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Carotenoid-binding Sites of the Major Light-harvesting Complex II of Higher Plants*

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Roberta Croce‡, Saskia Weiss, and Roberto Bassi

From the Dipartimento Scientifico e Tecnologico, Facoltà di Scienze Matematiche, Fisiche, Naturali, Biotecnologie Vegetali, Università di Verona, Strada Le Grazie, 37134 Verona, Italia

Recombinant light-harvesting complex II (LHCII) proteins with modified carotenoid composition have been obtained by *in vitro* reconstitution of the Lhcb1 protein overexpressed in bacteria. The monomeric protein possesses three xanthophyll-binding sites. The L1 and L2 sites, localized by electron crystallography in the helix A/helix B cross, have the highest affinity for lutein, but also bind violaxanthin and zeaxanthin with lower affinity. The latter xanthophyll causes disruption of excitation energy transfer. The occupancy of at least one of these sites, probably L1, is essential for protein folding. Neoxanthin is bound to a distinct site (N1) that is highly selective for this species and whose occupancy is not essential for protein folding. Whereas xanthophylls in the L1 and L2 sites interact mainly with chlorophyll *a*, neoxanthin shows strong interaction with chlorophyll *b*, inducing the hyperchromic effect of the 652 nm absorption band. This observation explains the recent results of energy transfer from carotenoids to chlorophyll *b* obtained by femtosecond absorption spectroscopy. Whereas xanthophylls in the L1 and L2 sites are active in photoprotection through chlorophyll-triplet quenching, neoxanthin seems to act mainly in $^1O_2^*$ scavenging.

Light energy for the photosynthesis of green plants is collected by an antenna system composed of many homologous proteins belonging to the *Lhc* multigene family (1). These pigment-protein complexes are organized around photosynthetic reaction centers to form supramolecular complexes embedded into the thylakoid membrane, accounting for ~70% of the pigment involved in plant photosynthesis. LHCII[1] is the most abundant light-harvesting complex in higher plants. The structure of this complex has been resolved at 3.4 Å by electron microscopy (2) and is formed by three hydrophobic transmembrane helices connected by hydrophilic loops and an amphipathic helix exposed to the luminal surface of the membrane. LHCII coordinates 7 Chl *a*, 5 Chl *b*, and 3–4 carotenoid molecules (lutein, neoxanthin, and a substoichiometric amount of violaxanthin) depending on the genotype (3) and the physiological state of the plant (4). In the structural model of LHCII (2), 2 xanthophyll molecules have been located in the center of the complex, forming an internal cross-brace interacting with helices A and B. These appear to be crucial for protein stabilization, as suggested by the fact that a stable LHCII complex cannot be obtained without lutein in refolding experiments (5, 6). Although the 2 central molecules were tentatively assigned to lutein (2), the structural resolution is insufficient for their identification and for the location of the xanthophyll molecule with respect to the 2 detected by structural analysis. The nature and location of the binding site for the third xanthophyll molecule are presently unknown. It is also unclear if the individual binding sites have different affinities for the three xanthophyll species.

Carotenoids have at least five different roles in photosynthesis: 1) light harvesting, 2) chlorophyll triplet quenching, 3) singlet oxygen scavenging, 4) excess energy dissipation, and 5) structure stabilization and assembly. In most cases, interaction with Chl molecules plays an important role. A tentative assignment of the chlorophyll type bound to individual sites was based on the proximity of 7 chlorophyll molecules to the 2 central xanthophyll molecules. It was argued that most of the triplet states will be formed on Chl *a* because of the subpicosecond energy transfer from Chl *b* to Chl *a*, which is faster than triplet formation. Therefore, only Chl *a* triplets need to be quenched and therefore in close contact with xanthophyll molecules. The above assignment is not in agreement with studies of the triplet activity in LHCII (7–10), suggesting the involvement of additional xanthophyll molecules. Accordingly, direct energy transfer between xanthophylls and Chl *b* was observed (3), suggesting that at least some of the Chl *b* sites are in close contact with xanthophylls. In this work, we report the identification of a third carotenoid-binding site within monomeric LHCII and on the selectivity of the three binding sites for the different xanthophyll species components of LHCII. The molecular structures of the xanthophylls investigated in this work are shown in Fig. 1. By using *in vitro* reconstitution of recombinant LHCII, overexpressed in bacteria, with different pigment preparations, we obtained LHCII complexes that bind either a single xanthophyll species or combination of two. Biochemical, spectroscopic, and functional characterization of these recombinant proteins provides evidence for distinct binding sites for lutein and neoxanthin and for strong interaction of carotenoid molecules with both chlorophylls *a* and *b*, thus affecting their spectroscopic properties.

EXPERIMENTAL PROCEDURES

DNA Constructions—A construct overexpressing LHCII was obtained by mutagenesis of the *Lhcb1* cDNA clone (11, 43) to obtain a *Bam*HI restriction site at nucleotide 155 and a *Hin*dIII site at position 880 immediately after the stop codon. The resulting fragment was inserted into the pQE52 expression vector (pDS series; QIAGEN Inc.) (11, 43).

The pDL2BH3 construct codes for a protein containing one additional Ile (which substitutes for the first Ala of the transit peptide) and a two-amino acid vector portion: Arg-Ile. The construct were controlled by automated cycle sequencing of both strands.

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‡ To whom correspondence should be addressed. Tel.: 39-045-8098915; Fax: 39-045-8098929; E-mail: croce@mpi-muelheim.mpg.de.

[1] The abbreviations used are: LHCII, light-harvesting complex II; Chl, chlorophyll; HPLC, high pressure liquid chromatography.

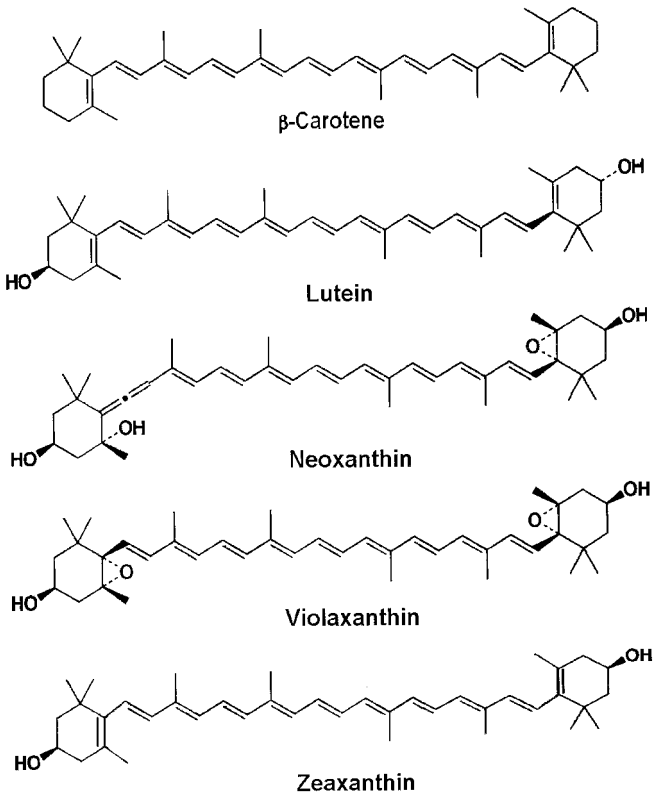

FIG. 1. **Molecular structure of xanthophylls bound to higher plant Lhc proteins and whose binding to LHCII was studied in this work.**

Isolation of Overexpressed LHCII Apoprotein from Bacteria—LHCII was isolated from the SG13009 strain transformed with the LHCII construct as described previously (12).

Reconstitution of Pigment-LHCII Complexes and Purification of Reconstituted LHCII—These procedures were performed as described (12) with the following modifications. The Chl/protein ratio in the reconstitution mixture was set to 20, and the carotenoid/protein ratio was set to 7. The Chl *a*/*b* ratio in the mixture was 2.3. Nondenaturing SDS-polyacrylamide gel electrophoresis was performed as described previously (13). Purification of reconstituted LHCII was performed by ion-exchange chromatography (12). For determination of pigment/protein stoichiometry, a fully purified protein was obtained, which did not contain any residual contamination by bacterial proteins, by preparative isoelectric focusing (14), followed by ultracentrifugation in a glycerol gradient (15–40% containing 0.06% β-dodecyl maltoside and 10 mM Hepes (pH 7.6); 12 h at 60,000 rpm in a Beckman SW 60 rotor) to eliminate ampholytes.

Protein and Pigment Concentration—The concentration of the LHCII apoprotein purified from *Escherichia coli* inclusion bodies was determined by the bicinchoninic acid assay (12). For stoichiometric (pigment/protein ratio) determination, the protein concentration was determined by the ninhydrin method (15). Chlorophyll concentration was determined as described (16). HPLC analysis was as described (17).

Spectroscopy—Absorption spectra were obtained using an SLM-AMINCO DW-2000 spectrophotometer at room temperature. Fluorescence emission spectra were obtained at room temperature using a Jasco FP-777 spectrofluorometer. CD spectra were obtained at 8 °C with a Jasco 600 apparatus. The samples were diluted in 10 mM Hepes (pH 7.6), 0.06% β-dodecyl maltoside, and 20% glycerol. Chlorophyll concentration was ~10 μg/ml for CD and absorption measurements and 0.01 μg/ml for fluorescence measurements.

The stability of the reconstituted protein was analyzed by recording its CD spectrum at increasing temperatures in the 600–720 nm range. Four measurements were accumulated for the spectrum, and the stability of each sample was measured twice. The absorbance at the peak was 0.75. The temperature was increased from 20 to 75 °C, recording a spectrum every 5 °C, allowing 6 min for temperature equilibration between measurements.

Photobleaching—The samples were diluted to an absorbance of 0.75 at the maximum in the Q_y region. The protein was then illuminated with white light (5500 microeinsteins m^{-2} s^{-1}) from a halogen lamp filtered through a water layer. After each time interval, the cuvette was removed from the light source, and the absorption spectrum was recorded with an SLM-AMINCO DW-2000 spectrophotometer in the range of 600–750 nm. The rate of photobleaching in the absence of carotenoid photoprotection was determined by treating the sample with 5% Triton X-100. When an oxygen-scavenging system (glucose and glucose oxidase (34 mg/ml) and catalase (11 mg/ml)) was used, no photobleaching was observed.

RESULTS

When purified from higher plant thylakoids, LHCII preparations bind lutein, neoxanthin, and violaxanthin in a ratio of 1.8:1:0.2, in addition to 7 Chl *a* and 5 Chl *b* molecules (Table I), in agreement with previous results (3, 18). LHCII is the product of *Lhcb1–3* genes. These can be overexpressed in bacteria, and the apoprotein refolded *in vitro* with pigments to obtain a pigment-protein complex (5, 12, 18). We have applied this procedure to maize *Lhcb1* cDNA (11, 43) using a pigment mixture containing Chl *a*, Chl *b*, β-carotene, violaxanthin, lutein, and neoxanthin and found that the pigments bind to recombinant LHCII in the same stoichiometry and relative amounts as in the native complex (Table I). We therefore repeated the reconstitution experiment by using individual xanthophylls or a combination of two, rather than the full pigment complement, to verify if the three carotenoids could freely exchange for each other. In addition, we attempted reconstitution with zeaxanthin. For this xanthophyll, in fact, there is considerable debate on its ability to bind to LHCII. In a preliminary experiment, the formation of a pigment-protein complex was analyzed by nondenaturing SDS-polyacrylamide gel electrophoresis. In all cases, it was possible to obtain a green band. However, the relative intensities of the bands were not equal, thus indicating differences in the efficiency of reconstitution. With respect to LHCII control samples (hereafter indicated as recombinant LHCII reconstituted using the full pigment set), a significant reduction in the yield of reconstitution was observed when zeaxanthin was used as the only carotenoid during refolding. In the case of neoxanthin, only a very faint band was obtained, suggesting a lower stability of the complex reconstituted with these xanthophylls, whereas violaxanthin and lutein yielded stable complexes with high yield (data not shown).

Pigment Composition and Stoichiometry of Recombinant LHCII

To characterize the LHCII complexes obtained with different xanthophylls, we prepared the protein in greater quantity by the method recently described for CP29 and CP24 (12, 19). The pigment composition of the recombinant proteins was determined by a combined approach of HPLC analysis and fitting of the acetone extract spectrum with the sum of spectra of purified pigments (3). The pigment/protein stoichiometry was also determined as described previously (20, 21). The results are reported in Table I. In all cases, the Chl *a*/*b* ratio obtained was 1.4 ± 0.02, essentially identical to the native complex extracted from leaves. Accordingly, 12 ± 0.3 Chl *a* + *b* molecules/polypeptide were bound for both the native and recombinant proteins, in agreement with previous results with native LHCII (2, 22) showing that, as in the native protein, 7 Chl *a* and 5 Chl *b* molecules are bound per recombinant LHCII polypeptide. This indicates that the differences in the xanthophyll content do not affect Chl binding. The only exception was the protein obtained with zeaxanthin as the only carotenoid. In this case, a Chl *a*/*b* ratio of 2.3 was obtained, suggesting that zeaxanthin affected chlorophyll-protein interactions in LHCII. Analysis of the carotenoid composition and stoichiometry showed that reconstitution with the complete xanthophyll complement yielded a

TABLE I
Pigment composition of recombinant LHC II complexes

Sample	Pigment mixture				Yield	Pigment composition (protein)					
	N[a]	V	L	Z		Chl *a*/*b*	N	V	L	Z	ΣCar.
	%										
Native	/	/	/	/	/	1.4	1 ± 0.1	0.2 ± 0.05	1.8 ± 0.1	—	3
Control	16	16	50	—	+++	1.4	1 ± 0.1	0.15 ± 0.03	1.85 ± 0.1	—	3.0
L	—	—	100	—	+++	1.41	—	—	2 ± 0.05	—	2.0
V	—	100	—	—	+++	1.42	—	2.2 ± 0.1	—	—	2.2
V	tr	100	—	—	+++	1.45	0.13 ± 0.02	2.2 ± 0.1	—	—	2.3
N	100	—	—	—	———	/	/	/	/	/	/
N	98	1	—	—	+	1.43	1.1 ± 0.1	1 ± 0.1	—	—	2.1
Z	—	—	—	100	+	2.3	—	—	—	2	2
L/V	tr	50	50	—	+++	1.42	0.13 ± 0.01	0.6 ± 0.01	1.6 ± 0.05	—	2.3
L/N	50	—	50	—	+++	1.4	1.0 ± 0.1	—	1.9 ± 0.1	—	2.9
N/V	50	50	—	—	+++	1.43	1 ± 0.1	1.3 ± 0.05	0.6 ± 0.05	—	2.9

[a] N, neoxanthin; V, violaxanthin; L, lutein; Z, zeaxanthin; ΣCar., total carotenoids; tr, trace; /, not available; —, not found.

protein with 3 bound xanthophyll molecules (1.8 lutein, 1 neoxanthin, and 0.2 violaxanthin molecules) as for native LHCII. This value of 3 was maintained in all cases in which neoxanthin was present together with comparable amounts of violaxanthin or lutein (or both) in the reconstitution mixture. When neoxanthin was absent, only 2 xanthophyll molecules were found bound to LHCII, strongly suggesting that neoxanthin binds to a distinct site, specific for this xanthophyll species, that could not be occupied by other xanthophylls. The value of 2 xanthophyll molecules/polypeptide was obtained when lutein, violaxanthin, and zeaxanthin were present together or alone, suggesting that all pigments can occupy two sites with similar specificity. On the contrary, it was not possible to obtain a reconstituted protein with only neoxanthin, indicating that the occupancy of the neoxanthin site is not sufficient for stabilization of the LHCII complex. Irrespective of the concentration of neoxanthin or of its proportion with respect to lutein or violaxanthin, the number of this xanthophyll species bound to the complex did not exceed 1 molecule/polypeptide, whereas samples with 2 lutein or 2 violaxanthin molecules could be readily obtained. This suggests that neoxanthin does not compete for the two lutein/violaxanthin sites. It is interesting to note that when small amounts of violaxanthin or lutein were added to the Chl *a*/Chl *b*/neoxanthin mixture, a stable complex was obtained, although with a very low yield. However, the yield increased with the amount of lutein or violaxanthin added. When the neoxanthin/violaxanthin ratio in the mixture was 100:1, the violaxanthin became limiting, and a complex was obtained binding only 2 xanthophyll molecules/polypeptide: 1 violaxanthin and 1 neoxanthin molecule. Although it was possible to reconstitute a complex binding only violaxanthin (2 molecules/polypeptide) when both lutein and violaxanthin were present in the same amount (1:1) in the reconstitution mixture, the complex obtained bound 2.7 times more lutein than violaxanthin. When the ratio was 3:1, the amount of lutein bound was 9 times higher than that of violaxanthin (Table I).

Spectroscopic Characterization

Fluorescence Emission

Fluorescence emission spectroscopy was used to probe energy transfer within the recombinant proteins. Fluorescence emission spectra were essentially identical (one major emission at 682 nm), irrespective of whether Chl *a*, Chl *b*, and xanthophylls were excited at 440, 475, and 500 nm respectively. This indicates an efficient energy transfer and equilibration between all pigments bound. Since energy transfer to Chl *a*, especially in the case of carotenoids, is strongly dependent on chromophore-chromophore distance and orientation (23), this result suggests that protein folding is very similar, if not identical, to the LHCII control. The only exception to this pattern was the LHCII zeaxanthin sample: the fluorescence emission spectrum strongly depended on the excitation wavelength, and part of the Chl *b* was incompetent for energy transfer with Chl *a* as shown by the direct Chl *b* emission at 660 nm. This was also the case for a Chl *a* subset that emitted at shorter wavelengths with respect to the LHCII control sample, whereas the Chl *a* emission excited by Chl *b* (475 nm) or xanthophyll (500 nm) wavelengths was red-shifted by several nanometers (Fig. 2*E*).

Absorption Spectra

The absorption spectra of selected recombinant proteins are shown in Fig. 3. Changes in the absorption spectra were detected not only in the Soret region, where the xanthophylls absorb, but also in the Q_y region, where only Chl absorption is expected. Difference absorption spectra are shown in Fig. 4 (*A* and *B*).

Soret Region—It is well known that the $S_0 \rightarrow S_2$ transition for carotenoids is strongly affected by the environment (24) and that the absorption peaks of these protein-bound molecules are shifted toward lower energy with respect to those in organic solvent. However, the actual absorption of xanthophylls in native LHCII proteins is difficult to determine due to superposition of the Chl *a*, Chl *b*, and xanthophyll transitions. The availability of recombinant LHCII proteins that bind a single carotenoid species allows for the determination of the energy level of the red-most $S_0 \rightarrow S_2$ transition of individual xanthophyll molecules within LHCII proteins by second derivative analysis of the difference spectra of LHCII control minus single xanthophyll LHCII proteins (Fig. 5). The values determined for the red-most transition of lutein, violaxanthin, neoxanthin, and zeaxanthin were 495, 492, 488, and 501 nm, respectively. Since the corresponding values in 80% acetone are 477.2, 472.8, 468.4, and 481.6 nm, it follows that the protein microenvironment causes a red shift of 18–20 nm upon xanthophyll absorption.

Q_y Transition—The difference spectra between the LHCII control and single xanthophyll proteins in the 600–720 nm range are shown in Fig. 4 (*A* and *B*). In the 630–660 nm range, corresponding to Chl *b* absorption (19, 21), LHCII lutein showed a strong decrease in the amplitude of the 652 nm peak, whereas the 640 nm absorption component was slightly increased. Differences were also observed in the Chl *a* absorption region (660–684 nm), where the amplitude of the 677 nm transition was decreased, whereas higher absorption was observed at 663 nm. Similar results were obtained in the case of the LHCII violaxanthin and LHCII lutein/violaxanthin proteins, although the effects were of lower magnitude. When

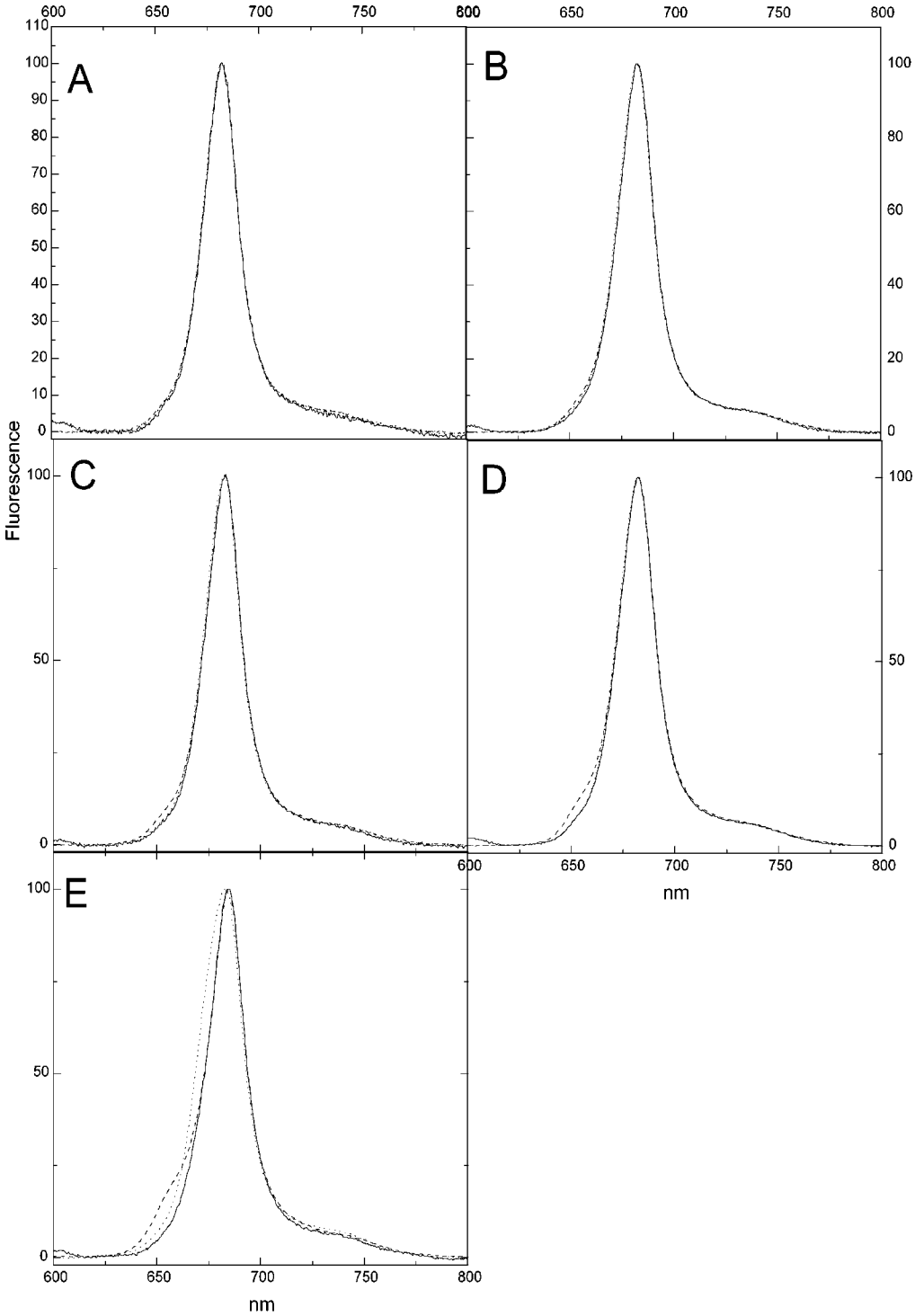

FIG. 2. **Fluorescence emission spectra at room temperature of the recombinant proteins with three different excitations: 440 nm (——), 475 nm (· · ·), and 500 nm (– – –).** The samples were diluted in 10 mM Hepes (pH 7.6) and 0.03% β-dodecyl maltoside at 0.005 μg/ml. *A*, LHCII control; *B*, LHCII lutein; *C*, LHCII violaxanthin; *D*, LHCII neoxanthin/violaxanthin (2 xanthopylls); *E*, LHCII zeaxanthin.

neoxanthin was present, proteins had spectra similar to that of the LHCII control with respect to the amplitude of the 652 nm Chl *b* peak. Nevertheless, small differences either in the Chl *a* or Chl *b* region could be detected. In the case of the complex binding 1 violaxanthin and 1 neoxanthin molecule/polypeptide, with respect to the LHCII control, a shift of a 678 nm absorption component to 666 nm was observed. In the Chl *a* region, the absorption in the Chl *b* region was almost unaffected. The major changes in absorption as detected by difference spectral analysis are reported in Table II.

CD Spectra

The CD spectra for the complexes are reported in Fig. 6. Clear differences in peak shape are observed in the Soret region. The typical CD spectrum of the LHCII control shows a major negative signal at 491 nm and a shoulder at 474 nm, in

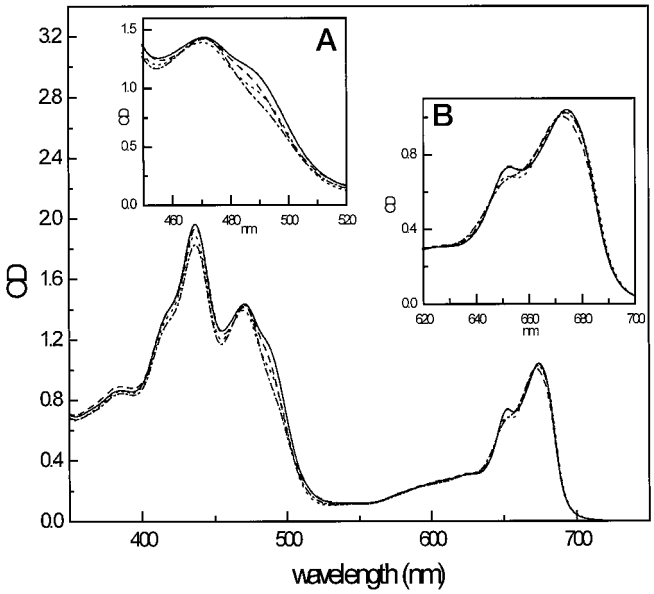

FIG. 3. **Absorption spectra at room temperature of the LHCII control containing three xanthophyll species and of LHCII proteins with modified xanthophyll content.** ——, LHCII control; – · – ·, LHCII lutein; · · · ·, LHCII violaxanthin; – – –, LHCII neoxanthin/violaxanthin. *A* and *B* show enlargements of the Soret and Q_y regions, respectively. The spectra were normalized on the area of the Q_y absorption region (630–750 nm) on the basis of the number of Chl *a* and Chl *b* molecules determined by biochemical analysis considering a value of 0.7 for Chl *b* extinction with respect to Chl *a*.

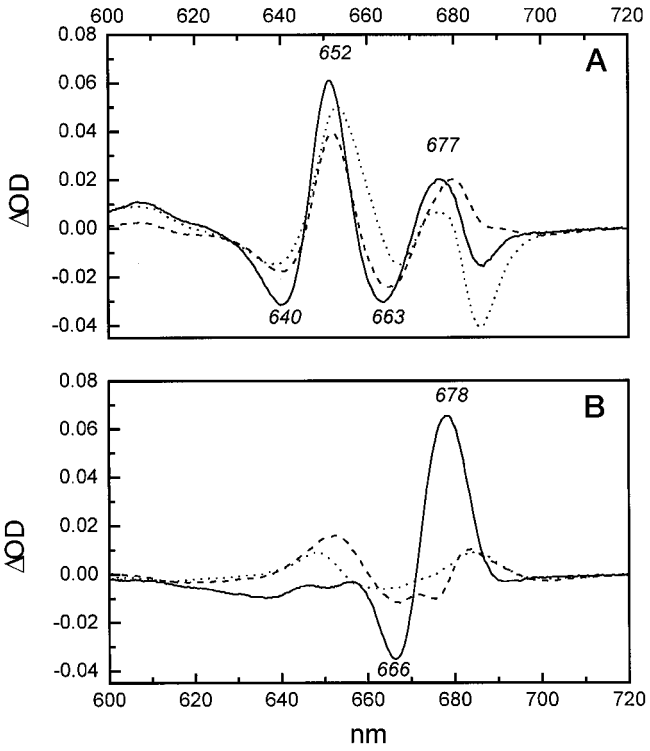

FIG. 4. **Difference spectra between the LHCII control and LHCII proteins with different xanthophyll composition.** *A*, LHCII control minus LHCII lutein (——), LHCII control minus LHCII violaxanthin (· · · ·), and LHCII control minus LHCII lutein/violaxanthin (– – –). *B*, LHCII control minus LHCII neoxanthin/violaxanthin (2 carotenoid molecules) (——), LHCII control minus LHCII neoxanthin/violaxanthin (3 carotenoid molecules) (– – –), LHCII control minus LHCII lutein/neoxanthin (· · · ·).

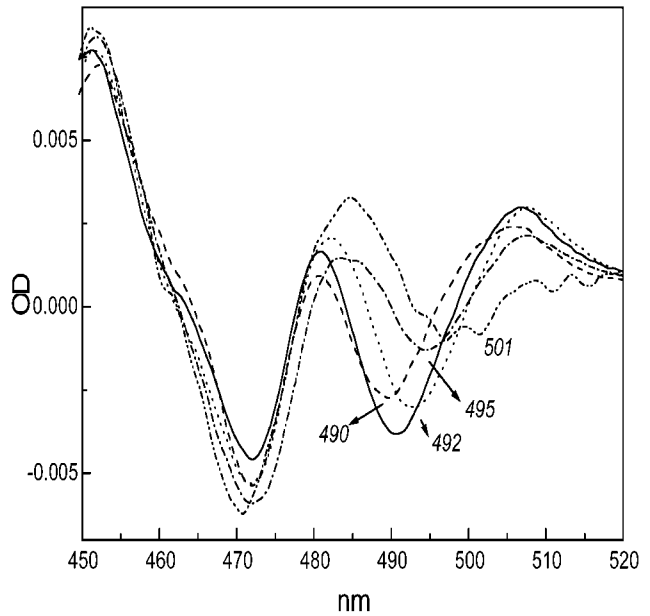

FIG. 5. **Second derivative of the absorption spectra of the complexes in the Soret region.** ——, LHCII control; – · – ·, LHCII lutein; – – –, LHCII neoxanthin/violaxanthin; · · · ·, LHCII violaxanthin; – · · –, LHCII zeaxanthin.

agreement with a previous report on LHCII in the monomeric state (25). In the case of the complex reconstituted with lutein only (LHCII lutein), the relative amplitude of these two peaks is reversed, with the 474 nm (−)-signal becoming predominant. A similar effect was also observed in LHCII violaxanthin and LHCII lutein/violaxanthin, suggesting that the amplitude of the 491 nm (−)-signal is enhanced by the presence of neoxanthin in the LHCII complex. Accordingly, the CD spectra of LHCII lutein/neoxanthin and LHCII neoxanthin/violaxanthin have a shape more closely resembling that of the LHCII control. Differences were also observed in the 600–700 nm region, where the LHCII control shows negative signals at 650 and 682 nm and a positive signal at 668 nm. The 650 nm (−)-signal and the 668 nm (+)-signal are due, at least in part, to a Chl *a*-*b* excitonic interaction (26) as supported by their concomitant change in amplitude among different samples (Fig. 6). In addition, the amplitude of the 652 nm (−)-signal is strongly dependent on the presence of neoxanthin. Accordingly, the 652 nm (−)-signal was reduced in amplitude in LHCII lutein with respect to the LHCII control, whereas it was restored in the neoxanthin-containing samples. The 682 nm (−)-signal was essentially unaffected by the carotenoid composition of LHCII, suggesting that it is mainly due to chlorophyll *a* alone.

Stability

The resistance of selected reconstituted complexes to heat denaturation was measured by recording the CD spectra at increasing temperatures. Spectra were registered from 620 to 720 nm, and the unfolding of the LHCII structure was observed as a decrease in the CD signals to a very low level, due to the intrinsic CD of free chlorophyll.

Temperature-dependent denaturation measurements were performed on three samples to probe the effect of site occupancy in LHCII on the stability of the pigment-protein complex. In particular, the LHCII control sample (in which all three sites are occupied), the LHCII lutein sample (in which the neoxanthin site is empty), and the LHCII neoxanthin/violaxanthin sample (in which the neoxanthin site is occupied, and one of the two sites (for which lutein, violaxanthin, and zeaxanthin compete) is empty) were examined. The decay of the 652 and 681 nm CD signals was fitted by a sigmoidal curve for

TABLE II
Summary of positive and negative components obtained from difference absorption spectra of the recombinant LHCII proteins

	Soret		Q_y	
	+	−	+	−
	nm		*nm*	
CT-L	437, 490		651.2, 677	640, 664, 686.4
CT-V	440, 488		653.2, 677	640, 668, 686
CT-L/V	440, 490		652, 680	640, 666
CT-N	432, 460, 494	447	678.4	666.4
CT-L/N			648	685
CT-N/V	467		653, 684	666, 676
CT-Z	434, 490	516.8	651, 676	664.4, 687.2

[a] CT, L, lutein; V, violaxanthin; N, neoxanthin; Z, zeaxanthin.

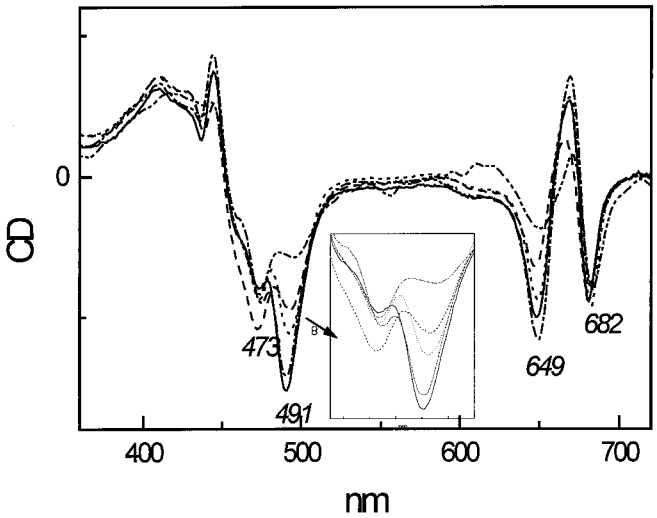

FIG. 6. **Circular dichroism spectra at 10 °C of the different LHCII samples.** ——, LHCII control; – · – ·, LHCII lutein; – – –, LHCII neoxanthin; · · · ·, LHCII violaxanthin; – · · –, LHCII zeaxanthin. The spectra are normalized at the same absorption in the Q_y Chl *a* transition.

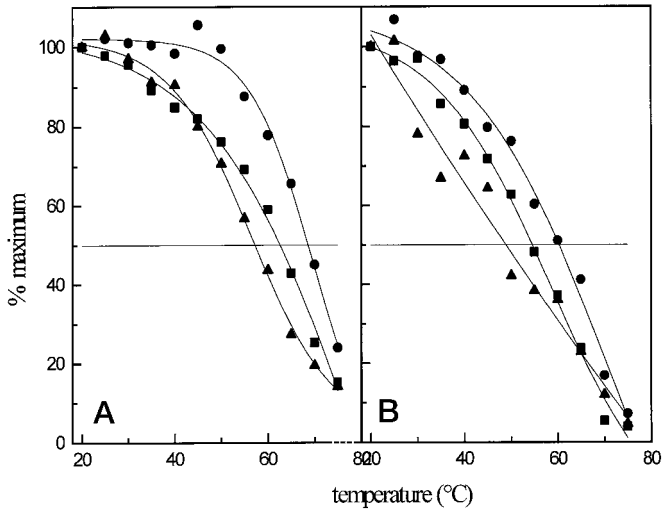

FIG. 7. **Decrease in the CD signals at 652 nm (*A*) and 681 nm (*B*) with temperature for three samples.** ■, LHCII control; ●, LHCII lutein; ▲, LHCII neoxanthin/violaxanthin. The data points are fitted to a sigmoid curve.

these three samples (Fig. 7, *A* and *B*). The temperature at which a 50% decrease in the 652 nm CD signal was observed was different with respect to the LHCII control sample. The value for LHCII lutein is ~5 °C higher (65 °C), and the value of LHCII neoxanthin/violaxanthin (55 °C) is ~5 °C lower. This observation was confirmed by analysis of the 682 nm signal, although the neoxanthin/violaxanthin sample had a more scattered distribution of the data due to the low amplitude of the 681 nm signal at 10 °C.

Photobleaching

We probed the photoprotection capacity of recombinant LHCII containing different xanthophyll complements by illuminating the complexes in the presence of O_2 with bright light (5500 microeinsteins m^{-2} s^{-1}) and determined the decrease in chlorophyll absorption caused by $^1O_2^*$ bleaching of these pigments. The measured effect is the sum of the direct ^{3}Chl* quenching and the ^{1}Chl* quenching, which, in turn, decrease the concentration of ^{3}Chl*. Moreover, direct $^1O_2^*$ quenching by xanthophylls within the protein cannot be excluded. After each consecutive time interval of bleaching, the absorption spectrum of the sample in the range of 600–750 nm was recorded (data not shown). The decrease in the peak area with each subsequent bleaching interval is reported in Fig. 8*A*. As a control, we measured the destruction of the chlorophyll molecules in the control sample treated with Triton X-100, which causes unfolding of the protein and disrupts the chromophore arrangement in the complex.

When an oxygen-scavenging system was added, no photobleaching of the chlorophyll chromophores was observed. The data points were fitted to a first-order exponential decay function (Table III). The bleaching time represents the time needed for a 1% decrease in the initial chlorophyll absorption in the range from 630 to 750 nm.

The samples tested clearly differed for their y_0 value and for their bleaching times. The y_0 value indicates the percentage of total absorption, which is protected from bleaching described by a monoexponential kinetic curve. With longer treatment (>1 h), the protein structure collapsed due to massive chromophore destruction.

When the total area of the Q_y absorption was considered (Chl *a* + *b*), it could be observed that the y_0 value decreases in the following order: lutein/neoxanthin ≥ neoxanthin/violaxanthin > zeaxanthin ≥ violaxanthin > lutein (Table III). The samples lacking neoxanthin showed values well below those of samples binding this xanthophyll. The 1% bleaching time decreased in the following order: lutein/neoxanthin > lutein > zeaxanthin > neoxanthin/violaxanthin > violaxanthin, with the violaxanthin sample clearly appearing to be less efficient than the lutein/neoxanthin sample in protection from photodamage.

As shown above, neoxanthin occupies one unique site, distinct from those (two) for which violaxanthin, lutein, and zeaxanthin compete. When the neoxanthin site is empty, the relative photobleaching protection efficiency of lutein, violaxanthin, and zeaxanthin (bound to two other sites) can be compared, thus showing that lutein is much more effective than violaxanthin (0.47 *versus* 0.30). The quenching time of zeaxanthin is intermediate between the two (0.39).

Similar results are obtained when the neoxanthin site is occupied. The effect of the presence of neoxanthin in the LHCII structure provided a significant increase in the resistance of

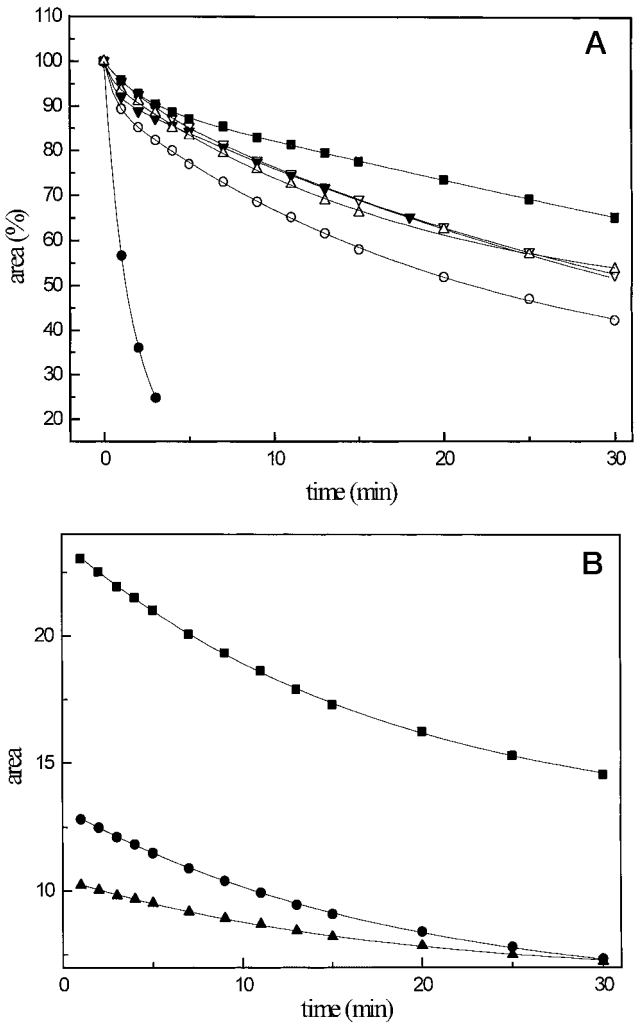

FIG. 8. **Decrease in the chlorophyll absorption due to photobleaching.** *A*, area decrease in the red absorption (600–750 nm) reported as a function of the time interval of high light treatment. The points represent the experimental data for different samples. ○, violaxanthin; ▽, lutein; ▼, LHCII zeaxanthin; △, LHCII neoxanthin/violaxanthin; ■, LHCII lutein/neoxanthin; ○, LHCII control + Triton X-100. *B*, decrease in the Chl *a* and Chl *b* absorption due to photobleaching in LHCII lutein/neoxanthin. ■, total Q_y transition; ●, Chl *a* (630–663 nm); ▲, Chl *b* (663–750 nm).

the complex to bleaching (bleaching time: lutein/neoxanthin, 0.62; and neoxanthin/violaxanthin, 0.34). Samples in which lutein was partially replaced by violaxanthin were more prone to photobleaching. Further insight into the photoprotection function is given by evaluation of the y_0 value and the bleaching times separately for Chl *a* and Chl *b*. The area from 630 nm to the isosbestic point at 663 nm is due to the Chl *b* absorption (19, 21) and decreases more slowly than the area from 663 to 750 nm, which corresponds to the Chl *a* absorption (Fig. 8*B*). In Table IV, the parameters of the monoexponential decrease in the Chl *a* and Chl *b* absorption are summarized. It is shown that the samples with the neoxanthin site occupied indeed protect more efficiently Chl *b* with respect to the samples without neoxanthin.

DISCUSSION

How Many Xanthophyll-binding Sites Are in LHCII?—In this study, we have characterized LHCII proteins reconstituted *in vitro* with an experimentally modified xanthophyll complement to determine the specificity of the binding sites and the effect of their occupancy on the function of LHCII as revealed from its spectroscopic, photoprotection, and protein stability properties. When refolded in the presence of a total thylakoid extract, recombinant LHCII bound 7 Chl *a*, 5 Chl *b*, 1.8 lutein, 1.0 neoxanthin, and 0.2 violaxanthin molecules/polypeptide chain, in agreement with the composition of native LHCII extracted from leaves (18), thus showing that three binding sites are present in each monomeric LHCII molecule. Moreover, the non-integer stoichiometry found for lutein and violaxanthin in the complex indicates that these two chromophores may bind to the same site. Structural determination (2) has revealed two binding sites, L1 and L2, interposed between the transmembrane helices A and B. The xanthophyll molecule in the L1 site thus cross-braces the stroma-exposed loop between helices C and A to the C-terminal domain of helix A, whereas the L2 site connects the N-terminal stretch to the helix B/helix C loop. According to their central location in the complex, these 2 xanthophyll molecules are involved in stabilizing the structure as shown by the absence of protein folding without xanthophylls (5, 6), not only in LHCII, but also in CP29 and CP24, which bind only 2 xanthophyll molecules/polypeptide (12, 13, 19). The third xanthophyll was not revealed by electron microscopy, and its location within the complex and its functional role are presently unknown.

The Neoxanthin-binding Site—In vitro, recombinant LHCII can be folded in the absence of neoxanthin, yielding proteins with 2 bound xanthophyll molecules rather than 3, showing that the site that binds neoxanthin (N1) is distinct from the other two xanthophyll sites. The LHCII proteins lacking neoxanthin bind a normal chlorophyll complement; are stable to heat denaturation; and show equilibration of energy among the 12 bound chlorophyll chromophores, indicating that the orientation and relative distances of chromophores are essentially conserved. The occupancy of the neoxanthin site is thus not necessary for structural stability. Neoxanthin-free LHCII proteins can be obtained in the presence of lutein, violaxanthin, or zeaxanthin or a combination of these xanthophylls. The resulting holoproteins exhibit several common features: (i) a decreased amplitude of the 652 nm absorption peak in the absence of any change in the Chl content and Chl *a*/*b* ratio, (ii) CD spectra with reduced amplitude of the conservative 652 nm (−)/668 nm (+)-signal due to the Chl *a*-*b* excitonic interaction (26) and a reduced ratio between the 491 nm (−)- and 474 nm (−)-signals, (iii) a reduced size of the chlorophyll pool efficiently protected in photobleaching experiments, and (iv) preferential photoprotection of Chl *a* with respect to Chl *b*. These common features suggest that lutein, violaxanthin, and zeaxanthin are bound to the central L1 and L2 sites, whereas the N1 site is located elsewhere in a Chl *b*-rich domain. This is consistent with the results of mutational analysis of the homologous protein CP29 (27), supporting the suggestion from structural work (2) that porphyrin sites belonging to the 2-fold symmetric core of the LHCII proteins, formed by transmembrane helices A and B, bind Chl *a*. Chl *b* is rather located in more peripheral sites near helices C and D.

Neoxanthin cannot provide protein stabilization when supplied as the only carotenoid, but can induce an increase in the carotenoid content of the protein from 2 to 3 molecules/polypeptide when provided together with lutein, zeaxanthin, and/or violaxanthin. We conclude that the N1 site is neither necessary nor sufficient for pigment-protein stability, contrary to a previous suggestion (28). The finding that LHCII with a vacant N1 site is more stable to heat denaturation with respect to the LHCII control, which binds 3 xanthophyll molecules, is somewhat surprising. The neoxanthin-binding site may be different with respect to the two other sites. A search in the primary sequence of LHCII for putative xanthophyll-binding sites (29) did not yield additional sequence motifs other than the two L

TABLE III
Parameters of the monoexponential decay functions describing the decrease in the chlorophyll absorption due to photobleaching (asymptote y_o, amplitude A, and decay time t)

Sample	y_o	A	t	Bleaching time
			min	*min/1% area decreased*
Viola xanthin	32.3	70.2	19.8	0.30
Lutein	26.7	72.6	34.2	0.47
Lutein/neoxanthin	50.2	52.6	30.6	0.62
Neoxanthin/violaxanthin	47.6	70.9	17.6	0.34
Zeaxanthin	34.9	68.4	25.5	0.39

TABLE IV
Parameters of the monoexponential decay funtions desribing the decrease in the chlorophyll a and b absorption due to photobleaching (asymptote y_o, amplitude A, and decay time t)

Sample	Chl *a*				Chl *b*				Chl *b* Bl.t./Chl *a* Bl.t.
	y_o	A	t	Bl.t[a]	y_o	A	t	Bl.t.	
Violaxanthin	28.0	75.1	17.8	0.25	36.3	65.3	24.8	0.39	1.56
Neoxanthin/violaxanthin	41.8	61.9	16.0	0.27	53.5	48.7	21.3	0.46	1.70
Zeaxanthin	36.4	65.6	23.4	0.37	46.6	54.4	30.7	0.57	1.54
Lutein	46.5	52.4	20.2	0.38	47.5	52.2	29.1	0.55	1.45
Lutein/neoxanthin	43.2	56.7	27.8	0.49	57.2	42.3	39.2	0.92	1.87

[a] Bl.t., bleaching time.

sites close to helices A and B. It can therefore be proposed that neoxanthin has its binding site made of pigment-pigment interactions rather than pigment-protein interactions as suggested by the changes in Chl *b* spectral properties in the neoxanthin-free protein. This may indicate that neoxanthin is interposed between several Chl *b* molecules, which modifies their environment. In its absence, Chl-Chl rather than xanthophyll-Chl interactions might induce an even more stable conformation than the LHCII control.

Luteinin, Violaxanthin, and Zeaxanthin Are Bound to the L1 and L2 Sites Identified by Electron Microscopic Analysis—The L1 and L2 sites are rather aspecific since they can accommodate lutein, violaxanthin, and zeaxanthin and, in their absence, even β-carotene (not present in native Lhcb proteins), but not neoxanthin. This selectivity is likely to be based on the peculiar molecular conformation of neoxanthin (30). The possibility of the rotation of the rings with respect to the polyene chain seems to be important for fitting the L1 and L2 sites as shown by the effect of reconstituting LHCII with zeaxanthin, whose rings lie in the plane of the polyene chain due to their participation in the delocalized π-orbital. The resulting protein shows an increased Chl *a*/*b* ratio (2.3 *versus* 1.4), suggesting that zeaxanthin interferes with the binding sites of two Chl *b* molecules for LHCII. Fluorescence emission spectra clearly show that zeaxanthin prevents efficient energy transfer between Chl *b* and Chl *a*, whereas short wavelength-absorbing Chl *a* molecules appear to be unable to transfer energy to longer wavelength forms, suggesting that the orientation and/or interchromophore distance (and therefore, protein folding) is affected. Zeaxanthin is very similar to lutein; however, the chirality of the two hydroxyl groups is the same for zeaxanthin and opposite for lutein. Moreover, the positioning of the double bond in the ε-ring alters the three-dimensional shape such that it lies at a different angle with respect to the conjugated backbone. The angle of the ε-ring of lutein is the same as that of the epoxy rings of antheraxanthin and violaxanthin, which seems to substitute effectively for lutein in *Arabidopsis* mutants (30). The xanthophylls have been proposed to have binding sites on the hydrophilic loops composed by hydrophobic sequences interrupted by a polar residue (29) that would interact with hydroxyl groups of rings. Changes in the geometry of the interaction might lead to the conformational changes affecting energy transfer in the complex. It should be noted that the minor Chl *a*/*b* protein CP29 binds zeaxanthin without affecting either Chl binding characteristics of the protein or energy equilibration (31, 44), in agreement with the suggestion that the zeaxanthin active in non-photochemical quenching is bound to Lhcb4–6 (CP29, CP26, and CP24) rather than to Lhcb1–3 (LHCII).

Although lutein, violaxanthin, zeaxanthin, and β-carotene can occupy the L1 and L2 site, the relative affinities are somewhat different: in the presence of violaxanthin/lutein ratios of 1:1 and 1:3 in the reconstitution mixture, the resulting complex bound 3 and 9 times more lutein than violaxanthin, respectively. Zeaxanthin and β-carotene were bound only when violaxanthin and lutein were either absent or present in limiting amounts during reconstitution. This result is in contrast with CP29, where violaxanthin and zeaxanthin, when present during reconstitution, are both bound to the complex (31, 44). This suggests that site affinity for xanthophyll species is distinct in each Lhc protein.

The Role of the L1 and L2 Sites in Protein Stability—In the presence of an excess of neoxanthin and a limiting amount of violaxanthin, a pigment-protein complex was obtained that bound only 2 xanthophyll molecules: 1 neoxanthin and 1 violaxanthin molecule. This result suggests that only 1 of the 2 xanthophyll molecules in the L1 and L2 sites is necessary for protein stabilization. With reference to the homologous Lhc protein CP29 (27), we suggest that the site required for the stabilization of the complex is L1.

Measurements of heat denaturation showed that the LHCII neoxanthin/violaxanthin sample, in which the N1 site is occupied and one of the L sites is empty, denatures at lower temperature with respect to the LHCII control sample, in which the three xanthophyll-binding sites are occupied. Thus, both L sites contribute to pigment-protein stability.

Does a Fourth Xanthophyll-binding Site Exist in LHCII?—The above results consistently support the view that violaxanthin is tightly bound to the L1 and L2 sites of LHCII, in agreement with the previous mutational analysis of CP29. However, it was reported that violaxanthin is bound to a peripheral site (18) and can be removed by low pH treatment (4). This apparent contradiction can be ascribed to the different sources of the proteins. Native LHCII from low light-grown *Vinca major* was reported to bind three carotenoids, while four were bound to the protein isolated from high light-grown plants. The additional site is occupied by acid-labile violaxanthin (4). After acid treatment (*e.g.* isolation by isoelectric focus-

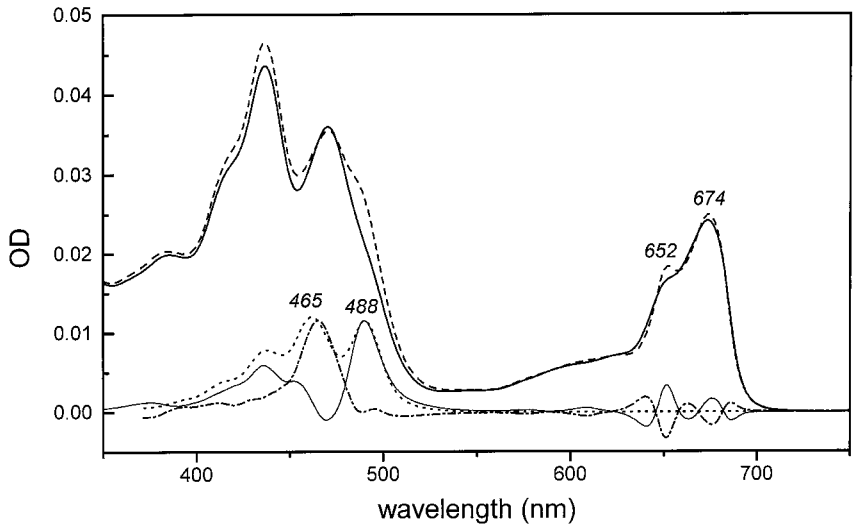

FIG. 9. The *two upper spectra* show the absorption spectra of LHCII lutein/neoxanthin (– – –) and LHCII lutein (——) at room temperature and are normalized to the total Q_y transition. The *three lower spectra* show the absorption spectrum of neoxanthin in 80% acetone shifted by 19 nm to mimic its absorption in the protein (· · · ·), the difference spectrum between LHCII lutein/neoxanthin and LHCII lutein (——), and the difference spectrum between the shifted neoxanthin spectrum and the LHCII lutein/neoxanthin minus LHCII neoxanthin difference spectrum (– · – · –), respectively. This latter spectrum shows that the dipole strength of the Chl *b* transition at 465 nm is increased in the sample without neoxanthin (LHCII lutein), whereas it is decreased in the case of the Q_y transition (652 nm peak in the *two upper spectra*). The *three lower spectra* were multiplied by a factor of 2 for better viewing.

ing), only 0.1–0.2 mol of violaxanthin/mol of polypeptide was still bound (4, 18). Recombinant LHCII bound only low amounts (0.1–0.2 mol/mol of polypeptide), which were acid-resistant. On this basis, we propose a dual location for violaxanthin in native LHCII: (i) the L1 and L2 sites in a small amount in competition with lutein and (ii) a peripheral V1 site, specific for violaxanthin. The reason why the V1 site is not found in recombinant LHCII is not yet clear. It might be that the binding site is stabilized by trimerization (31, 44). Another possibility is that this site may be present only in a subset of the many Lhcb1–3 gene products (32) whose expression is possibly enhanced under high light conditions (4).

Chlorophyll-Carotenoid Interactions Explain the Characteristic 652 nm Feature of the LHCII Absorption Spectrum and the Observed Energy Transfer from Xanthophylls to Chl b—The carotenoid composition has a strong influence on the spectral properties of monomeric LHCII. In the Soret region, the $S_0 \rightarrow S_2$ transition of carotenoid can be detected, allowing for the identification of the xanthophyll red-most transition. The shift of the carotenoid absorption upon binding to the apoprotein of light-harvesting complexes can be explained in terms of mutual polarization interactions between the carotenoid molecules and the surrounding medium (23, 24). Lutein, violaxanthin, and neoxanthin are shifted in LHCII by 18, 19, and 19 nm, respectively, with respect to the absorption in 80% acetone. These values indicate that the environment of the different xanthophylls in the three xanthophyll-binding sites is similar. Comparable red shift values were observed for spheroidene in LH2 proteins (33).

Biochemical analysis of recombinant proteins clearly shows that whereas the Chl *a* and Chl *b* complement is the same for all samples, the Chl Q_y transition is, nonetheless, clearly affected. Since the $S_0 \rightarrow S_1$ transition of carotenoids is forbidden (34), this transition is not apparent in absorption spectra. The changes in the 600–700 nm region of Chl *a* and Chl *b* thus represent modifications in the energy levels of the Chl transitions induced by xanthophyll proximity. This effect implies a strong interaction between xanthophylls and chlorophyll molecules. The most dramatic effect is an increase in the Chl *b* 652 nm absorption when the neoxanthin site is occupied. Among Lhc proteins, the prominent 652 nm peak is a unique feature of LHCII, whereas other members of the family exhibit a monotonic increase in absorption from 600 nm to the Chl *a* peak (35) even when the molar ratio between Chl *b* and Chl *a* is higher than in LHCII, as is the case in CP24 (19). This is likely to be due to the presence of only two carotenoid sites in Lhcb proteins other than Lhcb1–3 (36, 37) and therefore to the lack of the N1 site. Neoxanthin is also present in CP29 and CP26 (18). In the former protein, it was found to be bound to the L2 site (27).

Comparison of the absorption spectra of LHCII reconstituted with lutein/neoxanthin and with lutein only (Fig. 9) allows for the identification of the effect of neoxanthin-Chl interactions. The direct contribution of neoxanthin to the absorption spectrum is clearly observed in the increase of the 488 nm shoulder in the lutein/neoxanthin sample with respect to the lutein sample. The difference spectrum in the Soret region, however, does not yield the expected three peaks characteristic of neoxanthin: only the 488 nm peak is observed in the difference spectrum. Thus, the two contributions of neoxanthin at higher energies are presumably hidden by increased Chl absorption. Estimation of this absorption change was performed by the following procedures. (i) The neoxanthin contribution was obtained by shifting the 80% acetone spectrum by 19 nm toward lower energies, thus closely featuring the LHCII lutein/neoxanthin minus LHCII lutein difference spectrum in the 475–550 nm range. The amplitude of the 488 nm signal was consistent with 1 mol of neoxanthin/mol of LHCII polypeptide. (ii) The LHCII lutein/neoxanthin minus LHCII lutein difference spectrum was subtracted from the neoxanthin spectrum. This calculation yielded a positive band, peaking at 465 nm, which closely featured the Chl *b* Soret band. We can therefore conclude that the interaction between neoxanthin and Chl *b* induces not only a Chl *b* peak shift in the Q_y transition, but also a change in the relative amplitudes of the Q_y *versus* the Soret band. This could be due to a different orientation of the 7-formyl group of the Chl *b* molecule in the sample containing neoxanthin with respect to the LHCII lutein sample. It can be hypothesized that the formyl group of Chl *b* is bent with respect to the pyrrole plane and therefore cannot participate in the delocalized double bond system. This would make Chl *b* more similar to Chl *a*, in particular with respect to the ratio of the amplitude between the Q_y and Soret absorption bands. Alternatively, neoxanthin might provide a different environment for the several Chl *b* molecules localized between helix C and helices A and B (39), thus tuning their absorption to 652 nm and rendering the Chl *b* peak sharper. A combination of the two effects is also possible. This tight interaction between Chl *b* and neoxanthin supplies a structural ground for the energy transfer from xanthophyll to Chl *b* as observed by femtosecond transient absorption spectroscopy, which showed that the transfer from xanthophyll to Chl *b* was reduced in the mutant without neoxanthin (3).

The spectroscopic changes induced by the absence of neox-

anthin in LHCII monomers are similar to those induced by monomerization of LHCII trimers. This is particularly evident from CD spectra, which undergo major changes in the LHCII proteins without neoxanthin with respect to the LHCII control, which consist of a reduction in the amplitude of the 652 (−)/670 (+)-signal attributed to the Chl *a*-*b* excitonic interaction (26) and in the reversal of the relative amplitude of the two (−)-signals at 490 and 470 nm (Fig. 6). The LHCII lutein sample, although monomeric, has a CD spectrum that closely resembles the spectrum of native trimeric LHCII (27, 38). We therefore suggest that the interactions induced by trimerization cause changes in the neoxanthin-Chl *b* interactions.

Role of Individual Xanthophylls in Photoprotection—Carotenoids may function in photoprotection by quenching $^1O_2^*$ or by preventing its formation from ^{3}Chl*. Protection from photobleaching, which is the effect of either of the two processes or of both, clearly shows that xanthophylls in both the L1 and L2 sites and the N1 site are active in photoprotection. Triplet minus singlet spectra of LHCII showed that ^{3}Chl* quenching was afforded by lutein, but not by neoxanthin (10, 28). On this basis, we propose that the role of neoxanthin in photoprotection of LHCII is to scavenge the $^1O_2^*$ diffusing from Chl *a* chromophores to the Chl *b*-rich domain where neoxanthin is located. This is probably the main function of neoxanthin since this xanthophyll was shown to have the lowest efficiency of energy transfer to chlorophyll (3). Neoxanthin is likely to be capable of ^{3}Chl* quenching, but this function is unlikely to be useful in the N1 site since the probability of triplet formation by Chl *b* is low due to the fast singlet energy transfer to Chl *a* (40). Violaxanthin exhibited an unexpected behavior with respect to photoprotection, which consisted of decreasing the photoprotection capacity of LHCII.

The reasons for this effect are at present unclear. In principle, any xanthophyll with a number of conjugated double bonds more than or equal to 9 should exhibit an S_1 excited state level lower than singlet oxygen. Direct quenching should therefore occur. The photobleaching experiment, however, does not allow for a distinction between Chl singlet and triplet quenching. If the former process is relevant, then violaxanthin, which is suggested to have an S_1 state higher than that of Chl *a* (41), is likely to be less efficient. An anti-quenching effect of externally added violaxanthin was detected by Horton and co-workers (42).

Zeaxanthin has 11 conjugated double bonds and exhibits the lowest S_1 level among the xanthophylls considered in this study. The photobleaching experiment showed that LHCII reconstituted with zeaxanthin is more efficient in photoprotection than the sample with violaxanthin. It is somewhat surprising that its efficacy is lower than that of lutein, which has 10 conjugated double bonds. This might be due to the fact that zeaxanthin, not usually found in LHCII (18), induces some conformational change in the protein, causing incomplete energy equilibration and alteration in Chl binding. Thus, whereas lutein and violaxanthin samples are fully equilibrated, and therefore, chlorophylls and carotenoids have a fully functional positioning to each other for energy transfer, zeaxanthin causes a disturbance of the structure. We therefore conclude that zeaxanthin is not a genuine component of LHCII, at least not as a tightly bound chromophore. We confirm that zeaxanthin is a good quencher of ^{3}Chl*; in fact, despite incomplete equilibration, it is only slightly less efficient than lutein, the major xanthophyll component of native LHCII and the best ^{3}Chl* *a* quencher (10, 28). Accordingly, refolding *in vitro* in the presence of lutein as the only xanthophyll available yielded a fully equilibrated and functional complex.

Conclusions—In this report, we have constructed recombinant LHCII proteins with a modified carotenoid composition by *in vitro* reconstitution of the Lhcb1 protein overexpressed in bacteria. The monomeric protein possess three xanthophyll-binding sites: the L1 and L2 sites, localized by electron microscopy in the helix A/helix B cross, have the highest affinity for lutein, but can also bind violaxanthin and zeaxanthin with lower affinity. When incorporated into the complex, the latter xanthophyll causes disruption of excitation energy equilibration. The occupancy of at least one of these sites, probably L1, is essential for protein folding. Neoxanthin is bound to a distinct site that is highly selective for this species and whose occupancy is not necessary for protein folding. Whereas xanthophylls in the L1 and L2 sites interact mainly with Chl *a*, neoxanthin shows strong interaction with Chl *b*, thus inducing the hyperchromic effect of the 652 nm absorption band. This observation explains the recent results of energy transfer from carotenoids to Chl *a* through Chl *b* obtained by femtosecond absorption spectroscopy. Whereas xanthophylls in the L1 and L2 sites are active in photoprotection through ^{3}Chl* quenching, neoxanthin seems to act in $^1O_2^*$ scavenging. It is clear that individual xanthophylls are located in distinct sites in the different members of the Lhc family. Neoxanthin, for instance, is located in the L2 site in CP29 and probably in CP26, but in the N1 site in LHCII. Other Lhc proteins such as Lhcb4 and Lhca1–4 do not bind neoxanthin at all. Further work is needed for elucidation of the specific role of each carotenoid species in the photosynthetic apparatus.

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Roberta Croce, Saskia Weiss and Roberto Bassi

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