Stark effect measurements on monomers and trimers of reconstituted light-harvesting complex II of plants

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Abstract

The electric-field induced absorption changes (Stark effect) of reconstituted light-harvesting complex II (LHCII) in different oligomerisation states—monomers and trimers—with different xanthophyll content have been probed at 77 K. The Stark spectra of the reconstituted control samples, containing the xanthophylls lutein and neoxanthin, are very similar to previously reported spectra of native LHCII. Reconstituted LHCII, containing lutein but no neoxanthin, shows a similar electrooptical response in the Chl a region, but the Stark signal of Chl b around 650 nm amounts to at most ~25% of that of the control samples. We conclude that neoxanthin strongly modifies the electronic states of the nearby Chl b molecules causing a large electrooptical response at 650 nm stemming from one or more Chls b in the control samples. Ambiguities about the assignment of several bands in the Soret region [Biochim. Biophys. Acta 1605 (2003) 83] are resolved and the striking difference in electric field response between the two lutein molecules is confirmed. The Stark effect in the carotenoid spectral region in both control and neoxanthin-deficient samples is almost identical, showing that the neoxanthin Stark signal is small and much less intense than the lutein Stark signal.

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1. Introduction

Photosynthesis is the process in which sunlight energy is converted into organic compounds to be used as a source of energy by all living organisms on earth. To perform this process, plants are equipped with the so-called Photosystem I and II (PSI and PSII) [1,2]. Every photosystem is comprised of several pigment–protein complexes, whose main function is to absorb sunlight and to transfer excitation energy efficiently to the reaction center, where a charge separation is initiated [1,3]. In order to be able to absorb light, these light-harvesting complexes are disposed with Chl and carotenoid (xanthophyll) pigment molecules, which absorb light over a wide spectral range. Carotenoids also have a protective role by preventing the formation of harmful singlet oxygen and, in addition, a structural role has been suggested for some complexes [4,5]. Chl and carotenoid molecules work in a cooperative way and are often strongly coupled, influencing their mutual spectroscopic properties [6–8].

The light-harvesting complex II (LHCII) comprises more than 50% of the Chl content in PSII of green plants and algae [5]. Besides chlorophyll molecules, it binds four...
xanthophylls—carotenoids with oxygen functions—, namely two luteins, one neoxanthin and one violaxanthin per monomeric subunit, which is easily lost upon isolation (for a review about xanthophylls in LHCII, see Ref. [9]).

The crystal structure of LHCII was resolved at 3.4 Å resolution by Kühlbrandt et al. [5] and the obtained model allowed determination of the positions of most of the pigments, but at the same time their identities and exact orientation could not be determined, hampering a direct correlation between the spectroscopic features and the functioning of LHCII, although many of the experimental observations have been modeled quite successfully [6,10,11]. The model shows 12 Chl molecules and two central xanthophylls (lutein) having an important role in stabilizing the complex [12,13]. Concerning the identities of the pigments, three different research groups carried out point mutation and/or reconstitution experiments aimed at specific Chl binding sites [14–17], leading to the conclusion that for several Chls their initial assignment was questionable. Interestingly, mutation of the binding sites of Chls a6, b5 and b6 (notation as in Ref. [5]) led to a concomitant (partial) loss of neoxanthin molecules [14,15]. This led Croce et al. [18] to the conclusion that the neoxanthin binding site is located between the helix C and the helix A/B cross in the structural model of LHCII, in close contact with Chls b5 and b6. Furthermore, the authors concluded from linear dichroism (LD) measurements that neoxanthin makes an angle of 57 ± 1.5° with respect to the normal of the membrane plane [18–20]. The corresponding binding site appears to be highly specific for neoxanthin and not essential for protein folding [12,19].

The two central lutein molecules transfer singlet excited-state energy quite efficiently ( ~ 80–100%) to the Chl a molecules in trimeric LHCII from spinach [21–25]. Neoxanthin, which predominantly transfers excited-state energy towards the Chl b molecules shows a lower transfer efficiency [21,22,25], whereas violaxanthin, located at the outside of the complex, is unable to transfer excitation energy to the Chl molecules [26,27].

Apart from light-harvesting, carotenoid molecules are also essential for photoprotection, serving as quenchers of the excited triplet states of the Chl molecules and as singlet oxygen scavengers [28,29]. Both luteins quench Chl a triplets efficiently, but no triplet transfer has been observed towards the neoxanthin molecule [7,21,30,31]. This is easily explained by the fact that efficient and fast singlet energy transfer from Chl b to Chl a only leads to the formation of excited triplet states on the Chl a molecules [8,24,32], which are further away from the neoxanthin binding site than the Chl b molecules (see also above). It was recently proposed by Croce et al. [19] that neoxanthin is actively involved in the scavenging of singlet oxygen.

Recently, Stark effect measurements on native LHCII in different oligomeric states showed that the red most lutein absorbing at ~ 510 nm in trimers and oligomers exhibits a big Stark signal characterized by a large difference in dipole moment between ground and excited states (|Δμf| ~ 15 D/ f), whereas the lutein absorbing at 494 nm has a |Δμf| value of ~ 5 D/ f [33]. It was suggested that the enhancement of the Stark signal and red shift in absorption for one of the two luteins was probably caused by the presence of Mg2+ ions in trimers, since a trimer to monomer transition accompanied by the release of up to 2.5 Mg2+ ions has recently been reported [34]. Regarding neoxanthin, it appeared to be troublesome to ascertain its Stark signal due to strong overlap in the absorption and Stark spectra of the neoxanthin, lutein and Chl b absorption bands [8,35]. Moreover, the origin of the experimentally observed minima located at 457 and 427 nm in the Stark spectra, presumably due to one or more xanthophylls, could not unambiguously be deciphered. Exact knowledge of the electrooptical response of the xanthophylls present in LHCII is of great importance in understanding what is the difference between the two luteins and, for their assignment in the crystal structure of LHCII [5]. In an attempt to find out the contribution of lutein and neoxanthin to the Stark spectra, Olszówka et al. [36] tried to model the Stark signal of LHCII in the carotenoid region by taking the Stark spectrum of lutein in glassy solvents, and shifting it to match the approximate location of the vibrational absorption transitions of the S2 state of lutein and neoxanthin in LHCII. They concluded that lutein and neoxanthin exhibit a comparably strong Stark signal of similar magnitude as that of carotenoids in glassy solvents [37].

In the Qb absorption region marked changes occur around 650 nm (Chl b) in the absorption, circular dichroism (CD) and LD spectra upon “removal” of neoxanthin [18–20], suggesting strong interactions between neoxanthin and some Chl b molecules. The Stark spectrum of LHCII shows a remarkably strong Stark effect at 650 nm, which has been hypothesized to be due to a strong interaction between neoxanthin and one or more Chl b molecules [33]. However, in Ref. [36] it was hypothesized that the main Stark signal from Chl b is indirectly caused by neoxanthin through a structural modification on the protein leading to strong Chl b−Chl b coupling, rather than by direct coupling of neoxanthin with Chl b.

In order to obtain more insight into the interactions between xanthophylls and chlorophylls and to unravel the abovementioned unsolved questions, we have performed Stark measurements on reconstituted monomeric and trimeric LHCII samples with different xanthophyll contents (only lutein or both lutein and neoxanthin). Our results show that, whereas the Chl a molecules exhibit similar electrooptic properties in all the samples, the electrooptic response from the Chls b in the Qb absorption region is much less intense in neoxanthin-deficient samples, behaving like that of monomeric uncoupled Chl b molecules. In the Soret region, clear absorption changes were observed due to the absence of neoxanthin, whereas the Stark signals turned out to be similar for both neoxanthin-deficient and neoxanthin-containing samples. Finally, we have been able to distin-
guish between the two luteins, since they exhibit different behaviors upon optical excitation in the presence of an electric field.

2. Materials and methods

2.1. Protein expression and complex reconstitution

The DNA construct for overexpression of the LHCII apoprotein in *E. coli* was made using a barley gene (TC65915, BE411686), amplified for PCR (primers: CAGGATCCCGCAAGACGGCGGCAAGGC and GG-AAGCTTGGCCGGGCACGAAGGG) and cloned in a modified pQE50 vector carrying a sequence for an histidine tag at the C-term of the encoded protein.

Complex reconstitution was performed using a ratio of 420 µg of apoprotein, 240 µg of chlorophylls (Chls a/b ratio 2.9) and 60 µg of carotenoids (30 µg of lutein and 30 µg of neoxanthin for the control sample), modifying the procedure described in Ref. [38] for the Ni²⁺ column (ref. 2.9) and 60 Å.

Table 1 shows the pigment composition in every sample normalized to a total amount of 12 Chl molecules.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Sample</th>
<th>Mon-Cr</th>
<th>Mon-Lut</th>
<th>Trim-Cr</th>
<th>Trim-Lut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl a/b</td>
<td>Chl</td>
<td>1.8</td>
<td>1.8</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Neoxanthin</td>
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<td>0.00</td>
<td>0.91</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Lutein</td>
<td>1.82</td>
<td>2.37</td>
<td>1.79</td>
<td>2.38</td>
<td></td>
</tr>
<tr>
<td>Chl/Car</td>
<td>4.35</td>
<td>5.06</td>
<td>4.43</td>
<td>5.03</td>
<td></td>
</tr>
<tr>
<td>Total Chls</td>
<td>12.00</td>
<td>12.00</td>
<td>12.00</td>
<td>12.00</td>
<td></td>
</tr>
<tr>
<td>Total Cars</td>
<td>2.76</td>
<td>2.37</td>
<td>2.71</td>
<td>2.38</td>
<td></td>
</tr>
</tbody>
</table>

The pigment contents were normalized to the total chlorophyll content. For the measurements, all the samples were diluted in a medium containing 20 mM Hepes buffer at pH 7.7 and 60% (v/v) glycerol to ensure a transparent sample at low temperatures. The n-dodecyl-β-d-maltoside concentration is 0.03% (w/v).

2.2. Sample preparation

Table 1 shows the pigment composition in every sample normalized to the total chlorophyll content. For the measurements, all the samples were diluted in a medium containing 20 mM Hepes buffer at pH 7.7 and 60% (v/v) glycerol to ensure a transparent sample at low temperatures. The n-dodecyl-β-d-maltoside concentration is 0.03% (w/v).

2.3. Stark spectroscopy: basic principles

In the presence of an electric field, a molecule exhibits a shift in its transition energy. The magnitude of the shift depends on the difference in dipole moment and polarizability, Δμ and Δα, between the ground and excited states upon excitation (for a review about Stark spectroscopy and its applications, see for instance Ref. [43]).

Quantitatively, the observed absorption spectral changes experienced by randomly oriented and spatially fixed molecules in the presence of an electric field can be described by the equation [43, 44]:

\[
\Delta A(v) = (\overline{F_{ex}})^2 \left( A_{x} A(v) + B_{z} \frac{d}{dv} \left[ \frac{A(v)}{v} \right] \right) + C_{z} \frac{d^2}{dv^2} \left[ \frac{A(v)}{v} \right]
\]

In Eq. (1), \( \overline{F_{ex}} \) is the externally applied electric field and \( f \) is the local-field correction factor, which takes into account the enhancement of the applied electric field at the site of the molecule due to the environment. The terms \( A_{x}, B_{z} \) and \( C_{z} \) are dependent on the macroscopic angle \( \chi \) between the polarization direction of the light and the electric field. \( B_{z} \) and \( C_{z} \) are related to the molecular properties [Δμ] and Tr (Δα) according to the expressions:

\[
B_{z} = \frac{1}{2} Tr(\Delta \overline{\alpha}) \left[ 5 + (3 \cos^2 \chi - 1) \cdot 3 \left( \frac{\overline{\mu} \cdot \Delta \overline{\mu}}{Tr(\Delta \overline{\mu})} \right) - 1 \right]
\]

\[
C_{z} = [\Delta \overline{\mu}]^2 [5 + (3 \cos^2 \chi - 1) \cdot (3 \cos^2 \chi - 1)].
\]

Tr(Δα) and [Δμ] can easily be obtained by setting \( \chi \) to magic angle (54.7°). [Δμ] and Tr(Δα) values associated to the observed Stark signals were obtained by performing an analysis based on a nonlinear least-squares fitting program of the absorption and Stark spectra, as described before in Refs. [33, 45]. All the estimated values are given in terms of D/f and \( \Delta \overline{\alpha} / f^2 \), respectively, because the local-field correction factor \( f \) is not known exactly.

2.4. Stark setup

Stark measurements were performed at 77 K (Oxford cryostat, DN1704). The Stark cell consisted of two indium tin oxide (ITO)-coated glass plates glued together with double-sided sticky tape, resulting in a cell with an optical pathlength of 100 µm. Excitation was provided by a 150 W Xenon lamp (Oriel). The Stark effect was detected by lock-in amplification at 2ω, with ω being the frequency of the modulated applied field to the sample which was set to 310 Hz. Stark and absorption spectra were recorded simultaneously. Separate absorption spectra were obtained with the lock-in amplifier (EG&G Model 5210) locked to the frequency of a chopper at 312 Hz. The OD of the samples ranged from 0.45 to 0.60 at the Q, absorption maximum and
the Stark signal scaled quadratically with the applied field in the range 1.0–2.1 × 10^5 V/cm.

3. Results

3.1. Monomers

Fig. 1A shows the absorption spectrum of reconstituted monomeric LHCII containing lutein and neoxanthin (Mon-Ctr, solid line) and of reconstituted monomeric LHCII lacking neoxanthin (Mon-Lut, dashed line), together with the absorption difference spectrum enlarged by a factor of two (dashed-dotted line). Spectra were normalized at the Chl a Q_y maximum, the spectral region where the absorption spectrum is expected to be hardly affected by the lack of neoxanthin. Major differences are observed in the Chl b Q_y region and in the Soret region, as reported previously [18–20]. In the Q_y region, a blue shift for part of the 650 nm band seems to occur upon “removal” of the neoxanthin, whereas in the Soret region an overall decrease in absorption can also be observed for Mon-Lut, with the exception of a rise in absorption for the 472 nm peak. This can be seen most clearly in the absorption difference spectrum (Mon-Ctr minus Mon-Lut), which depicts three distinct peaks in the Soret region at 488, 453 and 433 nm. These peaks are roughly located close to the expected absorption bands associated with the three vibronic transitions of the S_2 excited state of neoxanthin [18,21,33,46]. However, the negative contribution of the 472 nm band to the absorption difference spectrum, probably makes the first two red peaks appear further apart than their actual separation. The hyperchromic effect at 472 nm is probably caused by a blue shift in the absorption of one or more Chl b molecules located in the surroundings of neoxanthin, i.e. from ~ 485 to ~ 472 nm. Inspection of the second derivative of both spectra reveals only small changes in the Chl b Q_y and Soret regions (450–500 nm), reflected by the change (gain or decrease) in intensity of some absorption bands, together with a slight red shift of the main Chl a Q_y band (< 1 nm) (not shown). No big changes are observed in the positions of the minima, which coincide with those of previously reported bands in LHCII [18,46–49].

The Stark spectra of Mon-Ctr (solid line) and Mon-Lut (dashed line) at 77 K recorded at \( \chi = 54.7^\circ \) are depicted in Fig. 1B (Q_y region) and Fig. 1C (Soret region). In the Q_y region, a decrease in intensity and a blue shift of the main Chl b signal is observed for Mon-Lut with respect to Mon-Ctr, whereas the Chl a Q_y region remains more or less unaltered. Similar observations were reported by Croce et al. [18] with regard to the LD of both samples. These two observations clearly point out that the lowest excited state of some of the Chl b molecules in LHCII is strongly influenced by the presence/absence of neoxanthin. The minimum at 649 nm in the Mon-Lut sample scales to only ~ 25% of the signal at 651 nm in the Mon-Ctr sample. The blue shift in the position of some of the minima is in agreement with the qualitatively described differences in absorption for both samples in the Q_y region (see above).

The Stark signal of the Chl b molecules in the Mon-Ctr sample resembles the second derivative of the Q_y absorption band as observed before for native LHCII, which is expected if the main contribution to the Stark spectrum stems mainly from a change in dipole moment, \( \Delta \mu \) upon excitation. Surprisingly, only small differences between the Mon-Ctr and Mon-Lut samples are observed in the Stark spectra in the Soret region. The slightly higher amount of lutein leads to a small increase of the lutein Stark signal. On the other hand, the absence of neoxanthin does not lead to the disappearance of any spectral features that are specific for neoxanthin. Therefore, it can be concluded that the Stark signal of neoxanthin is small and much less intense than that.

Fig. 1. (A) 77 K absorption spectra of reconstituted monomeric LHCII containing lutein and neoxanthin (Mon-Ctr, solid line), reconstituted monomeric LHCII containing only lutein (Mon-Lut, dashed line) and the difference between them enlarged by a factor of two (dashed-dotted line). Spectra were normalized at the Chl a Q_y peak and to a field strength of F = 1.0 × 10^5 V/cm. (B) Stark spectra in the Q_y region of reconstituted monomeric LHCII containing lutein and neoxanthin (Mon-Ctr, solid line) and reconstituted monomeric LHCII containing only lutein (Mon-Lut, dashed line) at 77 K recorded at \( \chi = 54.7^\circ \). Spectra were normalized to OD = 1 at the Chl a peak and to a field strength of F = 1.0 × 10^5 V/cm.
of lutein. The position of the minima and zero crossings are very similar to those in previously reported Stark spectra for LHCII in the Soret region \[33,36,50\]. Lutein, which absorbs at 494, 466 and 435 nm (\(S_2\) state, vibrational transitions \[33,46\]), dominates the observed Stark signal in this spectral region (see Discussion).

### 3.2. Trimers

The absorption spectra of reconstituted trimeric LHCII with lutein and neoxanthin (Trim-Ctr, solid line), reconstituted trimeric LHCII containing only lutein (Trim-Lut, dashed line) and the corresponding difference absorption spectrum enlarged by a factor of two (dashed-dotted line) are plotted in Fig. 2A. The spectra are again normalized to the Chl \(a\) \(Q_y\) maximum. As in the case of reconstituted LHCII monomers (see above), the main differences in absorption are the blue shift in the Chl \(b\) \(Q_y\) region and the relative decrease in absorption in the Soret region for the Trim-Lut sample.

Fig. 2B and C show the Stark spectra of Trim-Ctr (solid line) and Trim-Lut (dashed line) at 77 K recorded at \(\gamma = 54.7^\circ\) in the \(Q_y\) and Soret regions, respectively. Again, a strong decrease of the main Chl \(b\) signal at around 651 nm is observed for Trim-Lut, whereas the red part of the Chl \(a\) \(Q_y\) region exhibits no substantial changes, although a small red shift for the Trim-Lut sample with respect to Trim-Ctr is noticeable. In this case, the signal at \(\sim 651\) nm for Trim-Lut scales to only \(\sim 12\%\) of the observed signal for Trim-Ctr, which suggests that neoxanthin, when it is bound, modifies the electronic properties of some Chl \(b\) molecules, giving rise to an intense Stark signal at \(\sim 650\) nm. The enhancement of the signal at 642.5 nm probably corresponds to the blue shift observed in absorption for some Chl \(b\) spectral subbands (see above). Finally, the signal at \(\sim 651\) nm resembles the second derivative of the absorption spectrum in Trim-Ctr samples.

Both spectra are again very similar in the Soret region (Fig. 2C). Apart from a small increase of the lutein signal for the Trim-Lut sample, no difference is observed that can be ascribed to neoxanthin. It can thus be concluded that neoxanthin shows a rather small Stark signal. The positions of the intense Stark signals at 507 and 478 nm correspond with the pronounced minima observed at 509 and 481 nm in Stark measurements on native trimers and oligomers of LHCII, which are due to the red-most lutein absorbing at \(\sim 510\) nm \[33\]. Their strong electrooptic response in native LHCII becomes even stronger in reconstituted LHCII lacking neoxanthin. As in Refs. \[33,36,50\], minima are observed at \(\sim 492\), \(\sim 457\) and \(\sim 427\) nm for both samples, which should most likely be ascribed to lutein (see Discussion).

### 4. Discussion

#### 4.1. Chl \(a\) \(Q_y\) region

The Stark effect in the Chl \(a\) \(Q_y\) region shows an overall similarity in shape and intensity for all the samples. Only a slight red shift (\(\sim 0.5\) nm) can be observed for neoxanthin-deficient samples. Also the absorption spectrum is not markedly affected in the Chl \(a\) \(Q_y\) region when neoxanthin is not bound.

In order to estimate the magnitude of the electrooptic parameters of the chlorophylls in LHCII, we performed a simultaneous fit of the absorption and the Stark spectra (670–705 nm) with a B spline function \[45\] and its first and second derivatives. Fig. 3 depicts the fit for reconstituted trimers of LHCII lacking neoxanthin and the obtained values for \(|\Delta \vec{m}\)| and \(\text{Tr}(\Delta \vec{\alpha})\) for all the samples are summarized in Table 2. The \(|\Delta \vec{m}|\) and \(\text{Tr}(\Delta \vec{\alpha})\) values are in agreement with previously reported values for the

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![Image of Stark spectra](image_url)

**Fig. 2.** (A) 77 K absorption spectra of reconstituted trimeric LHCII containing lutein and neoxanthin (Trim-Ctr, solid line), reconstituted trimeric LHCII containing only lutein (Trim-Lut, dashed line) and the difference between them enlarged by a factor of two (dashed-dotted line). Spectra were normalized at the Chl \(a\) \(Q_y\) absorption maximum. (B) Stark spectra in the \(Q_y\) region of reconstituted trimeric LHCII containing lutein and neoxanthin (Trim-Ctr, solid line) and reconstituted trimeric LHCII containing only lutein (Trim-Lut, dashed line) at 77 K recorded at \(\gamma = 54.7^\circ\). Spectra were normalized to OD = 1 at the Chl \(a\) peak and to a field strength of \(F = 1.0 \times 10^5\) V/cm. (C) Stark spectra in the Soret region of reconstituted trimeric LHCII containing lutein and neoxanthin (Trim-Ctr, solid line) and reconstituted trimeric LHCII containing only lutein (Trim-Lut, dashed line) at 77 K recorded at \(\gamma = 54.7^\circ\). Spectra were normalized to OD = 1 at the Chl \(a\) peak and to a field strength of \(F = 1.0 \times 10^5\) V/cm.
change in dipole moment and polarizability in native LHCII [33,51] and close to the published values for monomeric uncoupled Chl \(a\) (see for instance Ref. [51]). Therefore, we conclude that the Chl \(a\) molecules in LHCII are weakly excitonically coupled and no charge-transfer states are present, in agreement with previously reported results [6,51–54].

The Stark signal at \(\sim 662\) nm in reconstituted trimers is more intense when compared to native trimers of LHCII. However, the relative absorption is also more pronounced at those wavelengths with respect to native trimers and the obtained \(|\Delta \mu^2|\) value using a Gaussian fit for a band located at this wavelength, is similar to the previously reported \(|\Delta \mu^2|\) value (1.4 D/f in reconstituted and 1.3 D/f in native trimers).

### 4.2. Chl \(b\) \(Q_y\) region

The Stark effect in the Chl \(b\) \(Q_y\) region for reconstituted control monomeric and trimeric LHCII is very similar to that of native LHCII, characterized by a pronounced minimum at \(\sim 650\) nm. The Stark signal for neoxanthin-deficient samples is much less intense and scales only up to 25% of the native. In addition, a shift of part of the Chl \(b\) absorption bands (mainly to the blue) is observed in reconstituted LHCII lacking neoxanthin. Although the most straightforward explanation is that in native LHCII neoxanthin strongly interacts with one or more Chls \(b\) giving rise to the experimentally observed intense Stark signal at \(\sim 650\) nm, it remains striking that no substantial changes are observed in the carotenoid region when neoxanthin is not bound. This fact leaves us with a second possibility: the Stark signal at \(650\) nm is due to strong Chl \(b\)–Chl \(b\) interactions. In a recent work, Olszówka et al. [36] reported a decrease and a blue shift in the Chl \(b\) Stark signal when this pigment is photobleached, whereas no changes below 500 nm were noticed, i.e. where neoxanthin absorbs. Therefore, they concluded that neoxanthin does not strongly interact with any Chl \(b\) molecule, ascribing the observed intense Chl \(b\) signal to strong Chl \(b\)–Chl \(b\) interactions. Our data clearly invalidate the argument of Olszówka et al. because neoxanthin does not contribute significantly to the Stark spectrum in the Soret region, thereby explaining that no changes would be noticeable if this pigment becomes photobleached. However, on the basis of the present data, we cannot actually rule out the possibility that Chl \(b\)–Chl \(b\) interactions cause the large Stark effect at 650 nm. In the atomic model proposed by Kühlbrandt et al. [5] for LHCII, the shortest Chl \(b\)–Chl \(b\) distance is 14.6 Å (B5–B6 pair). Nevertheless, recent reconstitution experiments have led to the conclusion that, probably, the original assignment of the identities of the Chl molecules is not fully correct and that there could be some binding sites where either a Chl \(a\) or a Chl \(b\) can be bound. This fact can cause the shortest Chl \(b\)–Chl \(b\) distance to become 10 Å (A7–B6 pair). Although the presence of mixed binding sites in LHCII and which ones they are is still a matter of debate, the fact that in some cases a Chl \(b\) can replace a Chl \(a\), implies that Chl \(b\)–Chl \(b\) interactions could account for the observed Stark signal at 650 nm. In such a case, only the pair of Chls bound at positions A7 and B6 could account for the pronounced Stark signal at 650 nm—Remelli et al. [14] reported that A7 is indeed a Chl \(b\) —, because the pair of Chls bound at sites A3 and B3 should give rise at the same time to strong Chl \(a\) and Chl \(b\) signals, since these sites bind either Chl \(a\) or Chl \(b\) [14] and according to Rogl et al. [15,55], B3 only binds Chl \(a\).

### Table 2

Stark parameters of Chl \(a\) in reconstituted trimers and monomers of LHCII estimated using a fit with a polynomial function, and its first and second derivatives

| Sample     | \(|\Delta \mu^2|\) (D/f) | Tr(Δ\(a\)) (Å^2/f^2) |
|------------|-------------------------|------------------------|
| Mon-Ctr    | 0.69 ± 0.06             | 47 ± 6                 |
| Mon-Lut    | 0.66 ± 0.06             | 49 ± 6                 |
| Trim-Ctr   | 0.63 ± 0.06             | 55 ± 6                 |
| Trim-Lut   | 0.60 ± 0.06             | 60 ± 6                 |

*The fitted spectral region ranged from 705 to 670 nm except for monomers, where a satisfactory fit could not be obtained when wavelengths below 675 nm were included.*
Fig. 4 shows a Gaussian fit of the whole $Q_y$ region for both absorption and Stark spectra for reconstituted trimers of LHCII. The $\Delta \mu^\alpha$ and $\Delta \alpha$ contributions from the Chl $a$ absorption band peaking at ca. 677 nm and from the main Chl $b$ located at ca. 650 nm to the Stark spectra are depicted to clearly show the difference in magnitude of both Stark signals. The resulting $|\Delta \mu^\alpha|$ and $\text{Tr}(\Delta \alpha)$ values for the bands associated with Chl $b$ are summarized in Table 3. Both (monomeric and trimeric) reconstituted control samples are characterized by $|\Delta \mu^\alpha| \sim 2.2 \ D/\ell$ for the band at $\sim 650$ nm. The neoxanthin-deficient samples show lower $|\Delta \mu^\alpha|$ values, which are typical for monomeric uncoupled Chl $b$ molecules [50]. These values are in agreement with the observed experimental Stark signals and clearly show that neoxanthin affects the electronic excited states of the nearby Chl $b$ molecules.

There are no reported values for $\text{Tr}(\Delta \alpha)$ of monomeric Chl $b$, but the obtained values are not much larger than the typical values for the Chl $a$ molecules bound to the protein LHCII ($\text{Tr}(\Delta \alpha) \sim 60 \ \AA^3/\ell^2$), with the exception of the band at 645 nm in reconstituted control trimers of LHCII, which is substantially higher. The fit also shows the broadening of the main Chl $b$ band, especially towards the blue side. In Fig. 5, the Stark difference spectrum for reconstituted monomeric LHCII (Mon-Ctr minus Mon-Lut) in the $Q_y$ region is plotted. It clearly shows the strong Stark effect at

Table 3

| Gaussian band (nm) | Relative area$^a$ (%) | $|\Delta \mu^\alpha|$ (D/\ell) (± 10%) | $\text{Tr}(\Delta \alpha)$ (\AA$^3$/\ell$^2$) (± 10%) | Gaussian band (nm) | Relative area$^a$ (%) | $|\Delta \mu^\alpha|$ (D/\ell) (± 10%) | $\text{Tr}(\Delta \alpha)$ (\AA$^3$/\ell$^2$) (± 10%) |
|-------------------|----------------------|-------------------------------|---------------------------------|-------------------|----------------------|-------------------------------|---------------------------------|
| Mon-Ctr           |                      |                               |                                 | Mon-Lut           |                      |                               |                                 |
| 658.5             | 13.3                 | –                             | –                               | 661.0             | 15.1                 | 1.1                           | –                               |
| 651.0             | 12.5                 | 2.1                           | 28                              | 652.5             | 14.3                 | 1.2                           | 99                              |
| 645.5             | 10.4                 | 1.7                           | 90                              | 646.0             | 10.1                 | 1.2                           | 20                              |
| 638.5             | 2.3                  | 0.8                           | –                               | 636.5             | 5.7                  | 1.4                           | 41                              |
| Trim-Ctr          |                      |                               |                                 | Trim-Lut          |                      |                               |                                 |
| 656.0             | 3.6                  | 1.7                           | 80                              | 656.0             | 6.6                  | 0.9                           | 107                             |
| 650.5             | 19.2                 | 2.2                           | 85                              | 650.5             | 14.6                 | 1.2                           | 47                              |
| 645.5             | 5.6                  | 0.9                           | 219                             | 643.5             | 10.8                 | 1.3                           | 84                              |
| 640.0             | 4.5                  | 0.8                           | 17                              | 638.0             | 3.1                  | 0.8                           | –                               |

$^a$ Only the values associated to the Chl $b$ bands are shown. The Chl $a$ bands showed typical values characterized by $|\Delta \mu^\alpha| \sim 0.6 \ D/\ell$ and $\text{Tr}(\Delta \alpha) \sim 60 \ \AA^3/\ell^2$, in agreement with previously reported results [33,51].

$^b$ The relative area was also estimated taking into account the contribution of the Chl $a$ bands to the fit.
\ (~ 650 nm) with a second derivative-like shape in monomeric LHCII containing neoxanthin, and the small red shift of a few Chl \(\text{a}\) subbands in Mon-Lut samples.

4.3. Carotenoid region

The main difference between the control and neoxanthin-deficient samples in the pigment stoichiometry is the presence/absence of neoxanthin and the relative higher amount of lutein when it is the only carotenoid bound (see Table 1). However, for all samples, the Stark effect in the carotenoid region is almost identical. This is quite surprising, because carotenoids usually give rise to strong Stark signals\,[37,56] and, therefore, if neoxanthin would exhibit a big Stark signal, pronounced differences in the Stark spectra should be readily noticeable. Thus, neoxanthin when bound to the protein LHCII, shows a much less intense Stark signal than for instance lutein. The result is at odds with the conclusion from a previous study on native trimers of LHCII, where it was concluded from a modeling of the Stark signal that the electrooptical parameters of lutein and neoxanthin do not substantially differ from each other\,[36].

We performed a simultaneous Gaussian fit of the absorption and Stark spectra in the carotenoid region for all the samples. The results of the fit for the three red-most bands are presented in Table 4. Fig. 6 shows the fits of the absorption and Stark spectra of reconstituted LHCII monomers lacking neoxanthin. Monomers of LHCII show a red-shifted absorption band located at ca. 507 nm when compared to the \(~ 494\) nm lutein band, but its relative area compared to that of the 494 nm band seems to be smaller. The \(\Delta \mu^+\) value of the 507 nm band is rather large for trimers, but lower than the previously reported \(\Delta \mu^+\) value for the 509 nm lutein of native trimers. However, for reconstituted LHCII the Tr\((\Delta \alpha)\) values are higher. The rest of the bands associated with the lutein molecules (\(~ 494\) and \(~ 506\) nm in monomers), show \(\Delta \mu^+\) values which are typical for monomeric lutein as measured in glassy solvents\,[37]. The absence of any additional trace of Mg\(^{2+}\) ions in the reconstitution procedure followed to form the trimers excludes the possibility pointed out in the Introduction that the large Stark signal and red shift in trimers for one of the luteins (most likely L2\,[57]), is indeed due to the presence of Mg\(^{2+}\) ions in the surroundings. Probably, a close charge residue in the protein backbone is responsible for the enhancement in the Stark signal for the red lutein. Still, it remains striking that the absorption band associated to the red lutein appears to be less intense than that of the other lutein. One explanation could be that not all the genes of which LHCII is composed, i.e. lhcb1, lhcb2 and lhcb3, lead to a red shift of one of the luteins. However, the reconstitution was performed with lhcb1 alone, which rules out this possibility. Alternatively, it might be possible that the extinction coefficient of the red-shifted lutein is lower that that of the other lutein. This effect, i.e. lower extinction coefficient when the absorption maximum shifts to longer wavelengths, has already been observed for lutein in solution\,[58].

![Fig. 5. 77 K Stark difference spectrum in the \(Q_y\) region of reconstituted monomeric LHCII containing lutein and neoxanthin (Mon-Ctr) minus reconstituted monomeric LHCII containing only lutein (Mon-Lut). Original spectra were normalized to OD = 1 at the Chl \(\alpha\) peak and to a field strength of \(F = 1.0 \times 10^5\) V/cm.](image-url)

<table>
<thead>
<tr>
<th>Table 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stark parameters for reconstituted monomeric and trimeric LHCII in the carotenoid region(^a)</td>
</tr>
<tr>
<td>Gaussian band (nm)</td>
</tr>
<tr>
<td>Mon-Ctr</td>
</tr>
<tr>
<td>555.0(^c)</td>
</tr>
<tr>
<td>507.5</td>
</tr>
<tr>
<td>496.0</td>
</tr>
<tr>
<td>487.0</td>
</tr>
<tr>
<td>Trim-Ctr</td>
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<tr>
<td>507.5</td>
</tr>
<tr>
<td>496.0</td>
</tr>
<tr>
<td>488.5</td>
</tr>
</tbody>
</table>

\(^a\) Only the values associated to the red-most carotenoid bands are shown.

\(^b\) The relative area was estimated also taking into account the contribution of the rest of the bands to the fit.

\(^c\) This Gaussian band was included to minimize the error due to the baseline and contributions from the Chl molecules.
A band between 486 and 489 nm was always needed for the fitting, which is slightly red-shifted in reconstituted control samples compared to reconstituted samples lacking neoxanthin. The $|\Delta \mu|$ values calculated for these bands are similar in all the samples, showing that the contribution of neoxanthin to the Stark spectra is at most of the same order of magnitude of that of the other chromophores absorbing mainly at 486 nm, i.e. Chl b [35]. The relative areas of these bands are higher in the control samples, reflecting the presence of neoxanthin. From the fits, it can be estimated that the Stark signal of neoxanthin is characterized by $|\Delta \mu|$ values which should be lower than 2 D/f.

Frank et al. [59] studied the effect of solvent environment on the lowest excited state of neoxanthin and several other carotenoids. It turned out that neoxanthin and spheroidene, both of them lacking carbonyl functional groups were hardly affected by the solvent environment, in contrast to the other carotenoid molecules with carbonyl functional groups. Furthermore, the dipole moment of the ground state of neoxanthin was calculated to be 2.1 D, which is rather low compared to that of other carotenoids (5.7 D for peridinin and 8.8 D for fucoxanthin [59]).

In Fig. 7 the Stark difference spectrum of reconstituted monomeric LHCII (Mon-Ctr minus Mon-Lut) in the Soret region is depicted. There are no indications of minima located at the presumably first vibrational levels of the $S_2$ excited state of neoxanthin, i.e. at 486–9 and 457 nm, confirming that neoxanthin shows a weak response to an applied electric field upon optical excitation. On the contrary, the Trim-Lut minus Mon-Lut Stark difference spectrum (Fig. 8) does show two pronounced minima. However, these are located at 507 and 479 nm, and should be ascribed to the red-most lutein.

Because of the small contribution of neoxanthin to the Stark spectra, it can be concluded that the Chl b molecules absorbing at ~ 486 nm [8,35] are mainly responsible for the Stark minimum at 485 nm observed in trimers. The minima at 457 and 429 nm should be ascribed to the blue lutein, which is reported to absorb in LHCII at 494, 466
and 435 nm [21,33,46]. Thus, a substantial contribution to the Stark signal from $\Delta\alpha$ should be expected, characterized by a first derivative-like shape, with a zero-crossing close to the maximum of the corresponding absorption band and a negative minimum towards the high energy side. However, a possible contribution from some Chl $a$ molecules absorbing at $\sim 435$ nm in the Soret region cannot be discarded.

5. Conclusions

The results in this study are completely consistent with the idea that neoxanthin is in close contact with Chl $b$ molecules, which could lead to strong interactions with them.

The red shift of one of the luteins (probably L2) when present in the trimer is not due to the close proximity of Mg$^{2+}$, and therefore is seems most likely that upon trimerization L2 comes in contact with a charged residue of a neighboring monomeric LHCII subunit.

The Stark signals and the electrooptical values ($|\Delta \mu|/\alpha$ and Tr($\Delta\tilde{\alpha}$)) obtained for all the pigments (Chl $a$, Chl $b$, lutein and neoxanthin) present in the control samples, are very similar to those reported previously for native LHCII.

The Stark effect of neoxanthin bound to LHCII is much less intense than that of lutein, which dominates the Stark signal in the carotenoid spectral region.

Neoxanthin, when it is bound, substantially modifies the electronic excited states of one or more Chl $b$ molecules and gives rise to a strong signal at 650 nm due to Chl $b$. The most straightforward explanation is that neoxanthin strongly interacts with the nearby Chl $b$ molecules, but on the basis of our present data we cannot rule out that Chl $b$–Chl $b$ interactions cause such a large Stark signal.

When neoxanthin is absent, some Chl $b$ absorption subbands shift from $\sim 486$ to $\sim 473$ nm, which agrees with previous conclusions stating that the band at 486 nm is due to both neoxanthin and Chl $b$ molecules [8] and with the concomitant blue shift observed in the Q$_y$ region.

All Stark spectra for LHCII trimers—native and reconstituted with and without neoxanthin—exhibited a strong signal around 507 nm, which is due to one of the lutein molecules. This Stark signal is characterized by a rather large change in dipole moment between ground and excited states.

We ascribe the observed Stark minima located at 457 and 428 nm to the blue lutein absorbing at $\sim 494$ nm. These Stark signals can be qualitatively described by a change in polarizability, rather than by a change in dipole moment, which clearly shows the different behavior of the two lutein molecules upon optical excitation in the presence of an electric field.

Finally, the Stark and absorption spectra for the Chl $a$ molecules are not substantially influenced by the presence/absence of neoxanthin.

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References


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