Configuration and Dynamics of Xanthophylls in Light-harvesting Antennae of Higher Plants

SPECTROSCOPIC ANALYSIS OF ISOLATED LIGHT-HARVESTING COMPLEX OF PHOTOSYSTEM II AND THYLAKOID MEMBRANES

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The light-harvesting antenna (LHA) of higher plants binds five types of xanthophylls: lutein, neoxanthin, violaxanthin, zeaxanthin, and antheraxanthin. The last three constitute the xanthophyll cycle, which has been suggested to participate in the process of dissipation of excess excitation energy, giving rise to nonphotochemical fluorescence quenching (1–3). Several important questions concerning these xanthophylls remain unanswered: why is there such a variety of xanthophyll types in an antenna; what is the exact molecular mechanism of zeaxanthin action in nonphotochemical fluorescence quenching; where are xanthophylls located; and what is the nature of their interaction with the protein and other pigments? LHA consists of a number of pigment-protein complexes accommodating different types and amounts of xanthophylls (5–8). The major and most characterized LHA complex, the trimeric LHCIIb, binds 2 luteins, 1 neoxanthin, and between 0.1 and 1 violaxanthin per monomer (7, 9). The amount of bound violaxanthin was found to depend on the treatment during purification (9), as well as on plant growth conditions (10). The two luteins of LHCIIb are thought to correspond to the two carotenoid molecules located near the transmembrane helices A and B in the inner core of the complex (11), thus being tightly associated with it and probably having a structural role. Site-directed mutagenesis experiments have suggested that neoxanthin is associated with helix C (12). Neoxanthin was found to have the highest affinity of binding to the complex (9). In contrast, the binding affinity of violaxanthin to the complex is the lowest, and it can be easily removed by various treatments. This biochemical work has therefore established that a large population of xanthophyll cycle carotenoids is peripherally bound to LHA complexes.

The structure of the minor PSII antenna complexes as well as all LHC I complexes is not known; therefore, it is not clear where xanthophyll molecules are bound to them, what the nature of this binding is, or what the carotenoid configuration is involved. The data on xanthophyll stoichiometry in the minor PSII antenna are controversial (4, 6, 8) and may well again reflect natural variation of xanthophyll ratios. However, it is clear that unlike LHCIIb, the minor antenna PSII complexes, CP24, CP26, and particularly CP29, contain at least one strongly bound xanthophyll cycle carotenoid. The efficiency of violaxanthin de-epoxidation—a prerequisite for the photoprotective energy dissipation state in thylakoid—was found to be the reciprocal of the relative affinity of binding of this xanthophyll to various antenna components, suggesting that only loosely bound molecules are accessible to the violaxanthin de-epoxidase (9). A similar tendency was observed for the PSI antenna, which contains at least 50% tightly bound violaxanthin that was not converted into zeaxanthin even under condi-
tions favoring maximum de-epoxidation (9, 13). Therefore, it may be argued that the "structural" violaxanthin molecules, which are strongly coordinated within the minor LHA complexes, do not play a role in the xanthophyll cycle.

It is clear that new methodologies are needed to identify, locate, and analyze xanthophylls and their configuration and function in LHA complexes. One powerful approach has been to reconstitute light-harvesting complexes from Lhcb polypeptides and pigment mixtures of varying composition. Site-directed mutagenesis of these polypeptides has been used to determine the location and specificity of carotenoid binding sites (12). The effects of carotenoid binding to peripheral sites in LHA complexes has allowed investigation of their role in energy dissipation (14–17). Along with the development of these biochemical techniques there has been progress toward the development of instrumental methods to analyze antenna xanthophylls. Absorption, linear and circular dichroism, and triplet state spectroscopies have been applied to identify their electronic absorption bands and energy transfer pathways to and from chlorophyll molecules (18–22). However, it remains difficult to make unambiguous assignments for even simpler systems, such as those containing only three xanthophylls, and impossible to make reasonable spectral assignments in the Soret absorption region of whole thylakoid membranes. Recently, we have combined optical absorption spectroscopy with selective excitation resonance Raman spectroscopy in order to identify the absorption transitions of lutein and neoxanthin in LHCIIb trimers (23). Unlike previous work (18), which used LHCIIb containing three types of carotenoids, we have prepared LHCIII, which contained only lutein and neoxanthin, a more simple system. The characteristic Raman feature for neoxanthin and lutein, the \( \chi_1 \) maximum position, was identified and used to build the Raman excitation profiles for the isolated complex. This allowed not only an estimation of the energies for the three absorption bands (0-0, 0-1, and 0-2) but also an observation of the dynamics of carotenoid configuration upon oligomerization of the complex.

The approaches mentioned above are susceptible to various artifacts and limitations, such as the removal of lipids and pigments by the detergents used in the isolation and reconstitution procedures and the resulting alteration in protein conformation, xanthophyll binding affinity, and xanthophyll environment. One way forward would be the isolation of a more integrated LHA, in which pigments are less perturbed by detergent treatment. For example, we have recently isolated an integrated LHA, in which pigments are less perturbed by detergent treatment. One way forward would be the isolation of a more integrated LHA, in which pigments are less perturbed by detergent treatment. For example, we have recently isolated an integrated LHA, in which pigments are less perturbed by detergent treatment. One way forward would be the isolation of a more integrated LHA, in which pigments are less perturbed by detergent treatment. For example, we have recently isolated an integrated LHA, in which pigments are less perturbed by detergent treatment. One way forward would be the isolation of a more integrated LHA, in which pigments are less perturbed by detergent treatment. For example, we have recently isolated an integrated LHA, in which pigments are less perturbed by detergent treatment. The first time the resonance Raman spectra of violaxanthin and zeaxanthin in vivo. It is concluded that zeaxanthin adopts a configuration that is likely to reflect its well defined binding within the antenna, rather than a free location in the membrane. This work establishes a new approach to the study of complex carotenoid-containing systems and offers a broad range of applications, from identification and assessment of xanthophyll configuration in reconstituted/isolated complexes to in vivo investigation of xanthophyll cycle carotenoids in order to establish their role in photoprotective mechanisms.

**MATERIALS AND METHODS**

Carotenoid samples were prepared as described in Ref. 24 by Dr. Denise Phillip (John Moores University, Liverpool, United Kingdom). LHCIIb was prepared from dark-adapted spinach leaves using isoelectric-focusing of PSI-enriched particles, as described in Ref. 8. Purification of LHCIIb trimers and removal of violaxanthin was carried out on a sucrose gradient (9). LHCIIb monomers were prepared by phospholipase A2 treatment of LHCIIb trimers for 36 h in the presence of 20 mM of CaCl2 at a chlorophyll concentration of 0.5 mM, followed by purification on a sucrose gradient as described previously (9). Intact thylakoid membranes were obtained by the procedure described in Ref. 8. To induce maximum violaxanthin de-epoxidation, thylakoids were incubated at room temperature at a chlorophyll concentration of 200 \( \mu \text{M} \) for 2 h in a medium containing 5 mM d-isoascorbate, 10 mM HEPES, and 10 mM sodium citrate at pH 5.5 with or without 5 mM Mg2+.

Absorption spectra were recorded on a Varian Cary E5 double-beam scanning spectrophotometer; measurements at 4 K were performed using a helium bath cryostat (Utreks). Low temperature resonance Raman spectra were obtained in a helium flow cryostat (Air Liquide, Paris, France) using a Jobin-Yvon U1000 Raman spectrophotometer equipped with a liquid nitrogen-cooled charge-coupled devices detector (Spectrum One, Jobin-Yvon, Paris, France) as described in Ref. 25. Excitation was provided by Coherent Argon (Innova 100) and Krypton (Innova 90) lasers (at 457.9, 476.5, 496.5, 488.0, 501.7, and 514.5 nm and at 528.7 and 413.1 nm, respectively) and a Liconix helium-cadmium laser (at 441.6 nm). The choice of this wavelength range was determined by the absorption profiles of the xanthophylls used. Fig. 1 displays absorption spectra of the four major xanthophylls that have been studied. The number of laser excitation lines (indicated in Fig. 1 by...
**RESULTS**

**Wavelength-selective Resonance Raman Spectroscopy of Isolated Carotenoids—**Carotenoids are very efficient Raman scatterers and exhibit a very strong resonance enhancement (26). Four main frequency regions have been observed, calculated, and assigned as follows: \( v_1 - C = C \) stretching vibrations; \( v_2 - C = C \) stretches coupled either to \( C - H \) in-plane bending or \( C - CH_3 \) stretching; \( v_3 - CH_3 \) in-plane rocking vibrations; \( v_4 - C = H \) out-of-plane bending modes. In RR spectra of carotenoid molecules, the position of \( v_1 \) varies according to the number of conjugated \( C = C \) bands that these molecules possess, being higher in the case of shorter conjugated chains (27). cis-Carotenoids also exhibit higher \( v_1 \) frequencies than all-trans-carotenoids (28, 29). We have found this frequency to be in general unaffected by the solvent type (pyridine, \( n \)-hexane, ethanol, and cyclohexane; data not shown), in agreement with the published data for \( \beta \)-carotene (30). Fig. 2 shows 488 nm excited RR spectra of the four main LHC antenna xanthophylls and \( \beta \)-carotene, all dissolved in pyridine. The different numbers of conjugated double bonds in these carotenoids (from 9 to 11) and the 9-cis conformation of neoxanthin give rise to a large \( v_1 \) variability from zeaxanthin at 1524 cm\(^{-1}\) to neoxanthin at 1533 cm\(^{-1}\). Other regions of the RR spectra are also specific for certain xanthophylls. The arrows in Fig. 2 show the characteristic frequencies for neoxanthin in the \( v_2 \) region at 1120, 1132, and 1203 cm\(^{-1}\), probably due to its 9-cis conformation, and for violaxanthin in the \( v_3 \) band at 1007 cm\(^{-1}\). The \( v_4 \) region was very low in intensity for all carotenoids, out-of-plane modes being formally resonance-forbidden for fully planar molecules. However, they can become significant under conditions in which the carotenoid undergoes configurational rearrangements leading to twisting of the molecule, due, for instance, to interaction with its environment. This situation is not frequently found in solvent or detergent media, but it has been observed in certain cases for carotenoids attached to antenna complexes (26, 23). Molecular distortion of this kind requires energy, which can be gained in the close contact with protein environments such as a hydrophobic helix. Thus, the \( v_4 \) region can be used as a marker for pigment-protein interactions and environmental perturbations involving the carotenoid molecule.

The position of the \( v_1 \) band for each carotenoid was determined for all excitation lines used (Fig. 3A). Each additional double bond produces an \(-3 \text{ cm}^{-1}\) downshift of \( v_1 \). Comparing lutein and neoxanthin, there is a difference of 8 cm\(^{-1}\) in the \( v_1 \) position. This parameter can therefore be used to identify xanthophyll absorption transitions in LHCII complexes containing these two types of xanthophylls (see below). Features in the \( v_2 \) and \( v_3 \) regions can be used to construct additional excitation profiles to confirm band assignments. For example, the \( v_1 \) frequencies for lutein and violaxanthin for some excitation lines can be very close (only 2 cm\(^{-1}\) apart) (Fig. 3A). However, additional analysis of the \( v_3 \) region can be used to make unambiguous assignments, because the corresponding frequency difference between lutein and violaxanthin is at least 4 cm\(^{-1}\) (Fig. 3B).

**Organization of Xanthophylls in LHCIIb: Lutein and Neoxanthin—**Neoxanthin and lutein have very strong binding affinities to LHCIIb compared with violaxanthin, which can be removed by detergent (9). Therefore, it was possible to prepare LHCIIb free from violaxanthin, containing only lutein and neoxanthin. The RR spectra of this LHCIIb sample, after monomerization, were obtained, and the \( v_1 \) maximum position was analyzed as a function of the excitation wavelength. Whereas this parameter was only slightly variable with the resonance wavelength for isolated xanthophylls (Fig. 3), for LHCIIb monomers, it was strongly dependent upon it (Fig. 4A, open circles). For 457.9 and 488.0 nm excitations, the \( v_1 \) position was close to that of isolated neoxanthin, whereas for other excitation wavelengths, it was similar to that of lutein. This indicates that neoxanthin contributions dominate the RR spectra obtained with 457.9 and 488.0 nm excitations. Fig. 4A also shows the second derivative of the absorption spectrum of the LHCIIb monomer, which shows two maxima, at 457 and 486 nm. The 29-nm shift between these bands is in a good agreement with that expected between 0-0 and 0-1 transitions of
xanthophylls. An additional diagnostic parameter was the relative amplitudes of the neoxanthin-specific bands at 1203 cm⁻¹ in the ν₂ region and at 1006 cm⁻¹ in the ν₃ band (see Figs. 2 and 3). Excitation profiles for both of these closely matched the ν₁ excitation profile, confirming the assignment of the 485 nm band to neoxanthin. We suggest that the 495 and 466 nm bands observed in the absorption spectrum most likely originate from lutein (the 476 nm band arises from chlorophyll b).

The absorption spectrum of the LHCIIb trimer is known to contain a new transition around 510 nm not found in monomers (18, 23) (see Fig. 4B). Although it was suggested to belong to violaxanthin (18), it was still present in preparations free from this carotenoid (23). Analysis of RR excitation profiles for ν₁ in LHCIIb trimers has been used as evidence that the 510 nm band belonged to lutein, because the 495 and 466 nm bands in the second derivative spectrum were significantly smaller than the 495 nm band. Because there are two luteins bound to each LHCIIb, if the 495 nm band corresponds to the 0-1 absorption band of one lutein molecule, then the 510 nm band should belong to that of the other one. Why then are their absorption amplitudes in the second derivative spectrum so different? A possible answer to this question is that the 510 nm band may have a smaller extinction coefficient. However, a more likely explanation lies in the observation that the 510 nm band is at least 70% broader than that at 495 nm. As the amplitude of peaks in a second derivative spectrum is proportional to their bandwidth, this results in the relative amplitude of the 510 nm component being lower.

Indeed, in the trimer minus monomer difference spectrum, the bandwidth of the band around 510 nm reaches 18 nm (Fig. 4B).

**Resonance Raman Spectroscopy of Thylakoid Membranes**—The Soret absorption band of the whole thylakoid membrane is more complex than that of LHCIIb in the carotenoid region (Fig. 5); this complexity arises from, among other factors, the presence of extra carotenoids, violaxanthin, and β-carotene. However, it is possible to do comparative spectroscopic studies on the membranes by replacing violaxanthin with zeaxanthin by activation of the xanthophyll cycle. Activation of de-epoxidation in thylakoids yielded about 80% replacement of violaxanthin with zeaxanthin. The 4 K absorption spectrum changes significantly after de-epoxidation of violaxanthin. In Fig. 5, arrows indicate a decrease in 488 and 460 nm regions and the appearance of new bands at 503 and 476 nm. This is consistent with the room temperature difference spectra-illuminated-minus-dark, measured on leaves (31, 32). However, the spectrum recorded at 4 K reveals more structure. The second derivative of the difference spectrum (+Zea)-(+Vio) resolves a complex picture (Fig. 5B). The three characteristic negative bands show a doublet structure at 4 K: 488/497, 452/460, and 429/423 nm. The doublet structure may arise from the two populations of violaxanthin (i.e. integrally bound or peripheral), both of which were de-epoxidized. There is also complexity in the positive bands, and the 503 and 511 nm positive bands may correspond to zeaxanthin 0-0 transitions. However, the de-epoxidation...
The process could have caused changes in antenna conformation, which could then affect other xanthophylls, altering their absorption parameters and giving rise to the complexity of the spectra shown in Fig. 5. Therefore, more evidence is required to identify the origin of the absorption changes observed upon violaxanthin de-epoxidation.

RR spectra were measured for the thylakoid membranes containing only violaxanthin and those enriched with zeaxanthin. The spectra were clearly different in the $n_1$ region, the position of $n_1$ maximum being 3 cm$^{-1}$ downshifted in thylakoids containing zeaxanthin (Fig. 6, circles). It was found that the RR spectra of thylakoids was much wider than the spectrum of LHCIIb (Fig. 6 A, broken line). This is most likely to be due to a more complex structure of thylakoid RR because of additional contributions of violaxanthin, zeaxanthin, $\beta$-carotene, and small amounts of antheraxanthin. A deconvolution of this region using the $n_1$ spectra of isolated carotenoids (Fig. 6 A, solid lines) provides evidence to support this view. The spectrum for violaxanthin-containing thylakoids can be explained as the sum of lutein, violaxanthin, neoxanthin, and $\beta$-carotene (Fig. 6A). The $n_1$ regions for $\beta$-carotene and zeaxanthin are almost identical and are added together in Fig. 6B; again, a good fit to the thylakoid spectrum was obtained. Thus, the downshift in the $n_1$ position after de-epoxidation can be explained by an increase in the low-frequency zeaxanthin/$\beta$-carotene signal (around 1522 cm$^{-1}$) and a decrease in the violaxanthin band at 1528 cm$^{-1}$. Lutein (1526 cm$^{-1}$) and neoxanthin (1534 cm$^{-1}$) bands remained almost unchanged after de-epoxidation. The domination of the spectrum by lutein is explained by neoxanthin being further out of resonance from 501.7 nm excitation compared with lutein, because it absorbs at 485 nm, whereas lutein absorbs at 485 nm (see Fig. 1 and discussion above).

An alternative approach to deconvolution of the spectrum is to calculate difference RR spectra (de-epoxidized-minus-epoxidized) following normalization at the 1540 cm$^{-1}$ region, where contribution of the zeaxanthin and violaxanthin signals is very low (see Fig. 6 A and B). Fig. 6C displays a number of such difference spectra for the $n_1$ region. The vertical dotted line indicates the normalization point, where the difference was always zero. It was found that the shape of the spectrum was strongly dependent on excitation wavelength. With 528.7, 514.5, and 501.7 nm excitation, an almost symmetrical positive band was found, but with 496.5 and particularly 488 nm exci-
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An attempt was made to compare absolute RR amplitudes for violaxanthin- and zeaxanthin-containing thylakoids on the same scale without using this normalization procedure. First, exactly the same amount of chlorophyll was used in each sample, and an average was calculated from several replicates. Second, the small but reproducible signals from chlorophyll at 1437, 1354, and 1327 cm$^{-1}$ due to a decrease at 1530 cm$^{-1}$ matched the two positive bands in the low temperature difference spectrum de-epoxidized minus epoxidized (Fig. 3A). Furthermore, the relative increase of intensity around 1520 cm$^{-1}$ matched the two positive bands in the low temperature difference spectrum de-epoxidized minus epoxidized (Fig. 3B). Therefore, it is concluded that the transitions at 505–510 and 476 nm belong to the 0-0 and 0-1 bands of zeaxanthin. On the other hand, the wavelength dependence for the intensity of the negative band around 1530 cm$^{-1}$ indicates that 488 and 460 nm absorption bands arise from violaxanthin. Even the 497 nm second derivative band shown in Fig. 5 may also belong to violaxanthin, because excitation at 496.5 nm produced a relative decrease in the $v_1$ intensity in the violaxanthin region. Unfortunately, the limited number of excitation lines available did not allow resolution of the 452 and 460 nm bands, but their distance from the 488 and 497 nm transitions suggests that they arise from 0-1 vibrational satellites.

An attempt was made to compare absolute RR amplitudes for violaxanthin- and zeaxanthin-containing thylakoids on the same scale without using this normalization procedure. First, exactly the same amount of chlorophyll was used in each sample, and an average was calculated from several replicates. Second, the small but reproducible signals from chlorophyll at 1437, 1354, and 1327 cm$^{-1}$ were used for normalization. Both methods gave very similar results. As expected, the intensity of the $v_1$ band was dependent upon excitation wavelength (Fig. 7C). The replacement of violaxanthin by zeaxanthin caused significant changes in the excitation profiles, particularly the decrease in intensity with 488 nm excitation and increases above 496.5 nm in the de-epoxidized thylakoids. The calculated difference spectrum (+Zea)-(+Vio) clearly showed the positive changes at 476.5 and 496.5 nm and the negative band at 488 nm, again similar to the shape of the corresponding absorption difference spectrum. This confirms that the band at 488 nm absorption is in a good resonance with 488.0 excitation. The formation of zeaxanthin enhances the RR signal above 500 nm, where zeaxanthin 0-0 absorption resonates with the 501.7, 514.5, and 528.7 nm lines.

The $v_1$ region of the RR spectrum was also investigated in the thylakoid samples. The $v_2$ for zeaxanthin is located at 1003 cm$^{-1}$, whereas for violaxanthin it is shifted down to 1007 cm$^{-1}$ (see Fig. 3B and Fig. 7D, inset). Therefore, the 1003/1007 cm$^{-1}$ amplitude ratio monitors the relative contribution of these xanthophylls. The dependence of this ratio upon excitation wavelength was determined (Fig. 7D). The lowest value of the ratio was found to be at 488 nm excitation, consistent with this resonance arising from violaxanthin. The ratio increased upon de-epoxidation for all excitations used, consistent with an increase in zeaxanthin content, whereas the larger differences between de-epoxidized and epoxidized samples observed for 514.5 and 528.7 nm excitation result from selective excitation of the zeaxanthin present.

Thus, excitation at 488.0 nm is selective for violaxanthin, whereas excitation above 500 nm (e.g. at 528.7 nm) is selective for zeaxanthin. With this information, it is possible to explore the state of violaxanthin and zeaxanthin in the thylakoid mem-
brane, compared with pigments dissolved in detergent/lipid micelles or in organic solvent. In Figs. 8 and 9, RR difference spectra were obtained for xanthophylls dissolved in pyridine (spectum 1), in the free pigment fraction following detergent treatment of thylakoid membranes (spectrum 2), and for thylakoid de-epoxidation treatment (spectrum 3) excited at 488.0 nm (Fig. 8) and 528.7 nm (Fig. 9). The spectra are $(+\text{Vio})-(+\text{Zea})$ for 488.0 nm excitation, respectively. For 488.0 nm excitation, the thylakoid spectra in the $\nu_1$, $\nu_2$, and $\nu_3$ regions matches very closely that for isolated violaxanthin in pyridine and for the free pigment fraction. This clearly identifies the RR difference spectrum for thylakoids as the violaxanthin spectrum with characteristic violaxanthin features at 1529, 1184, 1213, and 1006 cm$^{-1}$ and adds strength to the assertion that the 488 nm band originates from violaxanthin. The $\nu_4$ region for the thylakoid spectrum exhibits one sharp transition at 949 cm$^{-1}$ and another at 962 cm$^{-1}$, whereas the structure of this region for the spectrum of the isolated pigment, either in solvent or in detergent/lipid micelles, is almost absent, and intensity is reduced. The presence of features in the $\nu_4$ region suggests that violaxanthin in vivo is distorted, most likely due to its binding to antenna complexes.

The thylakoid spectra for 528.7 nm excitation are again similar to the spectra of isolated zeaxanthin, either in solvent or in detergent-lipid mycelles (Fig. 9). The $\nu_1$ position around 1522 cm$^{-1}$ and $\nu_3$ maximum at 1003 cm$^{-1}$ are identical in all three spectra. The $\nu_2$ regions of zeaxanthin in pyridine and the thylakoid spectrum are also similar apart from the downshift of the 1190 cm$^{-1}$ band to 1185 cm$^{-1}$. This downshift is also present in the spectrum of the free pigment fraction. The $\nu_4$ region for the RR difference spectra of thylakoid membranes is strongly enhanced and clearly structured compared with that for isolated zeaxanthin. For the free pigment fraction, this region is slightly enhanced but less structured in comparison to the thylakoid membrane spectrum. This suggests that in vivo zeaxanthin is in a well defined environment.

**DISCUSSION**

In this paper, we have demonstrated a new approach to the characterization of higher plant xanthophylls using comparison of absorption band structure and resonance Raman excitation profiles. The approach proved to be an effective methodology for identification and monitoring of the molecular conformation and configuration of carotenoids both in isolated pigment-protein complexes and, most significantly, in intact thylakoid membranes. This method is based on the identification of a number of characteristic fingerprints in the resonance Raman spectrum for each carotenoid involved. Analysis of iso-
lated LHClIb monomers and trimers containing only two types of xanthophylls, lutein and neoxanthin, allowed the identification of their corresponding absorption bands and molecular configuration.

In the monomeric state of LHClIb, the configuration and environment of the two LHClIb luteins are very similar, both absorbing at 495 nm. The broader full width at half maximum for the band at 495 nm in the monomer spectrum compared with that of the trimer (Fig. 4) may suggest that their maxima positions differ within this region and therefore that their environment is also slightly different. In the trimer there is a 510 nm band not found in the monomer; this band was assigned to a red-shifted lutein in the former. Its intensity was significantly reduced in the second derivative spectrum because of the much broader FWHM compared with the short wavelength form of the pigment. In the trimer, the 510 nm lutein undergoes significant twisting, as a result either of an influence of the protein or of interaction with the other pigments or lipids present. It is not clear from our data whether this lutein plays a specific role in, for example, stabilization of the trimer and/or photoprotection of chlorophyll. However, these large differences in environment of the two luteins are important from structural and spectroscopic viewpoints (11, 18–21). This approach has revealed the molecular dynamics of a bound xanthophyll as a function of the state of oligomerization of the complex, and this for the first time. These factors may (at least in part) be behind the existence of monomeric (minor antenna), trimeric (major LHClIb), and oligomeric states of antenna proteins in vivo. In addition, the methodology described here could also be used in the investigation of the LHC reconstitution process, using various types of xanthophylls (33, 34).

The application of RR spectroscopy to thylakoid membranes has revealed important new information about the xanthophyll cycle carotenoids, zeaxanthin and violaxanthin. The physiological role of these carotenoids is unclear, as is their location in the thylakoid membrane. Indirect measurements (correlating nonphotochemical fluorescence quenching with de-epoxidation state) have suggested that xanthophyll cycle carotenoids may be completely free in the thylakoid membrane and can only move to a very small number of quenching centers in LHClI proteins upon ΔpH formation (35). Some data on isolated LHClI have suggested that only the minor LHClI can bind violaxanthin and zeaxanthin tightly and that therefore these must be the functional proteins in nonphotochemical fluorescence quenching (36). In contrast, we have suggested that all of these carotenoids are bound, mostly to peripheral sites on each of the proteins. The strongly associated violaxanthin molecules located within minor complexes were found to be inaccessible to the de-epoxidase enzyme and therefore unlikely to be involved in photoprotection (9, 13). Therefore, we have suggested that the peripheral violaxanthin and zeaxanthin molecules play the key role in photoprotective energy dissipation. In this study, we have been able to obtain the first data on the state of the xanthophyll cycle carotenoids in the thylakoid membrane. The more structured RR spectrum for both violaxanthin and zeaxanthin in the thylakoid membrane compared with those free in solution indicate that both of these carotenoids are in fact in well coordinated environments in the thylakoid membrane, almost certainly bound to protein and not free to move. Zeaxanthin appeared to be more distorted than violaxanthin. Indeed, the ν2 region is more structured and intense for zeaxanthin than violaxanthin, suggesting that the former is in a tight association with the protein, consistent with estimations of binding affinities to LHClI proteins (9).

Ultra-low temperature absorption spectroscopy has also revealed new information about these carotenoids. The zeaxanthin-minus-violaxanthin absorption spectrum of thylakoids had a complex structure. The heterogeneity within this spectrum suggested that violaxanthin exists in two different populations. This suggestion is consistent with the observation that violaxanthin was found to be differently bound to the minor and major LHClI and LHClI complexes. It is possible that one population corresponds to violaxanthin associated with the minor LHClI or LHClI, whereas the other is peripheral bound to LHClIb. An alternative explanation is that the dual band structure of the difference spectrum zeaxanthin minus violaxanthin originates from excitonically coupled violaxanthin. In this case, the coupling energy (V) will be within 180 cm⁻¹ taking into account the Davidov's splitting (E) of approximately 360 cm⁻¹ (E = 2V). In the case of a moderate transition dipole moment and a parallel orientation of two interacting violaxanthin molecules, they should be located within 0.5–0.7 nm of each other (37, 38).

It is interesting to mention that acidification did not cause alteration in violaxanthin maximum positions, which one would anticipate in the case of pApH-induced detachment of violaxanthin from antenna to undergo de-epoxidation. Resonance Raman spectra were also found to be unaffected (data not shown). This indicates that acidification has no immediate effect on the state of violaxanthin.

Clearly, the application of spectroscopic methods to the analysis of xanthophylls in isolated complexes and intact thylakoids provides a new approach to understanding the structure and dynamics of these molecules. In particular, the selectivity of the RR technique and its freedom from the artifacts and problems normally associated with absorption and fluorescence measurements on complex intact systems have provided new information about violaxanthin and zeaxanthin in vivo. In the future, we hope to apply a similar methodology to whole leaves.

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