Photodynamics of the Bacteriochlorophyll–Carotenoid System. 1. Bacteriochlorophyll-photosensitized Oxygenation of β -Carotene in Acetone[¶]

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ABSTRACT

Carotenoids are well-known physical quenchers of chlorophyll excited states and reactive oxygen species both *in vivo* and *in vitro*. They may also be involved in chemical quenching undergoing, *e.g.* isomerizations or oxidations. We have found that β -carotene (Car) in aerobic acetone is rapidly oxygenated under strong illumination with red light ($\lambda_{\text{exc}} \geq 630$ nm) in the presence of bacteriopheophytin *a*. At the same time the photosensitizer undergoes only slight (<10%) photodegradation. By preparative high-performance liquid chromatography as many as seven major products of oxygen attachment to Car have been isolated. Their molecular masses show that Car sequentially accumulates up to six oxygen atoms while its C₄₀-skeleton remains intact.

INTRODUCTION

In photosynthetic organisms carotenoids are a ubiquitous and structurally very diverse group of isoprenoid pigments. Among their various functions (1) the most essential and indispensable one is probably that of photoprotection. It has been shown for many photosynthetic organisms, in particular under aerobic conditions, that their viability under phototropic growth is reduced by inhibition of carotenoid biosynthesis. By a variety of mechanisms carotenoids dissipate excess energy from light harvesting complexes and reaction centers

and neutralize reactive oxygen species (ROS)[‡] (2). In isolated reaction centers carotenoids quench triplet states of the primary donor chlorophylls (3–7) and similar processes may also occur in the antennae. In the latter carotenoids are also discussed as quenchers of chlorophyll excited singlet states, *e.g.* zeaxanthin, which in green plants is produced in the xanthophyll cycle from the antenna pigment, violaxanthin (8–11). Moreover, further down in the chain of events carotenoids can efficiently scavenge singlet oxygen (12,13) as well as other ROS (13,14). They also react readily with free radicals and are involved in protection against oxidative damage in nonphotosynthetic organisms (15–18).

In solution, β -carotene (Car) (1 in Scheme 1) very efficiently quenches chlorophyll *a* fluorescence at rates near to the diffusion limit and is able to protect chlorophylls from photobleaching (19,20). With its triplet energy lying below that of ¹O₂, Car and other carotenoids are excellent physical quenchers of singlet oxygen (¹Δ_g) (13,16,21,22).

Most studies on carotenoid photoprotection have been concerned with the fate of the organism or the pigment complex that is to be protected. Much less is known about the fate of the carotenoids in these processes. Their role as physical quenchers has been widely recognized. It relies both on the unusual properties of their S_n and T₁ states (23) and on the conformational flexibility which allows the rapid dissipation of excited state energy by internal conversion to the electronic ground state. There is, however, increasing evidence of chemical quenching as an alternative or additional processes. Transient *cis-trans* isomerization has been implicated in the protection of reaction centers (24). Carotenoids can also become irreversibly modified chemically. Several mono- and di-oxygenated carotenoid products have been characterized as early photosensitized products of Car, such as the β -carotene 5,6-epoxide 2, the 5,8-furanoid pigments (*e.g.* aurochrome 3) and β -carotene 5,8-endoperoxide 4 (25–28), see Scheme 1.

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[‡]Abbreviations: ACN, acetonitrile; BChl, bacteriochlorophyll *a*; BPhe, bacteriopheophytin *a*; Car, β -carotene; DAD, diode array detection; HPLC, high-performance liquid chromatography; ROS, reactive oxygen species; t_r, retention time; THF, tetrahydrofuran; TLC, thin-layer chromatography.

Scheme 1.

A variety of products carrying one oxygen and having a rearranged C_{40} -skeleton have been obtained from peroxidation of Car (28,29). More exhaustive photooxidation of Car leads eventually to a breakdown of its C_{40} -skeleton into a variety of small, colorless end products (29,30). Some of these breakdown products appear to be harmful, their accumulation may cause indirect oxidative damage, *e.g.* in plant tissues under light stress (17,31,32) or in solution (20). While some of these oxidations of Car have been recognized more than 30 years ago, their relevance in photoprotection and many points concerning the mechanism of Car interactions with singlet oxygen as well as the sequence of reactions and the structures of Car oxidation products still remain unclear.

In the course of a systematic study on the effect of Car on the photodegradation of bacteriochlorophyll (BChl) derivatives we have recently found that the protective capacity and the kinetics as well as selectivity of the reaction show a dramatic solvent dependence (Fiedor *et al.* personal communication, the results are partly published in Kammhuber [34]). In particular, in acetone, the putative protectand, *viz* Car, is rapidly degraded while the sensitizer is quite stable. In view of the potential of bacteriochlorins for photodynamic therapy (35), we wish to present results on the photooxidation kinetics and products of Car decomposition in acetone induced by the irradiation of the photosensitizer, bacteriopheophytin (BPhe), with red light ($\lambda_{irr} \geq 630$ nm). Under these conditions, Car undergoes rapid and multiple oxygenation to products bearing as many as six oxygen atoms while retaining the C_{40} -carotenoid skeleton.

MATERIALS AND METHODS

Pigments. BChl was isolated from the purple bacterium *Rhodospirillum rubrum* (carotenoidless mutant G9) following the method of Omata and Murata (36) with some modifications. BChl was extracted with methanol from the freeze-dried cells, transferred to acetone and purified on diethylaminoethyl-sepharose CL 6B (Pharmacia, Uppsala, Sweden) preequilibrated in acetone. BPhe was obtained by pheophytinization of BChl in acetic acid (37). β -Carotene (95% all-*trans*- β -carotene, Sigma, St. Louis, MO) was used as supplied. Its purity was confirmed prior to use by high-performance liquid chro-

matography (HPLC) and mass spectroscopy. The pigments were aliquoted, dried with a stream of Ar, and kept in the dark at -20°C under Ar. The purity of the pigments was routinely checked by diode array detection (DAD)-HPLC and thin-layer chromatography (TLC).

Solvents. Acetone, methanol (Baker, Mumbai, India) and acetonitrile (ACN) (Roth, Berlin, Germany) were of HPLC grade, *n*-hexane and tetrahydrofuran (THF) of spectroscopy grade (Merck, Darmstadt, Germany). THF was freed of peroxides by passage over alumina N (ICN, activity 1) and subsequent distillation over sodium hydroxide. Other solvents were used as supplied.

Sample preparation. Immediately before an irradiation experiment, aliquoted portions of solid Car (*ca* 10 mg) and BPhe *a* (*ca* 3 mg) were dissolved in acetone (up to 60 mL). To completely dissolve Car the solutions were sonicated for 10 min. In order to determine the exact pigment concentrations aliquots were taken from the batches, diluted in 2 mL of acetone and their absorption spectra recorded. The following extinction coefficients were used—BChl: $\epsilon_{770} = 7.15 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$; BPhe: $\epsilon_{750} = 4.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (37); Car: $\epsilon_{454} = 13.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (38).

Irradiations. A halogen cold-light source (Intralux 150H, Universal, Volpi, Zürich, Switzerland) with a light intensity of $4700 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in the wavelength range 350–850 nm was used for irradiations. For sensitization with BPhe a low-pass “cut-off” filter (RG 630, $\lambda \geq 630$ nm) was placed at the exit of the lightguide which reduced the light intensity to $850\text{--}900 \mu\text{mol m}^{-2} \text{ s}^{-1}$. In order to monitor the progress of the reaction the absorption spectra of the reaction mixture were recorded every 4 (analytical conditions) or 20 min (preparative conditions). The photoreaction was carried out in acetone at ambient temperature under equilibration with air (stirring). The illumination time (20–120 min) depended on the desired degree of photooxidation and the amount of unreacted pigments in the prepared solution. Usually, immediately after the reaction was completed (>90% of β -carotene reacted) the mixture was subjected to HPLC separation.

HPLC. The components of the reaction mixture were separated by HPLC (Abimed, Lagenfeld, Germany, Gilson, Villiers Le Bel, France) at ambient temperature on a semipreparative reverse-phase SPLC-18 column ($5 \mu\text{m}$ Supelcosil, $250 \text{ mm} \times 10 \text{ mm}$) monitored on-line using a model 8452A (Hewlett-Packard) diode array spectrophotometer. A gradient system according to Tregub *et al.* (20) with some modifications was applied (A: ACN/MeOH/THF 90:7.5:2.5, vol/vol/vol; B: ACN/MeOH/THF 32:60:8, vol/vol/vol; the gradient: neat A from 0 to 20 min, linear change to 100% B from 20 to 30 min; flow rate: 5 mL min^{-1}). The solvents were filtered and degassed before use. The carotenoid photodegradation products were collected and kept on ice in the dark. Fractions containing the same products were pooled, the solvent was evaporated under vacuum and samples stored at -20°C under Ar in the dark.

Analytical HPLC was done on a reverse-phase column ($5 \mu\text{m}$ LiChrospher 100 RP-8, $250 \text{ mm} \times 4.6 \text{ mm}$) using the same eluent as for SPLC-18 column. In both cases the samples were injected in solvent A. All separations were monitored by on-line recording of the absorption spectra (300–820 nm, HP model 8452A diode array detector with a $30 \mu\text{L}$ flow cell) in intervals of 2 s.

Spectroscopy. Steady state absorption spectra of the pigments isolated by HPLC were recorded in the 250–900 nm range in *n*-hexane with an ultraviolet/visible/near-infrared spectrophotometer (Lambda 2, Perkin-Elmer, Norwalk, CT). Mass spectra were obtained by atmospheric pressure chemical ionization method (APCI) (LCQ—mass spectrometer, Finnigan MAT, Bremen). For mass spectroscopy the dry pigments (*ca* 0.1 mg) were dissolved in acetone immediately before the measurements.

Kinetics of β -carotene degradation and appearance of its degradation products. A mixture of Car and BPhe in acetone was irradiated with red light ($\lambda \geq 630$ nm) for 40 min. Every 4 min, 1 mL aliquots were taken from the illuminated solution and subjected to HPLC analysis with on-line recording of absorption spectra by DAD.

Chemical tests. For a preliminary determination of the chemical character of the groups present in the examined pigments, the following chemical tests were performed (39). To test for epoxides Car and its degradation products were loaded (in acetone) on coated silica gel plates (Merck RP-8, $10 \times 10 \text{ cm}$) and developed in solvent A for HPLC. The dried plate was then exposed to HCl vapors for

Figure 1. Absorption spectra of the Car/BPhe mixture in acetone before (—) and after (---) 25 min of irradiation with red light ($\lambda \geq 630$ nm).

several minutes. The color changes (before and after exposure to HCl) were documented with an Olympus digital camera and the images were computer-processed using a Micrografx Photo Magic software package. To test for keto groups the pigments were dissolved in MeOH and their absorption spectra recorded. Under stirring, a small amount of solid NaBH_4 was added and the absorption spectra were recorded again after 5 min.

RESULTS

HPLC analysis of irradiated solutions

When a solution of Car and BPhe in acetone in equilibrium with air is irradiated with red light ($\lambda \geq 630$ nm), a gradual discoloration is observed. In this solvent, bleaching is mostly due to the degradation of Car as seen by the shifted and decreased absorption in the 400–500 nm range. In contrast, the absorption of BPhe ($\lambda_{\text{max}} = 746$ [Q_y] and 524 nm [Q_x]) decreased by less than 8% during the same time (Fig. 1).

By subjecting the reaction mixture to HPLC seven major Car photooxygenation products were identified (peaks I–VII, Fig. 2). The Car degradation products are all yellowish and more polar than Car itself, as judged from the gradual decrease of their retention times (t_r) (see Table 1) on the reverse-phase column and by the concomitantly increased R_f values on TLC (Fig. 3). In addition, the chromatogram showed unreacted Car ($t_r = 1645$ s) and an unidentified Car isomer ($t_r = 1671$ s). If judged by subsequent TLC analysis the fractions I–VII obtained by the semipreparative HPLC were chromatographically homogeneous and showed at the most only traces of colored contaminants. By repurification the products were shown to be stable under the conditions of TLC (*i.e.* in the presence of air) but they degraded slowly even during storage at -80°C under Ar.

The only detectable reaction product of BPhe was its 13²-epimer, BPhe *a'* which was identified by its absorption spectrum and slightly lower polarity ($t_r = 1440$ s *versus* 1358 s for BPhe) (Fig. 2). It amounted to as much as 20% of the BPhe which is close to the equilibrium value in acetone (40).

Figure 2. Reverse phase-HPLC chromatogram of the Car/BPhe acetone solution after 25 min illumination (see text for details of the HPLC separation). Peak assignments: I–VII, products of photosensitized Car oxygenation; BPhe *a/a'*, bacteriopheophytins *a/a'*.

Spectral changes

The absorption spectra of the seven Car degradation products show a gradual shift to shorter wavelengths which roughly parallels their increase in polarity (Table 1). The most intense absorption peak of the most polar product I shows a shift of its absorption maximum from $\lambda_{\text{max}} = 450$ (Car) to 315 nm ($\Delta = 9524 \text{ cm}^{-1}$) in *n*-hexane; the derivatives III and IV have nearly as large blueshifts while for products VII and V the shifts (compared to Car) are very small. In Fig. 4 the absorption spectra (in *n*-hexane) of the intermediates (II and IV), the most polar (I) of the isolated degradation products and of the educt Car are displayed.

Mass spectroscopy of β -carotene degradation products

Mass spectroscopy of the pigments documented a gradual increase in the molecular masses of the products in multiples of 16, indicating successive oxygenation without degradation of the C₄₀-skeleton (Table 2). The number of oxygens added again parallels roughly the increase in polarity (Table 1).

The mass spectrum of Car shows two signals: the major one can be assigned to the molecular ion (m/z 536), the minor one $[\text{M} - 92]^+$ derives from the loss of toluene from in-chain elimination (41,42). The molecular mass of derivative VII is 552 (one oxygen atom added), the only major frag-

Table 1. Absorption maxima of the isolated β -carotene, its oxygenation products and their HPLC retention times (λ_{max} in *italics*)

Pigment	Absorption peaks [nm] in <i>n</i> -hexane	HPLC retention times* [s]
β -carotene	450, 477	1647
VII	423, <i>444</i> , 472	1092
VI	380, <i>400</i> , 422	1040
V	404, <i>425</i> , 451	962
IV	447, 420, <i>364</i> , 348, 294	540
III	424, 397, <i>374</i> , 355, 337, 318	472
II	425, <i>400</i> , 378, 360	440
I	330, <i>315</i> , 302	276

*Retention times obtained with the reverse-phase analytical HPLC RP-8 column.

Figure 3. Photograph (black and white) of RP-8 TLC plate spotted with Car and its isolated photooxygenation products VII–I (from left to right) and developed in HPLC-solvent A. Spots VII, IV, III, II and I turn blue or green after exposure to HCl, derivatives VI and V remain yellow.

ment at m/z 535 corresponds to loss of water and addition of one hydrogen atom $[M - H_2O + H]^+$. The ion at m/z 551 represents formally the loss of one hydrogen atom. Similar signals are also seen for other ions in other products, they probably arise from dehydrogenation/protonation during spectroscopy. Derivative VI (m/z 568, two extra oxygens) is probably partly hydrogenated under the conditions of the measurement as judged by a prominent $[M + 2]^+$ peak. It gives one major fragment, corresponding to the loss of water and protonation (m/z 551) $[M - H_2O + H]^+$. Of the series of several smaller ions, the one at m/z 477 derives from loss of toluene and protonation $[M - 92 + H]^+$. The isomeric pigment V (m/z 568, two oxygen atoms attached) also shows partial hydrogenation and loses water (m/z 550) but no loss of toluene is observed. Derivatives II–IV (m/z 600, four attached oxygen atoms) lose up to two molecules of water leading to ions with m/z 583 $[M - H_2O, \text{protonated}]$ and 564 $[M - 2H_2O]^+$. Again, in addition to protonation, there occurs frequently the (formal) loss of one hydrogen (ions at

599 m/z). In the case of derivative II a fragmentation ion at m/z 523 $[M - 78 + H]^+$ appears which corresponds to a loss of a C_6H_6 moiety. Derivative I has the highest molecular weight (m/z 632, six oxygens added). It loses up to three molecules of water: 615 $m/z = [M - H_2O + H]^+$, 597 $m/z = [M - 2H_2O + H]^+$ and 579 $m/z = [M - 3H_2O + H]^+$. Fragment ions with m/z 527 $[M - 106 + H]^+$ and m/z 553 $[M - 79]^+$ are observed and assigned to a loss of a xylene and benzene fragments, respectively.

Except for Car and derivative VII, characteristic fragments of significant intensity are seen at $[M - 32]^+$, $[M - 2 \times 32]^+$ or $[M - 3 \times 32]^+$. They correspond formally to the (multiple) loss of two oxygens. Another characteristic fragment $[M - 138]^+$ with somewhat lower intensity appears in the spectra of derivatives VI, V, III and II and is typical to the cleavage of a 3-hydroxylated- ϵ -ionone ring (42). Some of the pigments showed peaks which may indicate contamination with the next higher oxygenated species, *e.g.* for V and VI with a triply oxygenated and for II by a penta-oxygenated species.

Figure 4. Normalized absorption spectra of Car (—), intermediates IV (····) and II (— · — · —) and the most polar photooxygenation product I (---). All spectra were recorded in *n*-hexane.

Chemical reactivity of β -carotene degradation products

Two reactivity tests were used to chemically characterize the isolated Car derivatives. Firstly, they were checked for the presence of epoxy- or vicinal diol-groups by subjecting them to TLC and subsequent exposure to HCl vapors (Fig. 3). The HCl treatment has no effect on Car and the derivatives VI and V which all remain yellow. Pigments VII and II turn blue under HCl, III and I turn green and pigment IV gives a greenish-blue color. The color change from yellow to blue or green is indicative of the presence of at least one epoxide or vicinal diol function (39,43) and hence, we assume such structures are present in pigments I–IV and VII.

The second test was for carbonyl groups. The methanolic solutions of the separated pigments were treated with $NaBH_4$. In no case there is any shift, apart from a small decrease in absorption, in the absorption spectra (data not

Table 2. Summary of the mass spectroscopy results and oxygen contents for the photooxygenation products of β -carotene

Pigment	(M + H) ⁺ (relative intensity) (%)	Significant fragmentation ions (relative intensities) (%)	Number of O atoms
β-carotene	536 (100)*	444 (50.5)	—
VII	553 (100)	535 (16)	1
VI	569 (100)	585 (7), 567 (15), 555 (15), 551 (46), 430 (9)	2
V	569 (100)	585 (16), 567 (32), 553 (24), 552 (27), 551 (65), 535 (8), 405 (8)	2
IV	601 (76)	599 (14), 585 (26), 583 (100), 569 (10), 567 (56), 565 (42), 551 (13), 549 (10)	4
III	601 (59)	599 (18), 583 (100), 581 (7), 567 (42), 565 (35), 549 (8), 419 (15), 379 (11), 165 (6)	4
II	601 (61)	617 (20), 615 (18), 599 (48), 597 (15), 591 (29), 585 (54), 583 (100), 567 (92), 565 (45), 551 (26), 549 (19), 435 (27), 419 (51), 165 (18)	4
I	633 (75)	631 (21), 629 (10), 617 (15), 615 (100), 614 (12), 613 (20), 611 (10), 601 (14), 599 (68), 597 (87), 595 (17), 585 (6), 583 (49), 581 (62), 579 (47), 569 (23), 567 (41), 565 (42), 563 (25), 553 (12), 551 (15), 549 (23), 547 (12), 537 (8), 527 (8), 475 (12), 433 (28), 419 (26), 351 (36), 313 (21), 303 (19)	6

*Unprotonated molecular ion.

shown). These results indicate the absence of carbonyl groups in all compounds examined.

Kinetics of degradation

The kinetics of β -carotene-photosensitized degradation and the appearance of the various degradation products have been monitored by sampling the reaction mixture during irradiation every 4 min and subsequent HPLC analysis. The time evolution of the reaction (Fig. 5) shows that most of the degradation products are formed only transiently and only the concentration of product I (six oxygen atoms added) keeps rising until the end of the experiment. This indicates a progressive oxygenation during photosensitized degradation of Car.

The first degradation products, *viz* derivatives VII (one oxygen), VI and V (two oxygens each) appear from the onset of illumination and reach their maxima after about 15 min. More highly oxygenated products appear only later; these are in the order of their times of maximum concentration: derivative II (four oxygens, $t_{\max} \approx 27$ min), derivative IV (four oxygens, $t_{\max} \approx 30$ min), derivative III (four oxygens,

$t_{\max} \approx 33$ min). The concentration profiles obtained were used to estimate optimal times for larger scale preparations of each of the Car photooxidation products.

In contrast to Car, the sensitizer BPhe is very stable against auto(photo)oxidation in acetone. After 40 min of irradiation, when >90% of Car is oxidized, BPhe ($t_r = 1358$ s) decreases by less than 25% as judged from HPLC. This is considerably more than that found spectroscopically, indicating product(s) with similar spectra. The decay profile is, however, not exponential and no significant decrease is observed during the first 12 min (Fig. 5). Subsequently, BPhe a' ($t_r = 1440$ s) accumulates rapidly to reach 20% of the BPhe peak and this ratio then remains constant. BPhe a' , the 13²-epimer of BPhe, is as good a sensitizer as BPhe (44,45). Its appearance would account for most of the decrease in BPhe estimated by HPLC. The combined loss of BPhe/BPhe a' is only <10% which agrees well with the degree of BPhe photodegradation observed by absorption spectroscopy (see above).

DISCUSSION

In spite of being a good photosensitizer and singlet oxygen generating agent, BPhe is surprisingly stable when irradiated into its Q_y absorption band ($\lambda_{\text{exc}} \geq 630 \text{ nm}$) in various solvents. The results of the comparative investigations of the solvent and BChl central metal effects on photosensitized reactions (34) will be published elsewhere in detail (33). In the present work we concentrate on the solvent acetone in view of a remarkably rapid oxygenation of β -carotene added to the solution. The stability of BPhe is only slightly enhanced if Car is added to BChl in acetone but the Car undergoes a relatively quick degradation, indicating the formation of ROS to which Car is much more sensitive than BPhe. Hence this solvent has been used to study the photosensitized oxidation of Car. In the course of the reaction Car is nearly spent after 30 min when $\leq 10\%$ of the sensitizer, BPhe (or its epimer, BPhe a'), has reacted. Obviously Car reacts quickly with ROS produced under these conditions but does not provide much further protection to the tetrapyrrole.

There are several reports in the literature on oxidation

Figure 5. Time evolution of BPhe-photosensitized oxygenation products of Car, monitored by the HPLC analysis of the reaction mixture (see text for details). Labels: Car (---), BPhe (—), photooxygenation products I (---), II (▲), III (■), IV (···), V (---), VI (◆), VII (●).

reactions of Car induced either chemically or photochemically (25–30) and some of the products of these reactions have been known and identified more than 30 years ago (25,29,46). Still more products of Car oxidation formed upon addition of peroxy radicals, most of them carrying only one oxygen atom, have been described more recently by Yamauchi *et al.* (27,28). The particular feature of the system used here is the accumulation of a series of products which are highly oxygenated and yet maintain the C₄₀-skeleton. The HPLC analysis of the irradiated acetone solutions show that: (1) the BPhe-sensitized degradation of Car results in a complex product mixture; (2) more than one product is formed in parallel; and (3) the primary product(s) are followed by secondary and probably tertiary ones in order of increasing polarity and degree of oxygenation. Since the in-line absorption spectra of the different fractions during HPLC are identical to the ones of the respective products isolated on a micropreparative scale, they represent products which are stable in the dark and survive the work-up procedure. A rough estimate of the mass balance, made using the same maximum extinction coefficients for all oxygenated carotenoids, indicates that formation of the carotenoid products I–VII is the main process for Car degradation in acetone and that there are, during the time of the experiment, only few unaccounted (*e.g.* colorless) products.

Guided by the order of their appearance (maximum peaking) and choosing appropriate irradiation times, it was possible to more selectively generate certain groups of the products and to purify the seven major ones for mass spectroscopy. All seven pigments have molecular masses higher than Car. The data show that they all retain the C₄₀-carotenoid skeleton and that the mass increase is due to the oxygenation. The absorption spectra of these pigments show a gradual blueshift of the maximum absorption from 450 to 315 nm. This shift to higher excitation energies is, therefore, not associated with the breakdown of the carotenoid C₄₀-skeleton but must be due to a shortening of the conjugated π -electron system in the course of oxygen attachments.

With the exception of the mono-oxygenated product VII, the molecular mass increase occurs in multiples of 32 indicating that they arise from gradual addition of molecular (singlet?) oxygen photogenerated by BPhe. In the case of 1 and 2 molecules of O₂ added, there is more than one product of the same mass. They may be stereoisomers but the different reactivities of II and III with HCl indicate that there exist several sites within the Car molecule which are attacked by oxygen at comparable rates and give rise to reaction branching. The intermediates VII–II seem to converge, however, to the common product I (six oxygen atoms) probably due to the saturation of the Car molecule with oxygen. However, no exhaustive photoreaction has been carried out to test this conclusively. From a pairwise symmetric addition of oxygens away from the center of the molecule, one would anticipate also the formation of a product carrying eight oxygens which has not been observed. There is, however, another possibility for the oxygenation sequence which accounts better for the first products: there are two different types of oxygenations, with the addition of one oxygen on either Car side in the first steps and a subsequent O₂ addition on either side.

We have been unable so far to isolate the products in

sufficient quantity for a complete structure determination and while the mass spectra indicate little (see above) contamination with products of different masses they do not preclude the formation of isomers. In particular, due to the introduction of chiral centers at the sites of oxygen attachment stereoisomers may be formed which have very similar spectroscopic and chromatographic properties (*i.e.* products V and VI).

In spite of this uncertainty, the electronic absorption and mass spectroscopy results in combination with chemicals tests and the comparison to the existing literature allow for preliminary structural assignments, at least for some of the isolated pigments. Car 1 and the putative structures of the derivatives VII, VI and V are shown in Scheme 1. Thus, product VII (C₄₀H₅₆O, m/z = 552, adduct of one oxygen atom) has an absorption spectrum which closely resembles that of β -carotene-5,6-epoxide 2. The fragmentation pattern (data not shown) further supports this assignment. Moreover, the same compound was found among Car oxidation products *in vivo* (47) and appears in chlorophyll-sensitized Car oxidation *in vitro* as one of the main products (27).

Likewise, the absorption spectrum of derivative VI (C₄₀H₅₆O₂, m/z = 568, two oxygen atoms) resembles that of β -carotene-5,8,5',8' diepoxide (*i.e.* aurochrome 3), also readily formed upon photosensitized Car oxidation (26). Accordingly, this compound is stable in HCl vapors.

Derivative V, an isomer of compound VI (also unaffected by HCl vapors), resembles β -carotene-5,8-endoperoxide 4, both in the absorption spectrum and partly in the fragmentation pattern. Moreover, together with the above-mentioned β -carotene-5,6-epoxide it was reported by Yamauchi *et al.* (28) as the main product of chlorophyll-sensitized oxidation of Car.

One should note that the comparison with the absorption spectroscopy data reported earlier is based on the positions of the absorption peaks and does not take into consideration the relative intensities of the bands (most reports do not present full spectra). Analogously, the mass spectroscopy results are only partly conclusive since the fragmentation patterns depend on the ionization method. Therefore the structural assignments are still preliminary and cannot yet be extended to the Car derivatives with higher degree of oxygenation.

The data are insufficient at the present stage to point out a detailed mechanism that the oxidation reactions are following. Two mechanisms can be envisaged: one is the formation of epoxides and perhaps the subsequent decomposition (hydrolysis) to vicinal diols. The other is 4 + 2 cyclic photoadditions (48) while 2 + 2 photoadditions can probably be excluded. Firstly, they are expected to yield ketones and aldehydes by fission, but CO-groups are unlikely from the lack of spectral changes upon addition of NaBH₄. Secondly, all reactions except the one with the Δ 5,6 and Δ 5',6'-double bonds in the β -ionone rings would lead to a fragmentation of the C₄₀-system which has not been observed.

The chlorophyll-sensitized oxidation reactions of Car may have a biogenetic/evolutionary relevance to *in vivo* systems such as the enzymatic formation of β -carotene-5,6-epoxide, observed in leaves under high light (47,49) and more generally to the formation of epoxycarotenoids leading to the evolution of the xanthophyll cycle (8). If epoxides are indeed

the early products, one might then even speculate about the recycling of such products outside the xanthophyll cycle. Another aspect of the sensitized Car oxidation in natural systems may concern the function of polar carotenoid pigments in the protection of biomembranes against oxidative destruction where oxygen binding to the β -ionone rings is supposed to enhance Car's protective efficiency (50).

In conclusion, the results show that chemical quenching of ROS can be an important pathway of protection by carotenoids, by scavenging of substantial amounts of oxygen onto the polyunsaturated molecules. Two-dynamic nuclear magnetic resonance studies are under way to fully elucidate the Car degradation product structures.

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REFERENCES

- Cogdell, R. J. and H. Frank (1987) How carotenoids function in photosynthetic bacteria. *Biochim. Biophys. Acta* **895**, 63–79.
- Frank, H. A., A. J. Young, G. Britton and R. J. Cogdell (1999) *The Photochemistry of Carotenoids*. Kluwer Academic, Dordrecht.
- Parson, W. W. (1967) Flash-induced absorbance changes in *Rhodospirillum rubrum* chromatophores. *Biochim. Biophys. Acta* **131**, 154–172.
- Mathis, P. (1969) Triplet-triplet energy transfer from chlorophyll a to carotenoids in solution and in chloroplasts. In *Progress in Photosynthesis Research* (Edited by H. Metzner), pp. 822–881, Laupp, Tübingen.
- Cogdell, R. J., W. W. Parson and M. A. Kerr (1976) The type, amount, location, and energy transfer properties of the carotenoid in reactions centres from *Rhodospseudomonas sphaeroides*. *Biochim. Biophys. Acta* **430**, 83–93.
- Frank, H. and R. J. Cogdell (1993) Photochemistry and function of carotenoids in photosynthesis. In *Carotenoids in Photosynthesis* (Edited by A. Young and G. Britton), pp. 253–326. Chapman & Hall, London.
- Frank, H. A. and R. J. Cogdell (1997) Carotenoids in photosynthesis. *Photochem. Photobiol.* **63**, 257–264.
- Demmig-Adams, B. and W. W. Adams III (1993) The xanthophyll cycle. In *Carotenoids in Photosynthesis* (Edited by A. Young and G. Britton), pp. 206–251. Chapman & Hall, London.
- Demmig-Adams, B., W. W. Adams III, V. Ebbert and B. A. Logan (1999) Ecophysiology of the xanthophyll cycle. In *The Photochemistry of Carotenoids* (Edited by H. A. Frank, A. J. Young, G. Britton and R. J. Cogdell), pp. 245–269. Kluwer Academic, Dordrecht.
- Frank, H. A., J. A. Bautista, J. S. Josue and A. J. Young (2000) Mechanism of nonphotochemical quenching in green plants: energies of the lowest excited singlet states of violaxanthin and zeaxanthin. *Biochemistry* **39**, 2831–2837.
- Schubert, H., B. M. A. Kroon and H. C. P. Matthijs (1994) *In vivo* manipulation of the xanthophyll cycle and the role of zeaxanthin in the protection against photodamage in the green alga *Chlorella pyrenoidosa*. *J. Biol. Chem.* **269**, 7267–7272.
- Borland, C. F., R. J. Cogdell, E. J. Land and T. G. Truscott (1989) Bacteriochlorophyll a triplet state and its interactions with bacterial carotenoids and oxygen. *J. Photochem. Photobiol. B: Biol.* **3**, 237–245.
- Kohen, E., R. Santhus and J. G. Hirschberg (1995) *Photobiology*. Academic Press, San Diego.
- Edge, R. and T. G. Truscott (1999) Carotenoid radicals and the interaction of carotenoids with active oxygen species. In *The Photochemistry of Carotenoids* (Edited by H. A. Frank, A. J. Young, G. Britton and R. J. Cogdell), pp. 223–234. Kluwer Academic, Dordrecht.
- Duthie, S. J., A. R. Collins and G. G. Duthie (1998) The role of carotenoids in modulating DNA stability and lipid peroxidation. Importance for human health. In *Subcellular Biochemistry* (Edited by F. J. Quinn and H. D. Kagan), pp. 181–207. Plenum Press, New York.
- Edge, R., D. J. McGarvey and T. G. Truscott (1997) The carotenoids as anti-oxidants—a review. *J. Photochem. Photobiol. B: Biol.* **41**, 189–200.
- Naguib, Y. M. A. (2000) Antioxidant activities of astaxanthin and related carotenoids. *J. Agric. Food Chem.* **48**, 1150–1154.
- Nakagawa, K., K. Fujimoto and T. Miyazawa (1996) β -Carotene as a high-potency antioxidant to prevent the formation of phospholipid hydroperoxides in red blood cells of mice. *Biochim. Biophys. Acta* **1299**, 110–116.
- Beddard, G. S., R. S. Davidson and K. R. Thethewey (1977) Quenching of chlorophyll fluorescence by β -carotene. *Nature* **267**, 373–374.
- Tregub, I., S. Schoch, S. Erazo and H. Scheer (1996) Red-light-induced photoreactions of chlorophyll a mixtures with all-*trans*- or 9-*cis*- β -carotene. *J. Photochem. Photobiol.* **98**, 51–58.
- Foote, C. S., Y. C. Chang and R. W. Denny (1970) Chemistry of singlet oxygen. X. *cis-trans* isomerisation of carotenoids by singlet oxygen and probable quenching mechanism. *J. Am. Chem. Soc.* **92**, 5218–5219.
- Chantrell, S. J., C. A. McAuliffe and R. W. Munn (1977) Excited states of protoporphyrin IX dimethyl ester: reaction of the triplet with carotenoids. *J. Chem. Soc. Faraday Trans. 1* **73**, 858–865.
- Christensen, R. L. (1999) The electronic states of carotenoids. In *The Photochemistry of Carotenoids* (Edited by H. A. Frank, A. J. Young, G. Britton and R. J. Cogdell), pp. 137–159. Kluwer Academic, Dordrecht.
- Koyama, Y. and R. Fujii (1999) *Cis-trans* carotenoids in photosynthesis: configurations, excited-state properties and physiological functions. In *The Photochemistry of Carotenoids* (Edited by H. A. Frank, A. J. Young, G. Britton and R. J. Cogdell), Kluwer Academic, Dordrecht.
- Tsukida, K., S.-C. Cho and M. Yokota (1969) Photosensitized oxygenation of all-*trans*- β -carotene. *Chem. Pharm. Bull.* **17**, 1755–1756.
- Seely, G. R. and T. H. Meyer (1971) The photosensitized oxidation of β -carotene. *Photochem. Photobiol.* **13**, 27–32.
- Yamauchi, R., M. Nobuyuki, H. Inoue and K. Kato (1993) Products formed by peroxy radical oxidation of β -carotene. *J. Agric. Food Chem.* **41**, 708–713.
- Yamauchi, R., K. Tsuchihashi and K. Kato (1998) Oxidation products of β -carotene during the peroxidation of methyl linoleate in the bulk phase. *Biosci. Biotechnol. Biochem.* **62**, 1301–1306.
- Isoe, S., S. B. Hyeon and T. Sakan (1969) Photo-oxygenation of carotenoids. I. The formation of dihydroactinidiolide and β -ionone from β -carotene. *Tetrahedron Lett.* **4**, 279–281.
- Stratton, S. P., W. H. Schaefer and D. C. Liebler (1993) Isolation and identification of singlet oxygen oxidation products of β -carotene. *Chem. Res. Toxicol.* **6**, 542–547.
- Pogson, B. J., K. K. Niyogi, O. Björkman and D. DellaPenna (1998) Altered xanthophyll compositions adversely affect chlorophyll accumulation and nonphotochemical quenching in *Arabidopsis* mutants. *Proc. Natl. Acad. Sci. USA* **95**, 13 324–13 329.
- Niyogi, K. K., A. R. Grossman and O. Björkman (1998) *Arabidopsis* mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. *Plant Cell* **10**, 1121–1134.
- Fiedor, J., L. Fiedor, N. Kammhuber and H. Scheer (2001) Photodynamics of the bacteriochlorophyll-carotenoid system. 2. Influence of central metal, solvent and β -carotene on photobleaching of bacteriochlorophyll derivatives. (unpublished)
- Kammhuber, N. (1999) Bacteriochlorophyll-derivate als neuartige sensibilisatoren für die photodynamische therapie von tumoren: darstellung und screening. Ph.D. thesis, Ludwig-Maximilians Universität.
- Rosenbach-Belkin, V., L. Chen, L. Fiedor, Y. Salomon and A. Scherz (1998) Chlorophyll and bacteriochlorophyll derivatives

- as photodynamic agents. In *Photodynamic Tumor Therapy. 2nd and 3rd generation photosensitizers* (Edited by J. G. Moser), pp. 117–125. Harwood Academic Publishers, Amsterdam.
36. Omata, T. and N. Murata (1983) Preparation of chlorophyll *a*, chlorophyll *b* and bacteriochlorophyll *a* by column chromatography with DEAE–Sephacrose Cl-6B and Sepharose Cl-6B. *Plant Cell Physiol.* **24**, 1093–1100.
 37. Rosenbach-Belkin, V. (1988) The primary reactants in bacterial photosynthesis modeling by in vitro preparation. Ph.D. thesis, The Weizmann Institute of Science.
 38. Davies, B. H. and H.-P. Köst (1988) Chromatographic methods for the separation of carotenoids. In *CRC Handbook of Chromatography, Plant Pigments* (Edited by H.-P. Köst), pp. 3–185. CRC Press, Boca Raton.
 39. Eugster, C. H. (1995) Chemical Derivatization: microscale tests for the presence of common functional groups in carotenoids. In *Carotenoids* (Edited by G. Britton, S. Liaaen-Jensen and H. Pfander), pp. 71–80. Birkhäuser, Basel.
 40. Katz, J. J., L. L. Shipman, T. M. Cotton and T. J. Janson (1978) Chlorophyll aggregation: coordination interactions in chlorophyll monomers, dimers, and oligomers. In *The Porphyrins* (Edited by D. Dolphin), pp. 401–458. Academic Press, New York.
 41. Vetter, W. and W. Meister (1985) Fast atom bombardment mass spectrum of β -carotene. *Org. Mass Spectrosc.* **20**, 266–267.
 42. Enzell, C. R. and S. Back (1995) Mass spectrometry. In *Carotenoids* (Edited by G. Britton, S. Liaaen-Jensen and H. Pfander), pp. 261–317. Birkhäuser, Basel.
 43. Liaaen-Jensen, S. (1971) Isolation, reactions. In *Carotenoids* (Edited by O. Isler), pp. 61–188. Birkhäuser, Basel.
 44. Katz, J. J., G. D. Norman, W. A. Svec and H. H. Strain (1968) Chlorophyll diastereoisomers. The nature of chlorophylls *a'* and *b'* and evidence for bacteriochlorophyll epimers from proton magnetic resonance studies. *J. Am. Chem. Soc.* **90**, 6841–6845.
 45. Watanabe, T., M. Nakazako and K. Honda (1986) Kinetic and thermodynamic parameters for the pheophytin *a/a'* epimerization in organic solvents. *Chem. Lett.*, 253–256.
 46. Hasegawa, K., J. D. MacMillan, W. A. Maxwell and C. O. Chichester (1969) Photosensitized bleaching of β -carotene with light at 632.8 nm from a continuous-wave gas laser. *Photochem. Photobiol.* **9**, 165–169.
 47. Ashikawa, I., M. Kito, K. Satoh, H. Koike, Y. Inoue, K. Saiki, K. Tsukida and Y. Koyama (1987) All-trans- β -carotene-5,6 epoxide in thylakoid membranes. *Photochem. Photobiol.* **46**, 269–275.
 48. Adam, W., C. R. Saha-Moller, S. B. Schambony, K. S. Schmid and T. Wirth (1999) Stereocontrolled photooxygenations—a valuable synthetic tool. *Photochem. Photobiol.* **70**, 476–483.
 49. Young, A. J., P. Barry and G. Britton (1989) The occurrence of β -carotene-5,6-epoxide in the photosynthetic apparatus of higher plants. *Z. Naturforsch. C* **44**, 959–965.
 50. Woodall, A. A., G. Britton and M. J. Jackson (1997) Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: relationship between carotenoid structure and protective ability. *Biochim. Biophys. Acta* **1336**, 575–586.