Mechanisms Underlying Carotenoid Absorption in Oxygenic Photosynthetic Proteins*

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Background: Tuning of carotenoid function, through modulation of their electronic properties, is seen throughout Nature.

Results: Two photosynthetic proteins are able to modulate the effective conjugation length of bound carotenoid cofactors.

Conclusion: Altering the conformation of conjugated end cycles via steric hindrance provides a means of tuning the electronic properties of carotenoids.

Significance: A novel mechanism tuning the functional properties of carotenoids is revealed.

The electronic properties of carotenoid molecules underlie their multiple functions throughout biology, and tuning of these properties by their in vivo locus is of vital importance in a number of cases. This is exemplified by photosynthetic carotenoids, which perform both light-harvesting and photoprotective roles essential to the photosynthetic process. However, despite a large number of scientific studies performed in this field, the mechanisms(s) used to modulate the electronic properties of carotenoids remain elusive. We have chosen two specific cases, the two luteins in the major photosystem II light-harvesting complex, to investigate how such a tuning of their electronic structure may occur. Indeed, in each case, identical molecular species in the same protein are seen to exhibit different electronic properties (most notably, shifted absorption peaks). We assess which molecular parameters are responsible for this in vivo tuning process and attempt to assign it to specific molecular events imposed by their binding pockets.

The combined use of electronic absorption and resonance Raman (RR) spectroscopies may help in determining the molecular parameters underlying the tuning of Car electronic properties during excitation energy transfer in light-harvesting proteins and/or after charge recombination in reaction centers (RCs; 6–8). Cars can additionally quench any \( ^1 \text{O}_2 \) that may nevertheless be formed. More recently it was shown that, in both plants and cyanobacteria, Cars play an essential role in regulating the amount of excitation energy reaching the RC in high light environments, thus preventing damage due to overexcitation of these proteins (9–11).

Cars achieve these functions through their electronic properties, which arise from their linear conjugated polyene chain. They exhibit a fairly simple structure, built from the assembly of isoprenoid units (see Fig. 1), and a number of their electronic properties have been successfully predicted. Their main absorption transition, which corresponds to a transition from the ground state to the second excited singlet state (\( S_0 \rightarrow S_2 \)), tightly depends on the number of conjugated carbon-carbon double bonds present in this chain (12–16) and on the refractive index of their local environment (17–22). However, predicting the full electronic structure of Car molecules has turned out to be extremely complex. Despite the intense level of research on Car properties over the last 40 years, several new, low energy excited states have been proposed for these molecules in the last decade alone (23, 24), and precise calculations of their electronic and vibrational properties still remain a challenge (25). In vivo, protein binding sites provide a highly anisotropic environment to Car molecules. In these conditions, it is extremely difficult to characterize the most important parameters that govern their electronic properties. However, determining these parameters would represent an important approach in our understanding of the role of the protein matrix in tuning the first steps of the photosynthetic process. Given that the role of carotenoids in other biological systems also generally involves their electronic properties (e.g. signaling functions, making specific use of their color), such an understanding should also prove more widely applicable throughout carotenoid research.

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§The abbreviations used are: Car, carotenoid; Chl, chlorophyll; DFT, density functional theory; LHCCI, light-harvesting antenna complex of PSII; lut1 and lut2, lutein 1 and lutein 2 Car bound to LHCCI; PSII, photosystem II; RC, reaction center; RR, resonance Raman.
transitions. In RR spectra, the frequency of the C=C stretching mode of these molecules (the \( \nu_1 \) band) gives direct access to the structure of the alternated system of their electronic ground state. As we recently showed (26) and as illustrated in Fig. 2, a different relationship is observed between the frequency of the \( \nu_1 \) band and the position of the \( S_0 \rightarrow S_2 \) transition, depending on the molecular mechanism tuning this transition. The frequency of the \( \nu_1 \) band may thus yield direct indications on these mechanisms.

We have studied the absorption properties of \( \beta \)-carotene and lutein (Fig. 1) bound to two photosynthetic proteins isolated from photosystem II (PSII): the RC and the major light-harvesting complex, LHCII, respectively. The PSII-RC binds two \( \beta \)-carotene molecules, which at low temperature have their main absorption transition at 489 and 507 nm (27–29), the former being perpendicular and the latter parallel to the membrane plane (27, 30, 31). These Car molecules exhibit only limited singlet-singlet energy transfer to Chls and essentially no quenching of Chl singlet excited states at low temperature (77 K) were performed using a nitrogen-flow cryostat (Air Liquide, Sassenage, France). Measurements at low temperature (77 K) were performed using a nitrogen-flow cryostat (Air Liquide, Sassenage, France).

**EXPERIMENTAL PROCEDURES**

**Carotenoids and Solvents**—\( \beta \)-Carotene (\( \beta \,\beta \)-carotene synthetic type I, C-9750) was obtained from Sigma-Aldrich. Lutein (\( \beta \,\epsilon \)-carotene-3,3‘-dil) was isolated and purified as described previously (40). The molecular structures of these molecules are displayed in Fig. 1.

**RESULTS AND DISCUSSION**

**Isolated \( \beta \)-Carotene and Lutein**—The position of the \( S_0 \rightarrow S_2 \) transition of Car molecules tightly depends on their molecular structure and in particular on the length of the \( \pi \)-electron-conjugated system. Increasing this length induces a progressive
loss of the double bond character of the C=\(\text{C}\) bonds. This double bond character can be directly probed by RR spectroscopy, as it influences the frequency of the \(\nu_1\) Raman band. As a result, for a given solvent, a linear correlation between this frequency and the position of the \(S_0 \rightarrow S_2\) transition exists, when expressed as a function of the length of the Car-conjugated chain (Fig. 2, black line). The frequency of the \(\nu_1\) Raman band for lutein and \(\beta\)-carotene does not correspond to that expected for Cars with 10 and 11 double bonds, respectively, whereas they nevertheless show the same relationship between \(\nu_1\) frequency and absorption position (Fig. 2 and Ref. 26). These cyclic Cars, when isolated in solvents, actually display effective conjugation lengths of 9.3 and 9.6, respectively. This result was attributed to rotation of the conjugated end cycles out of the plane, such that the ring C=\(\text{C}\)s are only partially conjugated (26, 42). The position of the main absorption transition of Car molecules also tightly depends on the properties of the solvent in which they are dissolved, and particularly on its refractive index (17–22). Again, there is a correlation between the position of the \(S_0 \rightarrow S_2\) absorption transition and the frequency of the \(\nu_1\) Raman band for a given Car molecule, when expressed as a function of solvent polarizability. This correlation is plotted in Fig. 2 for \(\beta\)-carotene (blue) and lutein (red), and displays a different slope to that for different Cars in the same solvent (black), as already observed and discussed for several Car molecules (26).

**\(\beta\)-Carotene in PSII Reaction Centers**—At low temperature, PSII-RCs exhibit two main peaks in the Car absorption region, at 489 and 507 nm (Fig. 3). Linear dichroism experiments showed that these peaks correspond to distinct Car molecules, with different orientations relative to the membrane plane (27), and considering their position, they must be attributed to the \(0 \rightarrow 0\) sublevel of the absorption transition of each molecule. Although the resolution between these peaks becomes much lower at room temperature, the overall position of the Car absorption transition appears not to shift by more than a few nanometers between low temperature and room temperature (see Fig. 3). This is not specific to PSII and was already observed in other photosynthetic complexes. For instance, in light-harvesting complexes from purple bacteria it was shown that, in general, the absorption transitions of the bound Car molecules are very poorly dependent on temperature (43).

Fig. 4A displays RR spectra of PSII-RCs obtained at room temperature with 514.5- and 488.0-nm excitation, chosen to be selective for the 507- and 489-nm-absorbing \(\beta\)-carotene, respectively. They contain four main groups of bands, denoted \(\nu_1\) to \(\nu_4\), typical of Car molecules. The \(\nu_1\) band around 1520 cm\(^{-1}\) arises from stretching vibrations of \(\text{C=\(\text{C}\)}\) double bonds. As mentioned above, its frequency depends on the length of the \(\pi\)-electron-conjugated chain and on the Car conformation. The \(\nu_2\) band at 1160 cm\(^{-1}\) arises from stretching vibrations of \(\text{C=C}\) single bonds coupled with \(\text{C-H}\) in-plane bending modes. This region may be used as a fingerprint for the assignment of Car configurations (trans-cis). The \(\nu_3\) band at \(\sim 1000\) cm\(^{-1}\) arises from in-plane rocking vibrations of the methyl groups attached to the conjugated chain, coupled with in-plane bending modes of the adjacent \(\text{C-H}\)s. Finally, the \(\nu_4\) band around 960 cm\(^{-1}\) arises from \(\text{C-H}\) out-of-plane wagging motions coupled with \(\text{C=C}\) torsional modes (out-of-plane twists of the carbon backbone). When the conjugated system of the Car is symmetrical and the molecule is planar, these out-of-plane modes will not be coupled with the electronic transition. As a result, these bands will not be resonance-enhanced upon excitation and will exhibit very low intensity in RR spectra. However, distortions around \(\text{C=C}\) single bonds will increase the coupling of these modes with the electronic transition, resulting in an increase in resonance enhancement, i.e. \(\nu_4\) gains intensity. Although both spectra are globally similar and typical for all-trans-\(\beta\)-carotene, at 514.5 nm the \(\nu_1\) frequency is down-shifted by 6 cm\(^{-1}\) compared with 488.0 nm whereas \(\nu_3\) and \(\nu_4\) are both slightly narrower. These differences are also observed at 77 K (Fig. 4B; see also Ref. 35), indicating that the detailed structure of each \(\beta\)-carotene molecule is the same at both temperatures. It is worth noting that the increase at 488.0 nm in the width of \(\nu_3\) at room temperature is translated into a splitting of this band into two components at 77 K.

In Fig. 5 we plot the \(\nu_1\) frequencies and absorption positions of the two \(\beta\)-carotenoids from PSII-RCs at room temperature.
(red triangles) compared with the frequencies and positions of different length carotenoid molecules (black line) and of β-carotene in various solvents (blue line) taken from Fig. 2. The values obtained for the blue-absorbing carotene fit on the blue slope, suggesting that the position of this absorption transition is mainly governed by the local polarizability of its binding site. It may be noted that the binding site of this molecule exhibits a rather low local polarizability (equivalent to that found in chloroform, blue open symbol labeled f, with a polarizability value, \( \alpha_r \), of 0.266). On the other hand, the difference in \( \nu_1 \) frequency of the two β-carotene molecules accompanying the shift in their \( S_0 \rightarrow S_2 \) transition (see dashed red lines in Fig. 5) is much too large to be accounted for by changes in the local polarizability of their protein binding sites. In this case, considering the difference in the position of their electronic transition, a change of \( \nu_1 \) frequency of approximately 2 cm\(^{-1}\) should be expected, i.e. three times smaller than actually observed. The 6 cm\(^{-1}\) change seen here corresponds to a sizeable change of the apparent length of the conjugated chain, probably through perturbation of its alternated system. Such a change in the alternated system (observed upon increasing the length of the conjugated chain) would indeed up-shift the position of the Car absorption by approximately 17 nm, i.e. it is sufficient to explain the difference in absorption between the two PSII-RC-bound β-carotene molecules. Thus the dashed line connecting these two points is parallel to the black line, which relates Cars of different chain length in the same solvent (Fig. 5).

The same relationship between absorption position and \( \nu_1 \) frequency is also shown for measurements of the PSII-RC at 77 K (Fig. 5, inverted red triangles). The dashed line between these two points exactly parallels that obtained at room temperature, but at the lower temperature there is a shift of about 5 cm\(^{-1}\) in the \( \nu_1 \) frequency for both β-carotene molecules. Exactly the same shift for this Raman band, between room and low temperature, has recently been described in RR spectra of Cars in solution and was explained by an intrinsic sensitivity of the Raman frequency to temperature (for details, see Ref. 44).

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Lutein Molecules in LHCII—The absorption spectrum of LHCII trimers in the Car region is rather complicated because these complexes bind up to four carotenoid pigments per monomer, namely two luteins, one 9-cis-neoxanthin and usually one violaxanthin/zeaxanthin (present in negligible amounts in our sample). Indeed, the position of the individual absorption transitions could only be determined through second derivative analyses of absorption spectra obtained at 4 K (3). However, as in PSII, comparing the absorption spectra of LHCII at low temperature and room temperature suggests that the main band positions of the LHClI-bound Car molecules are very poorly sensitive to temperature (Fig. 6).

RR spectra of the LHCII-bound luteins at 77 K are displayed in Fig. 7B. As documented extensively in the literature (3, 43, 44), at this temperature 496.5- and 514.5-nm excitations yield RR spectra dominated by contributions from lut1 (absorbing at 495 nm) and lut2 (absorbing at 510 nm), respectively. The frequency of the \( \nu_1 \) band, which arises from the C=C stretching modes of lutein molecules, is observed at 1531 and 1527 cm\(^{-1}\) for lut1 and lut2, respectively. In the \( \nu_3 \) region (around 1000 cm\(^{-1}\)), lut1 (at 496.5 nm) exhibits two overlapping components of similar amplitude, at 1003 and 1007 cm\(^{-1}\). The same two bands are seen for lut2 (514.5-nm excitation), but the intensity of the higher frequency component is less than one third that of the lower frequency one. The \( \nu_3 \) region exhibits higher intensity and structure for lut2 than for lut1 (514.5- and 496.5-nm excitation, respectively), indicating a higher degree of distortion for lut2 in its LHClI binding site (as previously concluded; 3, 45). At room temperature, the broadening of the Car electronic transitions must, at least in part, impair selective excitation of each Car. In room temperature spectra obtained using excitation at 514.5 nm (Fig. 7A, upper trace), the \( \nu_3 \) is quite narrow (full width at half-maximum ~16 cm\(^{-1}\)), and the structure of the \( \nu_4 \) region is very similar to that observed using the same excitation at 77 K. We may thus conclude that this wavelength still ensures selective excitation of the lut2 molecule at the higher temperature and that the distortion of this molecule, up to now only
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observed at low temperature, is also present in LHCII at room temperature. Thus this distortion does not result from temperature-induced reorganization of the binding site. In this spectrum the position of the \( \nu_1 \) band is at 1522 cm\(^{-1} \). As in PSII-RC, we thus observe a 5-cm\(^{-1} \) shift between experiments conducted at 77 K and room temperature. In contrast, the spectrum at 496.5 nm exhibits significant broadening of \( \nu_1 \) when the measurement is taken at room temperature (Fig. 7A, lower trace; full width at half-maximum \( \sim 18 \) cm\(^{-1} \)). The contributions of more than one carotenoid are thus present in this spectrum, due to a reduction in resonance selectivity as a result of broadening of the Car absorption transitions at the higher temperature. Indeed, in the \( \nu_2 \) region a small but significant increase in intensity is observed for the shoulder at \( \sim 1130 \) cm\(^{-1} \). This is consistent with an increase in neoxanthin contributions to the spectrum, as bands on the low frequency side of \( \nu_2 \) are quite typical for 9-cis-carotenoids. Contributions of lut1 thus cannot be selectively observed in RR spectra at room temperature. Indeed, this was found to be difficult even at 77 K, where a “pure” lut1 spectrum was only obtained after removing the Neo contribution by differential analysis (46).

In Fig. 8, the absorption position and \( \nu_1 \) frequency of luteins in LHCII are compared with those obtained for lut1 in various solvents (red line) and with the frequencies and positions of different length carotenoid molecules (black line). A large shift is observed in \( \nu_1 \) frequency between the two luteins at low temperature (4 cm\(^{-1} \); blue inverted triangles). As for \( \beta \)-carotene in PSII-RC, this shift, which reflects a change in the alternation of the conjugated C=C chain, is enough to account for the energy difference between their \( S_0 \rightarrow S_2 \) absorption transitions. As in PSII-RC, the \( \nu_1 \) frequency of lut2 at room temperature (the only one we could safely extract from the RR spectra) is shifted by approximately 5 cm\(^{-1} \) compared with its low temperature value. By analogy, we can deduce the expected \( \nu_1 \) frequency of lut1 at room temperature, by shifting its low temperature value by 5 cm\(^{-1} \). The resulting value fits perfectly with the in vitro relationship between the lutein \( \nu_1 \) frequency and the position of its \( S_0 \rightarrow S_2 \) transition according to the polarizability of the solvents (Fig. 8, red line), tending to validate this approach. As in PSII-RC, we may conclude that the position of the electronic transition of this molecule is mainly governed by the local polarizability of its binding site. However, the deduced polarizability for this lutein is much higher than that calculated for the \( \beta \)-carotene in PSII-RC, as it corresponds to a value slightly higher than that for nitrobenzene (j, red open symbol; Fig. 8) with a refractive index, \( n_\text{RT} \), of 0.319.

Mechanisms Tuning Carotenoid Absorption in PSII-RC and LHCII—In both PSII-RC and LHCII, RR spectroscopy unambiguously shows that the position of the absorption transition of the blue-absorbing Car molecule is governed mainly by the polarizability provided by the protein environment. Indeed, the position of this transition and the frequency of the \( \nu_1 \) mode of these molecules strictly obey the correlation obtained for both \( \beta \)-carotene and lutein according to solvent refractive index. The deduced average value of the environment polarizability of the blue-absorbing \( \beta \)-carotene in PSII-RC is lower than that of the blue-absorbing lutein in LHCII. This is consistent with the environment deduced by analysis of x-ray crystallographic structures (30, 31, 37). In PSII-RCs, Cars are mainly surrounded by amino acids and are quite distant from other cofactors; they exhibit only low rates of energy transfer to the bound Chl molecules (27, 33, 34). On the other hand, the luteins in LHCII are in very close contact with the LHCII-bound Chl molecules, both at the levels of their end cycles and of the conjugated C=C chain (37). Some of these Chls, such as Chl \( a_{603} \), are nearly in van der Waals contact with lut1 (closest distance 3.83 Å) and are likely to provide an environment of higher polarizability.

By contrast, it is quite clear that the energy shifts between the blue- and the red-absorbing Car molecules in both studied complexes are not induced by a variation in polarizability of their binding sites. If so, the position of the absorption transition of these Cars and their \( \nu_1 \) Raman frequency would obey a correlation similar to the blue/red lines in Fig. 2, whereas it is clear that they deviate from these lines (see Figs. 5 and 8). Again,
this conclusion is consistent with the description of the environment of these molecules provided by the crystallographic structures of the two pigment-protein complexes: the blue and red luteins in LHCI1, as well as the blue and red β-carotene molecules in PSII-RC, are embedded in quite similar protein environments, which are unlikely to display large changes in average polarizability (indeed, in LHCI1, the two binding pockets are related by the local 2-fold symmetry of the complex). Instead, the absorption transition of these molecules and the frequency of their ν1 Raman band behave as if the conjugated chain of the Car molecules was increased by nearly one C=C double bond at constant polarizability (Figs. 5 and 8). Note that for both red-absorbing Cars, the main ν2 band is also seen to shift to lower frequency in parallel with the downshift in ν1 (Figs. 4 and 7); this is exactly as expected for an increase in conjugation length (see e.g. 47). Thus the apparent lengths of the conjugated chain of the red-absorbing lutein and β-carotene in LHCI1 and PSII-RC (at room temperature) become 10 and 10.2, respectively (Figs. 5 and 8). The external parameters susceptible to induce such changes are not documented in the literature, and again, there is no dramatic change in the environment of these pigments which could be at the origin of such a change. It was shown that the luteins of LHCI1 and the β-carotenoids in PSII-RC experience different distortions at low temperature (3, 35), and we show in this work that these distortions also exist at room temperature. However, small distortions around C=C bonds are expected to have little influence on the structure of the C=C conjugated chain, and, whereas in LHCI1 the red-absorbing lutein is distorted (48), in PSII it is the blue-absorbing Car that exhibits the larger distortion (35).

However, lutein and β-carotene both exhibit shorter conjugation length in solvents than expected from their chemical structure (9.3 and 9.6, respectively; 26). This was explained in terms of out-of-plane rotations of the conjugated end cycles, resulting in a loss of conjugation. In the crystal structure of β-carotene, the β-ionone rings are indeed twisted out of the conjugated plane (dihedral angles −42°; 49). Although no solution structure currently exists for either carotenoid, density functional theory (DFT) simulations performed on β-carotene predict dihedral angles for the end cycles of ~47° (42, 50, 51; see Fig. 9), and this has been calculated to shorten the conjugation length by the exact amount predicted from the Raman ν1 position (42). Bringing one of these end cycles back into the plane of the C=C conjugated chain should accordingly result in a net increase of the effective conjugation length of these molecules of approximately 0.6 – 0.7, exactly as observed here for the red-absorbing lutein and β-carotene in LHCI1 and PSII-RC. We thus propose that these proteins are able to tune the absorption of their red-absorbing carotenoid via the rotation of conjugated end cycles toward the conjugated plane of the molecule, this rotation being imposed by their binding pocket through steric hindrance. As lutein only has one conjugated end cycle it must be this β-ring that is implicated in LHCI1, whereas for β-carotene in PSII-RC this rotation may involve one or both end cycles.

The initial crystallographic structure of LHCI1 led to the conclusion that the lutein end rings were all in a conformation perpendicular to the C=C chain (37), a conformation that would induce a further shortening of the C=C chain of the lutein molecules. This is at variance with the present vibrational analysis of these pigments and with the position of their electronic transitions. However, a more recent analysis led to the conclusion that these molecules display different conformations, with lut2 being more distorted than lut1 (39), fully in agreement with our previous conclusions (3). The progressive distortion of lut2 occurring from C9 to C13, as observed in the latter analysis, twists one of the end rings to orient it parallel to residue TRP46, ~3.4 Å away, to optimize van der Waals interactions with it (see Fig. 9E; note, however, that this figure was drawn using the earlier structure). As a result, this ring is brought back into the plane of the C=C chain, assuming that this ring contains the (partially) conjugated C=C bond (i.e. that it is the β-ring) then it would become more conjugated as a result of this distortion, entirely consistent with our proposed mechanism. It would also explain why the red shift of lut2 absorption only occurs when this molecule is distorted; in LHCI1 monomers, where the lut2 conformation is relaxed, its absorption transition coincides with that of lut1 (495 nm; 3).

Similarly, in the most recent crystallographic structure of photosystem II, a clear difference appears between the cycle geometries of the two β-carotenes bound to the PSII reaction center (31). This is illustrated in Fig. 9A–D, where the four end rings are compared with that of the DFT-calculated in vitro structure (42). Both cycles of the (blue-absorbing) β-carotene perpendicular to the membrane plane (Bcr645 in Protein Data Bank structure 3ARC) make a large angle with the conjugated C=C chain (dihedral angles 59° and 68°; Fig. 9, C and D). This is quite different for the (red-absorbing) β-carotene parallel to the membrane (Bcr651), where one of the cycles makes a dihedral angle of only 12° with the plane of the C=C chain, as seen in Fig. 9A. Once again, this twisting back into the plane allows the end ring to lie more or less parallel to an overlapping aromatic
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residue, this time PHE113 of the PsbD polypeptide (~3.9 Å away; Fig. 9A). Note that the second end ring, although lying out of the plane, also makes a smaller angle than those measured for the blue-absorbing Car (48°; Fig. 9B). These structural differences are perfectly in line with the conclusions of this study and would account for the difference in conjugation length measured by Raman between these two molecules.

Thus in both LHClI and PSII-RC, steric hindrance from a nearby aromatic residue forces an end ring of the red-absorbing Car back toward the conjugated plane of the molecule. This presents the possibility of testing our conclusions by site-directed mutagenesis; replacing this residue with a smaller, non-aromatic one (e.g. Ala) should allow the Car end ring to take up its relaxed conformation. As a result its conjugation length, absorption position and Raman ν1 frequency would all be similar to that of the blue-absorbing Car and in vitro. We are currently designing mutagenesis experiments to carry out this work in photosystem II of the cyanobacterium Synechocystis sp. PCC6803. Note that, in the absence of well established isolation protocols for cyanobacterial PSII-RC, the analysis will have to be performed on core preparations of photosystem II, containing approximately 50 pigment cofactors including >10 β-carotenes. Extracting the absorption and Raman signatures of two individual Cars from this large population, although substantially more difficult, should nevertheless be possible.

It is worth noting that, in the RR spectra of the red-absorbing β-carotene band to PSII, only a single, narrow contribution is seen in the ν3 region, whereas for the blue-absorbing β-carotene ν3 is relatively broad at room temperature and even splits into two components at 77 K (Fig. 4). Although this tendency is not so clear for LHClII, it is nevertheless seen; the red-absorbing lutein exhibits one major component with a satellite at higher frequency, whereas this higher frequency component is more prominent for the blue-absorbing lutein (Fig. 7). ν3 arises from in-plane rocking vibrations of the methyl groups attached to the conjugated chain, coupled with in-plane bending modes of the adjacent C–Hs. In Raman spectra of fucoxanthin the ν3 band is similarly composed of two components, which was attributed to differences in the methyl nearest neighbors in the chemical structure of the carotenoid (46). Along the same lines, the splitting of this band observed in the blue-absorbing Car may reflect the out-of-plane rotation of the end cycles, as this rotation is likely to perturb the rocking frequency of the neighboring methyl group. It is striking that in lutein, where only one rocking mode should be concerned, the intensity of the additional component is weaker than in β-carotene (where potentially two methyl groups may be concerned). In solvents, where it was concluded that the end cycles are at least partially out of the plane, the ν3 is also observed to be broader in Raman spectra at room temperature (26). The structure of this band could thus be a direct indication of the conformation of the end cycles in both β-carotene and lutein, representing a probe for this mechanism of conjugation-length modulation.

Finally, it is striking that Nature has generally used Cars with conjugated end cycles in oxygenic photosynthesis. Our results show that by playing on the conformation of these cycles, the position of the absorption transitions of these cyclic Car molecules may be tuned by up to 15 nm/ring. Note that for a Car without end rings it would be highly unfavorable, energetically, to rotate the ends such that a conjugated C=C became (partially) unconjugated. This is not the case when conjugated end rings are present; steric factors have already determined that these rings are poised in a partially conjugated configuration. It may be that this property explains the recruitment of Car molecules with conjugated end cycles in the photosynthetic process, allowing for an optimization of the excitation energy cascade in these complex light-transducing structures.

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REFERENCES

1. Cogdell, B. J., Gillbro, T., Andersson, P. O., Liu, R. S. H., and Asato, A. E. (1994) Carotenoids as accessory light–harvesting pigments. Pure Appl. Chem. 66, 1041–1046 DOI 10.1351/pac199466051041
2. Horton, P., Ruban, A. V., and Walters, R. G. (1996) Regulation of light harvesting in green plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 655–684
3. Ruban, A. V., Pascal, A. A., and Robert, B. (2000) Xanthophylls of the major photosynthetic light-harvesting complex of plants: identification, conformation, and dynamics. FEBS Lett. 477, 181–185
4. Polivka, T., and Frank, H. A. (2010) Molecular factors controlling photosynthetic light harvesting by carotenoids. Acc. Chem. Res. 43, 1125–1134
5. Foote, C. S. (1976) in Free Radicals in Biology (William Pryor, ed) pp. 85–133, Academic Press, Boca Raton, FL
6. Britton, G., Liaaen-Jensen, S., and Pander, H. (eds) (2008) Carotenoids, Vol. 4, Natural Functions, Springer Birkhauser, Berlin
7. Truscott, T. G., Land, E. J., and Sykes, A. (1973) In vitro photochemistry of biological molecules. III. Absorption spectra, lifetimes and rates of oxygen quenching of triplet states of β-carotene, retinal and related polynyes. Photochem. Photobiol. 17, 43–51
8. Niyogi, K. K., Björkman, O., and Grossman, A. R. (1997) The roles of specific xanthophylls in photoprotection. Proc. Natl. Acad. Sci. U.S.A. 94, 1167–1172
9. Niyogi, K. K., Björkman, O., and Grossman, A. R. (1997) The roles of specific xanthophylls in photoprotection. Proc. Natl. Acad. Sci. U.S.A. 94, 1167–1172
10. Ahn, T. K., Avenson, T. J., Ballottari, M., Cheng, Y. C., Niyogi, K. K., Bassi, R., and Fleming, G. R. (2008) Architecture of a charge transfer state regulating light harvesting in a plant antenna protein. Science 320, 794–797
11. Wilson, A., Punginelli, C., Gall, A., Bonetti, C., Alexandre, M., Routaboul, J. M., Kerfeld, C. A., van Grondelle, R., Robert, B., Kennis, J. T., and Kirilovsky, D. (2008) A photoactive carotenoid protein acting as light intensity sensor. Proc. Natl. Acad. Sci. U.S.A. 105, 12075–12080
12. Araki, G., and Murai, T. (1952) Molecular structure and absorption spectra of carotenoids. Progr. Theor. Physics 8, 639–654
13. Suzuki, H., and Mizuhashi, S. (1964) π-Electronic structure and absorption spectra of carotenoids. J. Phys. Soc. Jpn. 19, 724–738
14. Dale, J. (1954) Empirical relationships of the minor bands in the absorption spectra of polyenes. Acta Chem. Scand. 8, 1235–1256
15. Hemley, R., and Kohler, B. E. (1977) Electronic structure of polyenes related to the visual chromophore: a simple model for the observed band shapes. Biophys. J. 20, 377–382
16. Christensen, R. L., Barney, E. A., Broene, R. D., Galinato, M. G., and Frank, H. A. (2004) Linear polyenes: models for the spectroscopy and photophysics of carotenoids. Arch. Biochem. Biophys. 430, 30–36
17. LeRosen, A. L., and Reid, C. E. (1952) An investigation of certain solvent effects in absorption spectra. J. Chem. Phys. 20, 233–236
18. Hirayama, K. (1955) Absorption spectra and chemical structure. II. Solvent effect. J. Am. Chem. Soc. 77, 379–381
19. Andersson, P., Gillbro, T., Ferguson, L., and Cogdell, R. (1991) Absorption spectral shifts of carotenoids related to medium polarizability. Photochem.
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20. Kuici, M., Nagae, H., Cogdell, R., Shimada, K., and Koyama, Y. (1994) Solvent effect on spheroidene in nonpolar and polar solutions and the environment of spheroidene in the light-harvesting complexes of Rhodobacter sphaeroides. *J. Biol. Chem.* 269, 16437–16442

21. Chen, Z., Lee, C., Lenzer, T., and Oum, K. (2006) Solvent effects on the S-0:1(1)Ag(+) → S-2:1(1)B(u)(+)(-) transition of β-carotene, echinenone, canthaxanthin, and astaxanthin in supercritical CO_2 and CF3H. *J. Phys. Chem. A* 110, 11291–11297

22. Renge, I., and Sild, E. (2011) Absorption shifts in carotenoids: influence of index of refraction and submolecular electric fields. *J. Photochem. Photobiol. A Chem.* 218, 156–161

23. Papagiannakis, E., Kennis, J. T., van Stokkum, I. H., Cogdell, R. J., and van Grondelle, R. (2002) An alternative carotenoid-to-bacteriochlorophyll energy transfer pathway in photosynthetic light harvesting. *Proc. Natl. Acad. Sci. U.S.A.* 99, 6017–6022

24. Wang, P., Nakamura, R., Kanematsu, Y., Koyama, Y., Nagae, H., Nishio, T., Hashimoto, H., and Zhang, J. P. (2005) Low-lying singlet states of carotenoids having 8–13 conjugated double bonds as determined by electronic absorption spectroscopy. *Chem. Phys. Lett.* 410, 108–114

25. Wirtz, A. C., van Hemert, M. C., Lugtenburg, J., Frank, H. A., and Groenen, E. J. (2007) Two stereoisomers of spheroidene in the Rhodobacter sphaeroides R26 reaction center: a DFT analysis of resonance Raman spectra. *Biophys. J.* 93, 981–991

26. Mendes-Pinto, M. M., Sansiaume, E., Hashimoto, H., Pascal, A. A., Gall, A., and Robert, B. (2013) Electronic absorption and ground state structure of carotenoid molecules. *J. Phys. Chem. B*, in press

27. Van Dorssen, R. J., Breton, J., Plijter, J. J., Satoh, K., Yangkornk, H. J., and Amesz, J. (1987) Spectroscopic properties of the reaction center and of the 47-kDa chlorophyll protein of photosystem-II. *Biochim. Biophys. Acta* 893, 267–274

28. Kwa, S., Newell, W., Vangrondelle, R., and Dekker, J. (1992) The reaction center of photosystem-II studied with polarized fluorescence spectroscopy. *Biochim. Biophys. Acta* 1099, 193–202

29. Tomo, T., Mimuro, M., Iwaki, M., Kobayashi, M., Itoh, S., and Satoh, K. (1997) Topology of pigments in the isolated photosystem II reaction center studied by selective extraction. *Biochim. Biophys. Acta* 1321, 21–30

30. Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. *Nature* 438, 1040–1044

31. Umena, Y., Kawakami, K., Shen, J. R., and Kamiya, N. (2011) Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. *Nature* 473, 55–60

32. Takahashi, Y., Hansson, O., Mathis, P., and Satoh, K. (1987) Primary radical pair in the photosystem II reaction centre. *Biochim. Biophys. Acta* 893, 49–59

33. Durrant, J. R., Giorgi, L. B., Barber, J., Klug, D. R., and Porter, G. (1990) Characterisation of triplet states in isolated photosystem II reaction centres: oxygen quenching as a mechanism for photodamage. *Biochim. Biophys. Acta* 1017, 167–175

34. Mimuro, M., Tomo, T., Nishimura, Y., Yamazaki, L., and Satoh, K. (1995) Identification of a photochemically inactive phytochrome molecule in the spinach D1–D2–CyD125 complex. *Biochim. Biophys. Acta* 1232, 81–88

35. Teller, A., Frolov, D., Barber, J., Robert, B., and Pascal, A. (2003) Oxidation of the two β-carotene molecules in the photosystem II reaction center.

Biochemistry 42, 1008–1015

36. Caffarri, S., Croce, R., Breton, J., and Bassi, R. (2001) The major antenna complex of photosystem II has a xanthophyll binding site not involved in light harvesting. *J. Biol. Chem.* 276, 35924–35933

37. Liu, Z., Yan, H., Wang, K., Kuang, T., Zhang, J., Gui, L., An, X., and Chang, W. (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. *Nature* 428, 287–292

38. Standfuss, J., Terwisscha van Scheltinga, A. C., Lamborghini, M., and Kühlbrandt, W. (2005) Mechanisms of photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5 Å resolution. *EMBO J.* 24, 919–928

39. Yan, H., Zhang, P., Wang, C., Liu, Z., and Chang, W. (2007) Two lutein molecules in LHClI have different conformations and functions: insights into the molecular mechanism of thermal dissipation in plants. *Biochim. Biophys. Res. Commun.* 355, 457–463

40. Phillip, D., Ruban, A. V., Horton, P., Asato, A., and Young, A. J. (1996) Quenching of chlorophyll fluorescence in the major light-harvesting complex of photosystem II: a systematic study of the effect of carotenoid structure. *Proc. Natl. Acad. Sci. U.S.A.* 93, 1492–1497

41. Ruban, A. V., Lee, P. J., Wentworth, M., Young, A. J., and Horton, P. (1999) Determination of the stoichiometry and strength of binding of xanthophylls to the photosystem II light harvesting complexes. *J. Biol. Chem.* 274, 10458–10465

42. Liu, W., Wang, Z., Zheng, Z., Jiang, L., Yang, Y., Zhao, L., and Su, W. (2012) Density functional theoretical analysis of the molecular structural effects on Raman spectra of β-carotene and lycopene. *Chin. J. Chem.* 30, 2573–2580

43. Gall, A., and Robert, B. (1999) Characterization of the different peripheral light-harvesting complexes from high- and low-light grown cells from Rhodopseudomonas palustris. *Biochemistry* 38, 5185–5190

44. Andreeva, A., Apostolova, L., and Velitchkova, M. (2011) Temperature dependence of resonance Raman spectra of carotenoids. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* 78, 1261–1265

45. Robert, B., Horton, P., Pascal, A. A., and Ruban, A. V. (2004) Insights into the molecular dynamics of plant light-harvesting proteins in vivo. *Trends Plant Sci.* 9, 385–390

46. Ilioaia, C., Johnson, M. P., Liao, P. N., Pascal, A. A., van Grondelle, R., Walla, P. J., Ruban, A. V., and Robert, B. (2011) Photoprotection in plants involves a change in lutein 1 binding domain in the major light-harvesting complex of photosystem II. *J. Biol. Chem.* 286, 27247–27254

47. Merlin, J. C. (1985) Resonance Raman spectroscopy of carotenoids and carotenoid-containing systems. *Pure Appl. Chem.* 57, 785–792

48. Ruban, A. V., Pascal, A. A., and Robert, B., Horton, P. (2001) 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. *J. Biol. Chem.* 276, 24862–24870

49. Senge, M., Hope, H., and Smith, K. (1992) Structure and conformation of photosynthetic pigments and related compounds. 3. Crystal structure of β-carotene. *Z. Naturforsch.* 47, 474–476

50. Goldbruch, K., Ehlers, F., Scholz, M., Oswald, R., Lenzer, T., Oum, K., Kim, H., and Koo, S. (2011) Ultrafast excited state dynamics and spectroscopy of 13,13′-diphenyl-β-carotene. *Phys. Chem. Chem. Phys.* 13, 6340–6351

51. Cerezo, J., Zügiga, J., Bastida, A., Requena, A., Cerón-Carrasco, J. P., and Eriksson, L. A. (2012) Antioxidant properties of β-carotene isomers and their role in photosystems: insights from ab initio simulations. *J. Phys. Chem. A* 116, 3498–3506