Xanthophylls of the major photosynthetic light-harvesting complex of plants: identification, conformation and dynamics

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Received 5 May 2000; received in revised form 12 June 2000

Edited by Richard Cogdell

Abstract The electronic transitions of lutein and neoxanthin in the major light-harvesting complex, LHCIIb, have been identified for the first time. It was found that 0–0, 0–1 and 0–2 transitions of neoxanthin were located around 486, 457 and 430 nm, whilst those for lutein were dependent on the oligomerisation state. For the monomer, the absorption bands of lutein were found at 495, 466 and 437 nm. Trimerisation caused a decrease in lutein absorption and the parallel appearance of an additional absorption band around 510 nm, which was identified by resonance Raman excitation spectra to originate from lutein. Circular dichroism measurements together with analysis of the ν_4 resonance Raman region of xanthophylls suggested that this lutein molecule is distorted in the trimer. This feature is not predicted by the LHCIIb atomic model of Kühlbrandt and coworkers [Kühlbrandt, W., Wang, D.N. and Fugiyoshi, Y. (1994) Nature 367, 614–621] and is an important step in understanding pigment dynamics of the complex. Oligomerisation of trimers led to a specific distortion of the neoxanthin molecule. These observations suggest that the xanthophylls of LHCIIb sense the protein conformation and which may reflect their special role in the assembly and function of the light-harvesting antenna of higher plants.

1. Introduction

The major light-harvesting complex of higher plants, LHCIIb, is the most common pigment–protein complex on earth. It constitutes more than 40% of the photosynthetic membrane protein. Oxygenic photosynthesis is largely reliant upon the efficiency and adaptability of this complex to collect light energy and deliver it to the reaction centres of photosynthesis [1]. The complex is thought to exist in a trimeric state in vivo. However, recent biochemical and electron microscopic studies have revealed the occurrence of at least seven tightly associated trimers in the chloroplast membrane [2,3]. Some years ago, a structural model of LHCIIb was released, showing the positions of 12 chlorophylls, three transmembrane helices and two of the four known xanthophylls [4]. The latter have been the subject of intense investigation during the last decade. It was shown that two of xanthophylls are luteins, one is neoxanthin and one is violaxanthin, this latter carotenoid having a low binding affinity to the complex [2]. Reconstitution work has demonstrated the obligatory requirement of two luteins for the formation of a stable, folded complex [5]. Therefore the structural role of these xanthophylls has been the focus of wide multidisciplinary research, which has included biochemical, genetic and spectroscopic approaches [6–8]. The role of lutein in the photoprotective regulation of energy dissipation in LHCIIb has been under consideration [9]. The position of neoxanthin is unknown and its role is not understood.

One of the major difficulties in the study of LHCIIb xanthophylls in vivo is the lack of an unambiguous assignment of their multiple electronic transitions in the Soret band absorption region of the complex. This constitutes the central theme of the presented work. Combining different types of LHCIIb, and isolated xanthophyll preparations with a number of site-selective spectroscopies, such as resonance Raman, circular dichroism (CD) and ultralow-temperature absorption spectroscopy, we have identified directly, for the first time, the electronic transitions of lutein and neoxanthin in LHCIIb. The conformation and orientation of these two xanthophylls, as well as their interaction with their environments, were strongly affected by the oligomerisation state of the complex.

2. Materials and methods

LHCIIb was prepared from dark-adapted spinach leaves, using a procedure of solubilisation of photosystem II-enriched particles with *n*-dodecyl-β-D-maltoside and subsequent preparative isoelectric focussing as described in [10]. Further purification and removal of violaxanthin was carried out on a sucrose gradient as described in [2]. LHCIIb monomers were prepared by phospholipase A_2 treatment of trimers for 36 h in the presence of 20 mM $CaCl_2$ at a chlorophyll concentration of 0.5 mM (protocol modified from [11]), followed by purification on a sucrose gradient as described in [2]. Oligomeric LHCIIb was obtained by incubation of trimers with 50 mg of SM-2 Absorbent (Bio-Rad) in a 1 ml fluorescence cuvette, allowing fine control over the extent of aggregation using simultaneous fluorescence measurements by Walz fluorimeter.

Low-temperature absorption spectroscopy was carried out either in a helium bath cryostat (Utreks) using a Varian Cary E5 double-beam scanning spectrophotometer. Some room temperature absorption spectra have been measured using an Aminco DW2000 spectrophotometer. CD measurements were carried out as described in [12].

Low-temperature resonance Raman spectra were obtained in a helium flow cryostat (Air Liquide, France) using a Jobin-Yvon U1000 Raman spectrophotometer equipped with a liquid nitrogen-cooled CCD detector (Spectrum One, Jobin-Yvon, France) as described in [13]. Excitation was provided by Coherent Argon (Innova 100) and Krypton (Innova 90) lasers (457.9, 476.5, 488.0, 496.5, 501.7, 514.5

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Abbreviations: LHCIIb, the major light-harvesting complex of photosystem II; CD, circular dichroism

0014-5793/00/$20.00
PII: S0014-5793(00)01799-3

and 528.7 nm and 413.1 nm, respectively) and a Liconix helium–cadmium laser (441.6 nm).

3. Results and discussion

Fig. 1A shows absorption spectra of trimeric LHCIIb in the Soret region. At room temperature, only a few components can be distinguished between 450 and 520 nm (curve 1). Lowering the temperature down to 5 K leads to an increase in resolution, as one band and two shoulders become clearly visible (curve 2). To further increase resolution, the difference between curves 1 and 2 was calculated (curve 3). Such a procedure, called low-temperature line narrowing, can be performed provided that no significant temperature shifts occur upon cooling (see below). At least five bands can be detected in the computed spectrum in the carotenoid region, at 457, 472, 486, 495 and 510 nm, as well as two transitions in the chlorophyll *a* region around 430–440 nm. It is also possible to gain resolution by calculating the second derivative of the absorption spectra. At 5 K, the second derivative reveals six clear transitions at 457, 466, 472, 486, 495 and 510 nm (curve 4). The strong 472 nm band and a part of the 466 nm band can be assigned to chlorophyll *b* [12] and the remaining transitions arising from those carotenoid molecules. The derivative analysis performed on spectra recorded at room temperature shows that there are no significant temperature-induced shifts of these various transitions (data not shown).

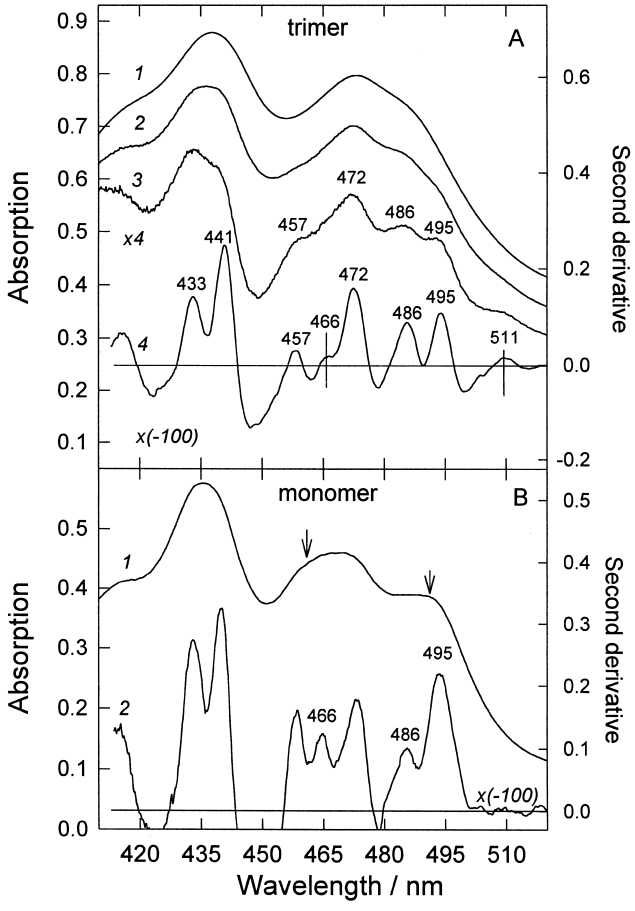

Fig. 1. Soret region absorption spectra of LHCIIb preparation. (A) Spectra of trimeric LHCIIb at 293 K (1) and 5 K (2), and the corresponding difference 2−1 (3). Also shown is the second derivative of the 5 K spectrum (4). (B) Spectra of monomeric LHCIIb at 5 K (1) and its second derivative (2). Arrows indicate the regions where the monomer spectrum differs most significantly from that of the trimer. All spectra are shown in offset.

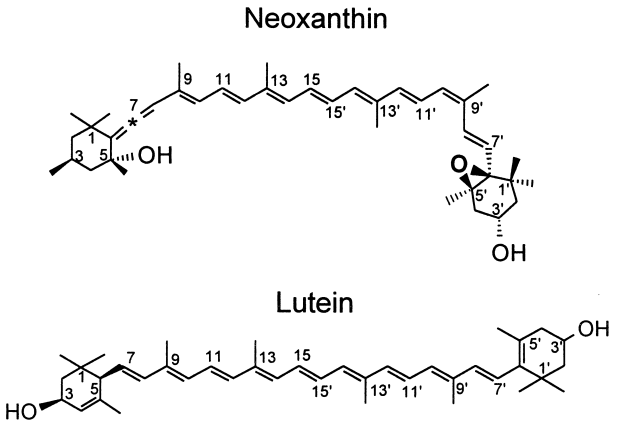

Fig. 2. Structures of two LHCIIb xanthophylls, lutein and neoxanthin.

Fig. 1B displays the absorption spectrum of LHCIIb monomers recorded at 5 K (curve 1), together with its second derivative (curve 2). Around 460 and 495 nm, this spectrum is clearly different from that of LHCIIb trimers. These changes are due to the enhancement of transitions at 466 and 495 nm, as can be seen in the second derivative. The other prominent change in monomer absorption is the disappearance of the trimer band at 510 nm, which cannot be attributed to differences in xanthophyll composition as the carotenoid composition of monomers and trimers was almost identical (data not shown). This transition thus depends on the association state of the LHCIIb subunits. The fact that there is an increase in 466 and 495 nm absorption in monomers, where the 510 nm band is absent, suggests that this latter transition in trimers may originate from the xanthophyll or xanthophylls absorbing at 466 and 495 nm in the LHCIIb monomer spectra.

It is however difficult to formally assign these bands to a particular carotenoid molecule. Since there are two lutein and one neoxanthin only per LHCIIb monomer and taking into account that the extinction coefficients of these molecules are similar [14], the lutein absorption bands, if all degenerate, should be twice as intense as those of neoxanthin. Indeed, the 495 nm band does appear twice as large as the 486 nm one in the second derivative absorption spectra. Moreover, these bands cannot arise from the same molecule as the distance between 0–0 and 0–1 vibrational bands is within 27–32 nm in organic solvents (data not shown). This would thus suggest that the 495 nm band arises from both lutein molecules, whilst that at 486 nm arises from neoxanthin. However, it is also possible that the 486 nm transition originates from a lutein being in a different environment, whilst the 495 nm one arises from both the other lutein and the neoxanthin molecule.

In order to assign these different transitions, resonance Raman spectra were measured at various excitation wavelengths throughout the 410–530 nm region. In resonance Raman spectra of carotenoid molecules, the position of the most intense band (ν_1) varies according to the number of conjugated C=C bands that these molecules possess, being up-shifted in the case of shorter conjugated chains. *Cis* carotenoids also exhibit higher ν_1 frequencies than all-*trans* carot-

enoids, the size of the up-shift being dependent on the *cis* position (see [15] for a review of carotenoid Raman spectra). As seen from Fig. 2, all-*trans* lutein has 10 conjugated C=C bonds while 9′-*cis* neoxanthin has nine. The ν_1 band, which arises from the C=C stretching mode, should thus help in determining which carotenoid(s) contribute to Raman spectra at a given excitation. The frequency of ν_1 for both lutein and neoxanthin was measured in various solvents (Fig. 3A, open circles). In the spectrum of neoxanthin, this band is located at ca. 1532 cm^{-1} whilst it is at ca. 1526 cm^{-1} for lutein – as expected, the shorter chain length and/or the presence of a *cis* conformation results in an up-shift in the case of neoxanthin. As shown in Fig. 3A, these positions vary a little with excitation wavelength in vitro (1532–1534 cm^{-1} for lutein and 1525–1527 cm^{-1} for neoxanthin). When the same experiment was performed on LHCIIb, it clearly appears that the frequency of the ν_1 strongly depends on the excitation wavelength (Fig. 3A, closed circles). Frequency variations of up to 5 cm^{-1} are observed depending on the excitation wavelength. The ν_1 frequency at 457 and 488 nm is close to that observed in spectra of neoxanthin in vitro, and close to that of lutein at 441, 476 and above 501 nm. This indicates that neoxanthin contributions dominate the resonance Raman spectra of LHCIIb trimers obtained with 457 and 488 nm excitation, and that these excitation conditions match the absorption transitions of this molecule. Therefore it is concluded that the transitions

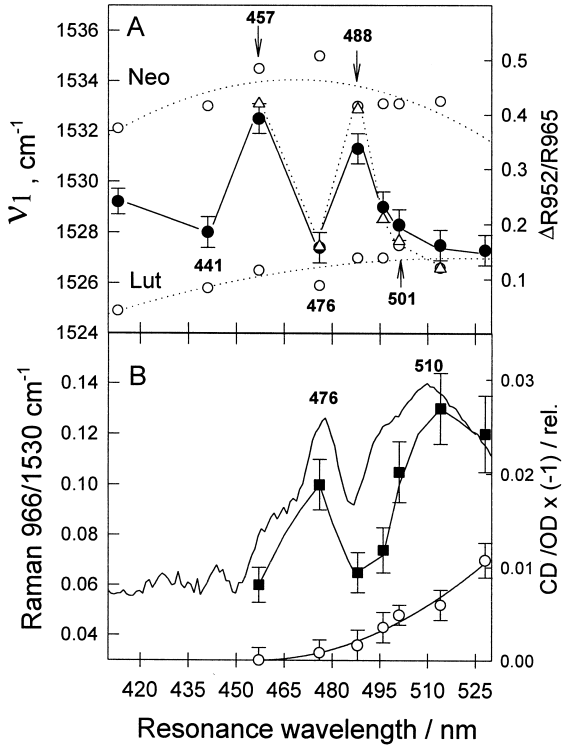

Fig. 3. Resonance Raman signals as a function of the resonance wavelength. (A) ν_1 dependency on the resonance wavelength in LHCIIb trimers (closed circles), and in isolated xanthophylls in pyridine (open circles; Neo – neoxanthin, Lut – lutein). Also shown is the relative increase in ν_4 952 cm^{-1} amplitude upon LHCIIb oligomerisation as a function of the resonance wavelength (open triangles). (B) Amplitude of ν_4 region (relative to ν_1) as a function of the resonance wavelength for LHCIIb trimers (closed squares) and monomers (open circles). Solid line – normalised CD (CD/OD) of trimeric LHCIIb multiplied by (−1).

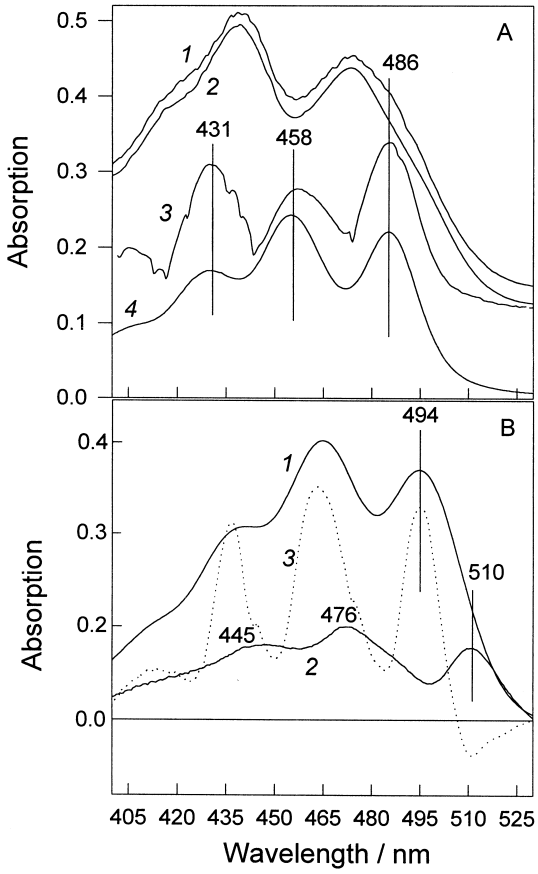

Fig. 4. (A) Room temperature absorption spectra of LHCIIb monomers prepared from *Spinacea oleracea* (1) and *C. reflexa* (2), and the corresponding difference 1−2 (3). 4 – absorption spectrum of neoxanthin in pyridine, up-shifted by 2 nm. (B) Absorption spectrum of lutein in pyridine up-shifted by 2 nm (1), and in glycerol (2). Also shown is the monomer−trimer absorption difference spectrum at 5 K (3) (spectra of trimer and monomer are taken from Fig. 1).

observed at 457 and 488 nm in Fig. 1 both arise from neoxanthin. The 29 nm shift between these transitions is in good agreement with that expected between 0–0 and 0–1 sub-levels of xanthophyll transitions [16].

Recently, we described the isolation and properties of LHCIIb from *Cuscuta reflexa*, which lacks neoxanthin [17]. Fig. 4A compares the absorption spectra of LHCIIb monomers isolated from spinach and from *Cuscuta*. Additional transitions may be observed in spinach LHCIIb at 431, 458 and 486 nm, and may be even more clearly seen in the difference spectrum (curve 3). The positions of the bands at 458 and 485 nm are all in perfect agreement with the Raman excitation wavelengths at which the neoxanthin contribution is maximum. Furthermore, all these bands also match with those of neoxanthin dissolved in pyridine, if a 2 nm red-shift is applied (curve 4).

From the analysis of the ν_1 frequency in Fig. 3A, it can also be concluded that the transition at 510 nm should be assigned to a lutein molecule. Indeed, the ν_1 frequency observed with 501 and 514 nm excitations is very close to that of lutein. Similarly, transition at 495 nm should also be attributed to lutein. Whilst the latter transition along with the band around 465 nm is likely to correspond to the 0–0 and 0–1 transitions

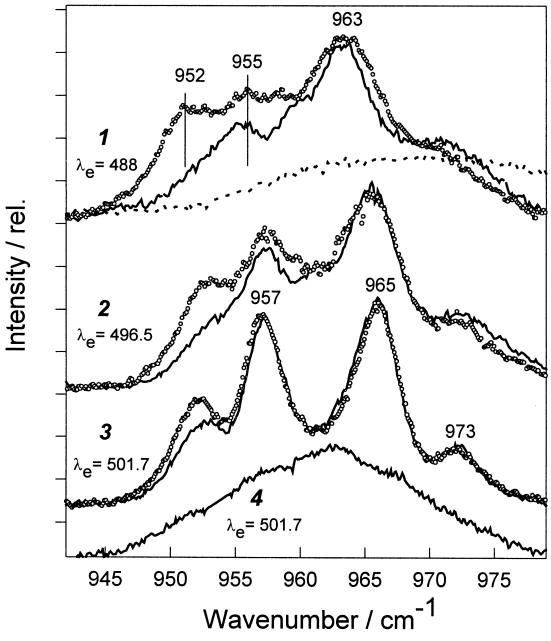

Fig. 5. ν_4 region of xanthophyll resonance Raman spectra, excited at 488.0 nm (spectral group 1), 496.5 nm (spectral group 2) and 501 nm (spectral groups 3, 4). Dotted line – lutein in pyridine, solid line – LHCIIb trimer, open circles – LHCIIb oligomer, 4 – LHCIIb monomer (at 501.7 nm excitation, closed circles).

of the same molecule (see Fig. 4B), the difference between the 510 and 495 nm band is only 15 nm, and is thus too small for these bands to account for two sub-levels of the same xanthophyll. It must thus be concluded that the 510 nm band originates from a different lutein molecule, possessing a particular binding site which induces a red-shift of its transitions. Such a red-shift may be induced by, for example, an extremely polarisable environment and/or chromophore–chromophore interactions [16]. Monomerisation of the LHCIIb trimers disengages this lutein from its binding site, and shifts its absorption transitions back towards the blue, where the other lutein absorbs – at 465 and 494–495 nm (Figs. 1 and 4B). Such a red-shift of lutein absorption may be observed upon molecular aggregation in highly polar media. Fig. 4B displays an example of such a red-shifted spectrum, obtained from lutein dispersed in glycerol. In this particular spectrum, the positions of the 0–1 and 0–2 bands are located around 476 and 445 nm.

Apart from the polarisability of pigment environment, red-shifted transitions usually arise from interaction-induced pigment distortion and/or pigment–pigment (excitonic) interactions [16], and thus should exhibit a stronger CD signal. Fig. 3B represents the CD spectrum normalised to the absorption spectrum of the LHCIIb trimer (note that the spectrum has been inverted for convenience). The major maximum is located at 510 nm, suggesting that this transition originates from the red-shifted lutein molecule. This is further supported by CD spectra of LHCIIb monomers, where this 510 nm band is completely absent and the main maximum is observed at 494 nm (data not shown). The second maximum in the trimer spectrum is located at 476 nm (i.e. exactly where the 0–1 transition of the red-shifted lutein is expected), suggesting that the 476 and 510 nm bands arise from the same lutein.

To further investigate the origin of the 510 nm band, the region of resonance Raman spectra containing out-of-plane C–H wagging modes was systematically analysed (ν_4 region, around 950 cm^{-1} [15]). This analysis is represented in Fig. 5. Normally, when the carotenoid is in a planar conformation, bands in this region are extremely weak as these out-of-plane modes are not coupled with the electronic transition. For example, this is the case for lutein dissolved in pyridine (curve group 1, dotted line). In spectra of the LHCIIb monomer (curve 4), bands in this region are also weak, suggesting that monomer-bound xanthophylls are in a more or less planar conformation. However, when the carotenoid molecules are twisted around a C–C bond, these out-of-plane modes become more strongly coupled to the electronic transitions, and their contributions to resonance Raman spectra are thus more intense [15]. Resonance Raman spectra of LHCIIb trimers and oligomers exhibit a rich structure in the ν_4 region, which is highly dependent on the resonance wavelength (Fig. 5). At 488 nm (group 1), only two distinct bands can be observed at 955 and 963 cm^{-1}, whereas in resonance conditions at 501 nm, additional maxima appear at 957 and 965 cm^{-1}, together with shoulders at 952 and 973 cm^{-1} (group 3). The intensity of the major transition in this region at 965 cm^{-1} relative to the ν_1 band (ca. 1530 cm^{-1}) was plotted against the resonance wavelength (Fig. 3B, closed squares). It matches, within experimental error, the normalised CD spectrum for the trimer. The main maximum is located in the 510–515 nm region with a second maximum at 476 nm. This again suggests that this transition should be attributed to the red-shifted lutein having its 0–0 transition at 510 nm. Note that no such behaviour is observed for monomeric LHCIIb (Fig. 3B, open circles), again indicating a more planar configuration for all xanthophylls in the monomer.

It has been shown that formation of aggregates of LHCIIb trimers influences the structure of the ν_4 region [18]. However, the preparation used in these early experiments was not homogeneous enough to allow unambiguous interpretation of the data. Fig. 5 shows how the oligomerisation of trimers enhances the 952 and 955 cm^{-1} modes. This enhancement is dependent on the resonance wavelength. The relative increase in the 952 cm^{-1} component induced by oligomerisation, when plotted against the resonance wavelength, follows quite closely the variation in ν_1 position (Fig. 3A, open triangles). As the ν_1 position reveals the absorption transitions of neoxanthin (see above), this suggests that the molecule whose conformation is distorted by oligomerisation is the LHCIIb-bound neoxanthin.

Table 1
Assignment of Soret absorption transitions in trimeric LHCIIb.

433 nm	441 nm	457 nm	466 nm	472 nm	486 nm	495 nm	511 nm
Neo 430 (0–2)	Lut*445 (0–2) Lut 437 (0–2)	Neo 457 (0–1)	Lut 466 (0–1)	Lut*476 (0–1)	Neo 486 (0–0)	Lut 495 (0–0)	Lut*510 (0–0)
chl *a*	chl *a*			chl *b*			

Lut* – long-wavelength lutein.

This work has allowed a direct assignment of the different electronic transitions of LHCIIb-bound lutein and neoxanthin. These assignments are shown in Table 1. Note that a similar assignment for the 0–0 transition of neoxanthin has been inferred from experiments in which the xanthophyll composition has been varied in reconstitution experiments [19]. The position of these transitions suggests that all xanthophylls are present in highly polarisable environments [16]. This is consistent with the position of lutein in close proximity to the hydrophobic helices A and B of the complex in the atomic model, as well as with the recent localisation of neoxanthin in the hydrophobic environment near to helix C [19]. Convincing evidence has been obtained that there is an additional, red-shifted transition arising from an all-*trans* lutein around 510 nm in trimeric, but not monomeric, LHCIIb. This transition exhibits a bandwidth 50% broader than those of the other xanthophylls as well as a distinct negative CD signal. Resonance Raman spectra indicate that this pigment possesses a slightly twisted configuration, as evidenced by the presence of two out-of-plane C–H modes around 955 and 965 cm^{-1} in the ν_4 region. Upon LHCIIb monomerisation, this twisting is relaxed, suggesting that either it arises directly from monomer–monomer interactions, or that it is induced by conformational changes in each monomer upon trimerisation.

Neoxanthin exhibits a less twisted configuration than that of lutein, as shown by resonance Raman spectroscopy, in LHCIIb trimers. However, it is specifically affected by oligomerisation of the trimers, resulting in the appearance of an out-of-plane C–H wagging mode at 952 cm^{-1} in resonance Raman spectra. It may thus generally be concluded that the configuration of xanthophylls in LHCIIb is modulated by each oligomerisation step (monomer → trimer → oligomer). The characterisation of such specific Raman 'fingerprints' for each type of xanthophyll will certainly be of great use in future research on the LHCII carotenoids in vivo.

Acknowledgements: A.V.R. thanks Professor Rienk van Grondelle and Dr Jan Dekker for use of the spectropolarimeter, Drs Andrew Young and Denise Phillip for samples of purified carotenoids and Dr Ralph Bungard for supplying *C. reflexa* plants. We thank Professor Peter Horton for critical reading of the manuscript. This work was supported by UK BBSRC. A.V.R. was in receipt of an ESF Fellowship and a grant from the Ministére des Affairs Éntrangéres, France. A.P. was funded by TMR Grant number ERBFMBICT983497.

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