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Scavenging of Benzylperoxyl Radicals by Carotenoids

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Carotenoids scavenge simple lipid-like alkylperoxyl radicals. However, the rate constant is too low to be determined directly and the mechanism is likewise not known with certainty [Mortensen, A. and Skibsted, L.H. (1998) *FEBS Lett*. **426**, 392–396]. It is demonstrated that carotenoids react with peroxyl radicals only slightly more reactive than lipidperoxyl radicals neither by electron transfer nor by hydrogen atom donation, but by adduct formation. Benzylperoxyl radicals are scavenged by the carotenoids β-carotene and canthaxanthin with a second-order rate constant of at least $1 \times 10^6\,\mathrm{M^{-1}\,s^{-1}}$ by formation of an adduct which decays in a first-order reaction.

Keywords: β-carotene; Canthaxanthin; Benzylperoxyl radical; Laser flash photolysis

INTRODUCTION

Carotenoids are a natural part of the photosynthetic apparatus in plants where they function as auxillary light harvesting pigments.[1] Carotenoids also exert antioxidant activity in plants by quenching reactive excited species like singlet oxygen and triplet photosensitizers (e.g. chlorophyll), both reactions usually occurring with close to diffusion-controlled rate.[2–7] Carotenoids may also be able to inhibit harmful oxidation in foods, man, and animals by scavenging reactive radicals. Of special interest is the reaction between carotenoids and peroxyl radicals, which are involved in the autoxidation of lipids.[8]

The mechanism of scavenging of peroxyl radicals by carotenoids has been studied by two different approaches: (i) direct detection of the intermediate(s) by laser flash photolysis or pulse radiolysis[7,9,10] or (ii) determination of the stable degradation products (autoxidation initiated by azo-initiators) to elucidate possible mechanisms.[11–15] Three different scavenging mechanisms may be envisaged

$$\text{Electron transfer} \quad \mathrm{ROO^\bullet + Car \rightarrow ROO^- + Car^{\bullet +}} \quad (1)$$

$$\text{Hydrogen abstraction} \quad \mathrm{ROO^\bullet + Car \rightarrow ROOH + Car(–H)^\bullet} \quad (2)$$

$$\text{Adduct formation} \quad \mathrm{ROO^\bullet + Car \rightarrow ROO–Car^\bullet} \quad (3)$$

Carotenoids react rapidly ($k \sim 2 \times 10^9\,\mathrm{M^{-1}\,s^{-1}}$) with the very reactive trichloromethylperoxyl radical (generated by pulse radiolysis) to generate two transient species: a carotenoid radical cation and what was suggested to be an adduct between carotenoid and trichloromethylperoxyl radical,[9] both exhibiting near infrared absorption. The equally reactive acetylperoxyl radical (generated by laser flash photolysis) is scavenged rapidly by β-carotene ($k = 9.2 \times 10^8\,\mathrm{M^{-1}\,s^{-1}}$) to form an acetylperoxyl–β-carotene adduct absorbing in the same spectral region as β-carotene itself.[7] In contrast, reaction between carotenoids and less reactive secondary alkylperoxyl radicals, mimicking lipidperoxyl radicals in reactivity, is too slow ($k < 10^6\,\mathrm{M^{-1}\,s^{-1}}$) to observe any transient species, and the mechanism is hence not known with certainty.[10]

A number of different products have been found in the product analysis studies,[11–17] most notably various carbonyl compounds and epoxides. Based on these findings, reaction (3) was invoked to explain

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the formation of the stable degradation products.[12–15] In a similar study it was suggested that carotenoids react with peroxyl radicals by two concurrent mechanisms: (i) by adduct formation (like the acetylperoxyl radical); and (ii) by donation of a hydrogen atom to the peroxyl radical.[11] However, these two intermediates-the carotenoid–peroxyl radical adduct and neutral carotenoid radical-could not be detected directly by the method employed (mass spectrometry). Based on the rate of degradation of a number of different carotenoids under peroxidising conditions[18] it was likewise suggested that hydrogen atom transfer plays a significant role in the scavenging of peroxyl radicals by carotenoids.

In this paper, the reaction between carotenoids and benzylperoxyl radicals is examined in detail in order to establish which mechanism(s) (reactions (1)–(3)) is/are operative. Based on reduction potentials,[19] the benzylperoxyl radical (0.78 V) is far less reactive than the trichloromethylperoxyl (1.13 V) and acetylperoxyl radicals (1.14 V) and only slightly more reactive than more simple alkylperoxyl radicals (0.64–0.75 V) similar to those involved in lipid autoxidation. The trichloromethylperoxyl and acetylperoxyl radicals are far too reactive to be good model compounds for lipidperoxyl radicals with respect to both mechanism and rate of reaction. The benzylperoxyl radical, on the other hand, is only slightly more reactive than lipidperoxyl radicals and thus a good choice for studying the mechanism and providing and estimate of the reaction rate by which carotenoids scavenge peroxyl radicals as this reaction cannot be studied directly with less reactive peroxyl radicals.[10]

MATERIALS AND METHODS

β-Carotene and canthaxanthin were supplied by Roche A/S (Hvidovre, Denmark). Di-*tert*-butyl peroxide (>98%), Merck–Schuchardt (Hohenbrunn, Germany), benzene p.a. and toluene p.a., both from Merck (Darmstadt, Germany), were used as received.

Laser flash photolysis experiments were carried out with an LKS.50 laser flash photolysis spectrometer from Applied Photophysics (Leatherhead, UK). The second harmonic at 532 nm of a pulsed Q-switched Nd:YAG laser, Spectron Laser Systems (Rugby, UK), was used to pump a dye laser generating emission at 594 nm which was subsequently frequency doubled to 297 nm. The energy of the laser pulse at 297 nm was approximately 26 mJ. The duration of the pulse was around 8 ns. A 1P28 photomultiplier tube from Hamamatsu (Hamamatsu City, Japan) was used to detect transient absorption below 550 nm, and an S1336-44BK silicon photodiode (Hamamatsu) was used for measurements above 550 nm. Long-pass cut-off filters were used when appropriate to minimize degradation of carotenoids by the monitoring light. Spectral slit widths were typically 4–5 nm. The aerated samples were excited in a flow cell from NSG Precision Cells, (Farmingdale, New York) with 1 cm optical path length. All samples were thermostated at 20.0 ± 0.5°C during laser flash photolysis. Solutions were used the same day they were prepared.

RESULTS

Laser flash photolysis of an aerated solution of carotenoid, di-*tert*-butyl peroxide and toluene in benzene leads to irreversible bleaching of the carotenoid (Fig. 1) due to reaction with the benzylperoxyl radical (*vide infra*). The bleaching is two-step with a fast component (until *ca.* 50 μs after the laser flash) followed by a slower reaction lasting around 1 ms after the laser flash (Fig. 1). In the absence of toluene no bleaching of carotenoid is observed.

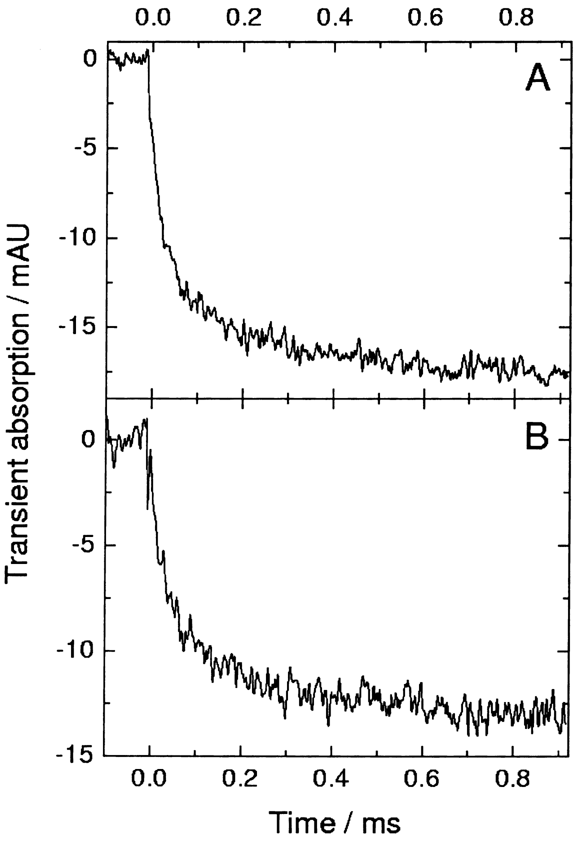

FIGURE 1 Time traces of absorption changes at 470 nm (A) and 490 nm (B) of an aerated solution of 10 μM β-carotene (A) or 10 μM canthaxanthin (B), 0.5 M di-*tert*-butyl peroxide and 10% toluene in benzene after laser flash photolysis at 297 nm.

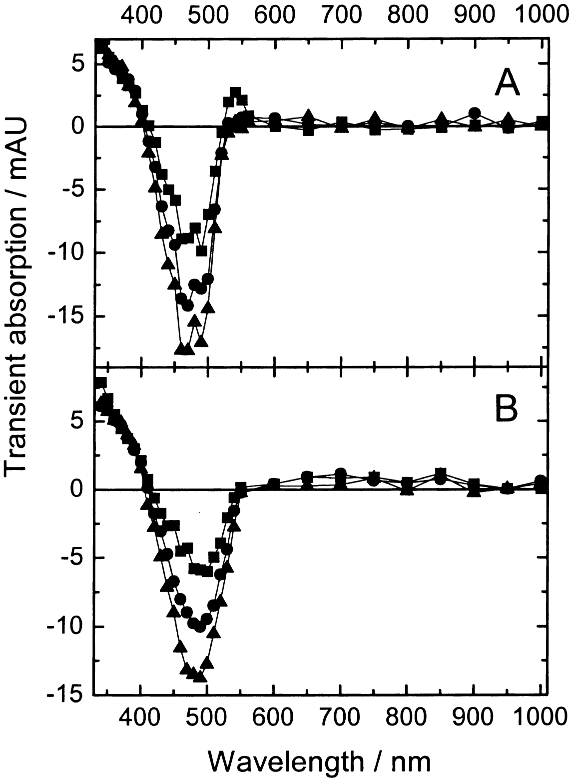

FIGURE 2 Transient absorption spectra of 10 μM β-carotene (A) or 10 μM canthaxanthin (B), 0.5 M di-*tert*-butyl peroxide and 10% toluene in aerated benzene at 20 μs (■), 100 μs (●), and 900 μs (▲) after laser flash photolysis at 297 nm. The positive transient at 540 nm in A is due to triplet β-carotene.[20]

By combining time traces to different wavelengths, time-resolved spectra were constructed (Fig. 2).

The first observation made is that the carotenoid radical cation absorbing around 900–1000 nm[2,9,21,22] is not formed by reaction with the benzylperoxyl radical (reaction (1)). Secondly, the two-step bleaching seen in Fig. 1 have been observed previously in the reaction between thiyl radicals and carotenoids[21,23] and in the reaction between the acetylperoxyl radical and β-carotene.[7] The two-step bleaching is due to the formation of an intermediate absorbing in the same spectral region as the carotenoid itself and thus not easily detected. The first fast step is hence due to reaction between carotenoid and radical and the second slower step is due to decay of the resulting intermediate (Fig. 1). The positive transient absorption below *ca.* 400 nm is due to both intermediate and the stable degradation product(s) formed by decay of the intermediate. This intermediate is known to be an adduct between carotenoid and radical.[7,21–23]

In the case of adducts between thiyl radicals and carotenoids, the radical adducts decay bimolecularly[21–23] whereas the decay of the acetylperoxyl–carotenoid radical adduct is unimolecular.[7] An attempt was made to determine the kinetics of this two-step reaction (Fig. 1) by varying the carotenoid concentration. However, due to the complex kinetics (*vide infra*) this was not possible. Also, varying the laser excitation energy changed the apparent rate constants determined by fitting time traces like those in Fig. 1 to a biexponential expression, showing that the kinetics are more complex than reaction (3) indicates. The decay of the radical adduct is clearly not second-order (though a second-order expression would easily fit the slower part of the time trace in Fig. 1): if a concentration of the adduct of 0.15 μM is assumed (roughly 1.5% of the carotenoid is bleached-Fig. 2) and a second-order rate constant of $10^9\,\mathrm{M^{-1}\,s^{-1}}$ is used, decay of the radical adduct would take place over 100 ms and not be essentially complete in 1 ms as observed (Fig. 1). Possible decay pathways of the radical adduct is discussed below.

DISCUSSION

Laser flash photolysis at 297 nm of di-*tert*-butyl peroxide leads to the formation of *tert*-butoxyl radicals

$$(CH_3)_3COOC(CH_3)_3 \rightarrow 2(CH_3)_3CO^\bullet \qquad (4)$$

The *tert*-butoxyl radical can abstract labile hydrogens for instance from toluene and di-*tert*-butyl peroxide

$$(CH_3)_3CO^\bullet + C_6H_5CH_3 \rightarrow (CH_3)_3COH + C_6H_5CH_2^\bullet \qquad (5)$$

$$(CH_3)_3CO^\bullet + (CH_3)_3COOC(CH_3)_3 \rightarrow (CH_3)_3COH + (CH_3)_3COOC(CH_3)_2CH_2^\bullet \qquad (6)$$

Direct reaction between *tert*-butoxyl radicals and carotenoids is not observed (no bleaching in the absence of toluene). Reaction (5) is rather slow (second-order rate constant $k = 2.3 \times 10^5\,\mathrm{M^{-1}\,s^{-1}}$[24]) whereas the rate constant of reaction (6) is not known. However, based on the reactivity of the *tert*-butoxyl radical towards "similar" (alkanes and ethers) substrates this rate constant is probably round $10^6\,\mathrm{M^{-1}\,s^{-1}}$.[24,25] In the presence of oxygen in the solution the two carbon-centered radicals will rapidly add oxygen to become peroxyl radicals

$$C_6H_5CH_2^\bullet + O_2 \rightarrow C_6H_5CH_2OO^\bullet \qquad (7)$$

$$(CH_3)_3COOC(CH_3)_2CH_2^\bullet + O_2 \rightarrow (CH_3)_3COOC(CH_3)_2CH_2OO^\bullet \qquad (8)$$

The second-order rate constant of reaction (7) is $2–3 \times 10^9\,\mathrm{M^{-1}\,s^{-1}}$[26,27] and, though not known, can be expected to be similar for reaction (8). In the absence of a suitable substrate, these peroxyl radicals will undergo second-order decay to form non-radical species

$$2C_6H_5CH_2OO^\bullet \rightarrow \text{products} \quad (9)$$

$$2(CH_3)_3COOC(CH_3)_2CH_2OO^\bullet \rightarrow \text{products} \quad (10)$$

$$(CH_3)_3COOC(CH_3)_2CH_2OO^\bullet + C_6H_5CH_2OO^\bullet \rightarrow \text{products} \quad (11)$$

Reaction (9) proceeds with a second-order rate constant of $2k = 3 \times 10^8\ M^{-1}\ s^{-1}$ (in neat toluene),[28] whereas the other two rate constants have not been determined. In the presence of a radical scavenger, like carotenoids, the peroxyl radicals may also disappear by reaction with the scavenger. However, carotenoids react too slowly with $(CH_3)_3COOC(CH_3)_2CH_2OO^\bullet$ to give rise to a time trace like that shown in Fig. 1 (no bleaching observed in the absence of toluene), meaning that bleaching of carotenoid is solely due to reaction with the benzylperoxyl radical

$$\text{Car} + C_6H_5CH_2OO^\bullet \rightarrow C_6H_5CH_2OO\text{–Car}^\bullet \quad (12)$$

Carotenoids do not scavenge benzylperoxyl radicals by electron transfer (reaction (1), Fig. 2). However, the possibility of hydrogen atom abstraction (reaction (2)) has not been ruled out. The resulting neutral carotenoid radical (the deprotonated radical cation) has never been observed in pulse radiolysis or laser flash photolysis experiments. However, a re-evaluation of all the existing data suggests[29] that the unidentified intermediate (absorbing in the near infrared region) formed by reaction between carotenoids and trichloromethyperoxyl radicals[9] or phenoxyl radicals[30,31] is not an adduct as originally suggested, but may indeed be the neutral carotenoid radical.[29] This species, absorbing at a slightly shorter wavelength than the radical cation, is similarly not observed in the reaction between benzylperoxyl radicals and carotenoids (Fig. 2).

Previously, it has been argued that hydrogen atom transfer (reaction (2)) was involved, together with adduct formation, because β-carotene degrades faster than canthaxanthin under peroxidising conditions,[18] and the detection of certain degradation products[11,18] seems to indicate hydrogen atom transfer as well. However, canthaxanthin is only bleached to a slightly lesser extent than β-carotene (Fig. 2), indicating that β-carotene reacts slightly faster (but only very little) with benzylperoxyl radicals than canthaxanthin does (the structure of the carotenoid has only a minor effect on the rate constant of reaction with radicals forming adducts with carotenoids,[23] whereas this is generally not the case with radicals scavenged by electron transfer[31]). The fact that β-carotene degrades faster than canthaxanthin[18] must hence be due to secondary reactions and not the primary scavenging action (reaction (12)). In fact, it has been shown that xanthophylls with carbonyl groups, e.g. canthaxanthin, autoxidizes much more slowly than carotenes, like β-carotene.[32] This was believed to be due to a higher reactivity of the carotene radicals (like the one formed in reaction (12)) compared to the xanthophyll radicals[32] (the authors suggested hydrogen atom transfer in stead of adduct formation but the relative order of reactivity would also hold for radical adducts) rather than a difference in reactivity towards peroxyl radicals (as the present results also indicate). Hydrogen atom transfer may thus take place as well but its importance seems to be much smaller than scavenging by adduct formation, i.e. the rate constant of reaction (2) is smaller than the rate constant of reaction (3).

The so-formed benzylperoxyl–carotenoid radical adduct (reaction (12)) can disappear in a number of reactions. Firstly, the radical adduct could undergo second-order decay

$$2C_6H_5CH_2OO\text{–Car}^\bullet \rightarrow \text{products} \quad (13)$$

However, this possibility has already, based on the kinetics (*vide supra*) been ruled out. As a carbon-centered radical it may add oxygen

$$C_6H_5CH_2OO\text{–Car}^\bullet + O_2 \rightleftarrows C_6H_5CH_2OO\text{–Car–}OO^\bullet \quad (14)$$

This reaction is reversible but neither the equilibrium constant nor the forward and reverse rate constants are known. Addition of oxygen destroys the resonance stabilisation of the benzylperoxyl–carotenoid radical adduct making it likely that this reaction is not very important in the decay of the radical adduct except at high oxygen concentrations.[33] Finally, the radical adduct could undergo unimolecular decomposition to give epoxides or carbonyls and a benzyloxyl radical

$$C_6H_5CH_2OO\text{–Car}^\bullet \rightarrow C_6H_5CH_2O^\bullet + \text{CarO} \quad (15)$$

This reaction has been invoked to explain the formation of carotenoid epoxides and carbonyls under peroxidising conditions.[11–15]

It is the fact that reaction (9) is rather fast which makes it difficult to determine the rate constant for reaction between carotenoid and benzylperoxyl radical (reaction (12)) because (pseudo) first-order conditions cannot be attained even at low excitation energies, i.e. low concentration of benzylperoxyl radicals. However, an estimate of the magnitude of this rate constant can be obtained. If only reactions (9) and (12) are considered, then the concentration of the benzylperoxyl–carotenoid radical adduct (P), if its decay is disregarded (reactions (13)–(15)), as a function of time is given by:[34]

$$[P] = \frac{k_{12}}{2k_9}[C]\ln\left[1 + \frac{2k_9[B]_0}{k_{12}[C]} - \frac{2k_9[B]_0}{k_{12}[C]}e^{-k_{12}[C]t}\right]$$

where $[C]$ is the concentration of carotenoid and $[B]_0$

is the concentration of the benzylperoxyl radical at time $t = 0$. At time $t \rightarrow \infty$, the concentration of P will reach the value corresponding to the total bleaching of carotenoid, i.e. roughly 1.5% (Fig. 2) or *ca.* 0.15 μM. Inserting the values for $2k_9(3 \times 10^8\,\mathrm{M}^{-1}\,\mathrm{s}^{-1})$ and $[C]$ (stays virtually constant at 10 μM throughout the reaction) gives:

$$[P]_\infty = 0.15\,\mu\mathrm{M}$$

$$= 3.33 \times 10^{-14} k_{12} \ln(1 + 3 \times 10^{13}[B]_0/k_{12})$$

Independent experiments have shown that the initial concentration of benzyl radicals, and thereby benzylperoxyl radicals, under the experimental conditions employed is a few μM. If this value is used, an approximate value of k_{12} is between $1.5 \times 10^6([B]_0 = 1\,\mu\mathrm{M})$ and $1.0 \times 10^6\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}([B]_0 = 3\,\mu\mathrm{M})$. This should be compared to the slightly less reactive alkylperoxyl radicals for which an upper limit of $10^6\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}$ was determined[10] but no bleaching of carotenoid could be observed under laser flash photolysis. The fact that the rate constant of reaction (9) is two orders of magnitude higher than that of reaction (12), together with the low concentration of carotenoid that can be employed in this type of experiments, easily explains why a direct determination of the rate constant is not possible. This is only a rough estimate as reaction (11) has not been taken into account. Another decay mechanism for the benzylperoxyl radical would mean that the estimated rate constant of reaction (12) is a lower limit, though probably not much higher than this.

Finally, one could argue that in homogeneous apolar systems (like oils and the present system), peroxyl radicals are bound to react by addition and not by electron transfer because the carotenoid radical cation cannot be solvated, whereas in heterogeneous systems carotenoid radical cations could be formed because they could be solvated at the lipid-water interface. However, recently the reduction potential of the β-carotene radical cation has been determined to be 1.06 V[35] making electron transfer from β-carotene to peroxyl radicals (reduction potential around 0.7 V[19]) highly unfavourable.

In conclusion, carotenoids scavenge peroxyl radicals that are not highly reactive be adduct formation and not by electron transfer. Carotenoids may also possibly scavenge peroxyl radicals by hydrogen atom transfer though this reaction seems to be of much lesser importance than adduct formation.

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