Zeaxanthin and violaxanthin radical cations and neutral radicals

most stable neutral radical
proton loss off the ends

unstable neutral radical
not observed by EPR

**Chemistry of Carotenoid Neutral Radicals:
An Additional Photoprotection Pathway**

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Abstract

Proton loss from the carotenoid (Car) radical cations ($\text{Car}^{\bullet+}$) to form neutral radicals ($\text{Car}^\bullet$) was investigated by numerous electrochemical, EPR and DFT studies described herein. The presence of carotenoid neutral radicals was recently confirmed by studies in *Arabidopsis Thaliana* plant and Photosystem II samples. Deprotonation at the terminal ends of a carotenoid radical cation could provide an additional photoprotection pathway which involves quenching excited state chlorophyll by the long-lived carotenoid neutral radicals formed.

Keywords: carotenoid neutral radicals, quenching, zeaxanthin radical cation, photoprotection

Introduction

An extensive literature from Professor Kispert's lab since 1986 has been generated on the physico-chemical properties of carotenoids (Car) with special emphasis on carotenoid radicals. Various reviews^{1,2,3,4,5,6,7,8,9} summarize these studies. By combining all these results it is only now clear that carotenoid neutral radical ($\text{Car}^\bullet$, # indicates proton loss), long considered an unfortunate decomposition product of the radical cation ($\text{Car}^{\bullet+}$) preventing the reversibility of cyclic voltammograms (CVs), can be an important photo-protection component in plants.¹⁰ The electrochemical, optical and Electron Paramagnetic Resonance (EPR) measurements of carotenoids in solution or adsorbed on solid supports combined with Density Functional Theory (DFT) calculations have established parameters that may elucidate the behavior of carotenoid radicals in photosynthetic systems. This review presents the chemistry of carotenoid neutral radicals and highlights a possible role in photoprotection.

Involvement of Carotenoid Neutral Radicals in Photoprotection of Photosynthetic System:¹⁰ New Pathway, Not Previously Recognized.

Plants have several redundant mechanisms for protection from excess light, collectively known as nonphotochemical quenching (NPQ).^{11,12,13,14} One important form of NPQ known as qE which results in thermal dissipation of excess energy, is characterized by a decrease in chlorophyll (Chl) fluorescence. *Arabidopsis* xanthophyll cycle mutants show a strong connection between zeaxanthin (Zea) and the qE component of NPQ.¹⁵ It was also shown^{16, 17} by femtosecond transient absorption spectroscopy that in qE proficient plants Zea forms a charge transfer complex with Chl, namely $\text{Zea}^{*+} \dots \text{Chl}^{\bullet-}$. In light-harvesting complex (LHC) proteins CP24, CP26 and CP29 the excited Chl is quenched by the charge transfer complex, producing a zeaxanthin radical cation (Zea^{*+}) and a chlorophyll radical anion ($\text{Chl}^{\bullet-}$) which then recombines to the ground state in 200 ps. Thus, further proton loss from Zea^{*+} , presumably occurring in femtoseconds^{18,19,20} (35 fs proton transfer was detected from an amine to oxygen in photoexcited 2-(2'-hydroxyphenyl) benzothiazole with the donor/acceptor groups separated by a distance of 2.8 Å.)²¹, could produce zeaxanthin neutral radical ($\# \text{Zea}^{\bullet}$) when proton acceptors are available. This formed neutral radical could be a potent quencher of the excited state Chl by electron exchange induced quenching. Free radicals have been shown to be quenchers of excited states, quenching being the basis for fluorescence detection of reactive oxygen species.^{22,23}

Carotenoid radical cations are weak acids (pKa 4-7) as shown by our earlier electrochemistry work^{1,2} and in the solid state, $\# \text{Zea}^{\bullet}$ forms from Zea^{*+} very efficiently.²⁴ A crystal structure of the qE active protein CP29 (Protein Data Bank 3PL9) in Figure 1 shows proton acceptors in close proximity to the most acidic protons of the C4(4') position of Zea^{*+} . A network of water molecules lies within hydrogen bonding distances of each other as well as the hydroxyl at C3(3'), suggesting that water plays a functional role in the protein. Deprotonation during the 200 ps time would drastically shift the redox potential of Zea^1 preventing

recombination of the charges, and would leave $\text{Chl}^{\bullet-}$ and $\#Zea^{\bullet}$ in the protein. The radical ions $\text{Chl}^{\bullet-}$ undergo facile electron transfer with other Chls in photosystem I and II, as well as in light-harvesting complexes.^{25,26,27,28,29,30,31,32}

In order to investigate whether $\#Zea^{\bullet}$ is formed during qE in the model organism *Arabidopsis thaliana*, an H/D exchange method¹⁰ was developed and analyzed by ESI mass spectrometry. Leaves from *A. thaliana* were saturated in D_2O and exposed to levels of sunlight either below or above the threshold to activate qE. When Zea^{++} deprotonates to form $\#Zea^{\bullet}$, it would eventually gain a deuteron which causes a shift in the isotopic profile. H/D exchange was observed only for those plants in the presence of sunlight intense enough ($> 300 \mu\text{E m}^{-2}\text{s}^{-1}$) to trigger qE. These results demonstrate that Zea^{++} deprotonated as predicted to form $\#Zea^{\bullet}$ during qE. A model of the experimental procedure and proton transfer mechanism is given in Figure 2.

β -carotene neutral radicals ($\#\beta\text{-car}^{\bullet}$) were also detected optically in photosystem II (PS II).³³ A previously unassigned absorption peak at 750 nm was detected in PS II samples using near-IR spectroscopy after illumination at 20 K. With increasing pH of the PS II sample the intensity of the absorption peak at 750 nm relative to the absorption of β -carotene radical cations ($\beta\text{-car}^{++}$) peak at 990 nm increased, providing evidence that the absorption peak was due to the deprotonation of $\beta\text{-car}^{++}$ forming $\#\beta\text{-car}^{\bullet}$, possible when proton acceptors are adjacent to β -carotene molecules. The X-ray crystal structure of photosystem II showed an electron-transfer pathway from a β -carotene molecule with an adjacent proton acceptor to P680^{++} . In the same study, Electron Nuclear Double Resonance (ENDOR) has shown that β -carotene neutral radicals are produced in humid air under ambient fluorescent light in the hexagonal pores of the molecular sieve Cu(II)-MCM-41, while visible/near-IR spectroscopies gave the maximum

absorption wavelengths of the neutral radicals formed, also predicted by time-dependent density functional theory (TD-DFT) calculations.

Loss of proton on the terminal ends, correlated with quenching in LHC II

Pulsed EPR techniques used to study radical cations and neutral radicals formed on solid supports in combination with DFT calculations on the four carotenoids present in light-harvesting complex II (LHC II) have shown a possible correlation between proton loss from the carotenoid radical cation to form neutral radicals and the quenching ability of the carotenoid.^{24,34} It is known that during xanthophyll cycle, in reduced light, Zea is converted into violaxanthin (Vio) which does not support qE. In intense light, also triggered by excess protons and a low pH, Vio is converted back into Zea. Furthermore, during qE, Zea can form the charge transfer complex $\text{Zea}^{\bullet+} \dots \text{Chl}^-$ leading to formation of $\text{Zea}^{\bullet+}$ and $\# \text{Zea}^\bullet$. Zeaxanthin, known as quencher of the excited state Chl, forms neutral radicals by deprotonation of the radical cation at positions situated on its terminal rings as determined by pulsed ENDOR on a silica-alumina support in combination with DFT calculations. Our DFT calculations, also described herein in a separate section, show²⁴ that the most acidic protons for $\text{Zea}^{\bullet+}$ are the methylene protons from C4(4') position or the methyl protons attached at the C5(5') position, with proton loss at the C4(4') methylene being the most favorable. Lutein, known as a quencher, was also shown to form neutral radicals losing protons off its terminal rings. Oppositely, violaxanthin and 9'-*cis* neoxanthin were not found to form neutral radicals on a silica-alumina support. It was suggested³⁴ that their inability to form neutral radicals was consistent with their nonquenching properties. DFT calculations have indicated that the epoxy groups on both ends in violaxanthin, and the epoxy at one end and the allene bond at opposite end in 9'-*cis* neoxanthin prevent protons loss at the terminal rings by reducing the conjugation so crucial for the neutral radical stability.

Also, the orientation of zeaxanthin and lutein in the thylakoid membrane of LHC II, with one end towards stroma and the opposite end towards lumen suggests possible proton loss to these hydrophilic proton-accepting regions, while the polyene chains having less acidic protons are embedded into the hydrophobic area of the membrane. According to DFT calculations,^{24,34} the most favorable proton loss for violaxanthin and 9'-*cis* neoxanthin would occur at the C9(9')-methyl proton position, a central location, situated in the hydrophobic region of the thylakoid membrane where proton loss would be unfavorable. This structural difference has been shown¹⁰ to be critical to the quenching of chlorophyll excited states by the presence of carotenoid neutral radicals and provides photoprotection needed by the plants during intense sunlight.

Electrochemical Studies:

The ability to lose terminal protons is dependent on the oxidation potential of the carotenoid: the higher the oxidation potential of the carotenoid, the greater the acidity of the terminal proton. Electrochemical studies can be used to predict quenching properties of the various carotenoids.

Carotenoids are known antioxidants and their extensive electrochemical studies^{1,2,35,36,37,38,39,40,41,42,43,44,45,46,47,48,49,50} in solution have established the following reactions upon electron transfer (Scheme 1). In equation 1 of Scheme 1, the radical cation $\text{Car}^{\bullet+}$ is formed at an oxidation potential E_1^o , the dication Car^{2+} in equation (2) at potential E_2^o and the neutral radical $^{\#}\text{Car}^{\bullet}$ at potential E_3^o . Carotenoid neutral radical is formed by reducing the cation $^{\#}\text{Car}^+$ obtained by proton loss from the dication (equation 5) which is a strong acid ($\text{pK}_a = -2$) and thus easily deprotonated in the presence of any proton acceptor. Carotenoid neutral radical can also form from deprotonation of the radical cation which a weak acid ($\text{pK}_a = 4-7$) according to equation 6.

Scheme 1. Reaction Mechanism of Carotenoids

Heterogeneous electrode reactions:

Homogeneous electrode reactions:

Side reactions:

(Note: Car neutral species of the carotenoid)

$\text{Car}^{\bullet+}$ carotenoid radical cation

Car^{2+} carotenoid dication

$\# \text{Car}^+$ deprotonated carotenoid cation (# indicates proton loss)

$\# \text{Car}^\bullet$ carotenoid neutral radical (# indicates proton loss)

The homogeneous electrode reactions 4-6 establish the comproportionation equilibrium (K_{com}) between the radical cation, dication and neutral carotenoid species. The value of K_{com} varies over 3 orders of magnitude, from 1 for β -carotene (which contains cyclohexene donor groups and has low oxidation potential) to 1000 for the strong acceptor dicyano-substituted carotene, 7'-apo-7',7'-dicyano- β -carotene. This means dications are formed in solution for electrolyzed β -carotene (97%), while radical cations (97%) are formed for those carotenoids with higher oxidation

potential. A recent publication⁵⁰ has reviewed the care with which electrochemical measurements must be carried out so that oxidation potentials reported from different labs can be compared. Calibrating the electrochemical measurements with ferrocene (0.528V vs. SCE)⁴⁸ was necessary as the conditions of the electrodes can vary from day to day and carotenoids are known to strongly adsorb on the electrode surface⁴⁶ making each measurement dependent on the electrode surface. The CVs are recorded over many orders of magnitude of sweep and fitted as a function of sweep rate to achieve accurate oxidation potentials. Depending on oxidation potential, carotenoids can scavenge toxic free radicals^{51,52} and be used in the design of improved artificial solar cells.^{53,54}

EPR Measurements:

The electrochemical studies have shown that carotenoid radicals are formed upon oxidation followed by proton loss. The structures of these radicals were further elucidated by EPR measurements coupled with DFT calculations. Details of these measurements have been outlined in Chapter 9 of the book *Carotenoids*.⁹ The carotenoid radicals contain a large number of different proton hyperfine couplings (~18) which results in approximately 300,000 unresolved EPR lines for symmetrical carotenoids, or greater for asymmetrical carotenoids. Fortunately, the methyl groups are rapidly rotating so the methyl group exhibits one set of proton couplings. In spite of this, the large number of proton couplings results in one unresolved, unhomogeneously broadened powder EPR line of 14 Gauss peak-to-peak linewidth.

To resolve the couplings, continuous wave (CW) electron-nuclear double resonance (ENDOR) measurements have been carried out.^{55,56,57,58} For each set of equivalent protons, only two ENDOR lines separated by the hyperfine coupling constant A occur. For best resolution, ENDOR measurements should be carried out in solution where proton dipolar anisotropy is

averaged out. However, the carotenoid radical cations are short-lived in chlorinating (electron acceptor) solvents^{36,59} with lifetimes on the order of 10-200 s and even shorter in other solvents. Thus a steady-state mM concentration needed for ENDOR measurements in a sample tube lasting 30 min. to 1 hr. could not be achieved. Producing the radical electrochemically in situ is not possible as the rf from the ENDOR instrument would heat up the samples to boiling. Only one ENDOR experiment by electrolyzing a sample of canthaxanthin external to the cavity was possible.⁶⁰ A large $K_{\text{com}} > 200$ and a steady state concentration occurring for 30 min enabled the measurement and thus only radicals were formed. By contrast, for β -carotene, K is approximately 1, favoring formation of the diamagnetic dication instead. The EPR signal of β -carotene radical cation in solution appears too weak for an ENDOR measurement.

Due to the fact that β -methyl groups rapidly rotate relative to the microfrequency even at 5 K and 670 GHz,⁶¹ resolved ENDOR spectra of carotenoid radicals have been observed in frozen solutions and in powders for carotenoids adsorbed on Nafion film,^{55,58} silica gel and on silica-alumina.⁵⁷ Further study⁶² has shown that the electron is transferred from the carotenoid molecules adsorbed on the activated alumina or silica-alumina to the surface Al^{3+} Lewis acid site, since ^{27}Al couplings were detected. Adsorption of carotenoids in activated silica-alumina^{63,64} results in the formation of radical cations by electron transfer between carotenoid molecules and the Al^{3+} electron acceptor. Subsequent loss of a proton from the weak acid forms the neutral radical. The three-pulse ESEEM spectrum and the HYSCORE spectrum of canthaxanthin/ AlCl_3 contain a peak at ^{27}Al Larmor frequency (3.75 MHz), proving the existence of electron transfer interactions between Al^{3+} Lewis acid site and carotenoids on silica-alumina.⁶⁵ Tumbling of carotenoid molecules adsorbed on the solid support is restricted, increasing the lifetime of the radicals to days from the usual 100 seconds in solution.⁶⁶ But each

methyl groups rotates, and this rotation is the only type of dynamic processes which is evident in the CW ENDOR spectra.^{62,65} Davies pulsed ENDOR, 330 GHz EPR⁶¹, ESEEM and HYSCORE measurements⁶⁵ have been used in combination to examine the structures and features of these adsorbed radicals. Davies pulsed ENDOR and 330 GHz EPR⁶¹ measurements from 3.3 to 80 K showed no lineshape changes, implying very rapid rotation of the methyl groups down to 3.3 K. Intense ENDOR spectral lines that appear in frozen solution or powders are due to proton couplings between 0.3 and 8.3 MHz assignable to the β -methyl protons on the carbons 5(5'), 9(9') and 13(13') as well as the α -proton couplings.^{56,57} The INDO calculated proton couplings of carotenoid radical cations were found to be overestimated.⁵⁶ As shown later by DFT calculations,⁶³ the error was sufficient that the formation of the carotenoid neutral radical via the loss of a proton from the carotenoid radical cation was missed in early studies.⁵⁶ Carotenoid neutral radicals are formed upon photoirradiation and detectable by the observation of an ENDOR line at 7-9 and 21-23 MHz on either side of the free proton frequency.^{63,64}

EPR techniques that been used to characterize carotenoid radicals to resolve g-anisotropy (HF-EPR, located at the National High Magnets Field Lab at Florida State University), anisotropic coupling constants due to α -protons (CW, pulsed ENDOR, HYSCORE), isotropic coupling constants due to β -methyl protons (CW, pulsed ENDOR), to determine distances between carotenoid radicals and electron acceptor site (ESEEM, relaxation enhancement), etc., are listed with references in Table 1.

Table 1. Various EPR Techniques Used to Study Radical Cations and Neutral Radicals of Carotenoids.

Technique	References
SEPR detection of the transient radical during electrochemical	Khaled et al. (1991) ³⁷

preparation in solution	Jeevarajan et al. (1994) ³⁸
TREPR measurements	Jeevarajan et al. (1993) ⁴
Photoinduced electron transfer in frozen solutions carotenoid radical cations formed by chemical oxidation with I ₂	Konovalova et al. (1997) ⁶⁷ Ding et al. (1988) ⁶⁸
Carotenoid radical cations formed by chemical oxidation With FeCl ₃	Jeevarajan et al. (1996) ⁶⁹
EPR spin trapping methods	Polyakov et al. (2001- 2006) ^{51,70,71,72}
EPR studies of host-guest complexes of carotenoids	Polyakov et al. (2004- 2009) ^{5,6,72,73,74,75,76,77}
Measuring distances between carotenoid radicals and distant metals in matrices by using ESEEM methods and pulsed EPR relaxation techniques	Gao et al.(2005) ⁷⁸
EPR studies of radical cation on activated alumina silica-alumina, MCM-41, metal MCM-41	Konovalova et al. (1999, 2001a,b, 2003) ^{61,65,79,80} , Gao et al. (2002, 2003) ^{81,82}
Use of high-frequency/high-magnetic field technique to resolve g-anisotropy	Konovalova et al. (1999) ⁶¹
Identify high-spin metal complexes of carotenoids	Konovalova et al. (2003) ⁸⁰
Establish the effect of distant high-spin metals on π -radicals properties	Konovalova et al. (2004) ⁸³ , Gao et al. (2005) ⁷⁸
Use of CW ENDOR techniques to detect β -proton hyperfine couplings and matrix nuclei	Kevan and Kispert(1976) ⁸⁴ , Piekara-Sady et al (1994) ⁵⁸ , Kispert and Piekara-Sady (2006) ⁸⁵
Pulsed ENDOR techniques to detect β -proton hyperfine couplings and matrix nuclei	Konovalova et al (2001) ⁶⁵ , Focsan et al. (2008) ²⁴ Lawrence et al. (2008) ⁸⁶
HYSCORE techniques to detect α -proton anisotropic couplings	Konovalova et al (2001) ⁶⁵ Focsan et al.(2008) ²⁴
Density functional theory (DFT) calculations to interpret the powder ENDOR and HYSCORE spectra	Gao et al. (2006) ⁶³ , Focsan et al. (2008-2012) ^{24,34,64,87}

Polyakov et al. (2010)⁸⁸
 Konovalova et al.(2009)⁸⁹

Establish the use of the g-tensor parameters to detect
 the presence of dimers

Petrenko et al. (2005)⁹⁰

β -Methyl Protons From Pulsed ENDOR: Use of Davies and Mims ENDOR

Pulsed Davies and Mims ENDOR have been used to detect carotenoid radicals that are formed upon photo-irradiation of carotenoids adsorbed on silica-alumina^{24,63,64,65,88} or on the molecular sieves MCM-41,^{34,88} Ti-MCM-41⁸⁹ or Cu-MCM-41.^{33,86} Davies and Mims ENDOR complement each other because hyperfine coupling are shown using one method but not in the other due to different pulse delay times.

The best fit for the calculated⁶³ β -carotene radical cation spectrum with the experimental powder ENDOR spectrum has been found by using both the DFT-derived isotropic β -methyl protons and anisotropic α -protons hyperfine couplings for simulation. The outer peaks due to neutral radical were simulated in a similar manner. Examples have been published for β -carotene radicals on silica-alumina,⁶³ zeaxanthin and violaxanthin on silica alumina,²⁴ for lutein radicals formed in Cu-MCM-41 matrices,⁸⁶ 9'-cis neoxanthin radicals on MCM-41,³⁴ lycopene on activated silica-alumina,⁶⁴ and astaxanthin on silica-alumina,⁸⁷ MCM-41 and Ti-MCM-41.⁸⁸

α -Protons From HYSCORE Analysis of Powder Spectra

α -Proton hyperfine couplings of carotenoid radicals can be determined from 2D-HYSCORE analysis of the contour line-shapes of the cross-peaks^{91,92,93} which provide the principal components of the tensors. The tensors for the radicals of zeaxanthin and violaxanthin photo-generated on silica-alumina²⁴ were rhombic characteristic of planar conjugated radicals with the unpaired spin in a p_z orbital of the carbon of the C-H group. Details of the analysis

procedure have been summarized in chapter 9, p. 174-175 of the book entitled Carotenoids edited by John T. Landrum.⁹

DFT Calculations Used to Interpret EPR Spectra

DFT calculations of the minimum energy and structure for proton loss of various carotenoid radicals and EPR detection of the carotenoid neutral radicals stabilized on various matrices are summarized in Table 2.

Table 2. Carotenoid Neutral Radicals Isolated on Various Matrices, detected by EPR and characterized by DFT.

Carotenoid	Matrix	Refs.
β -Carotene	Silica alumina Cu-MCM-41	63 33,78,86
Zeaxanthin	Silica alumina	24
Violaxanthin	Silica alumina	24
9'- <i>cis</i> neoxanthin	MCM-41	34
all- <i>trans</i> neoxanthin	MCM-41	34
Astaxanthin	Silica alumina, MCM-41 Ti-MCM-41	87, 88
Astaxanthin mono and diester	Silica alumina	87
Linear Carotenoids: (in photosynthetic bacteria)		64
Lycopene	Silica alumina	
Neurosporene		
Spheroidene		
Spheroidenone		
Spirilloxanthin		
Anhydrorhodovibrin		

DFT calculations⁶³ were used to correct a previously missed assignment^{42,55,56,57,58,79} for the carotenoid radical cation by the only available RHF INDO/SP calculations (pre-2006). Using different DFT functionals and basis sets as a fit to experimental couplings, it was confirmed that the isotropic β -methyl proton hyperfine couplings do not exceed 9 MHz for the carotenoid radical cations.⁶³ DFT calculations of carotenoid neutral radicals formed by proton loss of the most acidic proton from the radical cation were shown to exhibit isotropic β -methyl couplings up to 16 MHz.^{24,63,86} This explained the large isotropic couplings observed in ENDOR measurements of irradiated carotenoids supported on silica gel, Nafion films, silica alumina matrices or incorporated in molecular sieves.^{55,56,58,62,79,81}

The most energetically favorable proton loss from the radical cations occurs from methylene or methyl groups usually situated on the terminal rings, extending the conjugated system. As a consequence of deprotonation, the unpaired electron spin distribution changes so that larger methyl proton hyperfine constants occur for the neutral radicals (13-16 MHz) than for the radical cation (7-10 MHz). Several papers have been published on the unpaired distribution that occurs for various carotenoids as a function of substituent, symmetry and function.^{24,34,63,64,86} The unpaired spin density for the carotenoid neutral radicals increases at carbons along the chain distant from the position from where the proton was lost. As an example, in Figure 3 are shown the most energetically favorable neutral radicals of zeaxanthin, #Zea $\cdot$ (4) (lowest energy structure) and #Zea $\cdot$ (5), formed by proton loss at the C4(4') and at C5(5') positions at the terminal ends of zeaxanthin radical cation which extends conjugation. In contrast, for violaxanthin, the presence of the epoxy groups localizes the unpaired spin density on the C4(4') and C5(5') atoms respectively resulting in neutral radical structures that are energetically unfavorable.²⁴ These structures which are 15 to 20 Kcal/mole higher than the most favorable

structure formed by proton loss at C9(9') situated in the polyene chain, have never been observed by EPR measurements of violaxanthin supported on molecular sieves²⁴ even though a large 16 MHz coupling would be easily observed in a powder EPR spectrum.

Conclusion

Deprotonation of carotenoid radical cations to form neutral radicals observed in electrochemical measurements in solution has been extensively studied using advanced EPR techniques in combination with DFT calculations. It was revealed under intense light and in the presence of proton acceptors, carotenoid neutral radicals $\text{Car}^\bullet$ form from radical cations $\text{Car}^{+\bullet}$ as detected in *Arabidopsis thaliana* and in PSII samples. It has been recently shown that carotenoid neutral radicals can be involved in quenching excited state Chls, a photoprotect pathway previously not recognized, provided that the lifetime of a formed charge transfer complex ($\text{Car}^{+\bullet} \dots \text{Chl}^\bullet$) is sufficient for charge transfer between Chl molecules and for proton loss from $\text{Car}^{+\bullet}$ to nearby acceptors.

In artificial matrices, resolved ENDOR peaks due to neutral radicals formation were differentiated from those of radical cations because of their much larger isotropic β -methyl proton couplings (of ~ 16 MHz) providing a means to identify possible carotenoid radicals in biological systems. The various structures of carotenoids and their environment allow or block formation of long-lived neutral radicals that can participate in energy transfer and quenching roles in photosynthetic systems.

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Figure 1: Orientation of (all-*E*)-violaxanthin/(all-*E*)-zeaxanthin in qE active protein CP29 (Protein Data Bank 3PL9). A charge transfer complex would be formed with zeaxanthin and chlorophyll at one end, followed by proton transfer from zeaxanthin radical cation to nearby proton acceptors (shown) at the other end. The structure inside the protein is crucial for this energy quenching mechanism to occur, and is not likely to happen with a (*Z*)-isomer of zeaxanthin present instead of the (all-*E*)-zeaxanthin.

Figure 2. Model of experimental procedure and reaction mechanism demonstrating the quenching of chlorophyll excited state Chl^* by the zeaxanthin neutral radical $\# \text{Zea}^{\bullet}$. The presence of the neutral radical was shown using isotopic labeling and LC/MS detection.

Figure 3. Unpaired spin distribution for zeaxanthin (left) and violaxanthin (right) radicals. From up to down: Zea^{*+} , $\# \text{Zea}^{*}(4)$ (lowest energy structure, proton loss from terminal ends at C4(4') positions), $\# \text{Zea}^{*}(5)$ and Vio^{*+} , $\# \text{Vio}^{*}(4)$, $\# \text{Vio}^{*}(5)$ (high energy structures, not observed by EPR), respectively. The black represents excess α and the dark gray excess β unpaired spin density (Adapted from reference 24 Focsan, A. L. et al., *J. Phys. Chem. B*, 112, 1806, 2008. With permission).

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