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Functional architecture of photosynthetic light harvesting complexes
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Chapter 6

PHOTOPROTECTION IN THE ANTENNA COMPLEXES OF PHOTOSYSTEM II: ROLE OF INDIVIDUAL XANTHOPHYLLS IN CHLOROPHYLL TRIPLET QUENCHING*

ABSTRACT

In this work the photoprotective role of all xanthophylls in LHCII, 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 O2 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 O2. Like 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.

* This chapter is based on the article: M. Mozzo, L. Dall’Osto, R. Hienerwadel, R. Bassi and R. Croce, in press in Journal of Biological Chemistry; Epub 13-12-2007
Chapter 6

INTRODUCTION

Under normal light conditions, the photosynthetic apparatus is very efficient in harvesting light energy and transferring excitation energy to the reaction centre, 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) 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 photoinhibition 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:

- by quenching of Chl triplets ($\tau = 500$ ps (8)) and
- 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 (PSI) and Photosystem II (PSII) core complexes bind $\beta$-carotene while the outer antennae, composed of nucleus-encoded light-harvesting complexes (Lhc) accommodate lutein, neoxanthin and violaxanthin in moderate light, while zeaxanthin is produced (12) via de-epoxidation of violaxanthin, under light-stress conditions. The structure of LHCII (13), the major antenna complex of PSII, shows the location of four carotenoids. Two xanthophylls are bound in the centre 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
Chlorophyll triplet quenching in Lhcb

purification. Like 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.

Several carotenoid triplet studies on LHCII by absorbance-detected magnetic resonance (ADMR), fluorescence-detected magnetic resonance (FDMR) 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 bi-exponential 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, while a longer lifetime, around 7 μs, was found by Siefermann-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 which have partly lost their structural integrity, 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 are contributing 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 quenching, since it was not active in singlet 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* (ecot. Col-0) *npq2* (38) and *chy1chy2lut5* (39) were grown under controlled light conditions (photoperiod 8 hours light and 16 hours dark; 100 μmol of photons m\(^{-2}\) s\(^{-1}\) for WT and *npq2*; 30 μmol of photons m\(^{-2}\) s\(^{-1}\) for *chy1chy2lut5* plants, due to 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 previously reported (17). Trimeric WT LHCII was further fractionated by flatbed isoelectrofocusing (IEF) 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-D-maltoside (α-DM) and further fractionated on a 0.1 to 1 M sucrose gradient containing 0.06% α-DM and 10 mM HEPES, pH 7.5, for 24 h at 280,000g at 4°C.

**RECONSTITUTED COMPLEXES**

The apoproteins of Lhcb1, Lhcb4 from *Zea mays* (14;42) and Lhcb5 from *A. thaliana* (38) were overexpressed in the SG13009 *E. coli* strain transformed with constructs following a protocol described previously (43;44). Reconstitution and purification of pigment-protein complexes were performed as described in (42) using a Chl *a/b* mixture with ratio 2.4 for Lhcb1 and 3.0 for Lhcb4 and Lhcb5.
PIGMENT ANALYSIS

The pigments were extracted with acetone 80% and separated and quantified by HPLC, as previously described (45) and by fitting the spectra of the acetone extracts with the spectra of individual pigments (46).

SPECTROSCOPY

The absorption spectra at RT in 10 mM Hepes pH 7.5, 0.2 M sucrose and 0.06% β-DM were recorded using a SLM-Aminco DK2000 spectrophotometer. The wavelength sampling step was 0.4 nm, scan rate 100 nm/min, optical pathlength 1 cm. Fluorescence emission spectra were measured using a Fluorolog (Jobin Yvon) spectrofluorimeter and 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% α-DM.

Light-induced absorbance changes were recorded with a home-built high-sensitivity laser-based spectrophotometer as described in (47), in the presence and absence of oxygen. The anaerobic conditions were obtained incubating the sample with 20 μg/ml Glucose oxidase, 40 μg/ml Catalase and 0.02 mM/ml of glucose for 15 minutes. During the measurements in aerobic conditions the signal intensity at 510 nm was checked at regular time interval, 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 function of time, in order 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 from 5 ns to 9 ms between the actinic and the detection light pulses. The delay time was obtained by setting the electronic trigger for each laser light pulse. The delay of the light pulses were determined with fast silicon detectors (Thorlabs-Det210). For one kinetic, a set of measurements with increasing delay times was performed. The time between excitations of the sample was 300 ms. For aerobic conditions, a set of 29 different delays times were recorded, for anaerobic conditions, a set of 48 delay times. The kinetics are the average of 5-10 sets of measurements. For the global fit the data were analyzed in the time interval between 70 ns to 9 ms in the 420-580 nm range. The GraphPad PRISM program (GraphPad Software) was used for globally analyzing the kinetics.
RESULTS

LHCII trimers were purified from WT plants and mutant plants affected in the carotenoid biosynthesis: *npq2*, which is blocked at the level of the zeaxanthin epoxidase and accumulates only lutein and zeaxanthin (LHCII-LZ); *chy1chl2lut5*, 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 which differ in the carotenoid/protein ratio (17). Monomeric LHCII (Lhcb1), Lhcb4 and Lhcb5 were obtained by refolding *in vitro*.

Table I. Pigment composition.

<table>
<thead>
<tr>
<th>Sample preparation</th>
<th>Chlα/Chlb</th>
<th>Chls tot</th>
<th>Cars</th>
<th>Neo</th>
<th>Viola</th>
<th>Lut</th>
<th>Zea</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHCII-WT4 LHCII WT trimer native</td>
<td>1.4</td>
<td>14</td>
<td>4.0</td>
<td>1.0</td>
<td>0.4</td>
<td>2.6</td>
<td>-</td>
</tr>
<tr>
<td>LHCII-WT3 LHCII WT trimer IEF</td>
<td>1.3</td>
<td>13</td>
<td>3.0</td>
<td>1.0</td>
<td>0.2</td>
<td>1.8</td>
<td>-</td>
</tr>
<tr>
<td>LHCII-LZ LHCII <em>npq2</em> trimer</td>
<td>1.4</td>
<td>14</td>
<td>3.5</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>LHCII-L LHCII <em>chy1chy2lut5</em> trimer</td>
<td>1.5</td>
<td>14</td>
<td>2.7</td>
<td>-</td>
<td>-</td>
<td>2.7</td>
<td>-</td>
</tr>
<tr>
<td>Lhcb1 rLhcb1 WT monomer</td>
<td>1.3</td>
<td>12</td>
<td>3.0</td>
<td>1.0</td>
<td>0.3</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Lhcb4 rLhcb4 WT</td>
<td>2.7</td>
<td>8</td>
<td>1.9</td>
<td>0.5</td>
<td>0.5</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>Lhcb5 rLhcb5 WT</td>
<td>1.9</td>
<td>9</td>
<td>2.4</td>
<td>0.8</td>
<td>0.1</td>
<td>1.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Maximal standard deviation is 0.1.

PIGMENT CONTENT

The pigment composition of the complexes is reported in Table I. 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 IEF (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, while the amount of lutein is unchanged. The presence of zeaxanthin in LHCII trimers obtained by IEF (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, L2 and V1, while 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 I). The L1 and N1 sites of Lhcb5 have similar occupancy as those of Lhcb4, while the L2 site binds lutein (16;21).

**ABSORPTION SPECTRA**

The absorption spectra of the trimers with altered carotenoid composition are reported in Figure 1, where they are compared to the WT spectrum (WT4). The WT minus LHCII-L absorption difference spectrum (Figure 1B) is identical, both in the carotenoid and in the Chl absorption region, to the difference spectrum (WT minus Lhcb1-L) obtained for Lhcb1 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.

![Absorption Spectra](image)

**Figure 1. Absorption Spectra.** Absorption spectra at RT of, LHCII-LZ (A) and LHCII-L (B) (dot-dash lines). In all panels also 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.

In the difference spectrum WT minus LHCII-LZ (Figure 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 is still accommodating lutein. Moreover, the main absorption band of zeaxanthin was found at around 504 nm, 18 nm red-shifted as compared to the absorption in solution. This shift is compatible with the binding of zeaxanthin in sites L1 and N1, which induce a similar shift in lutein and neoxanthin absorption in the WT complex (15).

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.

A) LHCII TRIMERS

The decay of the carotenoid triplets in LHCII-WT4 under aerobic conditions could be best fitted by a bi-exponential 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 (Figure 2A, Table II). This indicates that one or more carotenoids absorbing around 490 nm are responsible for the fast decay while 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 Figure 2. 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 (Figure 2B, Table II). 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 LHCII-WT3, in which the V1 site is empty, were measured (Figure 2C-D, Table
Chlorophyll triplet quenching in Lhcb

II) and compared to those of LHCII-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 is not participating in triplet quenching.

![Triplet minus Singlet spectra of LHCII-WT4 (A-B) and LHCII-WT3 (C-D). The spectral components were obtained by global fit of the kinetics. (A) LHCII-WT4 measured in aerobic conditions: 2.3 µs component (circle), 3.6 µs component (square). (B) LHCII-WT4 in anaerobic conditions: 9 µs spectral component (circle), ms spectral component (square). (C) LHCII-WT3 in aerobic conditions: 2.1 µs spectral component (circle), 3.7 µs spectral component (square). (B) LHCII-WT3 in anaerobic conditions: 9.2 µs spectral component (circle), ms spectral component (square).](image)

To investigate the involvement of the neoxanthin in triplet quenching, the TmS spectra of LHCII-L, in which the N1 site is empty, were measured. The fitting of the triplet decay of LHCII-L under aerobic conditions only requires a single exponential showing a 2 µs lifetime (Figure 3A, Table II), at variance with the WT results. However, the TmS spectrum of LHCII-L is identical to the sum of the two spectral components obtained for the WT (data not shown), indicating that the different decay behaviour 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 (Figure 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 LHCII-L are identical to those of LHCII-W4, thus demonstrating that the neoxanthin is not participating to the triplet spectrum.

Table II: TmS spectral features and carotenoid triplet lifetimes ($\tau$)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aerobiosis</th>
<th>Anaerobiosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N °components</td>
<td>max T-S (nm)</td>
</tr>
<tr>
<td>LHCII-WT4</td>
<td>2 507</td>
<td>522</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.64± 0.22</td>
</tr>
<tr>
<td>LHCII-WT3</td>
<td>2 507</td>
<td>522</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.70± 0.29</td>
</tr>
<tr>
<td>LHCII-LZ</td>
<td>2 510</td>
<td>522</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.11± 0.07</td>
</tr>
<tr>
<td>LHCII-L</td>
<td>2 507</td>
<td>507</td>
</tr>
<tr>
<td>Lhcb1</td>
<td>1 507</td>
<td>507</td>
</tr>
<tr>
<td>Lhcb4</td>
<td>1 505</td>
<td>505</td>
</tr>
<tr>
<td>Lhcb5</td>
<td>1 507</td>
<td>507</td>
</tr>
</tbody>
</table>

Absorption maxima of the TmS (max T-S) spectra and carotenoid triplet lifetimes were obtained by global analysis of a data set consisting of time traces measured at different wavelengths. In addition to the components reported in the table all complexes under anaerobic conditions show a ms decay component, with the spectrum of Chl triplets.

Zeaxanthin is a component of LHCII under light stress conditions where it substitutes violaxanthin, thus inducing a conformational 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 verifying 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 bi-exponential 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-WT (Figure 3C, Table II).

In the absence of oxygen the lifetime of the carotenoid triplets increases to 10.2 µs and the spectrum shows a maximum at 515 nm whereas also a second component with a ms lifetime and a Chls TmS spectrum is detected (Figure 3D, Table II). The shift of the carotenoid spectrum, as compared to the WT, demonstrates the involvement of zeaxanthin in the triplet quenching and it suggests that this xanthophyll is partially occupying the L1 site. This is not
reflecting the situation in WT plants exposed to stress conditions, where the zeaxanthin in mainly accommodated in the V1 site (17). However, LHCII obtained from npq2 plants is widely used as a model system to determine the effect of zeaxanthin in the process of non-photochemical quenching (38;48;53). Knowledge about the location of the zeaxanthin in LHCII purified from these mutant plants is thus important for the interpretation of experimental results.

B) LHCB MONOMERIC COMPLEXES

For Lhcb1 the carotenoid triplet decay was fitted with a single exponential decay. The TmS spectrum shows a maximum at 507 nm and a lifetime of 2 µs in the presence of oxygen and 8.6 µs under anaerobic conditions (Figure 4A, Table II). As compared to the spectrum of trimeric LHCII, monomeric Lhcb1 lacks the 522 nm form, in agreement with an earlier report.
on monomerized native LHCII (18). This 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 (Figure 4B-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 II). 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 II). 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 (Figure 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 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 are participating 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 LHCII (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, while 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.
Figure 4. Triplet minus Singlet spectra of monomeric Lhcb. Lhcb1 (A), Lhcb5 (B) and Lhcb4 (C) were measured under anaerobic conditions: square: µs spectral component; circle: ms spectral component.
DISCUSSION

The comparison of LHCII trimeric complexes with different carotenoid composition allowed determining 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 are active in triplet quenching. The two components of the TmS spectrum at 507 nm and 522.5 nm can thus unequivocally be attributed to the luteins in sites L1 and L2, with the red lutein accommodated in site L2, as suggested previously (32). Similar results were obtained for Lhca4 which show that the blue carotenoid is in site L1 and the red one is in the L2 site (47).

The triplet transfer occurs via the Dexter mechanism (55;56) that requires close proximity between donor and acceptor, since the exchange coupling decreases exponentially with the distance. Calculations on LH2 complexes suggested that for efficient triplet transfer the distance should not be larger than 4 Å (56). The edge-to-edge distances between the Chls and the carotenoids can be calculated from the structure of LHCII and they are reported in Table III. Chls a 610, 612 and 613 are in close proximity of lutein-L1 assuring full photoprotection. Chls a 602 and 603 are located at less than 4 Å from lutein-L2 and are also efficiently protected. Chl 604 is located at 4.4 Å from lutein-L2 and at 4.24 Å from the neoxanthin (N1), but it seems to transfer energy to the former, although a very small amount of transfer to the neoxanthin cannot be excluded. The difference in amplitude for the TmS spectrum of lutein-L1 (507 nm) 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 LHCII. 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 LHCII, 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 is accommodating a high-energy Chl which 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 are not contributing to the triplet quenching. Thus, Chl 611 seems not to be protected in purified LHCII 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 neighbouring 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 LHCII-WT after exposure to strong illumination (59).

Table III. Carotenoid – Chlorophyll distances

<table>
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<tr>
<th>Chl</th>
<th>L1</th>
<th>L2</th>
<th>N1</th>
<th>V1</th>
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</table>

* Chl $b$ from adjacent monomer within a trimeric LHCII

Closest distances (Å) between the conjugated $\pi$-systems of chlorophylls and carotenoids in sites L1, L2, N1 and V1

The TmS spectrum of the Lhcb1 monomer is narrower than that of the LHCII 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 to the trimer; consequently also its triplet spectrum is blue shifted, as observed previously for LHCII 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 (Figure 5). The TmS spectrum of lutein-L1 can be obtained from the analysis of
WT LHCII under aerobic conditions. The TmS spectrum of Lheb4 is virtually identical to that of lutein-L1 (Figure 5A), suggesting that:

- only one xanthophyll is participating in the triplet quenching or
- all bound xanthophylls have similar spectra.

Figure 5. Analysis of the TmS spectra of monomeric complexes. Comparison of the TmS spectra of the monomeric complexes Lheb1 (A), Lheb5 (B) and Lheb4 (C) (open square) measured under aerobic conditions (see table II for the lifetimes) and the TmS spectrum of lutein-L1 (circle) as obtained from the analysis of LHCII-WT3 (fast component under aerobic conditions).
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 to the TmS spectrum, a component could be expected that is blue-shifted with respect to the L1 spectrum. Since 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 (Figure 5B), 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 to Lhcb4, is probably due to the different occupancy of the L2 site (lutein vs. 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 to that of L1, while 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 is not directly participating 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):

- the triplet lifetime of the lutein in the L2 site strongly decreases and becomes identical to that of lutein-L1;
- 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 is opening a channel in the structure, which directly points to the
end ring of the lutein in L2 (Figure 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 (Figure 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 Sieferman-Harms and Angerhofer observed only one longer lifetime is thus not compatible with the structural organization of the complex. Moreover, their argument that only intact LHCII will show one long lifetime (27) does not apply to our LHCII trimers which were purified by very mild detergent treatment and in one single step, in contrast to the preparation of Siefermann-Harms, 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 LHCII, limiting the access of oxygen.

Figure 6. Effect of the absence of neoxanthin. Model of LHCII trimer (13) in the presence (left) and in the absence (right) of neoxanthin.

The apoproteins of the three monomers are in different blue colors, Chls are in green, lutein-L1 in yellow, lutein-L2 in red and neoxanthin in magenta. It can be observed that while in the presence of neoxanthin the lutein-L2 is deeply buried in the complex (lutein-L2 in red is not visible), the absence of neoxanthin opens a channel which exposes lutein-L2 to the external environment (lutein-L2 in red is visible).

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 (21). Moreover, the triplet lifetimes of the monomeric complexes under aerobic conditions are shorter than observed for LHCII trimers, indicating that they are more accessible to oxygen, in agreement with the fact that they have a less packed structure.

However, it is likely that in the membrane, when the proteins are assembled around the reaction centre, they are oriented in such a way that the internal part of the complex is again protected and that also in these complexes neoxanthin is protruding into the membrane, acting as an oxygen barrier.

**CONCLUSION**

We have obtained a description of the role of the different xanthophylls binding sites in trimeric LHCII: only the xanthophylls bound at sites L1 and L2 have a direct role in Chl triplet quenching, while the occupancy of site N1 by neoxanthin controls the accessibility of molecular oxygen to the inner core of the complex. The trimeric organization of LHCII, is thus effective in screening the internal protein domain from molecular oxygen as long as the N1 site is occupied.

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 $^3\text{Chl}^*$ triplets is the reason for the need of singlet oxygen scavenging, not only by carotenoids bound to Lhc but also by carotenoids free in the lipid matrix (64;65) or present at the interface between the lipids and the proteins (66).
57. Ruban, A. V., Berera, R., Ilioiaia, C., van Stokkum, I. H., Kennis, J. T., Pascal, A. A., van Amerongen, H., Robert, B., Horton, P., and van...


