The molecular configuration of the xanthophyll cycle carotenoids, violaxanthin and zeaxanthin, was studied in various isolated photosystem II antenna components in comparison to intact photosystem II membranes using resonance Raman combined with low-temperature absorption spectroscopy. The molecular configurations of zeaxanthin and violaxanthin in thylakoids and isolated photosystem II membranes were found to be the same within an isolated oligomeric LHII antenna, confirming our recent conclusion that these molecules are not freely located in photosynthetic membranes (Ruban, A. V., Pascal, A. A., Robert, B., and Horton, P. (2001) J. Biol. Chem. 276, 24862-24870). In contrast, xanthophyll cycle carotenoids bound to LHII trimers had largely lost their in vivo configuration, suggesting their partial dissociation from the binding locus. Violaxanthin and zeaxanthin associated with the minor antenna complexes, CP26 and CP29, were also found to be in a relaxed configuration, similar to that of free pigment. The origin of the characteristic C-H vibrational bands of violaxanthin and zeaxanthin in vivo is discussed by comparison with those of neoxanthin and lutein in oligomeric and trimeric LHIII respectively.

Xanthophyll cycle (XC) carotenoids, violaxanthin and zeaxanthin, have been found to play an important role in the process of dissipation of excess excitation energy in the photosynthetic membrane giving rise to non-photochemical photo-chemistry (NPQ) (1-3). The molecular mechanism of their action in NPQ is not well understood. Several possibilities have been suggested. One is that zeaxanthin can act as a direct acceptor of excitation energy i.e. that it is a direct excitation quencher (4, 5). The other defines zeaxanthin as an allosteric quencher (4, 5). The affinity of violaxanthin binding to these complexes was much higher than to LHII, and the availability for deepoxidation. The minor PSII antenna complexes, CP24, CP26, and CP29, are highly enriched in XC carotenoids. The affinity of violaxanthin binding to these complexes was much higher than to LHII, and the availability for deepoxidation was lower (10). Recently, we have applied differential resonance Raman (RR) spectroscopy to identify the configuration of violaxanthin and zeaxanthin in vivo (11). It was found that they adopt very specific configurations in the thylakoid membrane, different from those in organic solvent or detergent micelles. The aim of the current work was to apply this new spectroscopic methodology to study the state of xanthophyll cycle carotenoids in isolated LHII, CP26, and CP29 in comparison with that in intact PSII membranes. We assess whether the configuration of XC carotenoids correlates with their binding affinity and determine which of the in vitro antenna preparations possesses a xanthophyll configuration similar to that in vivo. We also present a summary and analysis of the nature of the configuration-indicative resonance Raman modes of the four major xanthophylls of the PSII antenna. Comparative analysis of the \( \nu_4 \) region of the Raman spectra for all LHII-bound xanthophylls allows an assignment of some of the bands to particular molecular domains potentially involved in the interaction with the protein.

MATERIALS AND METHODS

Thylakoids and PSII-enriched membranes were prepared from dark-adapted spinach leaves as described in Ref. 9. To induce maximum violaxanthin deepoxidation, thylakoids were incubated at room temperature at a chlorophyll concentration of 200 \( \mu \)g/mL for 2 h in a medium containing 5 mM \( \beta \)-isosorbate, 10 mM HEPES, and 10 mM sodium citrate at pH 5.5 with or without 5 mM Mg\( \text{Cl}_2 \). Purification of LHII trimers and oligomers was carried out using sucrose gradient centrifugation of unstacked thylakoids or PSII-particles, solubilized by dodecyl-\( \beta \)-malto-side as described previously (10). Minor antenna complexes, CP26 and CP29, were isolated using preparative isoelectricfocusing (9). Treatment analysis was carried out by reverse phase high pressure liquid chromatography (12). For electron microscopy, freshly prepared samples were negatively stained with 2% uranyl acetate and applied to glow-discharged carbon-coated grids. Images were analyzed using a Phillips CM100 electron microscope.
microscope with an 80 kV accelerating voltage and 52,000 times magnification.

Absorption spectra were recorded on a Cary 500 (Varian) double-beam scanning spectrophotometer; measurements at 77 K were performed using an Oxford Optistat (Oxford Instruments). Low temperature resonance Raman spectra were obtained in a helium flow cryostat (Air Liquide) using a Jobin-Yvon U1000 Raman spectrophotometer equipped with a liquid nitrogen-cooled charge-coupled device detector (Spectrum One, Jobin-Yvon) as described in Ref. 11. Excitation at 488.0 and 514.5 nm was provided by a Coherent Argon laser (Innova 100).

The choice of these wavelengths was determined by the positions of the 1437, 1353, and 1327 cm⁻¹ bands of the chlorophyll bands at 1437, 1353, and 1327 cm⁻¹ observed as described in Ref. 11.

Infrared spectroscopy was performed on xanthophylls in the solid state using a Brucker Equinox 50 Fourier transform infrared (FTIR) spectrometer with a 3X reflection ATR sample applicator. Spectral resolution was 8 cm⁻¹.

**RESULTS AND DISCUSSION**

**Low Temperature Absorption Spectroscopy of Xanthophyll Cycle Activity**—The xanthophyll cycle content of thylakoids and PSII membranes was similar to those obtained previously (Table I) (10). Because of the gentle detergent treatment used, LHCl oligomers and trimers were found to contain around 20% xanthophyll cycle carotenoid of a total carotenoid content, whereas CP26 and CP29 exhibited even higher xanthophyll cycle content. The deepoxidation efficiency was nearly 80% in low pH-treated unstacked thylakoids and around 60% for PSII membranes prepared from light-treated leaves. Very high deepoxidation rates were found for LHClI oligomers and trimers, whereas significantly lower values were observed for CP26 and particularly CP29.

Measurements of the low-temperature absorption spectra revealed clear differences in the Soret band region between violaxanthin- and zeaxanthin-enriched samples (Fig. 1). The difference absorption spectrum (“deepoxidized-minus-dark-adapted”) for PSII membranes revealed a typical zeaxanthin-minus-violaxanthin spectrum, similar to that found previously for thylakoid membranes (11), with zeaxanthin and violaxanthin 0–0 transitions at 508 and 488 nm, respectively (Fig. 1A). To test how detergent treatment of the membrane can affect the spectral properties of these pigments, PSII preparations were treated with 1.5% dodecyl-β-D-maltoside for 15 min on ice. The difference spectrum was blue-shifted, with the violaxanthin and zeaxanthin bands appearing at 484 and 504 nm, respectively (Fig. 1A, dotted line). It is likely that detergent has deeply perturbed the binding sites of these xanthophylls. Violaxanthin and zeaxanthin absorption bands in the free pigment fraction are located near 484 and 505 nm, respectively (Fig. 1B). It is thus probable that, after detergent treatment, these molecules are either loosely bound to the complexes or removed from their binding sites.

The deepoxidation-induced absorption difference spectrum of the oligomeric LHClI preparation was very similar to that of PSII membranes (Fig. 1C), with bands at 488 and 508 nm. We analyzed this preparation by negative-staining electron microscopy to evaluate the state of the protein. Fig. 2 represents a typical view of freshly prepared LHClI oligomers, which appear as particles rather than membrane vesicles, have a predominantly round, disc-like shape, and are ~25 nm in diameter (indicated by horizontal arrows). Each particle accommodates ~eight LHCl trimer complexes. However, these oligomers were unstable, and even a brief desalting step and staining procedure were harsh enough to break their integrity. Therefore, a significant number of smaller particles are also seen on the micrograph. Average frequency of large particles was around 20%, suggesting that approximately every second oligomer was damaged, yielding a number of trimers (six-eight). Despite the glow discharging of the carbon-coated grids some of the oligomeric particles can be clearly seen positioned perpendicularly to the plane of the view (indicated by vertical arrows). This enables us to estimate their thickness to be about 6 nm, indicating single layers of LHClI. Because the isolation procedure also yielded monomeric PSII core complexes and some fraction of the minor antenna components, it is feasible that the detergent has selectively solubilized central parts of the PSII supercomplex (14) leaving intact tetrads of associated trimers and oligomeric peripheral antenna (15, 16).

Presented in Fig. 1, D–F are absorption spectra of LHClI trimers, CP26 and CP29, isolated from violaxanthin- and zeaxanthin-enriched preparations. These have progressively decreasing deepoxidation states (Table I), which we have suggested indicates the presence of a fraction of inaccessible
violaxanthin in the minor antenna (10). As expected, the amplitudes of the difference spectra (deepoxidized-minus-epoxidized) tend to correlate with the measured deepoxidation states. It was found that the absorption maxima for violaxanthin and zeaxanthin are all blue-shifted relative to those for oligomeric LHCII. Indeed for CP29 and CP26 in particular, violaxanthin and zeaxanthin exhibited similar maxima as for the free pigment fraction (compare Fig. 1, B with E and F).

**Resonance Raman Spectroscopy of Violaxanthin and Zeaxanthin in Different Antenna Preparations**—Resonance Raman spectra of all the preparations characterized by absorption spectroscopy (Fig. 1) were measured using two excitation lines, one to excite violaxanthin preferentially (488.0 nm) and the other for zeaxanthin (514.5 nm; see dotted arrows in Fig. 1). Because deepoxidation removes the major population of violaxanthin molecules, calculation of the difference spectrum dark-adapted-minus-deepoxidized for 488.0 nm excitation yields the RR spectrum of the violaxanthin present in the dark-adapted sample that was converted into zeaxanthin in the deepoxidized one. Similarly for zeaxanthin, a deepoxidized-minus-dark-adapted spectrum can be calculated for 514.5 nm excitation. We recently established this procedure, using the chlorophyll bands at 1437, 1353, and 1327 cm$^{-1}$ for normalization, and have already applied it successfully to the study of xanthophyll cycle carotenoids in thylakoid membranes (11).

The RR spectrum of carotenoids contains four different regions, termed $v_1$–$v_4$. The $v_1$ and $v_2$ regions were found to vary in their peak positions for violaxanthin and zeaxanthin (11, 17). The $v_1$ band, which arises from the stretching mode of the C=C conjugated bonds of the carotenoid molecules, is located around 1529 and 1522 cm$^{-1}$, whereas the main $v_2$ mode peaks at 1006 and 1002 cm$^{-1}$ for violaxanthin and zeaxanthin, respectively. For all of the difference spectra calculated for violaxanthin (at 488.0 nm), the position of the $v_1$ band was around 1530 cm$^{-1}$,
whereas the \( \nu_3 \) maximum was at 1005–1006 cm\(^{-1} \); for the calculated zeaxanthin spectra (at 514.5 nm), these values were around 1522 and 1002 cm\(^{-1} \), respectively (Fig. 3). Thus, it was possible to extract specific resonance Raman spectra of violaxanthin and zeaxanthin for all of the samples studied.

In the \( \nu_4 \) region of RR spectra of carotenoids contribute weak bands arising from the C–H out-of-plane wagging modes. These modes are not active in resonance Raman when the carotenoid molecule is planar. However, distortions of the backbone structure, induced by rotations around C–C bonds, result in an increase in coupling of certain C–H and C–CH\(_3\) wagging modes (which appear in this region) with the electronic transition, and the intensity of the bands arising from these modes increases (18). This region thus yields information about the configuration of the carotenoid studied. Our recent work has demonstrated that bands in the \( \nu_4 \) region in RR spectra of violaxanthin and zeaxanthin in thylakoid membranes exhibit a higher intensity and more structure compared with the same pigments in solvent solution (11). For Fig. 4, the \( \nu_4 \) region of the calculated RR difference spectra for violaxanthin and zeaxanthin in the various samples. For PSII membranes, violaxanthin reveals a new band at 950 cm\(^{-1} \) and an enhanced 964 cm\(^{-1} \) transition compared with that of violaxanthin in detergent/lipid micelles (Fig. 4A, traces 1 and 7). The corresponding spectrum for zeaxanthin exhibits an even more complex structure, with enhanced bands at 950, 955, 963, 965, and 970 cm\(^{-1} \) (Fig. 4B, trace 1). Both spectra look very similar to those for the intact thylakoid membrane (11). Disintegration of PSI particles with detergent results in the disappearance of all structural \( \nu_4 \) components (Fig. 4, traces 2).

The structural features of the \( \nu_4 \) region for the oligomeric LHClI preparation are very similar to those of PSII membranes, particularly in the case of zeaxanthin (Fig. 4, traces 3). In the case of isolated LHClI trimers, however, the \( \nu_4 \) region has clearly lost much of its structure for both calculated spectra (Fig. 4, traces 4), even though the xanthophyll cycle content was identical to that of the oligomeric LHClIb preparation. (Note that the zeaxanthin spectrum does, nevertheless, retain a few weak components in this region; Fig. 4B, trace 4.)

The structure of the \( \nu_4 \) region for the minor antenna proteins, CP26 and CP29, is even less pronounced than that of the LHClI trimer (Fig. 4, traces 5 and 6). In all antenna preparations zeaxanthin seems to retain the native configuration better than violaxanthin.

**Are Xanthophyll Cycle Carotenoids Free in Vivo?**—The location and binding of xanthophyll cycle carotenoids are central questions in the elucidation of the molecular mechanism of their action in protecting the photosynthetic apparatus against light stress and its consequences (2, 19, 20). Although there is no doubt about their association with the light-harvesting antenna in PSII, it had not been clear until recently whether they were relatively free to diffuse in the membrane or were tightly bound to pigment-protein complexes (8, 10, 22). The analysis of absorption and RR spectra presented here for various PSII preparations have yielded consistent results concerning the binding of xanthophyll cycle carotenoids. The blue shift of their 0—0 electronic transitions in trimeric LHClI, CP26, and CP29 when compared with PSII membranes and oligomeric LHClI correlates precisely with the decrease in amplitude and complexity of the \( \nu_4 \) region of their calculated RR spectra. This indicates that xanthophyll cycle carotenoids are not free in the thylakoid membrane, but rather that they are tightly bound to LHA proteins. However, it is clear that it is relatively easy to dislodge these pigments from their binding site. Thus, local (deepoxidase-aided) displacements cannot be excluded.

It should be pointed out that only the blue-shifted violaxanthin was accessible to deepoxidase in the minor antenna complexes CP26 and CP29, leaving the tightly bound pool out of reach for the Raman difference analysis. All attempts to resolve the maximum absorption wavelength of this violaxanthin have failed. This could be due to a strong spectral overlap/masking with the 490–495 nm lutein band, which would be consistent with the possible red shift of the violaxanthin maximum due to a more intrinsic location in the complex.
On the Origin of $\nu_4$ in Antenna Xanthophylls, Binding Fingerprints—The carotenoid $\nu_4$ region studied in this work and in our previous papers has revealed a complex structure for xanthophylls bound to their protein hosts (11, 17). Fig. 5 shows a summary of spectra in the 920–990 cm$^{-1}$ region for the four major antenna xanthophylls of higher plants. One of the luteins in trimeric LHCII is red-shifted and has a strong and complex structure, with bands at 950, 955, 965, and 970 cm$^{-1}$ (17) (Fig. 5A). Transient absorption measurements have demonstrated association of a carotenoid absorbing around 510 nm with the blue-shifted chlorophyll $a$, which is likely to be Chl $a_4$ (23). According to the structural model of LHCII, only one of two luteins, L2, is associated with Chl $a_4$ (24). Therefore, the 510 nm lutein can be tentatively assigned to site L2. The $\nu_4$ region for zeaxanthin in the intact membrane reveals a reasonably similar structure to that of L2, with slightly more complexity in the form of additional shoulders around 947 and 961 cm$^{-1}$ (Fig. 5C). On the other hand, the spectrum for violaxanthin in thylakoids resembles that shown by neoxanthin in the oligomeric form of LHCII (Fig. 5, D and C, respectively). Both spectra have a somewhat reduced structure, exhibiting a main band around 950 cm$^{-1}$ (with minor contributions at 965 and at 955 cm$^{-1}$ for neoxanthin only). The origin of these different frequencies may be understood by comparison with the normal coordinate analysis made for $\beta$-carotene (21). In this analysis it was concluded that the mode around 950 cm$^{-1}$ originates from out-of-plane C–H wagging vibrations at the C$_{15}$–C$_{16}$ atoms (see Fig. 6 for nomenclature). In our case the assignment seems to be consistent with the fact that the 950 cm$^{-1}$ frequency is present in the RR spectra of all four xanthophylls, most likely due to the similarity of the configuration and environment of the C$_{15}$–C$_{16}$ group to that of $\beta$-carotene. The 955 and 965 cm$^{-1}$ frequencies have been assigned to C$_7$–C$_8$ and/or C$_{11}$–C$_{12}$ (21). Hence, the variability of their amplitude for the four xanthophylls studied here can be reasonably explained. Indeed, the reduced intensity of these components in the neoxanthin and especially in the violaxanthin spectrum could simply be due to the fact that the C$_7$–C$_8$ region is strongly influenced by the presence of cis configuration allene and epoxy groups interfering with this mode (Fig. 6). To test this possibility we have recorded infrared spectra of all four xanthophylls in the solid state. Fig. 7 shows that the major vibrational components of the $\nu_4$ group do exist, though in different proportions, for all xanthophylls and especially for neoxanthin and violaxanthin. Thus, despite the differences in the composition and configuration of the end groups and their environment, the number of C–H vibrational modes of the xanthophylls remains constant. It is possible that epoxide, restricting the end group mobility, makes the C$_7$–C$_8$ environment more rigid, which in turn restricts the distortion induced by interactions with the protein, and therefore the characteristic C–H wagging electronically coupled modes are reduced. Alternatively, different binding loci on protein could distort only certain parts of each xanthophyll, thereby enhancing only some of the C–H modes.

The $\nu_4$ group structure can clearly serve as a precise and sensitive carotenoid-binding fingerprint in studies of xanthophyll location and conformational dynamics of pigment-protein complexes. For each xanthophyll the characteristic of the back-

**Fig. 5.** $\nu_4$ region for luteins, Lut 1 (1) and Lut 2 (2) of the LHCIIb trimer (A); neoxanthin in aggregates of LHCIIb (B); zeaxanthin and violaxanthin in intact PSII-containing membranes (C), and (D), respectively.

**Fig. 6.** Structural formulae of the major four PSII antenna xanthophylls.

**Fig. 7.** Infrared absorption spectra of lutein (1), zeaxanthin (2), neoxanthin (3), and violaxanthin (4) in solid state. The arrow indicates the progressive emergence of a complex structure in the infrared region overlapping with the RR $\nu_4$ band group.
bone distortion would depend on the initial configuration and the flexibility of the molecule and on the nature of the protein-binding site. Trimerization of LHCII monomers affects strongly the lutein L2 configuration, aggregation of LHCII trimers leads to distortion of the neoxanthin molecule, and the macrostructure of LHA in vivo modifies the configuration of violaxanthin and zeaxanthin. Clearly, because each carotenoid is binding at different sites, different protein domains are affected at different levels of the macromolecular organization of the photosynthetic antenna. This provides a first insight into the way in which the structure of the protein subunits is perturbed as a result of their assembly into the LHCII-PSII macrostructure. In turn, this approach provides a new way to understand in vivo the dynamic changes associated with the assembly and functioning of the LHA that occur under different physiological and developmental conditions, including nonphotochemical quenching, photoacclimation, state transitions, and photoinhibition.

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Molecular Configuration of Xanthophyll Cycle Carotenoids in Photosystem II Antenna Complexes

Alexander V. Ruban, Andy Pascal, Pamela J. Lee, Bruno Robert and Peter Horton

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