Photoprotection in the Antenna Complexes of Photosystem II

ROLE OF INDIVIDUAL XANTHOPHYLLS IN CHLOROPHYLL TRIPLET QUENCHING

Milena Mozzo$^{1,2}$, Luca Dall’Osto$^{3}$, Rainer Hienerwadel$^{1}$, Roberto Bassi$^{1}$, and Roberta Croce$^{1,4}$

From the $^1$Department of Biophysical Chemistry, Groningen Bimolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands; $^2$Istituto di Biofisica del Consiglio Nazionale delle Ricerche, via Sommarive 18, 38100 Povo-Trento, Italy; $^3$Dipartimento Scientifico e Tecnologico, Università di Verona, Strada Le Grazie 15-37134 Verona, Italy, and $^4$Università d’Aix-Marseille II, LGPB-Département de Biologie, Faculté de Sciences de Luminy, 163 Avenue de Luminy, 13288 Marseille, France

In this work the photoprotective role of all xanthophylls in LhcbII, Lhcb4, and Lhcb5 is investigated by laser-induced Triplet-minus-Singlet (TmS) spectroscopy. The comparison of native LHCII trimeric complexes with different carotenoid composition shows that the xanthophylls in sites V1 and N1 do not directly contribute to the chlorophyll triplet quenching. The largest part of the triplets is quenched by the lutein bound in site L1, which is located in close proximity to the chlorophylls responsible for the low energy state of the complex. The lutein in the L2 site is also active in triplet quenching, and it shows a longer triplet lifetime than the lutein in the L1 site. This lifetime difference depends on the occupancy of the N1 binding site, where neoxanthin acts as an oxygen barrier, limiting the access of O$_2$ to the inner domain of the Lhc complex, thereby strongly contributing to the photostability. The carotenoid triplet decay of monomeric Lhcb1, Lhcb4, and Lhcb5 is mono-exponential, with shorter lifetimes than observed for trimeric LHCII, suggesting that their inner domains are more accessible for O$_2$. As for trimeric LHCII, only the xanthophylls in sites L1 and L2 are active in triplet quenching. Although the chlorophyll to carotenoid triplet transfer is efficient (95%) in all complexes, it is not perfect, leaving 5% of the chlorophyll triplets unquenched. This effect appears to be intrinsically related to the molecular organization of the Lhcb proteins.

Under normal light conditions, the photosynthetic apparatus is very efficient in harvesting light energy and transferring excitation energy to the reaction center, where it is used to induce charge separation. In high light, when the amount of energy harvested by the system exceeds the capacity for electron transport to available sinks, other de-excitation mechanisms become important (1). Part of the excitations decay via intersystem crossing leading to the production of chlorophyll (Chl)$^2$ triplets, which live rather long ($\tau = 600–1100$ $\mu$s in different solvents (2, 3)) and can react with molecular oxygen producing singlet oxygen, a very reactive and harmful species (4). The damage includes oxidation of lipids (5), protein, and pigments (6), leading to photoinduction of the photosynthesis machinery and photobleaching. Light-harvesting complexes are protected against singlet-oxygen formation by carotenoids (7). These isoprenoids can act in two ways: (i) by quenching of Chl triplets ($\tau = 500$ ps (8)) and (ii) by directly scavenging singlet oxygen ($\tau = 0.7$ ns in benzene (9)); in both cases carotenoid triplets are formed that decay to the ground state, producing heat ($\tau = 9$ $\mu$s in benzene (10)). For these processes to occur, Chls and carotenoids need to be in close contact because triplets are transferred from Chls to carotenoids via the Dexter exchange mechanism (11). The close distances are maintained by the proteins, which coordinate the pigments, thereby allowing fast energy transfer and efficient photoprotection.

The carotenoid composition of higher plants is highly conserved; the chloroplast-encoded subunits of Photosystem I and Photosystem II core complexes bind $\beta$-carotene, whereas the outer antennae, composed of nucleus-encoded light-harvesting complexes (Lhc), accommodate lutein, neoxanthin, and violaxanthin in moderate light, whereas zeaxanthin is produced (12) via de-epoxidation of violaxanthin under light stress conditions. The structure of LHCII (13), the major antenna complex of photosystem II, shows the location of four carotenoids. Two xanthophylls are bound in the center of the molecule in sites L1 and L2 which accommodate mainly lutein (14). A third carotenoid binding site (N1), highly specific for neoxanthin, is located near helix C (15). The fourth site (V1) is at the periphery of the monomeric subunits, and it accommodates violaxanthin, lutein, or zeaxanthin depending on light conditions (16, 17). All carotenoids but the one in the V1 site (17) are involved in light-harvesting and singlet energy transfer (18–20).

Three carotenoid binding sites are present in Lhcb4 and Lhcb5, although these complexes seem to coordinate less than three xanthophylls, possibly due to some loss during purification. As in LHCII, site L1 binds lutein, N1 neoxanthin, and L2 violaxanthin (in Lhcb4) or lutein (in Lhcb5) (16, 21). Singlet energy transfer was observed from all three xanthophylls (22, 23), but the triplet energy transfer and the triplet quenching properties of the carotenoids bound to these complexes have never been investigated.

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1 To whom correspondence should be addressed. Tel.: 31503634214; Fax: 31503634800; E-mail: R.Croce@rug.nl.

2 The abbreviations used are: Chl, chlorophyll; Lhc, light-harvesting complex; Abs, absorption; TmS, Triplet-minus-Singlet; WT, wild type.
Several carotenoid triplet studies on LHCII by absorbance-detected magnetic resonance, fluorescence-detected magnetic resonance, and TmS have been reported in literature (8, 18, 24–33). Although there is general agreement on the lifetime values under anaerobic conditions, differences exist with respect to the values measured in the presence of oxygen. Peterman et al. (18) found a biexponential decay of the carotenoid triplets with components of 2 and 4 μs, associated, respectively, to the TmS spectra peaking at 505 and 525 nm; Schödel et al. (30) described the decay kinetics with a monoexponential decay of 2 μs, whereas a longer lifetime, around 7 μs, was found by Sieffermann-Harms and Angerhofer (27). Because the presence of oxygen enhances the intersystem crossing (34), it was suggested that this difference is due to the sample preparation procedure; it was assumed that the faster lifetimes are associated to LHCII complexes that have partly lost their structural integrity, which is required to prevent oxygen from diffusing into the complexes (27). Because most of the xanthophylls have similar absorption spectra, it has not been possible to unequivocally determine how many and which carotenoids contribute to the TmS spectrum. However, due to the very fast energy transfer from Chl $b$ to Chl $a$ (35), it is expected that triplets are mainly formed on Chls $a$, and thus, carotenoids should be located in their close proximity (36). At that time the available structure of LHCII (36) showed only the location of the two central xanthophylls, and carotenoid to Chl singlet energy transfer measurements have shown that these xanthophylls transfer their excitation energy primarily to Chl $a$ molecules, supporting the hypothesis that Chls $a$ are in close proximity of luteins L1 and L2 (18–20). Later, Lampoura et al. (32) speculated that the violaxanthin in the V1 site could not be involved in triplet energy transfer (17) and that also the neoxanthin was not involved since it was surrounded by Chl $b$ molecules (15). However, from the 2.72 Å resolution structure of LHCII (13) it is now known that at least one Chl $a$ molecule is in close proximity of each xanthophyll; L1 is the carotenoid closest to Chls 610, 612, 613, and 614 (all Chl $a$ according to the structure). L2 is the nearest neighbor of Chls 602 (a), 603 (a), 607 (b), and 609 (b), N1 of Chls 604 (a), 605 (b), 606(b), and 608(b), and V1 of Chls 601(b) and 611(a). Moreover, in the equilibrated system part of the energy, although small, is still located on Chls $b$ (37), which thus, might also require triplet quenching. In this work we have investigated the role of all individual xanthophylls in photoprotection by analyzing native LHCII preparations with different carotenoid composition. We also report for the first time on the carotenoid triplet properties of the minor antenna complexes Lhcb4 (CP29) and Lhcb5 (CP26). By combining spectroscopic measurements with structural data on the LHCII holocomplex, we derive detailed information about the functional role of the individual carotenoids in the Lhc antenna complexes.

**EXPERIMENTAL PROCEDURES**

Plant Material—The wild type (WT) and mutants of Arabidopsis thaliana (ecotype Col-0) npq2 (38) and chylchy2lut5 (39) were grown under controlled light conditions (photoperiod of 8 h of light and 16 h of dark; 100 μmol of photons m$^{-2}$ s$^{-1}$ for WT and npq2; 30 μmol of photons m$^{-2}$ s$^{-1}$ for chylchy2lut5 plants, because of their higher photosensitivity), temperature (23°C/20°C, day/night) and relative air humidity (60–70%).

Thylakoid Preparation; Solubilization and Sample Preparation—Unstacked thylakoids were isolated from leaves, as described previously (40). LHCII trimers from WT and mutants were purified by sucrose gradient as reported (17). Trimeric WT LHCII was further fractionated by flatted iso-electrofocusing at 4°C as described previously (41). Green bands were harvested and eluted from a small column with 10 mM HEPES, pH 7.5, and 0.06%, n-dodecyl-α- and-β-maltoside (α-DM) and further fractionated on a 0.1–1 M sucrose gradient containing 0.06% α-DM and 10 mM HEPES, pH 7.5, for 24 h at 280,000 × g at 4°C.

Reconstituted Complexes—The apoproteins of Lhcbb1, Lhcbb4 from Zea mays (14, 42), and Lhcbb5 from A. thaliana (38) were overexpressed in the SG13009 Escherichia coli strain transformed with constructs following a protocol described previously (43, 44). Reconstitution and purification of pigment-protein complexes were performed as described in Giuffra et al. (42) using a Chl a/b mixture with ratio 2.4 for Lhcbb1 and 3.0 for Lhcbb4 and Lhcbb5.

Pigment Analysis—The pigments were extracted with acetone (80%) and separated and quantified by high performance liquid chromatography, as described in Gilmore and Yamamoto (45) and by fitting the spectra of the acetone extracts with the spectra of individual pigments (46).

Spectroscopy—The absorption spectra at room temperature in 10 mM Heps, pH 7.5, 0.2 mm sucrose, and 0.06% D-maltoside were recorded using a SLM-Amino DK2000 spectrophotometer. The wavelength sampling step was 0.4 nm, the scan rate was 100 nm/min, and the optical path length was 1 cm. Fluorescence emission spectra were measured using a Fluorolog (Jobin Yvon) spectrophotometer and were corrected for the instrument response. The samples were excited at 440 and 475 nm. The spectral bandwidth was 5 nm (excitation) and 3 nm (emission). The chlorophyll concentration was about 0.02 μg/ml in 10 mM HEPES and 0.03% D-maltoside.

Light-induced absorbance changes were recorded with a home-built high sensitivity laser-based spectrophotometer as described in Croce et al. (47) in the presence and absence of oxygen. The anaerobic conditions were obtained by incubating the sample with 20 μg/ml glucose oxidase, 40 μg/ml catalase, and 0.02 μg/ml of glucose for 15 min.

During the measurements under aerobic conditions, the signal intensity at 510 nm was checked at regular time intervals to detect possible loss of intensity due to bleaching of the sample. This value was used to build a curve of the intensity variation as a function of time to correct the data. The correction was particularly important for LHCII-L and LHCII-LZ, which showed amplitude reduction during the experiment.

For a given wavelength the kinetics of the absorbance change were recorded with variable delay times between the actinic and the detection light pulses, ranging from 5 ns to 9 ms. The delay time was obtained by setting the electronic trigger for each laser light pulse. The delay of the light pulses was determined with fast silicon detectors (Thorlabs-Det210). For each kinetic trace, a set of measurements with increasing delay times.
TABLE 1

Pigment composition

| Sample preparation | Chl a/Chl b | Chl total | Cars | Neo | Viola | Lute | Zea |
|--------------------|------------|-----------|------|-----|-------|------|-----|
| LHCII-WT4          | 1.4        | 14        | 4.0  | 1.0 | 0.4   | 2.6  |     |
| LHCII-WT3          | 1.3        | 13        | 3.0  | 1.0 | 0.2   | 1.8  |     |
| LHCII-LZ           | 1.4        | 14        | 3.5  |     |       | 2.5  | 1.0 |
| LHCII-L            | 1.5        | 14        | 2.7  |     |       | 2.7  |     |
| Lhcb1              | 1.3        | 12        | 3.0  | 1.0 | 0.3   | 1.7  |     |
| Lhcb4              | 2.7        | 8         | 1.9  | 0.5 | 0.5   | 0.9  |     |
| Lhcb5              | 1.9        | 9         | 2.4  | 0.8 | 0.1   | 1.5  |     |
| LHCII-WT3 IEF      | 1.3        | 13        | 3.0  | 1.0 | 0.2   | 1.8  |     |
| LHCII-WT4 monomer  | 1.4        | 14        | 3.5  |     |       | 2.5  | 1.0 |
| LHCII-WT trimer    | 1.5        | 14        | 2.7  |     |       | 2.7  |     |
| LHCII-LZ trimer    | 1.3        | 13        | 3.0  | 1.0 | 0.3   | 1.7  |     |
| LHCII-LZ trimer    | 2.7        | 8         | 1.9  | 0.5 | 0.5   | 0.9  |     |

was performed. The time between excitations was 300 ms. For aerobic conditions, 29 different delay times were taken and 48 delay times for anaerobic conditions. The kinetics were measured 5–10 times and averaged. The GraphPad PRISM program (GraphPad Software) was used for globally analyzing the kinetics between 5–10 ns and 9 ms over the 420–580-nm wavelength range.

RESULTS

LHCII trimers were purified from WT plants and mutant plants affected in the carotenoid biosynthesis; that is, npq2, which is blocked at the level of the zeaxanthin epoxidase and accumulates only lutein and zeaxanthin (LHCII-LZ), and chylchl2lut5, which lacks all the xanthophylls in the β-β branch and contains only lutein (LHCII-L). The trimers from WT plants were purified under mild and more aggressive conditions, which led to complexes that differ in the carotenoid/protein ratio (17). Monomeric LHCII (Lhcb1), Lhcb4, and Lhcb5 were obtained by refolding in vitro.

Pigment Content

The pigment composition of the complexes is reported in Table 1. The two different preparations of LhcII WT, WT4 and WT3, bind 4 and 3 carotenoids, respectively. They differ in the amount of lutein and violaxanthin, in agreement with the absence of the xanthophyll in the V1 site in the complex purified by flatbed isoelectrofocusing (LHCII-WT3), as was shown previously (17). LHCII-LZ binds 3.5 carotenoids. The absence of neoxanthin and violaxanthin is partially compensated by the accumulation of zeaxanthin, whereas the amount of lutein is unchanged. The presence of zeaxanthin in LHCII trimers obtained by flatbed isoelectrofocusing (48) indicates that this xanthophyll also binds to the internal sites (L1 and L2), although it is not possible to discriminate between them. LHCII-L coordinates 2.7 luteins (39), which are likely to be accommodated in sites L1 and L2, whereas site N1 is empty (49). Monomeric Lhcb1 coordinates three carotenoids lacking the xanthophylls in the V1 site (50). Lhcb4 coordinates lutein, violaxanthin, and neoxanthin, which are accommodated in sites L1, L2, and N1, respectively (Table 1). The L1 and N1 sites of Lhcb5 have similar occupancy as those of Lhcb4, whereas the L2 site binds lutein (16, 21).

Absorption Spectra

The absorption spectra of the trimers with altered carotenoid composition are reported in Fig. 1, where they are compared with the WT spectrum (WT4). The WT minus LHCII-L absorption difference spectrum (Fig. 1B) is identical both in the carotenoid and in the Chl absorption region to the difference spectrum (WT minus Lhc1-L) obtained for Lhc1 monomers reconstituted in vitro in which the N1 site is empty (14). Note the decrease in the absorption of Chl b at 650 nm, which is typical for complexes lacking neoxanthin, and the lack of a 488 nm band, which is the lowest energy absorption maximum of the neoxanthin (15). It can be concluded that the environment of the neoxanthin does not change upon trimerization.

In the difference spectrum WT minus LHCII-LZ (Fig. 1A) a positive band around 490 nm and a negative around 504 nm were detected, corresponding, respectively, to the neoxanthin, which is absent in LHCII-LZ, and the zeaxanthin, which absorbs mainly around 504 nm, in agreement with previous results on recombinant complexes (15). This difference spectrum is very similar to that of LHCII-WT minus LHCII-L (the sample without neoxanthin), suggesting that the N1 site is at least partially empty, also in LHCII-LZ. No decrease in the absorption at 510 nm is observed, thus suggesting that site L2 still accommodates lutein. Moreover, the main absorption band of zeaxanthin was found at around 504 nm, 18-nm red-shifted as compared with the absorption in solution. This shift is compatible with the binding of zeaxanthin in sites L1 and N1, which induces a similar shift in lutein and neoxanthin absorption in the WT complex (15).

FIGURE 1. Absorption spectra. Absorption spectra at room temperature of LHCII-LZ (A) and LHCII-L (B) (dot-dash lines). In all panels the spectrum of LHCII-WT4 (thin solid line) is presented together with the difference spectrum (thick solid line) multiplied by 3. The spectra are normalized to the Chl content.
The WT4–WT3 difference spectrum (not shown) is identical to the one reported by Caffarri et al. (17) showing the loss of a xanthophyll band around 486 nm.

**Triplet Minus Singlet Spectra**

Triplet formation in native and recombinant Lhcb complexes was studied by flash-induced transient absorption under aerobic and anaerobic conditions; 5-ns flashes excited Chl b at 640 nm, and absorption changes were detected in the 420–580-nm range.

**LHCCI Trimers**—The decay of the carotenoid triplets in LHCCI-WT4 under aerobic conditions could be best fitted by a biexponential decay, the first component having a lifetime of 2.30 μs, a maximum at 507 nm, and a bleaching at 490 nm, and the second component having a lifetime of 3.6 μs, maximum at 522.5 nm, and bleaching at 505 nm (Fig. 2A, Table 2). This indicates that one or more carotenoids absorbing around 490 nm is responsible for the fast decay, whereas a red-shifted xanthophyll (absorption around 505 nm) is responsible for the slower decay. These results are in agreement with those of Peterman et al. (28), but in disagreement with other TmS experiments which showed a single decay component (8, 27). The amplitudes of the two components differ; 68% of the total spectrum is associated to the fast decay and 32% to the slow decay, as shown in Fig. 2A. Under anaerobic conditions, the triplet decay is also best fitted with two exponentials. The fast component has a lifetime of 9 μs, maximum at 510 nm, and a shoulder at 522 nm, and it corresponds to Car triplet decay (Fig. 2B, Table 2). The difference in the carotenoid triplet lifetime for aerobic and anaerobic conditions has been observed before (28), and it is due to the presence of oxygen, which enhances intersystem crossing (34). A second component with very small amplitude, ms lifetime, and a Chl TmS spectrum was also observed, indicating the presence of a small population of unquenched Chls.

To study the contribution to triplet quenching for the carotenoids in the V1 site, the TmS spectra of LHCCI-WT3, in which the V1 site is empty, were measured (Fig. 2C and D, Table 2) and compared with those of LHCCI-WT4. The shape of the spectra, the relative amplitudes of the components, and the lifetimes are virtually identical in both complexes, thus indicating that the carotenoid in the V1 site does not participate in triplet quenching.

To investigate the involvement of the neoxanthin in triplet quenching, the TmS spectra of LHCCI-L, in which the N1 site is empty, were measured. The fitting of the triplet decay of LHCCI-L under aerobic conditions only requires a single exponential showing a 2-μs lifetime (Fig. 3A, Table 2), at variance with the WT results. However, the TmS spectrum of LHCCI-L is identical to the sum of the two spectral components obtained for the WT (data not shown), indicating that the different decay behavior is due to a change in the lifetime of the red-shifted xanthophyll triplet rather than to a change in the energy distribution of the xanthophylls bound to the complex. Under anaerobic conditions, the decay time is 9.7 μs. A long-living (ms) Chl triplet component (Fig. 3B) with the features of both Chl a and Chl b (24, 51) was also detected, demonstrating the presence of unprotected Chls. The TmS carotenoid spectra of LHCCI-L are identical to those of LHCCI-W4, thus demonstrating that the neoxanthin does not participate in the triplet spectrum.

Zeaxanthin is a component of LHCCI under light stress conditions where it substitutes violaxanthin, thus inducing a con-

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**FIGURE 2. Triplet minus Singlet spectra of LHCCI-WT4 (A and B) and LHCCI-WT3 (C and D).** The spectral components were obtained by global fitting of the kinetics. A, LHCCI-WT4 measured under aerobic conditions; circle, 2.3-μs component; square, 3.6-μs component. B, LHCCI-WT4 under anaerobic conditions; circle, 9-μs spectral component; square, ms spectral component. C, LHCCI-WT3 under aerobic conditions; circle, 2.1-μs spectral component; square, 3.7-μs spectral component. D, LHCCI-WT3 under anaerobic conditions; circle, 9.2-μs spectral component; square, ms spectral component.

**TABLE 2**

TmS spectral features and carotenoid triplet lifetimes (τ)

| Sample     | Aerobiosis | An aerobiosis | No. components | Max T-S | τ (μs) | No. components | Max T-S | τ (μs) |
|------------|------------|---------------|----------------|---------|--------|---------------|---------|--------|
| LHCCI-WT4  | 2          | 507           | 2.30 ± 0.08    | 1       | 510    | 8.95 ± 0.06   |
| LHCCI-WT3  | 2          | 522           | 3.64 ± 0.22    | 1       | 507    | 9.17 ± 0.08   |
| LHCCI-LZ   | 2          | 507           | 2.14 ± 0.09    | 1       | 515    | 10.2 ± 0.3    |
| LHCCI-L    | 2          | 507           | 3.70 ± 0.29    | 1       | 507    | 9.71 ± 0.13   |
| Lhc1b      | 1          | 507           | 1.96 ± 0.02    | 1       | 505    | 8.61 ± 0.10   |
| Lhc4       | 1          | 505           | 1.53 ± 0.01    | 1       | 505    | 7.97 ± 0.05   |
| Lhc5       | 1          | 507           | 1.76 ± 0.11    | 1       | 507    | 8.29 ± 0.05   |
formational change of the complex, leading to a switch from a light-absorbing to an energy-dissipating mode (38, 52). The analysis of LHCII-LZ purified from npq2 plants allows verification of whether the conformational change induced by the binding of zeaxanthin influences the triplet-energy transfer properties of the system. The triplet decay under aerobic conditions can be fitted with a biexponential function; the spectrum is dominated by a fast component with maximum at 510 nm and a lifetime of 2.40 μs. A second component with a maximum at 522 nm and a lifetime of 4.11 μs is also present, but its amplitude accounts for only 10% of the total spectrum, showing a strong reduction with respect to the same component in LHCII-L under anaerobic conditions (17). How-  

FIGURE 3. Triplet minus Singlet spectra of LHCII-L (A and B) and LHCII-LZ (C and D). The spectral components were obtained by global fitting of the kinetics. A, LHCII-L measured under aerobic conditions; circle, 2-μs component; B, LHCII-L under anaerobic conditions; circle, 9.7-μs component; square, ms component. C, LHCII-LZ under aerobic conditions; circle, 2.4-μs spectral component; square, 4.1-μs spectral component. D, LHCII-LZ under anaerobic conditions; circle, 10.2-μs spectral component; square, ms spectral component.

indicates that the recombinant complex has the same triplet characteristics as the native one, as was previously reported for other spectroscopic characteristics (14, 50).

The carotenoid triplet decays for Lhcb4 (CP29) and Lhcb5 (CP26) were best fitted with single exponentials (Fig. 4, B and C). In the presence of oxygen, lifetimes of 1.53 and 1.76 μs were obtained for Lhcb4 and Lhcb5, respectively, and 8.0 and 8.3 μs under anaerobic conditions (Table 2). The maxima of the TmS spectra were 505 nm for Lhcb4 and 507 nm for Lhcb5. A ms component with small amplitude, corresponding to Chl triplets, can be observed for all monomeric complexes under anaerobic conditions (Table 2). Comparison of the spectra of the monomeric complexes shows a clear variation in their spectral width, the narrowest spectrum belonging to Lhcb4 and the broadest one to Lhcb1 (Fig. 4), indicating that the carotenoids participating in the triplet quenching have different properties in these antenna complexes.

**Chlorophyll Triplets**

In all complexes a small ms component corresponding to the decay of the Chls triplets was detected. The amount of Chl triplets was estimated in two independent ways. The first method compares the intensity of the TmS ms component to that of the TmS spectrum of Chl a in solution, measured under the same conditions and normalized to the absorbed number of photons. This comparison showed that around 5% of the triplets reside on Chls in most of the samples, corresponding to a 95% efficiency for Chl to Car triplet transfer. This value was slightly lower for Lhcb5 (88%) and still lower for LHCII-LZ (76%) and LHCII-L (70%). Similar results were obtained by calculating the amount of Chl triplets using the extinction coefficients of Chls and carotenoids (18, 28, 54). To check if the Chl triplet component was due to disconnected Chls, the fluorescence...
cience emission spectra of all samples after excitation of either Chl a or Chl b were measured (data not shown). The results show that all complexes are thermally equilibrated, indicating that all Chls participates in energy transfer, thus excluding the possibility that the observed triplets are formed on disconnected pigments. This result is at variance with previous room temperature TmS measurements, which suggested that there were no unprotected Chls associated to LHCCI (18, 24, 27, 30). The difference can be due to the time window used for the experiments, which is 9 ms for most of the measurements presented here, whereas it was in the μs range in the previous experiments. A difference in the signal to noise ratio between the experiments might also explain the discrepancy.

DISCUSSION

The comparison of LHCCI trimeric complexes with different carotenoid composition allowed determination of the role of the xanthophylls in photoprotection for all four binding sites. The results clearly show that neither the neoxanthin in the N1 site nor the xanthophylls in the V1 site is active in triplet quenching. The two components of the TmS spectrum at 507 and 522.5 nm can, thus, unequivocally be attributed to the lutein-L1 and lutein-L2 site, respectively. Closest distances (Å) between the conjugated π-systems of chlorophylls and carotenoids in sites L1, L2, N1, and V1.

| Chl | L1 | L2 | N1 | V1 |
|-----|----|----|----|----|
| 601 | 4.45 |
| 602 | 3.45 |
| 603 | 3.80 |
| 604 | 4.40 |
| 605 | 5.85 |
| 606 | 5.40 |
| 607 | 4.59 |
| 608 | 4.90 |
| 609 | 8.06 |
| 610 | 3.64 |
| 611 | 9.30 |
| 612 | 3.70 |
| 613 | 3.90 |
| 614 | 7.35 |

Chls a from the adjacent monomer within a trimeric LHCh-II.

The triplet transfer occurs via the Dexter mechanism (55, 56) and lutein-L2 (522 nm) can be explained based on the excited-state populations of the different chlorophylls at equilibrium. Indeed, Chls 610 and 612 participate to the low energy state of the system (37, 50) and, thus, are highly populated at equilibrium. This makes them a preferential sites for triplet formation and the lutein-L1 the major player in triplet quenching. Very recently it was proposed that lutein-L1 is involved in singlet excited state quenching in aggregates of LHCCI. However, it was shown that even under quenching conditions this xanthophyll is still active in triplet quenching (29, 57), although the amount of triplets is strongly reduced. Two more Chls a are present in LHCCI, namely Chls 611 and 614. Chl 614 is at 7 Å from lutein-L1 but it is in close proximity of Chl 613, and it is at least partially protected via downhill singlet energy transfer to Chl 613. Site 614 accommodates a high energy Chl that is not expected to be highly populated at equilibrium (37, 50). Chl 611 forms an excitonic pair with Chl 612, participating to the low energy state of the system, and it is, thus, populated at equilibrium. The nearest xanthophyll is located in the V1 site, but the distance is more than 7 Å, too large for efficient triplet transfer, as confirmed by the observation that the xanthophylls in the V1 site do not contribute to the triplet quenching. Thus, Chl 611 seems not to be protected in purified LHCCI, and it possibly contributes to the observed Chl triplet component. Chls b are located in close proximity of neoxanthin and lutein-L2, but in most cases the distance is larger than 4 Å. The Chls b are mainly protected in an indirect way, through fast singlet energy transfer to a neighboring Chl a (35). However, although their population at equilibrium is small, it is not zero (37), and this can explain the observed ms component Chl b contribution (58). It can be concluded that a small amount of Chl triplets is formed at physiological temperatures, explaining the bleaching of purified LHCCI-WT after exposure to strong illumination (59).

The TmS spectrum of the Lhcb1 monomer is narrower than that of the LHCCI trimer and slightly shifted to higher energy. This effect is mainly due a difference in the spectroscopic properties of the xanthophyll in the L2 site, which is blue-shifted in the monomers as compared with the trimer; consequently, also its triplet spectrum is blue-shifted, as observed previously for LHCCI native monomers (18). Previous data showed that the L1 site is occupied by a lutein molecule with absorption around 494 nm in all complexes (14, 18, 60–62). Therefore, it seems reasonable to assume that the triplet properties of lutein-L1 are similar in all complexes. Comparison of the L1 TmS spectrum with the spectra of the monomers provides information about the additional xanthophylls participating in the triplet quenching (Fig. 5). The TmS spectrum of lutein-L1 can be obtained from the analysis of WT LHCCI under aerobic conditions. The TmS spectrum of Lhcb4 is virtually identical to that of lutein-L1 (Fig. 5C), suggesting that (i) only one xanthophyll participates in the triplet quenching or (ii) all bound xanthophylls have similar spectra. It has been shown that neoxanthin in the N1 site of Lhcb4 absorbs at 483 nm (21), lutein-L1 at 494 nm, and violaxanthin in the L2 site at 492.6 nm (62). If neoxanthin would participate in the TmS spectrum, a component could be expected that is blue-shifted with respect to the L1 spectrum. Because this is not the case, we conclude that neoxanthin does not quench triplets in Lhcb4. The red part of the TmS spectrum of Lhcb5 is slightly broader than the spectrum of lutein L1 (Fig.

**TABLE 3**

Carotenoid-chlorophyll distances

Closest distances (Å) between the conjugated π-systems of chlorophylls and carotenoids in sites L1, L2, N1, and V1.
suggesting the involvement of a second carotenoid, probably accommodated in site L2 (notice that also in this complex the neoxanthin in N1 is at higher energy than lutein-L1 (21)). The larger amplitude in the red part of the spectrum of Lhcb5 when compared with Lhcb4 is probably due to the different occupancy of the L2 site (lutein versus violaxanthin). It appears that in all Lhcb the absorption spectrum of the xanthophylls bound to site L2 is 5–6 nm more red-shifted than that of the corresponding xanthophyll in site L1 (21, 61, 62). It can, thus, be expected that for Lhcb5, in which both sites accommodate lutein, the spectrum of L2 is red-shifted as compared with that of L1, whereas for Lhcb4, which accommodates violaxanthin in L2 and lutein in L1, the two spectra overlap, since the maxima of lutein and violaxanthin in solution differ 6 nm (63).

Although the neoxanthin does not directly participate in the triplet quenching, two striking effects can be observed in the TmS spectra of the complexes in which the N1 site is empty (LHCII-L) or partially empty (LHCII-LZ): (i) the triplet lifetime of the lutein in the L2 site strongly decreases and becomes identical to that of lutein-L1 and (ii) the Chl to carotenoid transfer efficiency decreases from 95 to 70%. The increased concentration of Chl triplets is in agreement with previous results which showed that complexes lacking neoxanthin are more sensitive to photobleaching (15, 49). Fluorescence emission measurements show that both complexes become quickly thermally equilibrated, indicating that all chromophores are active in singlet energy transfer but are not all equally active in transferring triplets. Because triplet transfer requires shorter distances between the pigments than singlet transfer, this suggests that the absence of neoxanthin has an effect on the structure of the complex, likely increasing the distance between some of the Chls and a xanthophyll molecule in site L1 or L2, thus leaving a small amount of Chls unprotected. The mechanism for such uncoupling can be suggested on the basis of a previous report (21) showing that in the absence of neoxanthin the strong excitonic interaction between Chls 604 and 606 is lost, pointing to a different organization of this protein domain.

The difference in triplet lifetime for the carotenoids in the L1 and L2 sites was attributed to a difference in oxygen accessibility (28). The occupancy of the N1 site thus appears to be important for creating a barrier against oxygen. Looking at the structure of trimeric LHCII, it can be observed that the lutein in the L2 site is deeply buried in the complex but that the absence of the neoxanthin opens a channel that directly points to the end ring of the lutein in L2 (Fig. 6), allowing oxygen to get into close contact. This effect can explain the results of Schödel et al. (8, 30) who found only one decay component in the triplet spectrum of LHCII under aerobic conditions with a 2-μs lifetime. As mentioned by the authors, the amount of neoxanthin varied extensively in their preparations (8), suggesting that the carotenoid in N1 was destabilized, thus explaining the absence of the long-decaying component. The structure also shows that the lutein in site L1 is partially exposed (Fig. 6) and, therefore, easily accessible for oxygen in the WT protein, explaining its shorter triplet lifetime. The structure, thus, explains the presence of two different lifetimes for the carotenoid triplet decay under aerobic conditions as was also found by Peterman et al. (28). The fact that Siefermann-Harms and Angerhofer (27) observed only one longer lifetime is, thus, not compatible with the structural organization of the
complex. Moreover, their argument that only intact LHCCI will show one long lifetime (27) does not apply to our LHCCI trimers, which were purified by very mild detergent treatment and in one single step, in contrast to the preparation of Sieffermann-Harms and Angerhofer (27), which required several steps in which a harsh procedure using Triton X-100 was needed as well as the elution from gel. Possibly, the digitonin used in their preparation acts as a screen around LHCCI, limiting the access of oxygen.

The protective effect of the neoxanthin is not visible in the Lhcb1 monomer, in agreement with the fact that in this complex the L2 site is easily accessible from outside. The same reasoning holds for Lhcb4 and Lhcb5, which only show a short lifetime, although both accommodate neoxanthin in the N1 site quenching, whereas the occupancy of site N1 by neoxanthin controls the accessibility of molecular oxygen to the inner core of the complex. The trimeric organization of LHCCI is, thus, effective in screening the internal protein domain from molecular oxygen.

A second important conclusion is that the xanthophylls only quench 95% of the Chl triplets, thus leaving 5% of the Chls unquenched. We propose that this incomplete quenching of Chl* triplets is the reason for the singlet oxygen scavenging not only by carotenoids bound to Lhc but also by carotenoids free in the lipid matrix (65, 66) or present at the interface between the lipids and the proteins (64).

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