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Xanthophyll Binding Sites of the CP29 (Lhcb4) Subunit of Higher Plant Photosystem II Investigated by Domain Swapping and Mutation Analysis*

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The binding sites for xanthophylls in the CP29 antenna protein of higher plant Photosystem II have been investigated using recombinant proteins refolded *in vitro*. Despite the presence of three xanthophyll species CP29 binds two carotenoids per polypeptide. The localization of neoxanthin was studied producing a chimeric protein constructed by swapping the C-helix domain from CP29 to LHCII. The resulting holoprotein did not bind neoxanthin, confirming that the N1 site is not present in CP29. Neoxanthin in CP29 was, instead, bound to the L2 site, which is thus shown to have a wider specificity with respect to the homologous site L2 in LHCII. Lutein was found in the L1 site of CP29. For each site the selectivity for individual xanthophyll species was studied as well as its role in protein stabilization, energy transfer, and photoprotection. Putative xanthophyll binding sequences, identified by primary structure analysis as a stretch of hydrophobic residues including an acidic term, were analyzed by site-directed mutagenesis or, in one case, by deleting the entire sequence. The mutant proteins were unaffected in their xanthophyll composition, thus suggesting that the target motifs had little influence in determining xanthophyll binding, whereas hydrophobic sequences in the membrane-spanning helices are important.

Carotenoids are involved in many aspects of higher plants photosynthesis. Reaction center complexes bind β-carotene for light harvesting, chlorophyll *a* (Chl *a*)[1] triplet quenching, and electron transport between cytochrome b_{559} and $P680^+$ (1, 2). In the peripheral antenna, Lhc proteins bind a number of xanthophyll species, namely lutein, neoxanthin, and violaxanthin, acting in the harvesting of light and transfer of excitation energy to Chls (3–6). The photoprotection function of xanthophylls is accomplished through multiple mechanisms including the quenching of Chl *a* triplet states, scavenging of singlet oxygen produced by the reaction of Chl triplets with O_2, and participation to non-photochemical quenching, a mechanism in which violaxanthin is de-epoxidated to zeaxanthin and excess energy is dissipated into heat (7). In addition, xanthophylls are essential for Lhc protein folding (8), whereas their binding to a specific allosteric site controls the transition between dissipative and conservative protein conformations (9, 10).

The reason why Lhc proteins require a number of xanthophyll species while reaction center proteins only require β-carotene is not completely understood; these carotenoid species have very similar physico-chemical properties, enabling, in each case, efficient light harvesting, triplet quenching, and singlet oxygen scavenging (11). Nonetheless, the pigment composition is one of the most conserved traits in higher plants, suggesting a specific function for each carotenoid species. The best known xanthophyll binding protein is the major light harvesting complex of Photosystem II (LHCII) for which four distinct binding sites have been reported to be bound into distinct domains of the protein. Sites L1 and L2 intersect the helix A/helix B cross-domain in the center of the Lhc structure (12). L1 is selective for lutein, whereas L2 can also bind violaxanthin (13). Site N1 is highly selective for neoxanthin and is located within the C helix domain of LHCII (14). Finally, a low affinity binding site has been named V1 after its major ligand (violaxanthin) in low light conditions (15, 16). Each binding site was shown to play a distinct functional role; structure stabilization and Chl *a* triplet quenching are provided by lutein in site L1 only (10, 17). Site V1 is not involved in singlet nor in triplet energy transfer and was suggested to accommodate a pool of readily available substrate molecules for the violaxanthin deepoxydase enzyme (16), whose product, zeaxanthin, can then be bound to the allosteric site L2 (18). Site N1 is active in light harvesting and singlet oxygen scavenging (13) and stabilizes the long lifetime conformation of LHCII (19).

Despite the high homology in the transmembrane regions, which suggests a similar folding, the number of xanthophyll binding sites, their selectivity, and their occupancy in the structure seem to be different in each Lhc gene product. Considering the different roles of individual sites and the complexity of the Lhc multigene protein family, it is possible that the function of individual Lhc gene products is largely determined by the presence/absence of individual xanthophyll sites and by their selectivity and strength.

Primary sequence analysis of structural determinants for xanthophyll binding sites has been less successful than in the case of Chl binding sites, possibly due to the contribution of neighbor Chls rather than of amino acid side chains, to the formation of binding pocket for xanthophylls (13, 14). Never-

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[1] The abbreviations used are: Chl, chlorophyll; Car, carotenoid; Lhc, light-harvesting complex; L, lutein; LD, linear dichroism; N, neoxanthin; V, violaxanthin; WT, wild type; PSII, Photosystem II; HPLC, high performance liquid chromatography; CTL, control.

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LHCII      ---RKTAAKAKPAAASGSPWYGP-DRVLYLGPLSGEPPSYLTGEFPGDYG
chimera    ---RKTAAKAKPAAASGSPWYGP-DRVLYLGPLSGEPPSYLTGEFPGDYG
CP29       RFGFGLGGKAKPAPKKVAKTSTSSDRPLWFPGAVA--PDYLDGSLVGDYG

LHCII      WDTAGLS-----------ADPETFAKN----------------------
chimera    WDTAGLS-----------ADPETFAKN----------------------
CP29       FDPFGLGKPVEYLQFELDSLDQNLAKNEAGGIIGTRFESSEVKSTPLQPY

LHCII      ---------RELEVIHSRWAMLGA-LGCVFPELLARNGVKFGEAVWFKAG
chimera    ---------RELEVIHSRWAMLGARLGALSVEWLT--G-----VTWQDAG
CP29       SEVFGLQRFRECELIHGRWAMLAT-LGALSVEWLT--G-----VTWQDAG

LHCII      SQIFSEGGLDYLGNPSLIHAQSILAIWACQVVLMGAVEGYRIAGGPLGEV
chimera    KVELVDG-SSYLGQP---LPFSISTLIWIEVLVIGYIEFQRNAELDP---
CP29       KVELVDG-SSYLGQP---LPFSISTLIWIEVLVIGYIEFQRNAELDP---

LHCII      VDPLYPGG-SFDPLGLADDPEAFAELKVKELKNGRLAMFSMFGFFVQAIV
chimera    VDPLYPGG-SFDPLGLADDPEAFAELKVKELKNGRLAMFSMFGFFVQAIV
CP29       EKRLYPGGSYFDPLGLAADPEKKERLQLAEIKHARLAMVAFLGFAVQAAA

LHCII      TGKGPLENLADHIADPVNNNAWAYATNFVPGK
chimera    TGKGPLENLADHIADPVNNNAWAYATNFVPGK
CP29       TGKGPLNNWATHLSDPLHTTIFDTFGGSSK--
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FIG. 1. **Sequence comparison between the chimeric complex, LHCII, and CP29.** The *bold* indicates the region of LHCII and CP29, which are present in the chimera. In the CP29 sequence, the putative carotenoid binding sequences are underlined.

theless, four short sequences, located in the hydrophilic domains and consisting into four hydrophobic amino acids and a charged residue, could be involved in the binding by forming a hydrophobic pocket hosting xanthophylls end-rings, whereas the charged side chain was supposed to interact with oxygenated ring substituents (20). Alternatively, binding might be performed by interactions between hydrophobic residues with the polyene chain located deeply in the membrane as observed in the bacterial LH2 complex (21). In this study, we report the results of a detailed analysis of the chlorophyll *a/b*/xanthophyll protein CP29 (Lhcb4) with respect to the xanthophyll stoichiometry, location, and function as well as of the sequence determinants involved in their interaction with the polypeptide chain.

EXPERIMENTAL PROCEDURES

DNA Construction and Mutation of CP29—CP29-WT and mutants were obtained as reported in Bassi *et al.* (17). The chimeric complex was build using two restriction sites, *Bss*hII and *Sal*I, which are present in the nucleotide sequence of Lhcb1 gene of *Zea mays*. These sites are, respectively, upstream and downstream from the region coding for helix C portion of LHCII apoprotein. The region between these sites was changed with the corresponding sequence of CP29 cloned using two primers (GGGGCGCGCGCCTCGGCGCCCTCTCCGTCGAG and GGGGGGTCGACCGGGTCGAGCTCGGCGTTGCG) in which there are the same restriction sites. In Fig. 1 the sequence of the chimeric complex is presented together with the sequences of LHCII and CP29. The apoproteins were overexpressed and isolated as reported in Bassi *et al.* (17).

Reconstitution and Purification of CP29-WT, Mutants, and Chimera-Pigment Complexes—Complex reconstitution and purification procedures were performed as described in Giuffra *et al.* (22) but using for CP29 a chlorophyll *a/b* ratio of 4.5. The chimera was reconstituted in the same conditions using a chlorophyll *a/b* ratio of 4.5 as CP29 and 2.3 as LHCII and the full carotenoid complement present in the thylakoid membrane. Samples with different carotenoid complement were reconstituted in the same way but with a changed xanthophyll composition in the pigment mixture. Native CP29 was purified from *Z. mays* as reported in Croce *et al.* (23).

Pigment Analysis—The pigment complement of the holoprotein was analyzed by HPLC (24) and fitting of the acetone extract with the spectra of the individual pigments (25).

Spectroscopy—Absorption spectra were measured by an SLM-Aminco DW-2000 spectrophotometer at room temperature. Fluorescence excitation and emission spectra were obtained using a Jasco-FP-777 spectrofluorimeter. Circular dichroism (CD) spectra were recorded at 10 °C with a Jasco 600. Samples were in 10 mM Hepes, pH 7.6, 0.06% β-D-dodecylmaltoside, 20% glycerol. Chlorophyll concentration was ≈10 μg/ml for CD and absorption measurements and 0.01 μg/ml for fluorescence measurements. LD spectra are the same reported in Simonetto *et al.* (26). Photobleaching kinetic was measured as described in Formaggio *et al.* (10).

Stability Measurements—The stability of the complexes was determined after the decrease of the CD signal at 490 nm induced by the temperature. A temperature range between 20 and 80 °C was used. The temperature was changed continuously by 1 °C/min. The thermal stability of the protein was determined by finding the $t_{1/2}$ of the signal decay.

Data Analysis—Deconvolution of spectra in the Soret (350–550-nm wavelength) range was performed as previously described (27) using a homemade program. Energy transfer efficiency from Cars to Chls was estimated from the ratio of contributions in fluorescence excitation with respect to absorption of individual pigment pools. In all the samples the energy transfer from Chl *a* was normalized to 100%.

RESULTS

The first step of this work consisted into the determination of the number of carotenoids bound to each CP29 polypeptide. To this end, the complex was purified by preparative isoelectrofocalization from *Z. mays* grana membrane as previously reported (28). The pigments were extracted with 80% acetone, and the absorption spectrum is shown in Fig. 2. The spectra of individual pigments were used for reconstructing the spectrum of the acetone extract from two hypothetical CP29 complexes binding 8 Chls (6 Chl *a* plus 2 Chl *b*) (17, 29) and either 2 or 3 xanthophylls in the ratio shown by HPLC analysis: 0.85 lutein, 0.5 neoxanthin, and 0.65 violaxanthin (30). The "synthetic" spectra are shown in Fig. 2 together with the experimental spectrum. An almost perfect fit was obtained considering two Cars per polypeptide, whereas the hypothesis of three Cars was discarded.

Reconstitution of Recombinant CP29 with Different Xanthophyll Species—Although the Car to polypeptide stoichiometry in CP29 is 2:1, three xanthophyll species were found in the pigment-protein complex, indicating promiscuity of at least one of the binding sites. To investigate the affinity of the binding sites for the different xanthophylls, recombinant CP29 apoprotein, overexpressed in *Escherichia coli*, was reconstituted in the presence of individual carotenoids or a combination of two. Stable complexes were obtained with all xanthophylls alone except neoxanthin, similar to what was observed in LHCII and CP26 (13, 25, 31).

The pigment composition of the reconstituted products was analyzed by HPLC and fitting of the acetone extracts. The results are reported in Table I. All samples showed a Chl/Car ratio around 4.0 implying two xanthophylls per polypeptide. The complex reconstituted with zeaxanthin was the only exception; although a complex with two zeaxanthin molecules per polypeptide was obtained (CP29-Zb) in some experiments, in other preparations a single *Zea* molecule was found (CP29-Za). Neoxanthin in the complex never exceeded one molecule per polypeptide, suggesting that only one site can accommodate this xanthophyll. Clearly, the occupancy of this site alone could not sustain the folding of the pigment-protein complex.

Stability—It was previously shown that reconstitution in the absence of carotenoid does not yield into a folded Chl-protein

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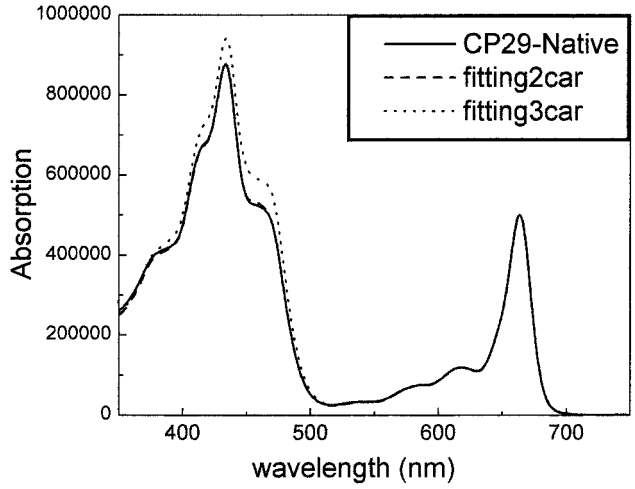

FIG. 2. **Number of carotenoids present in CP29.** Comparison between the absorption spectrum of the acetone extract of CP29-WT (*solid*) and the reconstituted spectra with 2 (*dotted*) and 3 (*dashed*) carotenoids.

complex (8), implying that carotenoids play a primary role in stabilizing Lhc holoproteins. To study the influence of each of the two Car binding sites and of individual xanthophyll species therein in the stabilization of the structure, denaturation experiments were performed. The data are reported in Table I. The control CP29 complex containing lutein, violaxanthin, and neoxanthin had a denaturation temperature of 64 °C. Among the complexes reconstituted in the presence of two xanthophyll species, CP29-LV and CP29-VN were slightly less stable, whereas CP29-LN was somewhat more stable than CP29-control, thus showing that neoxanthin has a stabilizing effect with respect to violaxanthin. Complexes containing only one carotenoid species were the less stable, their denaturation temperature ranging between 53 and 49 °C.

Light Harvesting—The absorption spectra of all complexes were recorded at room temperature. The spectra were almost identical in the Q_y region, indicating that the binding of different xanthophyll species does not strongly influence the Chl absorption (data not shown). Major differences were observed in the 400–520-nm region, where the Cars directly absorb by their S_0-S_2 transition (Fig. 3, *A*–*F*).

In the antenna complexes of higher plants carotenoids have a light-harvesting function. To determine the energy transfer efficiency of individual Cars in the two sites, the absorption spectra of the complexes were described in terms of absorption of individual pigments using two Chl *a* forms, two Chl *b* forms, and two Cars forms. Once the best fit was chosen for the absorption spectrum, the same set of data was used to describe the correspondent fluorescence excitation spectrum (Fig. 3, A′–F′). The integrated areas of the pigment bands in the absorption and excitation spectra were used to calculate the efficiencies of the energy transfer in the complex. The error is around 5% assuming for Chl *a* 100% transfer efficiency. The data are reported in Table II.

The overall Car to Chl *a* energy transfer depends on the xanthophyll composition. Among complexes with a single xanthophyll species, CP29-L was the complex with lowest transfer efficiency (60%), whereas CP29-V had the highest (73%). The two samples reconstituted with zeaxanthin only, had very different energy transfer efficiency: CP29-Za (with a single xanthophyll) showed an efficiency of 62%, whereas for CP29-Zb (with two xanthophylls) this value dropped to 37%, thus indicating that the additional zeaxanthin molecule is unable to transfer energy to Chl *a* to a significant extent (<5%). When two xanthophyll species were allowed, the highest efficiency was obtained in the case of CP29-VN (81%), the lowest with CP29-LN (69%). In the control CP29 sample, three xanthophyll species were present, with the lutein transferring with 75% efficiency and violaxanthin and neoxanthin with 60–65% (6).

Photoprotection—Xanthophylls in Lhc complexes act in quenching ^{3}Chl and scavenging of $^1O_2$, thus providing photoprotection. Photoprotection can be measured from the ability in preventing Chl photobleaching under strong white light illumination in the presence of oxygen (13). The results of this measurement for the recombinant CP29 complexes are shown in Fig. 4. CP29-control and the complexes with two xanthophyll species show high resistance to photooxidation, whereas complexes with only one xanthophyll species are more sensitive, thus yielding a behavior according to the following series: CTR = LN = LV > V > L > Z. It is interesting to note that violaxanthin is more effective in photoprotection than lutein; it should also be noted that CP29-Za contains a single carotenoid molecule per polypeptide, whereas the CP29-V sample has two.

Occupancy of the Xanthophyll Binding Sites: CP29 Versus LHCII—Three tightly bound xanthophyll binding sites have been described in LHCII, sites L1 and L2 (12), accommodating mostly lutein in the native complex, and N1, which is selective for neoxanthin (13, 14). The data presented above show that only two of these sites are conserved in CP29. Previous work showed that site L1 is conserved in CP29 (17). However, it is not clear if either site N1 or L2 is conserved. Alternatively, both sites could be present but only partially occupied. To discriminate between the above hypotheses, a chimeric complex was produced, including the central domain (helices A + B) of LHCII and the C helix domain of CP29. The helix A-helix C lumenal loop and part of the helix C-helix B stromal loop were also included in the swapped domain since the loops can affect Lhc protein folding (32) (Fig. 1). The apoprotein was overexpressed in *E. coli*, and the complex was reconstituted *in vitro* with pigments. A pigment-protein complex was obtained, which had the same mobility in a sucrose gradient of CP29 and LHCII monomeric complexes and a denaturation temperature of 66 °C, intermediate between that of CP29 (64 °C) and LHCII (73 °C). The absorption spectrum of the complex along with its second derivative is presented in Fig. 5. The maximum in the Q_y region is at 676 nm, whereas a strong Chl *a* contribution was detected at 666 nm. In the blue region the minima at 495, 468.4, and 437.2 nm in the derivative spectrum were attributed, respectively to Car, Chl *b*, and Chl *a*. HPLC pigment analysis showed that the chimeric complex has a Chl *a*/*b* ratio of 2.7 when reconstituted in the same condition of LHCII-WT (Chl *a*/*b* 2.3 in the mixture) and 4.0 when reconstituted in the condition of CP29-WT (Chl *a*/*b* 4.5 in the mixture). The Chl to Car ratio was 4.0, and the xanthophyll species bound were lutein and small amount of violaxanthin. The chimeric LHCII-CP29 complex never binds neoxanthin irrespective of the xanthophyll availability in the pigment mixture and other reconstitution conditions (Table III).

Orientation of Xanthophyll Transition Moments in CP29—Xanthophylls are bound to the L1 and L2 sites of LHCII, their polyene chain forming an angle of, respectively, 56.4 and 59.4° with respect to the normal to the thylakoid membrane plane (12). We have used LD to determine the orientation of xanthophyll transition moments, lying within a few degrees from the plane of the polyene chain (33) in CP29. To distinguish between the contribution of xanthophylls in sites L1 and L2 to the LD signal, we have used the CP29 mutants E166V and E174V previously shown to have either complete or partial emptiness of site L2 (17). The major contribution of xanthophylls to the CP29-WT LD spectrum (Fig. 6) appears as a positive signal at 495 nm, whose amplitude is reduced in the E174V mutant and

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TABLE I

Pigment composition and denaturation temperature of complexes reconstituted in the presence of different carotenoids

The samples were named after the carotenoid species used in the reconstitution mixture (*e.g.* CP29-L complex reconstituted in the presence of only lutein in the pigment mixture). Data are the average of four independent reconstitution experiments. The errors in the measurements are all less than 0.05. For the denaturation temperature, the error in the measurements is around 2 °C.

Sample	% in the mixture				Pigment composition of the complex						Den *T*
	L	N	V	Z	Chl *a*	Chl *b*	L	N	V	Z	
											°C
CP29-native					6	2	0.85	0.5	0.65		
CP29-cont	45	20	35		6	2	0.9	0.5	0.58		64.0
CP29-L	100				6.1	1.9	1.85				53.6
CP29-V			100		6	2			1.8		49.1
CP29-Za(b)[a]				100	6	2				1.2 (1.8)[a]	49.1
CP29-LN	50	50			6	2	1.2	0.8			56.5
CP29-LV	25		75		6.1	1.9	0.7		1.2		64.9
CP29-NV		50	50		5.9	2.1		0.8	1.2		57.8

[a] Two CP29-Z samples containing different amounts of carotenoids have been obtained, which are called Za and Zb.

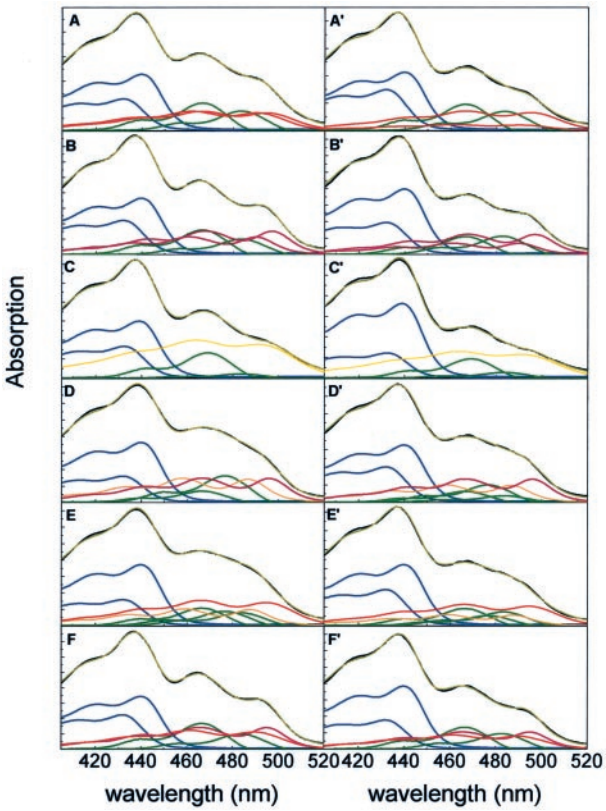

FIG. 3. **Fitting of absorption and excitation spectra in the Soret region of samples with different carotenoid composition.** *A*, CP29-L; *B*, CP29-V; *C*, CP29-Z; *D*, CP29-NV; *E*, CP29-LN; *F*, CP29-LV. *Black lines* correspond to experimental absorption (*left*) and excitation (*right*). The *dark yellow line* represents the fitting. The spectra were fitted with the spectra of individual pigments in protein environment: *blue*, Chl *a*; *green*, Chl *b*; *red*, lutein; *magenta*, violaxanthin; *yellow*, zeaxanthin; *orange*, neoxanthin.

becomes negative in the E166V mutant. This suggests that the xanthophyll bound to the site L1 has its dipole moment transition forming an angle smaller than 54.7° with the normal to the membrane plane.

The decreased intensity of the positive signal in CP29-E174V complex confirms that the positive contribution is related to carotenoid in the L2 site. In this case, the angle between the dipole moment transition axis and the normal to the membrane plane is larger than 54.7°. Using an internal standard it is possible to determine the exact values for these angles. In CP29 the orientation of the Chls has been determined from the LD signal after normalization. We therefore analyzed the spectra previously presented in Simonetto *et al.* (26), and we calculated the orientation of the Cars transition moments. The orientation of the xanthophyll located in the L1 site was calculated from the spectrum of the E166V mutant in which the only xanthophyll present is located in site L1. The analysis of L2 was performed on the WT − E166V difference spectrum. The spectra were fitted with the absorption spectra of the Cars to calculate the carotenoid LD signal as reported in Croce *et al.* (14). Then, using the factor of normalization value obtained from the analysis of the Chls transition moments (26), the values for L1 and L2 were calculated. These values are, respectively, 50 and 70°.

L1 and L2 Putative Binding Sequences—By primary structure analysis within the Lhc family, four consensus sequences have been identified in the hydrophilic domains adjacent to trans-membrane-helix regions A and B, two on the stromal loops and two on the lumenal loops, which could be involved in xanthophyll binding (20) (Fig. 1). These sequences are characterized by an hydrophobic stretch of four residues with a polar residue insertion, thus allowing the formation of a hydrophobic pocket for hosting end-rings of xanthophylls, whereas the interaction of the polar residue with the oxygenated ring substituents could have a stabilizing effect (20). To check the involvement of these consensus sequences in xanthophyll binding, mutation analysis was performed. In three cases the charged amino acids in the center of the hydrophobic sequence were either substituted by a non-charged one or by a residue with an opposite charge; in one case the putative binding sequence was deleted together with the whole N-terminal domain. In the case of the putative lumenal ligand for the carotenoid in L1 site, the mutation at the central AA (Gln-230, ligand for Chl A3) (17) has previously been performed with no effect on xanthophyll binding. Close inspection of the LHCII structure (Kühlbrandt *et al.* (12)) indicates that the end ring of the xanthophyll in the L2 site appears to be closely spaced with respect to the carbonyl of proline 238. This residue could then interact with -OH xanthophyll ring substituents. This residue was mutated to arginine. In Fig. 7*A* a scheme representing the structure of CP29 with indication of the putative carotenoid binding sequences and of the mutations performed is shown. The mutant sequences were expressed in *E. coli* and the apoproteins were reconstituted into complexes using a pigment mixture in which all xanthophylls were available together with Chl *a* and Chl *b*. The mutant Lhc complexes were purified and characterized by biochemical and spectroscopic methods. All complexes showed a high yield of reconstitution, similar to WT complex. Heat denaturation experiments accordingly show that all mutant proteins have the same stability of the WT (64 ± 1 °C). The only exception was the CP29-P238R mutant, which has a denaturation temperature of 57 °C (see "Discussion").

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TABLE II
Energy transfer efficiency for individual Cars in different sites

The absorption wavelength of the red-most peak of the xanthophylls is also reported. All the spectra were described with two carotenoid absorption forms. The transfer efficiency for each of them is reported. The error in the values is within 5%. Abs, absorbance.

Sample	% transfer total Car	Lutein 1		Lutein 2		Viola 1		Viola 2		Neo	
		Abs	%	Abs	%	Abs	%	Abs	%	Abs	%
CP29-Control	70	494.5	75					492	58	486.5	65
CP29-L	60	494.2	91	489.7	28						
CP29-V	73					496.8	80	489.8	60		
CP29-Za(b)	62 (37)[a]										
CP29-LV[b]	73		71				74				
CP29-LN	69	494.2	76							487.4	59
CP29-VN	81					496.8	90			485.4	68

[a] The value in the parenthesis is referred to the complex with 2 cars.
[b] For this sample four different Car absorption forms were needed for the best fitting: for simplicity, only the averaged values for transfer efficiency are reported.

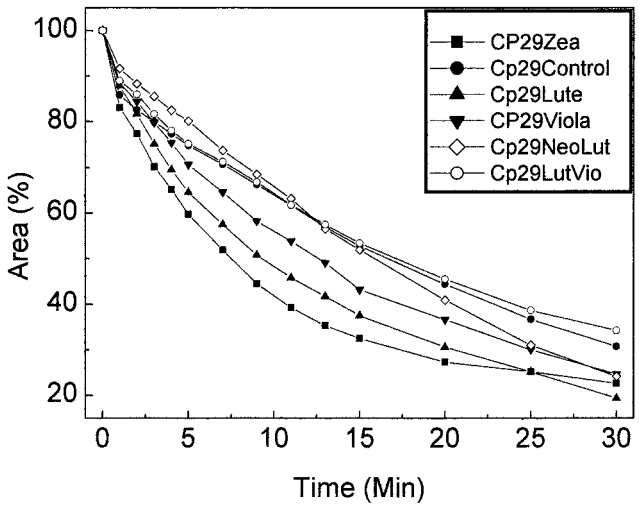

FIG. 4. **Photobleaching of recombinant CP29 proteins altered in the xanthophyll content.** The decay curves show the total Q_y absorption relative to a 100% initial value. Points refer to experimental data. Error was lower than 3% for each values as assessed from three independent measurements.

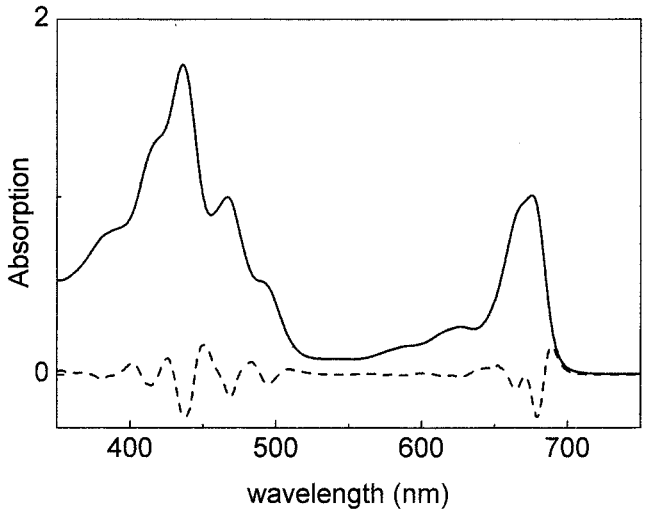

FIG. 5. **Absorption spectrum of the chimeric complex.** Absorption spectrum and second derivative at room temperature of LHCII-CP29-chimeric complex reconstituted with Chl *a/b* 4.5.

In Table IV the pigment analysis of the reconstituted complexes is presented. The differences in the pigment binding in the mutants with respect to the WT are rather small; CP29-D50L and CP29-N-term showed Chl *a/b* and Chl/carotenoid ratios identical to the CP29-WT reconstituted in the same conditions, whereas a small decrease in neoxanthin and violaxanthin was compensated by an increase in lutein content. CP29-D138L was identical to the WT. CP29-D195L and CP29-P238R complexes showed a lower Chl *a/b* ratio than CP29-WT, especially in the case of the P238R mutant, but not variation in the carotenoid composition, suggesting that these changes were due to the loss of a Chl binding site. These data clearly indicate that protein motifs including the mutations here performed do not play a major role in xanthophyll binding.

DISCUSSION

CP29 is the simplest chlorophyll-binding protein of the Lhc superfamily since it binds eight Chls per polypeptide (29), each coordinated by a specific amino acid residue (17). The binding of xanthophylls to CP29, however, is still a matter of discussion. Consensus results have been obtained on the binding of three xanthophyll species, violaxanthin, lutein, and neoxanthin (34), but different hypotheses have been proposed for the location of each species to a particular binding site. Although there is consensus on the binding of lutein to site L1, violaxanthin and neoxanthin have either been proposed to bind to different sites, as in LHCII (15), or to compete for site L2 only, thus implying that site N1 is absent or empty in CP29 (17).

In this work we performed an extensive analysis of xanthophyll binding in CP29. In particular, we address four questions, (i) how many carotenoids are tightly bound to this complex, (ii) in which sites they are located, (iii) which is the role of individual sites and individual xanthophylls, and (iv) how the putative binding sequences can influence the binding. We approached the problem by using recombinant pigment-protein complexes in which either the carotenoid complement was biochemically modified or the apoprotein was engineered at the putative carotenoid binding sites.

How Many Carotenoids Are Bound to CP29?—The data presented clearly indicate that both native and recombinant CP29 tightly bind two carotenoids per polypeptide. One mole of lutein and substoichiometric amounts of neoxanthin and violaxanthin together summing up to one per polypeptide were found in all preparations, thus suggesting low selectivity of at least one of the binding sites. This is also supported by the analysis of CP29 purified from different plant sources, showing variations in the ratio between the xanthophylls (5, 35, 36).

Reconstitution experiments in the presence of either one xanthophyll species or a combination of two xanthophyll species indicate that lutein, violaxanthin, and zeaxanthin can enter both binding sites, although with different affinity and binding strength. The neoxanthin, instead, can be accommodated in only one of the two sites, and its inability to drive the folding process alone testifies that the occupancy of this site is not sufficient to stabilize the pigment-protein complex.

Which Binding Sites Are Present in CP29?—Four xantho-

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TABLE III

Pigment composition of chimeric complex reconstituted in two different conditions

The values for LHCII and CP29 reconstituted in the same conditions are also reported. The number after the sample name indicates the Chl *a*/*b* ratio in the reconstitution mix used. The data are obtained from the average of two independent experiments showing less than 5% differences in the values.

Sample	Chl *a*/*b*	Chl/Car	Pigment composition			
			Chl b	N	V	L
			(mol/100 mol Chl a)			
Chimera-2.3	2.7	4.0	37.1	1.4	3.2	26.8
Chimera-4.5	4.0	4.0	26.3	1.3	3.2	27
LHCII-2.3	1.4	4.0	71	14	3	25.6
CP29–4.5	3.0	4.0	34	7.2	10.1	15

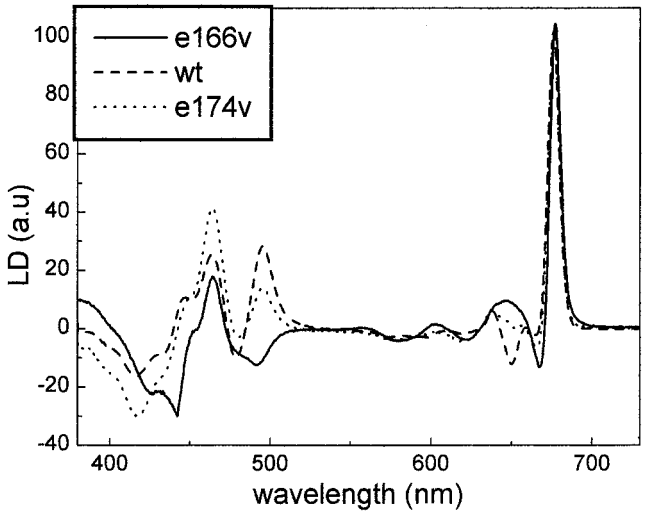

FIG. 6. **Linear dichroism.** LD spectra at 100K of CP29-control (*dash*), CP29-E166V (*solid*), and CP29-E174V (*dotted*).

phyll binding sites have been described in LHCII; that is, three tightly bound, L1, L2, and N1, and a loose one (V1). In CP29 only two of the three tight sites are conserved. Previous results show that the L1 site is present in CP29, where it accommodates mainly lutein (17). The second carotenoid binding site present in CP29 shows high affinity for neoxanthin, but it does not participate to the protein stability, and it can be either the L2 or N1 site. To discriminate between these two sites, we constructed a chimeric LHCII·CP29 protein in which the two central helices (A and B) derive from LHCII, whereas the C helix domain, previously shown to host the N1 binding site, derives from CP29 (Fig. 1). In Fig. 7*B* a schematic representation of the complex is presented. The chimeric complex was stable and had spectral and pigment binding properties intermediate between CP29 and LHCII. However, no neoxanthin was found in the complex despite high amounts of this carotenoid used during the reconstitution. We conclude that the N1 site is not present in CP29 and that the second carotenoid binding site in this complex is homologous to the L2 site of LHCII.

The LD analysis indicates that the dipole moment transition axis of L1 and L2 forms, respectively, an angle of 50 and 70° with the normal to the membrane plane. Although it is not possible from these data to determine the exact orientation of the polyene chain, it seems quite clear that the xanthophylls are oriented differently compared with LHCII, where a perfect symmetry was observed. Although models of CP29 structure have been proposed based on LHCII, the only available Lhc structure (12), it appears that Lhc proteins may carry significant structural differences. New structural data are of critical interest for the further understanding of Lhc protein structure and function.

Stability—The results presented indicate that neoxanthin can enter only the L2 site, whereas all other Cars can be accommodated in both sites. This implies that when a complex contains one molecule of neoxanthin and one molecule of another xanthophyll, the latter has to be located on site L1. This allows studying the role played by individual xanthophylls in the two sites.

We applied this model to the stability measurements to determine the influence of different xanthophylls in the L1 and L2 sites. We considered the denaturation temperature of CP29-L, CP29-V, CP29-NV, CP29-NL, and CP29-VL, and we resolved a system of five equations with five variables. This calculation does not take into account the contribution of Chls to the stability since the Chl content of all the samples was identical.

The calculation yields a value of 50.3 °C for the contribution of lutein in site L1 to the stability of the protein, whereas the same xanthophyll contributed only for 3.2 °C when located in site L2. For violaxanthin the corresponding values were respectively 42.9 °C (for L1) and 6.2 °C (for L2), whereas neoxanthin in site L2 contributes for 14.5 °C. As an example, we can calculate the stability of CP29-control (62.33 °C) and verify that it matches with the measured denaturation temperature (64 °C) of the CP29-control complex. The good match indicates the independence of all variables (*e.g.* lutein in L1 site stabilizes the complex in the same way independently from which xanthophyll is present in site L2). The results confirm that site L1 is the most important in Lhc protein stabilization, as previously assessed in Lhcb1 (10), and lutein in this site seems to confer the highest stability to the complex. Lutein in the L2 site has almost no effect on the stability, whereas the occupancy of this site by violaxanthin or, even better, by neoxanthin increases the temperature of denaturation.

Energy Transfer—The excitation energy transfer efficiency from individual binding sites has been determined by analyzing the absorption and excitation spectra in terms of the spectral contribution of individual pigments. The possibility of comparing samples, which differ only for the carotenoid composition, allowed quantitative information to be obtained from this analysis. It is worth mentioning that all samples were fully equilibrated with the only partial exception of CP29-Za and CP29-Zb, which showed a small emission contribution from Chl *b* fluorescence at 660 nm when the complex was excited at 475 nm.

Again, we applied the model used for the stability analysis (see "*Stability*"). It has been demonstrated that binding to either one or the other of the two sites yields into a different modulation of the S_2 energy level of the xanthophylls (4). This indicates that the same xanthophyll in the two sites is spectroscopically different. In agreement, two distinct values for the red-most peak were found for both lutein and violaxanthin, corresponding to the absorption in the L1 and L2 sites. In contrast, a single neoxanthin spectral form was needed for the best fit of both absorption and fluorescence excitation spectra consisting with its exclusive binding to the L2 site. Considering

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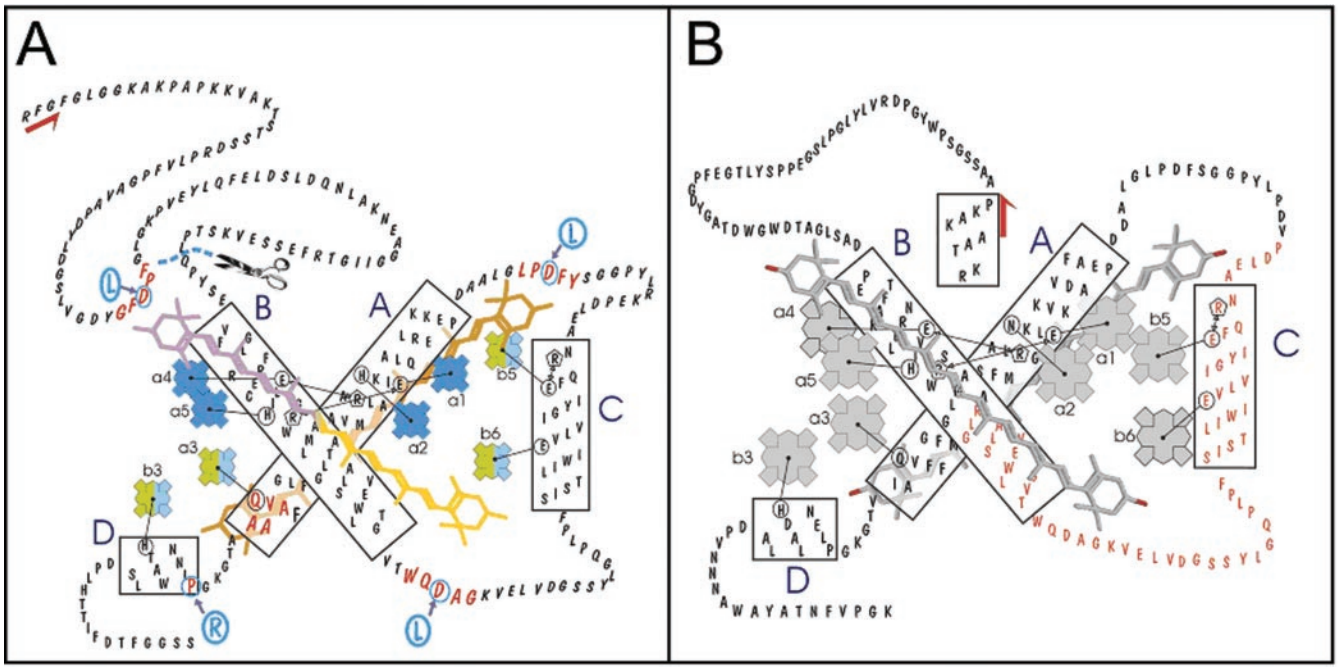

FIG. 7. **Schematic representation of the structure of CP29 and the chimera.** *A*, scheme for the structure of CP29. The putative carotenoid binding sequences are indicated in *red*. The mutated amino acid is indicated by the *blue circle*, and the substituted amino acids is indicated in *blue*. In the scheme Chls *a* are indicated in *blue*, Chl *b* in *green*, lutein in *orange*, violaxanthin in *magenta*, and neoxanthin in *yellow*. Two colors on the same pigment indicates mixed occupancy. *B*, schematic representation of the chimerical complex: *black*, the domain of LHCII; *red*, the domain of CP29.

TABLE IV

Pigment composition of mutants at the putative carotenoid binding sites relative to the WT composition

The samples were reconstituted three times. WT and mutant proteins were reconstituted *in vitro* using aliquots from the same pigment mixture. In the table the difference in pigment content in each mutant is compared to the WT reconstituted in the same conditions. Lutein, neoxanthin, and violaxanthin were calculated considering the same number of Chls in all complexes.

Sample	Chl *a*/*b*	Chl/Car	Lute	Neo	Viola
CP29-D50L	−0.01 ± 0.01	0.02 ± 0.01	+0.08 ± 0.01	−0.03 ± 0.02	−0.06 ± 0.02
CP29-D138L	−0.09 ± 0.08	0.03 ± 0.04	−0.02 ± 0.03	−0.03 ± 0.02	0.05 ± 0.01
CP29-D195L	−0.26 ± 0.04	0.01 ± 0.04	−0.04 ± 0.002	0.02 ± 0.005	0.01 ± 0.005
CP29-P238R	−0.57 ± 0.12	−0.2 ± 0.03	−0.05 ± 0.04	+0.05 ± 0.04	0.003 ± 0.007
CP29-N-term	−0.06 ± 0.05	−0.01 ± 0.06	+0.1 ± 0.02	−0.02 ± 0.05	−0.07 ± 0.01

that in the samples containing neoxanthin, violaxanthin and lutein can only be located in the L1 site, it is possible to associate the two absorptions at site L1 or L2 based on the pigment composition of the samples. Thus, lutein in the L1 site absorbs at 494 nm, whereas in the same site violaxanthin absorbs at 496 nm. The same description found for the absorption spectra was applied to the analysis of excitation spectra, thus allowing discrimination between the transfer from Car in L1 and L2. It is therefore possible to read the data of Table II with the understanding that notations "1" and "2" actually correspond to xanthophyll binding sites L1 and L2. We can conclude that lutein transfers with high efficiency (up to 91%) when in site L1, while its efficiency in exciting Chl *a* fluorescence is very low (down to 28%) when it is located in the L2 site. Violaxanthin transfers efficiently from the L1 site, and the efficiency is around 60% for both violaxanthin and neoxanthin from the L2 site. Both violaxanthin and lutein show high (80–90%) efficiency from the L1 site, while the decrease in the transfer efficiency from the L2 site is different; lutein decreases its efficiency by a factor of 3.2, whereas viola decreases its efficiency by a factor of 1.3. This suggests that the arrangement of the two xanthophylls in the L2 site is different. The case of zeaxanthin is even more extreme: its energy transfer efficiency from the L1 site is around 60%, whereas from the L2 site it is very low, if at all. These results suggest that the energy transfer from a single site is dependent from the site occupancy.

In general the L2 site seems to be less active in the energy transfer as compared with the L1 site. Moreover, the efficiency of excitation energy transfer to Chl is modulated by the xanthophyll species bound there in the following gradient: V = N > L > Z. The lower transfer efficiency from the L2 site, common to all xanthophyll species, indicates that in this site the distance/orientation between pigments is not optimal for excitation energy transfer. It has been recently observed that most of the transfer from Car to Chl occurs via the S_2 Car state in less than 100 fs. Considering the short carotenoid S_2 lifetime, a perfect geometry between donor and acceptor is required for highly efficient energy transfer (3, 4, 6), and small changes in this geometry can strongly influence the process. Moreover, the finding that the xanthophylls behave differently when bound to the L2 site suggests that this protein domain may change its folding in the presence of different xanthophylls, supporting the proposal that the L2 site has allosteric nature (10). It is thus possible to suggest that when, for example, lutein enters the site, which is usually occupied by neoxanthin and violaxanthin, a conformational change occurs, changing the relative chromophore orientation and, thus, decreasing the transfer efficiency. The picture in CP29 is thus different as compared with the case of LHCII, where both L1 and L2 sites showed high efficiency of excitation energy transfer to Chl *a* (4). This suggests the L2 site has a different function in the two proteins. In the case of CP29, a Ca^{2+} and dicyclohexylcarbodiimide bind-

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LHCII                                GLSADPETFAKNRELEVIHCRWAMLGAL---
CP26                                 GLGKKPEDFAKYQAYELIHARWAMLGAA---
cp29                                 -PYSEVFGLQRFRECELIHGRWAMLATLGAL
                                        .   : : :  *:** *****.:
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FIG. 8. **Sequence comparison between CP29, CP26, and LHCII in the B helix.**

ing site in the loop between helix B and C was suggested to work as a sensor for pH changes in the lumen (37, 38). Dicyclohexylcarbodiimide binding sites have been also found in CP26 (39). At low lumenal pH the substitution of the Ca^{2+} with an H^+ would "open" the structure, allowing violaxanthin to exit and zeaxanthin to enter the site (18). The high flexibility, which is required for this process, can thus explain the low energy transfer efficiency from this site. In the case of LHCII, zeaxanthin never enters the central sites, and thus, the structure could be optimized for the function of excitation energy transfer.

Photoprotection—The photobleaching measurements show that samples with two or more xanthophyll species are more resistant to photooxidation. Among the samples containing only one xanthophyll species, CP29-L and CP29-Z are the most sensitive to photo-oxidation. It has been proposed above that lutein in site L2 is organized differently compared with the other xanthophylls and in effect does not transfer energy efficiently. CP29-Z has only one xanthophyll molecule located in the L1 site. The results thus indicate that the L2 site in CP29 can play an important role in photoprotection and, again, that this role is modulated by the site occupancy. In this respect, CP29 differs strongly from LHCII for which it has been clearly shown that L1 is the only site active in preventing photooxidation (10).

Do Putative Carotenoid Binding Sequences Participate in Actual Xanthophyll Binding?—In the last part of this work, the putative binding sequences for the carotenoids in the L1 and L2 sites have been studied by mutation analysis. Comparison between the primary structures of Lhc multigenic family members has shown in the loops conserved sequences composed by one charged amino acid hydrophobic residues in between four. Considering that Lhc proteins cannot bind β-carotene, it was argued that these sequences are involved in the xanthophyll binding by establishing an H-bond between the polar residue in the polypeptide stretch and the ring -OH substituent (20). However, our mutation of the four putative binding sequences not only did not decrease pigment-protein stability but also did not change the affinity of binding sites for the different xanthophyll species. The changes in Chl composition observed for the P238R mutant are probably due to changes in the D helix domain and loss of the Chl in A3 site, as suggested by the similar effects of mutation P238R (this work) and Q230L (17) detected by pigment analysis, CD spectroscopy (data not shown), and the heat denaturation kinetics. The two mutations, which affect the putative stromal binding sequences for xanthophylls in the L2 site (CP29-D50L and CP29-N-term), support the view that pigment-protein stabilization by xanthophyll binding is not provided by hydrophilic sequences. A small but reproducible decrease of violaxanthin and neoxanthin balanced by an increase of lutein was, however, consistently found. This result may indicate that the sequence encompassing the Asp-50 residue plays a minor role in determining the selectivity of the L2 site.

The pigment binding properties of chimeric LHCII-CP29 complex further support the conclusion that lumenal sequences have little effect on site selectivity: despite the presence of the CP29 sequence in the lumenal region between B and C helices, it still accommodates mostly lutein in the L2 site, as is the case in LHCII (13) rather than violaxanthin and neoxanthin, as in CP29. Nevertheless, the absence of a clear phenotype for this chimeric complex can be useful in the search of the structural determinants for the affinity of the Car binding sites for individual xanthophyll. In fact, no effect in xanthophyll composition was observed by either deleting the N-terminal domain down to residue 96 (CP29) or by substituting half of the B helix and the lumenal loop. We conclude that the structural determinant for the selectivity of the L2 site has to be located in between these two regions, namely within a 28-amino acid sequence (*e.g.* from residue 50 to residue 77 in the LHCII sequence). It has been recently demonstrated that CP26 has the same L2 occupancy as in CP29 (25); this means that the determinant has to be conserved in CP29 and CP26 and not in LHCII. The three sequences are reported in Fig. 8. The only candidate is the N-61 in LHCII. An aromatic amino acid in both CP29 and CP26 occupies this position. In the structure of LHCII this asparagine is within 5 Å from the carotenoid in L2. The presence in this position of a Phe or Tyr may change the steric interaction between pigments, possibly favoring the binding of violaxanthin and neoxanthin with respect to lutein. This may be due to the difference in the ring orientation between lutein and violaxanthin and neoxanthin; lutein is in its low energy conformation when the ϵ-cycle is oriented parallel to the polyene chain, whereas the β-cycles of violaxanthin and neoxanthin are oriented perpendicularly. This difference can play a role in the selectivity of the binding site; an aromatic group in this site has higher steric hindrance and can select against lutein. However, the stringency cannot be very high as lutein is able to enter the L2 site when the reconstitution is performed in the absence of violaxanthin and neoxanthin. Consistent with this hypothesis is the finding that the CP29-L sample is less stable than the control CP29, and lutein in site L2 is differently oriented in the complex, as proposed above.

Although the putative carotenoid binding sequences on the stroma-exposed side of the protein can be somehow involved in the selectivity of the sites for the different xanthophylls, it is quite clear that the stabilization of the carotenoid binding cannot be attributed to these sequences. Similar results have been obtained for the major light-harvesting complex, LHCII, where the deletion of the N-terminal domain, which contains the putative binding sequence for L1 site, does not influence the carotenoid composition of the complex. The dragging force for the carotenoid binding is most probably to be found in hydrophobic interaction in the portion of the complex inside the membrane in a similar way to that observed in bacteria (40). Aromatic residues present in A and B helices can be involved in this binding. The position of these residues is not conserved in different members of the Lhc family but maintained in the same gene products from different species. Mutation analysis on the Chl binding residues both in CP29 and LHCII have shown that the interactions between Chls and carotenoids can play a major role in the xanthophylls binding. In particular, the binding of neoxanthin in the N1 site of LHCII is stabilized by interaction with Chls (14). This is consistent with CP29 lacking the N1 site; if the number of Chls in the domain in between helices A and C is lower than in LHCII, then critical Chl-neoxanthin interactions might be lacking. The loop in between helix A and helix C of CP29 is shorter with respect to the corresponding domain in LHCII; this may bring helix C closer

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to helix A and, thus, prevent Chl A6, A7, and B1 binding in both CP29 and the chimeric LHCII-CP29 complex.

CP29 is a member of the Lhc family, which is conserved in higher plants and green algae and is involved in light harvesting and protection from abiotic stress. Knowledge of the biochemical and functional properties of the isolated proteins will contribute to the understanding of the physiological roles of individual gene products in providing stress resistance. Recent work showed that during light stress the xanthophyll ligand of site L2 (violaxanthin) is changed into zeaxanthin (18), thus inducing a conformational change leading to fluorescence quenching, whereas the binding of neoxanthin in site N1 stabilizes the unquenched conformation (9, 19). The absence of site N1 in CP29 qualifies this antenna subunit for prompt response to light-induced photoprotective conformational change.

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Xanthophyll Binding Sites of the CP29 (Lhcb4) Subunit of Higher Plant Photosystem II Investigated by Domain Swapping and Mutation Analysis

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