Radicals formed from proton loss of carotenoid radical cations: a special form of carotenoid neutral radical occurring in photoprotection

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Abstract

In an organized assembly in *Arabidopsis thaliana* plant, proton loss from the radical cation of zeaxanthin ($\text{Zea}^{+\bullet}$) was found to occur under intense illumination, a possible component in photoprotection. A stable neutral radical is formed because of the favorable proton loss at C4(4') position(s) of the terminal ends of $\text{Zea}^{+\bullet}$ that extends the unpaired spin density distribution (notation $\text{Zea}^{\bullet}(4)$ or $\text{Zea}^{\bullet}(4')$ by symmetry). Proton loss from the radical cation of β -carotene ($\beta\text{-car}^{+\bullet}$) to available proton acceptors was also detected in a PSII sample upon irradiation. A controversial optical absorption peak at 750 nm predicted by DFT was attributed to β -carotene neutral radical formed by proton loss at C4(4') position(s) situated on the terminal end(s) (notation $\beta\text{-car}^{\bullet}(4)$ or $\beta\text{-car}^{\bullet}(4')$ by symmetry), also detected in solid copper-containing MCM-41 molecular sieves (Cu-MCM-41) and by hydrogen atom transfer from β -carotene to hydroxyl radical. Unlike the PSII organized assembly where proton loss occurs from the terminal ends of the radical cation, in the Cu-MCM-41 unorganized assembly proton loss occurs from all positions including the methyl groups on the polyene chain forming neutral radicals with different EPR couplings, different unpaired spin density distribution and different optical absorption peaks. We emphasize here the properties of these neutral radicals for various carotenoids formed under high-light conditions and in different media (solution, solid siliceous materials, and photosynthetic samples).

Graphical abstract

Highlights

- Properties of carotenoid neutral radicals Car* formed by proton loss from radical cations Car^{•+}.
- In organized assemblies proton loss from terminal ends of Car^{•+} to maximize delocalization.
- In unorganized assemblies proton loss occurs from all positions (C4, C5, C9 and C13).
- Different unpaired spin density distributions and optical spectra of Car*.
- Car* formed under irradiation could provide ps lifetime photoprotective role in plants.

Keywords: carotenoid neutral radical; carotenoid radical cation; photoprotection; unpaired spin density distribution; optical spectrum; surface oxygen.

1. Introduction

Carotenoids are integral components of light-harvesting centers and photochemical reaction centers in photosynthetic organisms. A variety of functions have been proposed for carotenoids including scavenging of free radicals, auxiliary light-harvesting pigments, and protective agents to quench and dissipate excess energy

Scheme 1: Carotenoid structures (circled in red are the positions for the most favorable proton loss from the radical cation-predicted by DFT calculations)

present under high-light conditions when photosynthetic process is saturated. In non-polar solvents peroxy radical ($\text{ROO}^\bullet$) addition to carotenoids (Car) occurs exclusively to give $\text{ROOCar}^\bullet$ addition radicals and adsorption occurs only in the visible region around 500 nm like that of parent carotenoid.¹ This addition is favored by the long conjugated length of carotenoids such those given in Scheme 1.

In polar solvents however, due to their antioxidant property, carotenoids react by an electron transfer mechanism generating the radical cation $\text{Car}^{+\bullet}$ and ROO^- . This property of carotenoids in polar media has been used to form and stabilize radical cations $\text{Car}^{+\bullet}$ for long term study (days, rather than the short transient lifetime in solution) on electron acceptor matrices such as silica-alumina, and the catalysts MCM-

41 and metal-substituted MCM-41.^{2,3,4,5,6,7,8,9}

EPR measurements show that Car^{++} is formed in the absence of light by electron transfer to the Lewis acid sites of the matrix surface, and upon light irradiation, neutral radicals ($\text{Car}^\bullet$) were detected. There was an order of magnitude increase in the concentration of carotenoid radicals, including both radical cations and neutral radicals, when a metal was present.¹⁰ A possible cause for increasing neutral radical concentration¹⁰ is the formation of superoxide radical anion $\text{O}_2^{\bullet-}$ from peroxy anions O_2^{2-} reacting with the photo-generated electrons trapped (e_{tr}) at the metal-oxygen (M-O) bonds. The superoxide radical anion $\text{O}_2^{\bullet-}$ then can abstract a proton from Car^{++} to form $\text{Car}^\bullet$ according to: $\text{O}_2^{\bullet-} + \text{Car}^{++} \longrightarrow \text{Car}^\bullet + \text{OOH}$. The superoxide radical anion $\text{O}_2^{\bullet-}$ could also be formed by reaction of O_2^{2-} with holes or hydroxide radical, or by reduction of the molecular oxygen on the surface by the photo-generated electrons.¹¹

EPR and DFT studies have identified the radicals formed on these matrices, both Car^{++} and $\text{Car}^\bullet$, for numerous carotenoids including symmetrical β -carotene, zeaxanthin, violaxanthin, astaxanthin, canthaxanthin, and asymmetrical lutein in Scheme I. Upon light illumination, the matrix surfaces are activated forming proton acceptors. Also electrochemical studies present¹² radical cations as being weak acids, enabling proton donation and thus forming the carotenoid neutral radical $\text{Car}^\bullet$. In such assemblies like the solid matrices described above or in solution, proton loss detected by advanced EPR occurs from different positions of the radical cation (See Table 1). DFT calculations predict^{2,3,4,5,6,7,9} the preferred location of the proton loss from the radical cation given by the minimum energy and other higher energy proton loss locations (Table 1). For example, for zeaxanthin radical cation Zea^{++} the preferred proton loss (indicated by the minimum energy $\Delta E = 0$) occurs from the C4, or by symmetry at C4' methylene positions on the cyclohexene rings (see Scheme 1, zeaxanthin), followed by loss of the methyl proton attached at the C5(or C5'), C9(or C9') or C13(or C13') positions on the polyene chain with relative energies $\Delta E = 3.15$, $\Delta E = 8.39$ and $\Delta E = 10.21$ kcal/mol, respectively⁴ (see relative energies for zeaxanthin neutral radicals in Table 1). The relative energies increase from proton loss at C4(4') methylene to C5(5') to C9(9') to C13(13') methyl group, similarly to β -carotene, for example. We use

notations $\text{Zea}^{\bullet}(4)$ or $\text{Zea}(4')$, $\text{Zea}^{\bullet}(5)$ or $\text{Zea}(5')$, $\text{Zea}^{\bullet}(9)$ or $\text{Zea}(9')$, $\text{Zea}^{\bullet}(13)$ or $\text{Zea}(13')$ for the specific neutral radicals formed, proton loss position being indicated in parenthesis.

Car	$\Delta E(3)$	$\Delta E(4)$	$\Delta E(5)$	$\Delta E(9)$	$\Delta E(13)$	$\Delta E(13')$	$\Delta E(9')$	$\Delta E(6')$	$\Delta E(5')$	$\Delta E(4')$	$\Delta E(3')$
β -carotene ^{2,6}	-	0 (24)	4.92 (24)	10.29 (19)	12.18 (15)	12.18 (15)	10.29 (19)	-	4.92 (24)	0 (24)	-
Zeaxanthin ⁴	-	0 (24)	3.15 (24)	8.39 (19)	10.21 (15)	10.21 (19)	8.39 (15)	-	3.15 (24)	0 (24)	-
Lutein ³	-	6.68 (22)	6.70 (22)	15.16 (18)	17.06 (14)	17.04 (14)	14.72 (18)	0 (23)	22.69 (4)	45.44 (4)	-
Violaxanthin ⁴	-	15.33 (4)	19.43 (4)	0 (17)	1.78 (13)	1.78 (13)	0 (17)	-	19.4 (4)	15.33 (4)	-
Astaxanthin ⁹	0 (26)	-	13.7 (24)	14.0 (19)	15.9 (16)	15.9 (16)	14.0 (19)	-	13.7 (24)	-	0 (26)
Canthaxanthin ³	-	-	0 (24)	4.32 (19)	5.83 (15)	5.83 (15)	-	-	4.32 (19)	0 (24)	-

Table 1. Relative energies $\Delta E(n)$ in kcal/mol (calculated relative to the energy minimum) of carotenoid neutral radicals formed by proton loss from the radical cation. Letter “n” indicates the position from which the proton was lost. For example, $\Delta E(4)$ is the relative energy for $\text{Car}^{\bullet}(4)$, the neutral radical formed by proton loss from the C4 position and $\Delta E(5)$ is the relative energy for $\text{Car}^{\bullet}(5)$, the neutral radical formed by proton loss from the methyl group attached at C5 position. In parenthesis below each relative energy, delocalization length is indicated by counting the number of C atoms over which the spin is delocalized. For proton loss at a methyl group (attached at positions C5, C9 and C13 and by symmetry at C5', C9' and C13'), the number of carbon atoms was counted from the methyl group in the direction of delocalization.

In *Arabidopsis thaliana* model plant proton loss from Zea^{++} to form $\text{Zea}^{\bullet}$ occurred only from one position, the methylene position at either C4 or at C4' by symmetry, thus at one end of the radical cation.¹³ In solution, proton loss from a chemically-generated radical cation Zea^{++} to form $\text{Zea}^{\bullet}$ was shown to occur at both ends suggesting that *A. thaliana* plant is an organized assembly allowing proton loss only at one end. The neutral radical species formed has been shown¹³ to be involved in the photo protection

of the plant in the intense sun light on a clear sunny day. Without Zea^{++} and $\text{Zea}^{\bullet}$ being formed in the presence of the intense sunlight, the photoprotection mechanism needed for photosynthesis would be destroyed.

Carotenoid neutral radicals of β -carotene ($\beta\text{-car}^{\bullet}$) were also observed in PSII sample where the organized assembly provided the aspartic acid⁶ as the proton acceptor, and detected by the presence of an optical spectrum at 750 nm. Further confirmation of the 750 nm spectrum was reported¹⁴ for the hydrogen atom transfer from β -carotene to the hydroxyl radical to form the carotenoid neutral radical. This species had a lifetime of approximately 15 ns consistent with the lifetime of the zeaxanthin neutral radical lifetime found in *Arabidopsis thaliana*¹³ estimated to be longer than 150 ps but less than the microsecond timescale for which reactions with diffusing oxygen occur. Careful preparation of an optical sample containing the β -carotene neutral radicals formed on copper-containing MCM-41 silicate (Cu-MCM-41) produced the experimental spectrum displayed in Figure 1a which has been deconvoluted in Figure 1b into the optical peaks due to β -carotene ($\beta\text{-Car}$) molecule at 520 nm, neutral radical $\beta\text{-car}^{\bullet}(4)$ due to proton loss of the methylene proton from the radical cation at 752 nm, and neutral radicals, $\beta\text{-car}^{\bullet}(5)$, $\beta\text{-car}^{\bullet}(9)$ and $\beta\text{-car}^{\bullet}(13)$ formed by loss of the methyl protons attached at the C5(5'), C9(9') and C13(13') positions of the radical cation occurring at 715, 675, and 536 nm respectively. No radical cation $\beta\text{-car}^{++}$ was detected indicating complete hydrolysis of the radical cation from the preparation. The arrows indicate the optical positions predicted by DFT, confirming the assignment.

Figure 1. (a) Optical spectrum of β -carotene neutral radicals on Cu-MCM-41⁶ and (b) the deconvolution of (a) into the separate overlapping optical spectra. The arrows indicate the DFT assignment.

The different adsorption maxima for the different neutral radicals formed on Cu-MCM-41 are consistent with the difference in delocalization length (conjugation length) (Figure 2). Proton loss at C4(4') gives a neutral radical with longer conjugation length (24 C atoms) has an absorption maximum at 750 nm, while the shortest conjugation length

(15 C atoms) generates an absorption peak at 530 nm (see Table 1). The β -carotene radical cation, with longer conjugation length than the lowest energy neutral radical has an optical spectrum at 950 nm. DFT also predicts extinction coefficients lower for the neutral radicals than for the radical cation.⁶

Figure 2: Deprotonation of radical cation $\beta\text{-car}^{+\bullet}$ at different positions (circled in red): at C4-methylene position and at C5, C9 and C13-methyl groups to form the neutral radicals $\beta\text{-car}^{\bullet}(4)$, $\beta\text{-car}^{\bullet}(5)$, $\beta\text{-car}^{\bullet}(9)$ and $\beta\text{-car}^{\bullet}(13)$, respectively (by symmetry $\beta\text{-car}^{\bullet}(4')$, $\beta\text{-car}^{\bullet}(5')$, $\beta\text{-car}^{\bullet}(9')$ and $\beta\text{-car}^{\bullet}(13')$ can be formed, not shown).

Investigation of crystal structure of light-harvesting complex II (LHCII) also shows that the location of preferred loss position from a radical cation is correlated to the quenching properties for carotenoids zeaxanthin and lutein.^{4,5} The orientation of zeaxanthin (I) and the two lutein (II) molecules in LHCII (see Figure 3, only one lutein molecule shown here, the other one makes a cross with II), having one terminal end towards aqueous stroma and the opposite end towards aqueous lumen, suggests that the radical cation formed upon light irradiation can lose protons off its terminal ends to these proton accepting aqueous regions, and form the neutral radical. The higher energy methyl protons of the radical cation are located on the polyene chain and imbedded in the hydrophobic area of the membrane, and thus they are not prone to be lost.

Figure 3: Orientation of the four carotenoids in LHCII^{4,5}: I-zeaxanthin, II-lutein, III-violaxanthin, IV-9'-cis neoxanthin

For the other two carotenoids present in LHCII, violaxanthin (III) and 9'-cis neoxanthin (IV), neutral radicals cannot be formed. Proton loss from the radical cation at both terminal ends is prevented by the epoxide groups in violaxanthin, and the epoxide and the allene bond in 9'-cis neoxanthin.⁵ These two carotenoids, violaxanthin and 9'-cis neoxanthin, that are unable to form neutral radicals from the radical cation in

LHCII (nor in silica-alumina⁴ or in MCM-41 molecular sieves⁵), were not observed to be quenchers in the literature. Figure 4 shows the predicted unpaired spin density distribution for the energetically preferred neutral radical, the zeaxanthin neutral radical formed by proton loss at the C4 (or C4') methylene position that extends conjugation. A radical cation formed upon light irradiation would readily lose protons off methylene position C4 (or C4') when zeaxanthin is oriented like in Figure 3. However, when plant is switched to dark (Figure 3) and zeaxanthin is converted into violaxanthin, its epoxide groups at both terminal ends prevent proton loss at C4 and C4' methylene position, or even at C5 and C5'-methyl groups, and a neutral radical would not form. Figure 4 shows the localized unpaired spin on the ring, a structure that DFT predicted to be energetically unfavorable. Such neutral radical species were not detected for violaxanthin on silica-alumina.⁴

Figure 4: Proton loss in zeaxanthin radical cation at methylene position C4 produces Zea*(4), while proton loss from this position in violaxanthin is prevented by the epoxide group (also preventing proton loss of the C5-methyl proton-not shown)

In minor LHCs a charge transfer complex $\text{Zea}^{2+} \cdots \text{Chl}^{\cdot-}$ that separates into Zea^{2+} and $\text{Chl}^{\cdot-}$ and then recombines in 150 ps was detected.^{15,16,17,18} This hundred ps timescale is sufficient to allow proton transfer (taking place in tens of fs to a few ps^{19,20,21,22}) from a small fraction of Zea^{2+} to pre-assembled proton acceptors to form $\text{Zea}^{\cdot}$.²³ Deprotonation of Zea^{2+} would prevent recombination of the charges $\text{Chl}^{\cdot-}$ and $\text{Zea}^{\cdot}$ and extend their lifetimes because the redox potential of Zea would be drastically shifted.¹ During this time, the $\text{Zea}^{\cdot}$ neutral radicals could quench excited states of the neighboring Chl, while the $\text{Chl}^{\cdot-}$ radical anions can migrate through the

lattice^{24,25,26,27,28,29,30,31} undergoing facile electron transfer with other chlorophylls in the complex. After 150 ps, Zea[•] needs to be protonated and the charge on Chl^{•-} needs to migrate back to the initial site to reform the charge transfer complex and return to the molecular ground state.²³

2. Special properties of neutral radicals critical for photoprotection

Summary of the supporting data for the properties of carotenoid neutral radical formed by proton loss from the lowest energy carbon position of a radical cation using instructive figures from the literature is provided in this section. All geometries for carotenoid radicals described in subsection 2.1 were optimized at the DFT level with the B3LYP exchange-correlation functional and the 6-31G** basis set. The unpaired spin density distributions of carotenoid radicals in subsection 2.1 were obtained using the AGUI interface from the wave functions and spin densities produced by Gaussian 03. The spin density is defined as the difference in α and β spin densities. Single point calculations on the B3LYP/6-31G** optimized geometries were used to predict the hyperfine couplings at the B3LYP level with the TZP basis set from the Ahlrichs, used in ENDOR simulations in subsection 2.2. The B3LYP/TZP(Ahlrichs)//B3LYP/6-31G** method proved to be in good agreement with our experimental data. In fact, DFT-predicted hyperfine couplings were so accurate that upon their simulation and using certain theoretical ENDOR parameters, experimental ENDOR parameters could be set to obtain a similar Mims ENDOR spectrum showing the outer peaks due to neutral radicals that otherwise would have been omitted or thought of as part of baseline.²³

2.1 Density Functional Theory methods for molecular orbital calculations

Zeaxanthin is a symmetrical molecule with the axis of symmetry between C15 and C15' (see Scheme 1). The prime positions are equivalent by symmetry with the unprimed positions thus yielding the same values for DFT calculations of proton loss. Shown here are calculations for proton loss from the unprimed positions only. Radical cation of zeaxanthin Zea^{•+} is formed by electron transfer from zeaxanthin molecule to an

electron acceptor. The DFT-predicted unpaired spin is distributed along the polyene chain like shown below.

Upon proton transfer from $\text{Zea}^{\bullet+}$ to proton acceptors, neutral radicals can be formed from methylene positions C4 (or 4'), and from methyl groups attached at positions C5(5'), C9(9') and C13(13'). Proton loss from C4(or C4') was shown to be the preferred geometry, with the lowest energy assigned as zero. When a proton is lost from $\text{Zea}^{\bullet+}$, the unpaired spin density increases at carbons along the chain distant from where the proton was lost. The unpaired spin density for $\text{Zea}^{\bullet}(4)$ shows long delocalization starting around C4 methylene and extending over the whole polyene chain towards the opposite cyclohexene end.

Proton loss from the methyl groups is predicted to be energetically less favorable than proton loss from C4. The relative energies increase for proton loss from the C5- to C9- to C13- methyl group by 3.15, 8.39 and 10.21 kcal/mol, respectively, relative to proton loss from C4 (See Table 1). This is in accordance with the predicted delocalization length shown to decrease from $\text{Zea}^{\bullet}(5)$ to $\text{Zea}^{\bullet}(9)$ to $\text{Zea}^{\bullet}(13)$.⁴

Lutein (see Scheme 1), isomer with zeaxanthin, has the double bond from C6'-C5' in zeaxanthin shifted to C5'-C4' in lutein, making lutein an asymmetric molecule. Upon formation of the radical cation of lutein (shown below is its unpaired spin density distribution) and upon proton donation to an acceptor, proton loss from similar unprimed positions as in zeaxanthin is possible, generating neutral radicals shown below, with proton loss at C4 being more favorable than C5, C9 and C13.³

However, shifting the double bond to C5-C4' makes proton loss at C4' highly unfavorable (45 kcal/mol, see Table 1) and instead, proton loss occurs at C6' position which results in the preferred geometry. Followed by a 6 kcal/mol difference, proton loss occurs at the opposite end, at C4 (see Table 1).³

Violaxanthin (see Scheme 1) has a similar structure to zeaxanthin, the difference being the replacement of the double bonds at C5-C6 and C5'-C6' with epoxy groups. This prevents proton loss at C4 (and C4') and C5 (and C5') indicated by localized unpaired spin at these positions and large relative energies predicted by DFT. Also,

such neutral radicals that would give very large couplings were not detected on solid supports by EPR.⁴

The most favorable geometry in this case becomes #Vio*(9) followed by #Vio*(13), the unpaired spin density distributions being shown next.

DFT-predicted hyperfine coupling constants for the carotenoid neutral radicals described above, and for other carotenoids, reach much larger (15 - 16 MHz) values than those of the radical cation (7-10 MHz). A summary of the largest hyperfine coupling for the radical cation and the largest coupling for the neutral radical for different carotenoids is published in Table 2 of reference 5. These values are quite similar to those measured on solid support including silica-alumina and molecular sieves.

2.2 ENDOR spectra edges to monitor neutral radicals

Stable carotenoid neutral radicals that can be studied for longer than minutes (for up to hours and even days if stored in liquid nitrogen) were prepared by adsorbing a methylene chloride solution of the carotenoid on the siliceous material^{3,4,5,6,7,8,9} in an EPR tube that was evacuated under high vacuum and sealed. Carotenoid radical cations are formed by electron transfer to the Lewis acid sites of the solid matrix and detected by EPR/ENDOR techniques. Upon light irradiation, proton transfer occurs from the C4 position or at higher energy barriers from the C5-, C9- or C13-methyl proton for example, to the $O_2^{\bullet-}$ acceptor species formed. Continuous-wave (cw) ENDOR in Figure 5 below, for example, shows the presence of the astaxanthin radical cation only, when a solution of the carotenoid is adsorbed on silica-alumina.

Figure 5. cw ENDOR for non-irradiated astaxanthin sample.⁷ No isotropic couplings larger than 9-10 MHz were observed indicating that no neutral radicals are formed, just the radical cation whose largest isotropic coupling for methyl proton attached at C9 (or 9') is 9.2 MHz. ENDOR lines occur at the ends of the arrows at low and high frequency, at half distance from the nuclear frequency at 14.8 MHz. Note: baseline is not corrected.

Only upon light irradiation neutral radicals are formed from the radical cation, identified as larger isotropic coupling constants than that of the radical cation (>7-10 MHz), placing the ENDOR lines characteristic for neutral radicals at the edges of the spectrum at high- and low-frequency (Figure 6). In a Mims ENDOR spectrum, peaks (indicated by * in Figure 6) given by those larger coupling would occur below 10 MHz (low-frequency) and above 20 MHz (high-frequency), and situated at the same distance from the proton nuclear frequency at 14.8 MHz in the middle of the spectrum.

Figure 6. Simulated spectra for various astaxanthin radicals including the radical cation, radical anion and neutral radicals formed by proton loss at different positions. Neutral radicals are predicted to occur at the edges indicated by star (*) symbol given by much larger isotropic coupling than that of the radical cation simulated in black. Radical cations and neutral radicals peak positions were identified using DFT-predicted couplings.

Mims ENDOR spectra of astaxanthin on Ti-MCM-41 (Figure 7a) shows that the presence of the metal, Ti(IV) in this case, greatly increases (66 fold increase) the concentration of the carotenoid radicals stabilizing both radical cations and neutral radicals, while in the absence of the metal (Figure 7b) the concentration of the radical cations indicated by the gray area exceeds the concentration of the neutral radicals.⁷ Although it is clear that the presence of a metal ion increases the photoyield of carotenoid radicals, it has not been possible^{3,6,7,10,11} to explain the variation in yield as a function of metal ion.

Figure 7. a) Mims ENDOR of astaxanthin adsorbed on irradiated Ti-MCM-41; b) Mims ENDOR of astaxanthin adsorbed on irradiated MCM-41, no Ti(IV) metal present. Note: radical cations are represented by the gray area combined with neutral radicals contribution, and the areas under the symbol * represent solely the neutral radicals. Radical cations and neutral radicals peak positions were identified using DFT-predicted couplings.

The increase in concentration of neutral radicals can be attributed to formation of the superoxide radical anion $\text{O}_2^{\bullet-}$ more prevalent in metal-MCM-41 than in MCM-41 without a metal.¹¹ Upon light irradiation at 77K, metal-oxygen (M-O) centers trap electrons on the surface of M-O (e_{tr}) and create holes. The holes can react with peroxy anions O_2^{2-} and form $\text{O}_2^{\bullet-}$, or the trapped electrons (e_{tr}) can reduce molecular oxygen on the surface to create $\text{O}_2^{\bullet-}$ that can abstract protons (see Figure 8). In fact, it was shown at high frequencies, that molecular oxygen is hard to remove from the surface despite evacuation of the sample (see Figure 9).¹¹

Figure 8: The 110 GHz spectrum of Ni(II)-MCM-41 prepared under high vacuum, high intensity UV-irradiated at 5 K and its (dotted) spectral simulation that determined the g tensors for generated $O_2^{\bullet-}$ species as $g_1 = 2.0115$, $g_2 = 2.0049$ and $g_3 = 2.00$. Tensors $g_1' = 2.0154$, $g_2' = 2.0058$, $g_3' = 1.996$ were assigned to V-centers or trapped holes on the framework oxygens.¹¹

Figure 9: High field 220 GHz spectrum measured at 5K of Ni(II)-MCM-41 (no carotenoid is adsorbed here, and no light) under high vacuum shows a narrow signal around $g = 2.00$ due to molecular oxygen.¹¹

It is important to mention that for carotenoids adsorbed on a solid matrix there is some random order for proton loss (the exact ratio of the specific neutral radicals cannot be determined due to the fact that ENDOR is a nonlinear method which depends on relaxation) that includes all neutral radicals generated by proton loss at different positions. Figure 10 shows β -carotene neutral radicals being all formed just by a few kcal/mol difference on Cu-MCM-41 upon light irradiation. Thus, the peaks indicated by * in Figure 10 indicate a mixture of neutral radicals formed by proton loss at C4 methylene, C5-, C9 -and C13-methyl positions; there is not just one type of proton loss.

Figure 10: Presence of neutral radicals in Cu-MCM-41: green-experimental Mims ENDOR spectrum, red-simulated spectrum of radical cation using DFT-predicted couplings; and blue-simulated spectrum of both radical cation and all neutral radicals using DFT-predicted couplings showing that neutral radicals appear at the edges of the spectrum indicated by stars *.⁶

2.3 Experimental methods of sample preparation

2.3.1 In vitro study on solid matrices

Normally, exposure of the activated metal-MCM-41 powder sample containing carotenoids to light and water results in a mixture of radical cations and neutral radicals.^{3,6,7} In order to prepare a sample for optical measurements that would contain

only neutral radicals, first a radical cation needs to be generated, then entirely hydrolyzed in order to form the neutral radicals by deprotonation.

The carotenoid radical cation was generated when 4 mL of an anhydrous solution of 0.1 M carotenoid in methylene chloride was added to activated Cu-MCM-41.⁶ Cu-MCM-41 was activated by placing it in an oven at 200 °C for 24 h then transferred to a dry box before reaching 100 °C in order to prevent the accumulation of water that would deactivate the sample. However, water or simply a humid air added after generating the radical cation functions as a base to deprotonate it. Thus, the mixture of activated Cu-MCM-41 and carotenoid solution which resulted in the formation of the radical cation by electron transfer from the carotenoid to the matrix, was then transferred to a beaker on the benchtop in humid air and stirred under fluorescent light for about 5 min to evaporate the solvent. There was a color change from light yellow to blue green, indicating the hydrolysis of the radical cation took place forming neutral radicals. The residual solvent was removed under vacuum and 20 mg of the sample was ground to a very fine powder, made into a large pellet using a pellet press to be used for optical measurements. Another 20 mg of activated Cu-MCM-41 without the carotenoids was also ground to very fine powder, a pellet was prepared as above and used as the background sample.⁶

Preparation of the sample as described above in Cu-MCM-41 generated all types of neutral radicals, and optical peaks at 752, 715, 675, and 536 nm. The spectrum (Figure 1) was consistent with predicted from DFT calculations and assigned to proton loss from the C4 methylene position, and loss of a methyl proton attached at C5, C9 and C13 positions.

2.3.2 In vivo study on solid matrices

Formation of the neutral radicals by deprotonation from the radical cation was confirmed in a PSII sample⁶ by looking at changes in pH. In Table 2, as the pH range changes from 6.5 to 8.5 both the concentration of the neutral radical and radical cation decreases overall, but the ratio of the neutral radicals to that of the radical cation increases, indicating neutral radicals being formed from the radical cation as the medium becomes more basic.

Table 2: Ratio of radical yields in a PSII sample after 15 minutes of irradiation at 20 K over the pH range 6.5-8.5 (from reference 6).

pH	Neutral radicals (%)	Radical cations (%)	Neutral radicals/radical cation ratio (%)
6.5	0.57	21	2.7 ± 0.1
7.0	0.54	19	2.8 ± 0.1
7.5	0.49	16	3.0 ± 0.1
8.0	0.36	12	3.0 ± 0.1
8.5	0.36	11	3.1 ± 0.1

In a different study,¹³ H/D exchange¹³ at both ends of the radical cation, possibly at positions C4 and C4', took place when a radical cation was first chemically made by oxidation of a carotenoid solution by FeCl₃, followed by D₂O addition to the sample.

2.4 Correlation between quenching ability and neutral radical formation

It is very interesting to note that the quenching ability of zeaxanthin^{15,16,17,18} and lutein³² in LHCII is correlated to their favored loss of the terminal protons of the radical cation. This does not occur for violaxanthin and 9'-*cis* neoxanthin, the other two carotenoids present in LHCII, where loss of terminal protons is prevented. This suggests that the neutral radicals formed can play a quenching role under special conditions.

Table 3. Formation of neutral radicals for zeaxanthin and lutein only, suggesting a quenching role under special conditions

Carotenoid	Reported quenching role and radical cation formation in LHCII	Energetically favorable proton loss at terminal end of the radical cation to form the neutral radical
zeaxanthin	yes ^{15,16,17,18}	yes
lutein	yes ³²	yes
violaxanthin	no	no
9'- <i>cis</i> neoxanthin	no	no

Quenching of singlet (Figure 12 shown below, opposite spins) or triplet states of excited neighboring chlorophyll Chl^* by a zeaxanthin neutral radical $\text{Zea}^\bullet$ could possibly be done through electron exchange induced quenching as a secondary quenching mechanism. This would follow the well-characterized quenching mechanism¹⁷ that involves formation of a charge-transfer state $\text{Zea}^{+\bullet} \dots \text{Chl}^{\bullet-}$ in minor LHCs with the charge separation forming Zea^{++} . A small fraction of Zea^{++} can deprotonate at C4-methylene terminal end to form a neutral radical $\text{Zea}^\bullet$ that would be long-lasting and available for quenching excited states in the same way a stable free radical within 10 Å was shown³³ to quench excited states of a fluorescing species in a number of biological materials by J exchange.

Figure 12: Electron-exchange induced quenching (J exchange) of excited singlet chlorophyll Chl^* by the zeaxanthin neutral radical $\text{Zea}^\bullet$

2.5 Electrochemistry

Carotenoid neutral radicals have been first detected in solution at room temperature. Carotenoid solutions in methylene chloride examined using cyclic voltammetry have indicated that neutral radicals form along with radical cations and dications. Their presence as a fifth peak in a cyclic voltammogram (CV) (see Figure 13, peak 5) was considered rather annoying as it was thought of as some side reaction. It was noticed that the intensity of the fifth peak increases in the presence of traces of water or simply during a moist environment, and can be formed only upon dication (peak 2) formation.

In a cyclic voltammogram (Figure 13), the first peak corresponds to the oxidation of carotenoid molecules to form the radical cations by transferring one electron, with potential E_1^0 (see equation 1 in Scheme 2). Upon transferring of the second electron, the oxidation of the radical cations leads to formation of dications (peak 2) with oxidation potential E_2^0 (see equation 2 in Scheme 2). The two cathodic peaks 3 and 4 are due to

the reduction of the dications and radical cations, with formation of radical cations and the carotenoid molecules, respectively (see the reverse reactions for equation 1 and 2 in Scheme 2).

The fifth peak present in the low potential region near 0.1–0.3 V was attributed to the formation of neutral radicals with a reduction potential, E_3^0 . Its intensity is related to the amount of dication formed from the oxidation of radical cation, and switching the potential before the dication can form results in no neutral radicals formed. So what is the explanation for the fifth peak formation? Numerous simulations of the fifth peak have shown that deprotonation of the radical cation, along with that of the dication (see equations 5 and 6 in Scheme 2) were crucial in the simulation. The dication, which is a very strong acid ($pK_a = -2$) and the radical cation which is a weak acid ($pK_a \sim 4-7$), both tend to lose a proton in the presence of water. Upon deprotonation, the dication forms cations (see equation 5), which upon reduction, form neutral radicals (see equation 3). Also, deprotonation of the radical cation forms the neutral radicals in solution according to eqn. 6. It was shown that under a dry atmosphere, by working in a glove box, it is possible to reduce the presence of the fifth peak to the extent that it is almost absent, so proton acceptors like water need to be present to drive the deprotonation of the dication and radical cation.

Figure 13: Typical cyclic voltammogram of a symmetric carotenoid in methylene chloride.

Scheme 2. Reaction mechanism of carotenoids

Heterogeneous electrode reactions:

Homogeneous electrode reactions:

Side reactions:

(Note: Car carotenoid neutral molecule

Car^{*+} carotenoid radical cation

Car²⁺ carotenoid dication

Car⁺ deprotonated carotenoid cation

Car[•] carotenoid neutral radical)

In a plant, proton transfer from the weak acid radical cations of zeaxanthin and lutein to available proton acceptors found nearby can form neutral radicals that can quench excited states of chlorophyll. Some carotenoids like astaxanthin indicate a preference for radical cation formation, while electron transfer from the radical cation to form dications is more likely for carotenoids like β -carotene. The ability of carotenoids to scavenge free radicals like $\bullet\text{OH}$, $\bullet\text{OOH}$ and $\bullet\text{CH}_3$, generated in a Fenton reaction increases significantly with increasing first oxidation potential. As the conjugation length shortens the oxidation potential increases and pKa of the radical cation increases for example, pKa for astaxanthin is 7-8, pKa for zeaxanthin is 4. Ability to lose the proton from the radical cation decreases with increasing oxidation potential, thus zeaxanthin

radical cation would form neutral radicals easier than astaxanthin. It is also easier for zeaxanthin to make a dication, which being a strong acid ($pK_a \approx -2$) can deprotonate and form a cation (eqn 5) whose reduction forms a neutral radical (eqn 3). It was also shown that the higher the oxidation potential the higher the scavenging ability of the carotenoid molecule, for example astaxanthin is a better scavenger than zeaxanthin.

2.6 Optical studies

Carotenoid radical cations have methyl groups attached at positions C5, C9 and C13, and by symmetry at C5', C9' and C13'. Proton loss from these positions is energetically less favorable than proton loss at the C4(4') methylene groups and the relative energies increase from proton loss at C5(5')- to C9(9')- to C13(13')-methyl group as described above. This generates neutral radicals with various unpaired spin density distributions, the conjugated length decreasing from C4(4') to C5(5') to C9(9') to C13(13'). Stable neutral radicals prepared by Gao et al.⁶ by adsorbing a carotenoid solution on activated Cu-MCM-41 in humid air under ambient fluorescent light were optically analyzed.⁶ The different proton loss sites yielded different optical spectra and the experimental work was complemented by electronic structure calculations. TD-DFT calculations in gas phase scaled by 0.4 eV to accommodate for the solvent effect and the errors in the TD-DFT calculations confirmed the detected absorption peaks and set the maximum absorption wavelengths of β -car(4), β -car(5), β -car(9) and β -car(13) being around 752, 715, 675, and 536 nm, respectively (Figure 1). The different adsorption maxima are consistent with the difference in conjugated chain length: the longer the conjugation length, the higher the absorption maximum. Proton loss at C4-methylene position which gives a neutral radical with longer conjugation length has an absorption maximum at 750 nm, while the shortest conjugation length generates an absorption peak at 530 nm. Radical cation with longer conjugation length than the lowest energy neutral radical has an optical spectrum at 950 nm. DFT also predicts extinction coefficients much lower for the neutral radicals than for the radical cation.

3. Optical studies of neutral radicals (proton loss versus addition) Recently, the influence of pH on the decay of β -carotene radical cation in aqueous Triton X-100 to form a β -carotene neutral radical was reported.³⁴ In that article information on the

reactivity toward O_2 was considered and the authors did not agree that the β -carotene neutral radical adsorbs at 750 nm but rather near 535 nm. It must be pointed out that these are two different types of carotenoid neutral radical being discussed. We have shown experimentally using EPR and by DFT calculations that proton loss at C4(4') of the radical cation produces a stable neutral radical in Cu-MCM-41. Such neutral radical produced by hydrolysis of the initially formed radical cation absorbs at 750 nm, also correlated with DFT calculations of absorption maximum, and tracked experimentally in PSII as a function of acid.⁶ An important point that was missed from column 3 in Table 2, is that the ratio of neutral radicals to radical cations concentration increases with increasing basicity under illumination. We suspect that oxygen must be present in Triton X solution enough to be dealing with oxygen addition radicals. This is a possibility considering our experience with evacuating O_2 from carotenoid solutions adsorbed on molecular sieves, the fact that despite extensive evacuation, we could not get rid of all O_2 from the surface. Forming a carotenoid neutral radical by O_2 addition to the polyene chain does not form a neutral radical that adsorbs at 750 nm but instead, it would yield an optical spectrum near 535 nm, consistent with shorter radical conjugation length of such species. This would be similar to the length of a neutral radical formed by proton loss at C13(13'), that we have shown to absorb at 535 nm. We believe that getting rid of O_2 entirely would be extremely difficult, and an oxygen addition radical was formed. In our studies of proton loss species formed on siliceous matrices and stabilized for long term study, avoiding the question of life time in solution.

Our DFT and EPR measurements show that neutral radical formation by loss of a proton from the carotenoid radical cation at C4 gives rise to an optical spectrum that adsorbs at approximately 750 nm where O_2 addition did not occur but rather H abstraction in the presence of light activation of the metal-oxygen (M-O) center or/and the formation of $O_2^{\bullet-}$ species. We want to point out is that the term carotenoid neutral radical with a similar optical spectrum cannot be used to cover all such radicals; addition to the polyene chain versus proton loss from the carotenoid radical cation both generate neutral radical species but with important differences in structure indicated by EPR and DFT predictions, and thus differences in optical properties.

Further confirmation of the 750 nm spectrum was reported for the hydrogen atom transfer from β -carotene to the hydroxyl radical to form the carotenoid neutral radical.¹⁴ This species had a lifetime of approximately 150 ns consistent with the lifetime of the zeaxanthin neutral radical lifetime found in *Arabidopsis thaliana*¹³ estimated to be longer than 150 ps but less than microsecond²³ for diffusing oxygen.

4. Conclusion:

The different structures and special possible photoprotective properties of carotenoid neutral radicals formed by proton loss from the radical cation are described herein. Proton loss possible at different locations generates stable neutral radicals (not transient species) with different unpaired density distributions, different EPR couplings and different optical spectra. Important to note is that methyl proton loss at C13 position of a radical cation forms a neutral radical with similar absorption spectrum (530 nm) to that of an addition radical formed when O₂ adds to the conjugated polyene chain of the carotenoid. One cannot use a generic term to cover all neutral radicals because of the differences in structure, unpaired spin distribution and optical spectrum.

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