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

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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 mainl 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 \(^1\)O\(_2\) 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 BamHI restriction site at nucleotide 155 and a HindIII 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 plDL2BH3 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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‡ The abbreviations used are: LHCII, light-harvesting complex II; Chl, chlorophyll; HPLC, high pressure liquid chromatography.
at the maximum in the Qy 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 (9, 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, 49) using a pigment mixture containing Chl a, Chl b, \(\beta\)-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 non-denaturing 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/polyepitope 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
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/polymer peptide 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/polymer peptide, 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/polymer peptide: 1 violaxanthin and 1 neoxanthin molecule. Although it was possible to reconstitute a complex binding only violaxanthin (2 molecules/polymer peptide) 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 iden

tical, 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. 2E).

**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 Qy 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 S0 → S1 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 S0 → S1 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.

**Qy 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

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**Table I**

| Pigment composition of recombinant LHC II complexes |
|-----------------------------------------------|
| **Sample** | **Pigment mixture** | **Yield** | **Pigment composition (protein)** |
|-----------|-------------------|-----------|---------------------------------|
| Native    | /  /  /  /  /   | 1.4       | Chl a/b  | N  V  L  Z  ΣCar. |
| Control   | 16  16  50       | 1.4       | 1 ± 0.1 | 0.2 ± 0.05 | 1.8 ± 0.1 | — | 3 |
| 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  —           | 1.43      | 1.1 ± 0.1 | 1 ± 0.1 | —       | — | 2.1 |
| Z         | — 100 +         | 2.3       | —       | —       | —       | 2 | 2 |
| LV        | tr  50 50        | 1.42      | 0.13 ± 0.01 | 0.6 ± 0.01 | 1.6 ± 0.05 | — | 2.3 |
| LN        | 50  — 50        | 1.4       | 1.0 ± 0.1 | —       | 1.9 ± 0.1 | — | 2.9 |
| LNV       | 50  50          | 1.43      | 1 ± 0.1 | 1.3 ± 0.05 | 0.6 ± 0.05 | — | 2.9 |

* N, neoxanthin; V, violaxanthin; L, lutein; Z, zeaxanthin; ΣCar., total carotenoids; tr, trace; /, not available; —, not found.
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
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
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 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 LH-CII 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 3Chl$^*$ quenching and the 1Chl$^*$ quenching, which, in turn, decrease the concentration of 3Chl$^*$. Moreover, direct 1O$_2^*$ quenching by xanthophylls within the protein cannot be excluded. After each consecutive time interval of bleaching, the absorption spectrum 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. 8A. 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.

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 LHCCI structure provided a significant increase in the resistance of 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.

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**TABLE II**

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

|                  | +              | –               |                  | +              | –               |
|------------------|----------------|-----------------|------------------|----------------|-----------------|
| Soret            | 437, 490       | –               | 651.2, 677       | 440, 488       | –               | 653.2, 677      |
| CT-L             | 440, 490       | –               | 652, 680         | 432, 460, 494  | 447             | 653, 680        |
| CT-V             | 516.8          | –               | 640, 664         | 467            | –               | 666.4          |
| CT-L/V           | 434, 490       | –               | 640, 664, 686.4  | 434, 490       | 516.8           | 666.4, 676      |
| CT-N             | –              | –               | 651, 676         | –              | –               | 644.4, 687.2   |
| CT-L/N           | –              | –               | –                | –              | –               | –              |
| CT-N/V           | –              | –               | –                | –              | –               | –              |
| CT-Z             | –              | –               | –                | –              | –               | –              |

*CT, L, lutein; V, violaxanthin; N, neoxanthin; Z, zeaxanthin.*
How Many Xanthophyll-binding Sites Are in LHCII?—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
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 LHClII control.

**Lutein, 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 LHClII 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 LHClII. 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 e-ring alters the three-dimensional shape such that it lies at a different angle with respect to the conjugated backbone. The angle of the e-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 (LHClI).

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 LHClII 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 LHClII 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 LHClII?**—The above results consistently support the view that violaxanthin is tightly bound to the L1 and L2 sites of LHClII, 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 LHClII 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-

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**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 $t$) at a different angle with respect to the conjugated backbone.

| Sample                  | $y_o$ | $A$  | $t$ | Bleaching time |
|-------------------------|-------|------|-----|---------------|
| 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 functions describing the decrease in the chlorophyll a and b absorption due to photobleaching (asymptote $y_o$, amplitude $A$, and decay time $t$) at a different angle with respect to the conjugated backbone.

| Sample                  | $y_o$ | $A$  | $t$ | $t$  |
|-------------------------|-------|------|-----|-----|
| Violaxanthin            | 28.0  | 75.1 | 17.8| 0.25|
| Neoxanthin/violaxanthin | 41.8  | 61.9 | 16.0| 0.27|
| Zeaxanthin              | 36.4  | 65.6 | 23.4| 0.37|
| Lutein                  | 46.5  | 52.4 | 20.2| 0.38|
| Lutein/neoxanthin       | 43.2  | 56.7 | 27.8| 0.49|

| Sample                  | $y_o$ | $A$  | $t$ | $t$  |
|-------------------------|-------|------|-----|-----|
| Violaxanthin            | 36.3  | 65.3 | 24.8| 0.39|
| Neoxanthin/violaxanthin | 53.5  | 48.7 | 21.3| 0.46|
| Zeaxanthin              | 46.6  | 54.4 | 30.7| 0.57|
| Lutein                  | 47.5  | 52.2 | 29.1| 0.55|
| Lutein/neoxanthin       | 57.2  | 43.3 | 39.2| 0.92|

*Bl.t., bleaching time.*
shows that the dipole strength of the Chl all samples, the Chl Q_y transition is, nonetheless, clearly affected. Whereas the Chl proteins (33).

neoxanthin in LH2 phylls in the three xanthophyll-binding sites is similar. Com-
parison red shift values were observed for spheroidene in LH2.

neoxanthin are shifted in LHCII by 18, 19, and 19 nm, 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 transition (652 nm peak in the two upper spectra). The three lower spectra were multiplied by a factor of 2 for better viewing.

FIG. 9. The two upper spectra show the absorption spectra of LHCI lutein/neox-
anthin (—) and LHCI lutein (—) at room temperature and are normalized to the total Q 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 LHCI lutein/neoxanthin and LHCI lutein (—), and the difference spectrum between the shifted neoxanthin spectrum and the LHCI lutein/neoxanthin minus LHCI 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 transition (652 nm peak in the two upper spectra). The three lower spectra were multiplied by a factor of 2 for better viewing.

Comparison of the absorption spectra of LHCI 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 LHCI lutein/neoxan-
thin minus LHCI 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 LHCI polyamide. (ii) The LHCI lutein/neoxanthin minus LHCI 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 transition, but also a change in the relative amplitudes of the Q, 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 LHCI 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, 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-

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 transition is, nonetheless, clearly affected. Since the S_0 → 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 in LHCCI, 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), only 0.1–0.2 mol of violaxanthin/mol of polypeptide was still bound (4, 18). Recombinant LHCI 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 violax-
anthin in native LHCI: (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 LHCI 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 Lhcbs1–3 gene products (32) whose expression is possibly enhanced under high light conditions (4).

Chlorophyll-Carotenoid Interactions Explain the Characteristic 652 nm Feature of the LHCI 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 → 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 LHCI by 18, 19, and 19 nm, respectively, with respect to the absorption in 80% acetone. These values indicate that the environment of the different xanthophy-
l 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 transition is, nonetheless, clearly affected. Since the S_0 → 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 in LHCCI, 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).
anthin in LHClI monomers are similar to those induced by monomerization of LHClI trimers. This is particularly evident from CD spectra, which undergo major changes in the LHClI proteins without neoxanthin with respect to the LHClI 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 LHClI lutein sample, although monomeric, has a CD spectrum that closely resembles the spectrum of native trimeric LHClI (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 1O2 or by preventing its formation from 3Chl*. 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 LHClI showed that 3Chl* quenching was afforded by lutein, but not by neoxanthin (10, 28). On this basis, we propose that the role of neoxanthin in photoprotection of LHClI is to scavenge the 1O2 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 3Chl* 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 LHClI.

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 S1 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 S1 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 S1 level among the xanthophylls considered in this study. The photobleaching experiment showed that LHClI 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 LHClI (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 LHClI, at least not as a tightly bound chromophore. We confirm that zeaxanthin is a good quencher of 3Chl*; in fact, despite incomplete equilibration, it is only slightly less efficient than lutein, the major xanthophyll component of native LHClI and the best 3Chl* 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 LHClI proteins with a modified carotenoid composition by *in vitro* reconstitution of the Lhc1 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 3Chl* quenching, neoxanthin seems to act in 1O2 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 LHClI. Other Lhc proteins such as Lhc4 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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