2 (36), and can both be refolded *in vitro* with zeaxanthin inducing fluorescence quenching (9, 25). These characteristics of CP26 and CP29 suggest

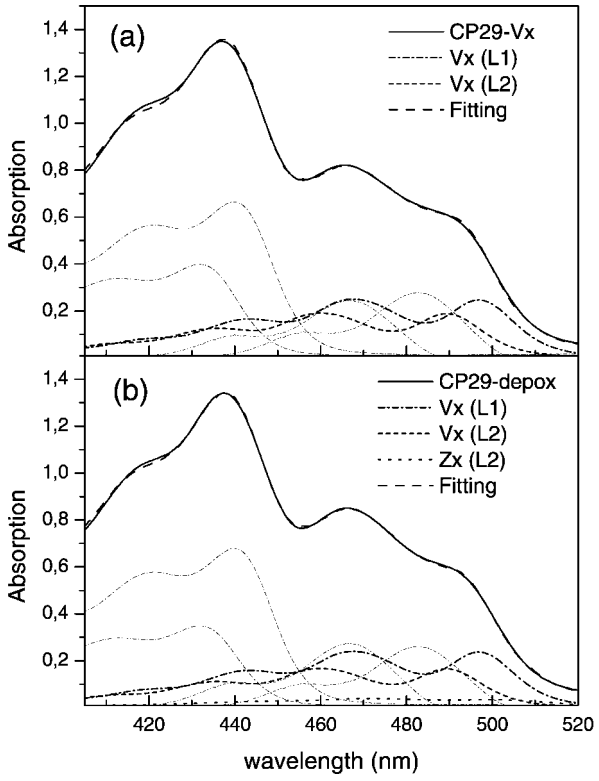

FIG. 5. **Spectral reconstruction of CP29 before and after the de-epoxidation *in vitro*.** Spectral deconvolution of *CP29-Vx* (*a*) and *CP29-depox* (*b*) is shown. Also, Chl *a* (*dash dotted*) and Chl *b* (*dash dot dotted*) forms are indicated.

that the major difference between the two proteins is the capacity of exchanging violaxanthin *versus* zeaxanthin in site L2. A possible physiological significance of this difference is that CP26 and CP29 might be involved in short *versus* long-term acclimation to excess light. Protonable residues have been detected as dicyclohexylcarbodiimide-binding sites in both CP29 and CP26 although in different domains of the protein (37, 38). Because violaxanthin-to-zeaxanthin exchange occurs in conditions of low lumenal pH *in vivo* and *in vitro*, it might be that the distribution of DCCD-binding sites exposed to the lumenal surface of Lhc proteins controls the xanthophyll exchange rate. DCCD binding has been recently reported for PsbS (39), a Lhc-like protein whose deletion in *Arabidopsis* strongly decreased the capacity for light-induced thermal dissipation (40). Because PsbS was shown to be unable to bind pigments in a stable manner, the function of protonable residues in this protein might induce a conformational change. Such conformational change could then be transferred to neighbor Lhc proteins, inducing transition to the quenched conformation (1).

The Major LHCII Antenna Complex—Analysis of pigment binding data of the IEF fractions containing LHCII only show that this trimeric complex can exchange violaxanthin for zeaxanthin, although to a lower extent with respect to CP26 and CP24. The study of recombinant Lhcb1, Lhcb2, and Lhcb3 gene products *in vitro*, however, indicates that the three components of LHCII differ in their capacity for binding zeaxanthin, Lhcb2 and Lhcb3 scoring better with respect to Lhcb1. This is consistent with the high zeaxanthin level in IEF fraction 1, containing almost pure Lhcb3. Lhcb3 was shown to contain a low energy Chl *a* ligand (Chl *a*, 686 nm), which is absent in Lhcb1, making it a local sink for excitation energy in a trimeric LHCII complex (41). The higher zeaxanthin exchange rate in Lhcb3 might be strategic for the control of the lifetime of excited states in the whole trimeric LHCII complex.

The PSI-LHCI Complex—Analysis *in vivo* shows that zeaxanthin is bound to the PSI-LHCI complex at a level of 0.2 mol/100 mol of Chl *a*. Xanthophylls are only bound to the LHCI moiety, which accounts for 34% of Chl *a* in the PSI-LHCI complex.[2] We can thus estimate a zeaxanthin content of ~0.6 mol/100 mol of Chl *a* in LHCI. This is consistent with the high level of zeaxanthin binding by Lhca1 and Lhca4 *in vitro*, considering we have assayed monomeric Lhca complexes, whereas LHCI is dimeric *in vivo* (43, 44). Oligomerization has been suggested to decrease the capacity for xanthophyll exchange in trimeric LHCII (21), but the finding of zeaxanthin in LHCI upon both *in vivo* and *in vitro* experiments suggests that xanthophyll exchange involves both PSI and PSII, confirming previous results with *Vinca major* (45).

Mechanism of De-epoxidation—The *in vitro* analysis of de-epoxidation also provides useful information about the mechanism of de-epoxidation of violaxanthin bound to Lhc proteins. It can be asked whether de-epoxidation occurs on violaxanthin still bound to Lhc proteins or in a free xanthophyll pool. The control samples incubated in the absence of VDE underwent the loss of a fraction of its bound violaxanthin, which might be the actual substrate for the reaction. This hypothesis is supported by two findings: first, the incubation of violaxanthin containing complexes with free zeaxanthin in the absence of VDE yielded incorporation of zeaxanthin into Lhc proteins and second, the capacity of individual Lhc proteins to release violaxanthin in the medium when incubated in the absence of VDE is related to the violaxanthin-to-zeaxanthin exchange capacity during VDE reaction.

We conclude that de-epoxidation occurs in a free-pigment pool dissolved in MGDG. This can explain the early finding that in the *Chlorina f2* mutant of barley (lacking Lhc proteins) de-epoxidation in high light occurs faster and to a higher final level than in wild type (46). The limiting steps of the reaction of violaxanthin-to-zeaxanthin exchange in Lhc proteins, therefore, are the liberation of violaxanthin from and the rebinding of zeaxanthin to their binding sites.

It is worthwhile to emphasize the pH-dependence of the xanthophyll exchange process. Excess light conditions lead to low lumenal pH, which is known to activate VDE. Our findings suggest that Lhc proteins might be the targets of an independent effect of lumenal pH, thus regulating their xanthophyll exchange capacity. Further studies are needed to assess the details of pH-dependence for individual Lhc proteins and also to verify the role of individual protonable residues in mediating the pH effect. However, it appears that pH-dependence might reflect conformational changes whose immediate effect is detected here as the efficiency of xanthophyll exchange but might also affect other properties of Lhc proteins such as their fluorescence yield. Such a hypothesis would be consistent with the residual level of NPQ detected in *npq1* and *npq2* mutants in which the xanthophyll cycle is disrupted (47).

Carotenoid Exchange Involves the Violaxanthin in One Site—Only a fraction of the Lhc-bound xanthophyll could be exchanged, in agreement with previous results with Lhcb1 (21) and with the previously described (48) limited availability of the violaxanthin substrate for de-epoxidation. We have further analyzed the changes in the spectral properties of Lhc proteins upon de-epoxidation *in vitro* to assess the role played by individual xanthophyll-binding sites in the exchange. This is possible because of the different tuning of xanthophyll optical transition energy by the binding to different sites (20, 49). In the simple case of CP29, for example, it was possible to assess

[2] Croce, R., Morosinotto, T., Castelletti, S., Breton, J., and Bassi, R. (2002) *Biochim. Biophys. Acta* **1556/1,** 29–40.

TABLE III

Carotenoid spectral forms identified in CP29 before (CP29-Vx) and after (CP29-depox) the de-epoxidation

Amplitude and shift of the spectral forms of xanthophylls bound in different sites, as identified by spectral deconvolution, is shown.

	Violaxanthin (L1)		Violaxanthin (L2)		Zeaxanthin (L2)		Total
	Amplitude	Shift	Amplitude	Shift	Amplitude	Shift	Amplitude
CP29-Vx	0.93	24	0.97	17			1.9
CP29-depox	0.82	24	0.54	17	0.1	17	1.46

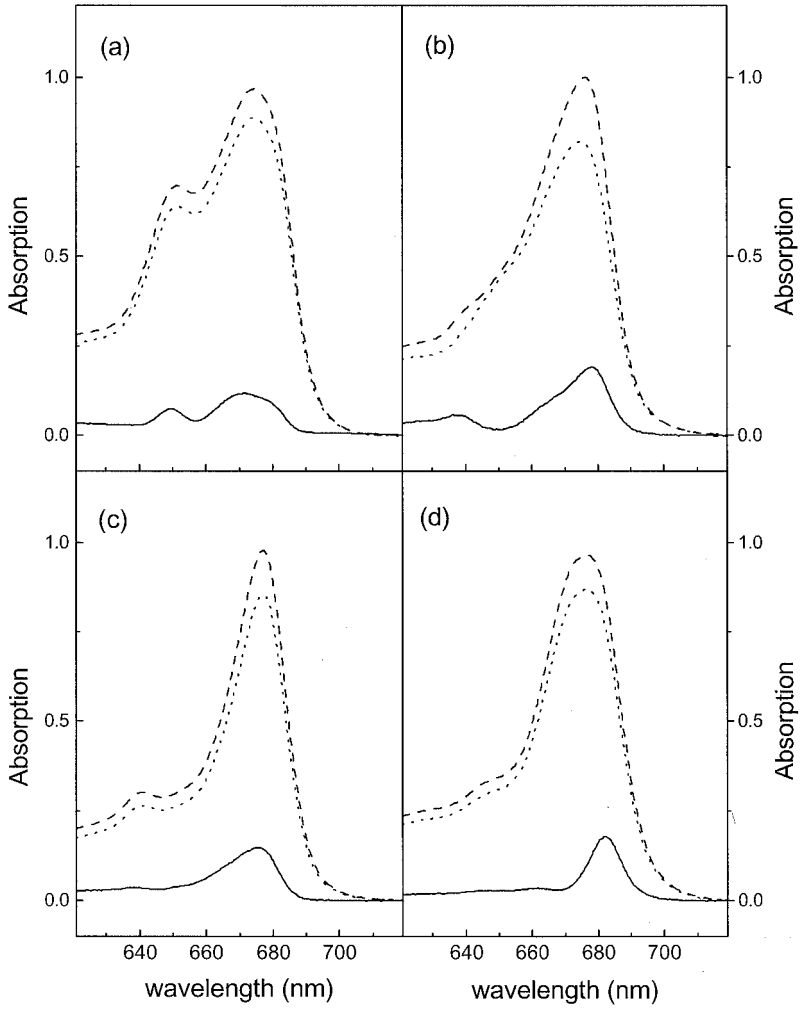

FIG. 6. **Spectroscopic differences upon de-epoxidation *in vitro*.** Difference spectra (*solid line*) of Lhcb1 (*a*), CP26 (*b*), CP29 (*c*), and Lhca1 (*d*) before (*dashed line*) and after (*dotted line*) the de-epoxidation are shown. Spectra were normalized to the number of chlorophylls as in Ref. 42.

that binding of violaxanthin to site L1 or site L2 yielded a red-shift of 24 or 17 nm, respectively, with respect to the absorption of the pigment in 80% acetone (20).[2] Fig. 5*A* shows such a deconvolution. It is worth noting that the spectral contributions closely fit the chromophore stoichiometry determined biochemically; in particular, two different spectral forms of violaxanthin with similar amplitude corresponding to the pigment bound to either site L1 or L2 (Table III). After de-epoxidation *in vitro*, the absorption spectrum was analyzed by the same method, but the spectral form of zeaxanthin was also included (Fig. 5*B*). Table III shows the amplitude of carotenoid absorption forms resulting from the analysis of CP29 spectra before and after the de-epoxidation. The amplitude of violaxanthin adsorbing at 489.8 nm was reduced with respect to the 497-nm form, suggesting the former is the species preferentially replaced by zeaxanthin. Consistently, a zeaxanthin form with a 17-nm red-shift was obtained, implying the newly incorporated zeaxanthin is located in site L2.[3] Similar results were obtained with other Lhc proteins, showing that one violaxanthin spectral form with characteristics consistent with binding to site L2 was preferentially reduced with respect to the others. These data strongly suggest that violaxanthin *versus* zeaxanthin exchange occurs in site L2 in all Lhc proteins analyzed. Our hypothesis is also supported by previous data on Lhcb1 showing that site L1 occupancy is fundamental for protein stability (10), whereas Lhc proteins with an empty L2 site maintain their folding (27).

Xanthophyll Exchange Involves the Chl a in Site a4—One additional issue emerging from experiments *in vitro* is the involvement of Chls in xanthophyll exchange. In fact, all complexes show the loss of a Chl *a* chromophore upon de-epoxidation. To verify whether or not the lost chromophore was derived from a particular binding site, we calculated difference spectra in the Qy band where Chl chromophores exhibit fine tuning of their S0-S1 transition energies depending on the particular binding site (27, 50). Examples of the difference spectra obtained in the case of Lhcb1, CP29, CP26, and Lhca1 are shown in Fig. 6. For all seven Lhc proteins analyzed, the lost chromophore absorbed at wavelengths between 675 and 682 nm.

[3] R. Croce, M. Gastaldelli, G. Canino, and R. Bassi, unpublished observations.

These results can be compared with the results obtained by mutation analysis of Lhcb1 and CP29 in which the absorption of individual chromophores was determined (27, 50) to identify the binding site made empty during the process of xanthophyll exchange. Results are consistent with the loss of Chl *a* in site a4, whose absorption is tuned at 676 and 673/681 nm, respectively, in CP29 and Lhcb1 (27, 50). Structural data from LHCII (51) shows that the Chl in site a4 is in close vicinity with the xanthophyll in site L2, thus allowing the hypothesis that occupancy of site a4 might affect the rate of xanthophyll exchange in site L2. The ligand of Chl a4 is a conserved glutamate in all Lhc complexes (4), and this acidic group has a p*K* of 4.28, so it is possible that in acidic conditions it can be protonated and lose the ability to coordinate the Mg^{2+} of chlorophyll. Proton flow though Lhc proteins was previously proposed to be activated in conditions of low lumenal pH that also lead to de-epoxidation (52). We do not think that Chl is actually freed in the membrane during operation of the xanthophyll cycle; however, a transient/partial disconnection of Chl a4 from its binding site cannot be excluded and might be involved in the mechanism of xanthophyll exchange.

CONCLUSION

In this study we have analyzed the phenomenon of the exchange of xanthophyll chromophores bound to Lhc proteins during the operation of the xanthophyll cycle *in vivo* and *in vitro*. The results show that CP26 and CP24 are the components of the PSII supercomplex that exhibit the highest rate of xanthophyll exchange. We found that xanthophyll are specifically exchanged in the L2 site, one of the 2/3 tight xanthophyll-binding sites found in Lhc proteins. This site was previously found not to be essential for Lhc protein folding but rather to be an allosteric binding site affecting the fluorescence yield of Lhc proteins (10) by controlling the equilibrium between conformations characterized by different fluorescence yield and thus having conservative *versus* dissipative characteristics with respect to the excitation energy (1). Analysis *in vitro* shows that the xanthophyll exchange occurs through the intermediate release of violaxanthin and rebinding of newly formed zeaxanthin in agreement with previous results (21). The exchange rate is thus determined by the characteristics of individual Lhc proteins that is, in turn, determined by the effect of low pH on protein structure. This determines a dynamic distribution of violaxanthin *versus* zeaxanthin in different subunits of photosystem II complexes that might regulate the excitation energy flow in PSII antenna to prevent over-excitation of reaction centers and photoinhibition. The present findings are consistent with recent results (7) showing the presence of a loosely bound violaxanthin pool in the major LHCII antenna protein, which is available for de-epoxidation. The xanthophyll cycle thus appears to have the characteristics of a signal transduction pathway for the light stress signal, constituted by low lumenal pH, activating VDE, and synthesizing a messenger molecule, zeaxanthin, which diffuses in the thylakoid membrane and affects the functional characteristics of Lhc proteins, CP26 and CP24, mediating excitation energy transfer from the major LHCII antenna to the PSII reaction center.

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Dynamics of Chromophore Binding to Lhc Proteins *in Vivo* and *in Vitro* during Operation of the Xanthophyll Cycle

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