Rapid report

Cyclic endoperoxides of β-carotene, potential pro-oxidants, as products of chemical quenching of singlet oxygen

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Abstract

Photoprotection by carotenoids is generally considered to be based on the photophysical quenching of triplets and singlet oxygen. There is also accumulating evidence of an alternative, chemical quenching of triplets and singlet oxygen by carotenoids. We report the identification of relatively stable cyclic mono- and diendoperoxides as first products of such an alternative reaction. Nevertheless, these species remain reactive and in the dark cause autooxidation of β-carotene in our model system. Their formation could explain the intriguing pro-oxidant and cytotoxic activity of carotenoids.
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Keywords: Carotenoid; Chemical quenching of ROS; Cycloaddition; Peroxide; Singlet oxygen

Carotenoids (Crts) are important natural antioxidants, found in both animal and photosynthesizing types of cells; for the latter, their photoprotective activity is essential. Physical quenching of harmful chlorophyll (Chl) triplets and singlet oxygen ($^1O_2$) by Crts has in particular been recognized as a major factor of photoprotection in photosynthesis [1,2]. It involves the symmetry-allowed energy transfer to Crt, either directly from Chl triplets, or indirectly from singlet oxygen (Fig. 1). The resulting Crt triplets are low in energy, relatively short-lived [3] and generally not considered as harmful. Recently, evidence has been obtained that Crts may also act as chemical quenchers [4–6]. The photoprotective action is markedly medium-dependent [5–7]. For instance, β-carotene in methanol shows no protective effect towards self-sensitized photo-degradation of Chla. In contrast, a solvent change to acetone prevents degradation of Chla, at the expense of rapid photodegradation of β-carotene. There is furthermore evidence that the Crt oxidation products can act subsequently as oxidants [5]. Such pro-oxidant activity of Crts was also reported in lipid peroxidation studies [8–10]. Neither the mechanism and the products of this intriguing reactivity of Crt are firmly established, nor its contribution to light-induced cellular damage and oxidative stress in living tissues [11–15]. Following the tentative identification of a cascade of oxidation products of β-carotene (structure 1 in Fig. 2), generated in bacteriochlorophyll (BChl)-sensitized reaction in acetone, we now report the structural assignment of the major four products (structures 2–5 in Fig. 2) and propose a mechanism of their formation.

The photosensitized oxygenation of β-carotene (all-*trans*, Sigma) was performed by irradiating a solution (5×10^{-3} M) in acetone for 60–120 min with red light ($\lambda > 630$ nm, 850 μmol m^{-2} s^{-1}) in the presence of bacteriopheophytin a (1×10^{-3} M). The solution was stirred

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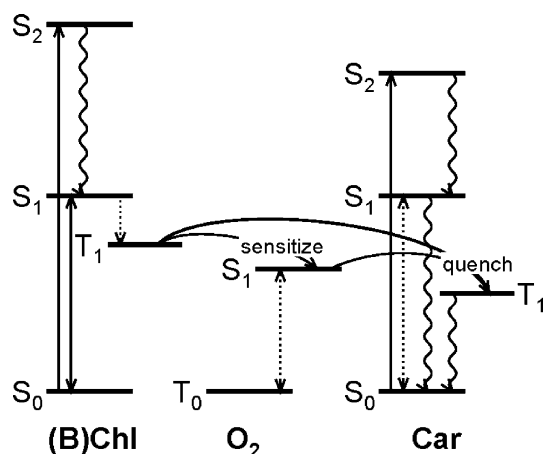
Fig. 1. Jablonski diagram illustrating the transfer of excitation energy between (bacterio)chlorophyll [(B)Chl], oxygen and carotenoid (Crt) with ≥11 conjugated double bonds. Solid arrows depict the allowed transitions, dotted arrows the forbidden transitions, and wavy arrows internal conversion. Only the spin allowed sensitizing energy transfer from T_1–(B)Chl to S_1–O_2 and the quenching energy transfers from T_1–(B)Chl to T_1–Crt, and from S_1–O_2 to T_1–Crt are shown in this diagram, as well as the relevant excited states.

in an open vessel to maintain oxygen equilibrium with air. After work-up, the product mixture was subjected to two rounds of semi-preparative HPLC [6] and analyzed immediately by NMR [in C_6D_6 at 281 K, Bruker DMX750 operating at 700.13 MHz (^{1}H) and 188 MHz (^{13}C)]. The chemical shifts given are relative to benzene as secondary reference (^{1}H = 7.28 ppm, ^{13}C = 12.85 ppm). Structures were elucidated using DQF-COSY, HMQC and HMBC NMR techniques.

For the kinetic studies, aliquots of an acetone solution of β-carotene (1.6×10^{-5} M) and bacteriopheophytin a (1.6×10^{-5} M) were irradiated with red light ($\lambda > 630$ nm) for 90 min (a) without any additions; (b) in the presence of 1,4-diazabicyclo-[2.2.2]octane (DABCO; 0.01 M, Sigma); and (c) in the presence of 2,6-di-tBu-p-cresol (BHT; 0.01 M, Fluka), and the reactions followed by absorption spectroscopy. A sample containing only β-carotene (1.6×10^{-5} M in acetone) served as dark control. UV/VIS absorption spectra were recorded every 10 min on a Lambda25 spectrophotometer (Perkin Elmer).

All products of the photooxygenation reaction are relatively stable peroxides; they can be worked-up and chromatographed under standard conditions. However, as exemplified in Fig. 3, the autooxidation of β-carotene continues for hours in samples standing in the dark at ambient temperature. The control shows no decay in the dark.

The previous tentative assignment [6] of the known 2 was verified by 2D NMR and three of the previously unassigned products have now been assigned as: 7,10-endoperoxide (3), 7,10,5′,8′-diendoperoxide (4) and 5,8,5′,8′-diendoperoxide (5) (Fig. 2). The monoendoperoxides 2 and 3 show characteristic signal patterns in the 5 ppm region of the proton spectra, which served to identify the substitution

pattern of the higher oxygenated compounds 4 and 5 (see the Supplementary material in Appendix A). The two cyclic diendoperoxides 4 and 5 are, to our knowledge, the first stable members of this class of pigments.

The photosensitized degradation of β-carotene is strongly inhibited by the $^1O_2$-quencher, DABCO [16], while the radical quencher, BHT [17], shows only little effect (Fig. 4). This confirms the role of $^1O_2$ as the reactive intermediate, most likely in its lowest singlet state $^1\Delta_g$, because diffusion from the sensitizer to β-carotene is necessary for the reaction to occur.

Based on this identification of $^1O_2$ as reactive intermediate and the structural assignment of the products 2–5, a 2:4 cycloaddition can be proposed as the oxygenation mechanism of β-carotene, considering the following features: (A) All isolated products have their oxygens near the ends of the conjugated system, whereas there were no products of oxygenation near the center of the molecule. The concerted mechanism requires the presence of an s-cis-diene conformation of the Crt educt. The oxygenation of all-trans-β-carotene to the 5,8-endoperoxides can proceed without prior conformational change because of the sterically favored 6-s-cis conformation [18]. The formation of 7,10-endoperoxides (3 and 4) requires, however, an s-trans to s-cis conversion at the C-8/C-9 single bond. C-7 and C-10 carry hydrogens and hence this s-cis conformation is not
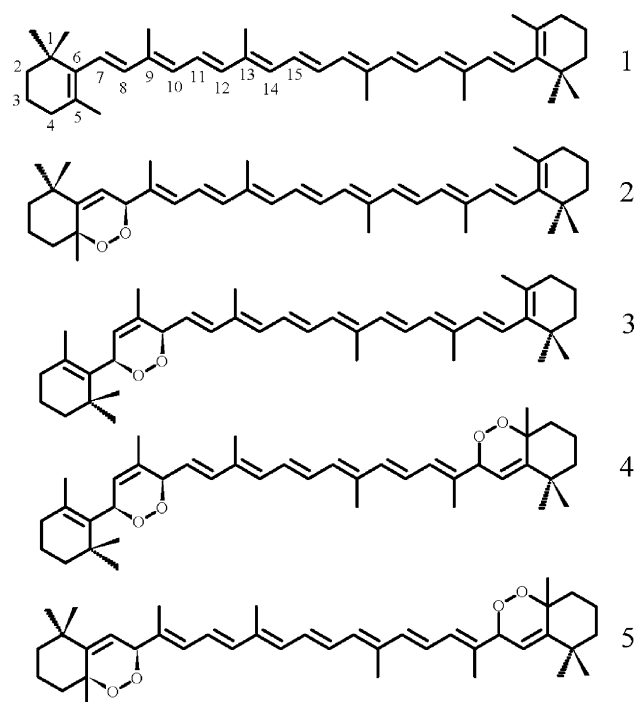

Fig. 2. Chemical structures of β-carotene 1 and the products of its photosensitized oxygenation: 5,8-endoperoxide 2 (V); 7,10-endoperoxide 3 (VI); 7,10,5′,8′-diendoperoxide 4 (III), and 5,8,5′,8′-diendoperoxide 5 (II). Numbers in brackets are as in [6]. Relative configurations of stereogenic centers in peroxides 2 and 3 and partially of 4 and 5 are also shown; compounds 4 and 5 are expected to be present as mixtures of diastereoisomers with respect to the two 'ends' of the molecules.
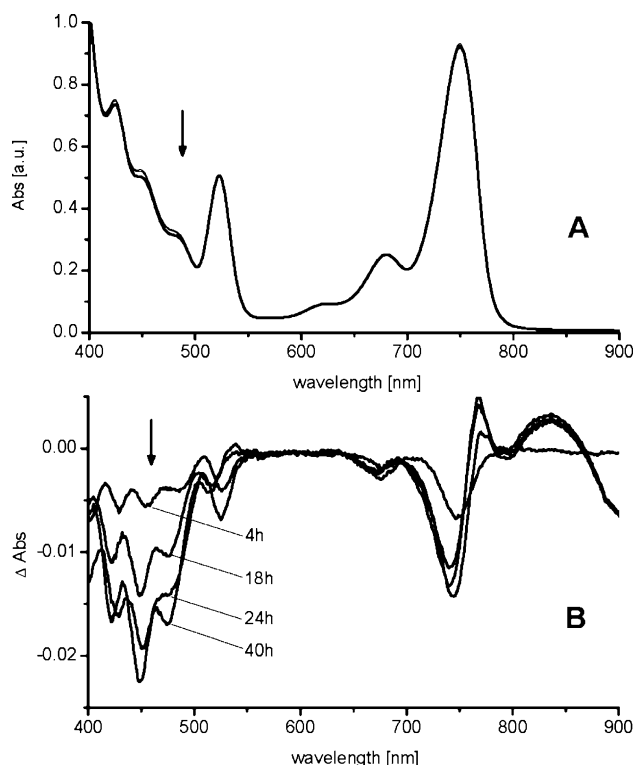
Fig. 3. Decay of β-carotene in the dark after previous irradiation. (A) Absorption spectra of an acetone solution of β-carotene and bacteriopheophytin after irradiation with red light ($\lambda > 630$ nm) and after standing in the dark for 0, 4 and 40 h; (B) difference absorption spectra after standing in the dark, spectrum at $t = X$ *minus* spectrum at $t = 0$, $X = 4$ h, 18 h, 24 h, and 40 h. The β-carotene peroxidation products, which accumulate during the irradiation, have blue-shifted absorption maxima and do not contribute significantly to the spectrum above 400 nm [6].

severely sterically hindered. Theoretical studies on bond order in polyenes have shown that while such conformational changes are particularly allowed at the ends of the conjugated systems, they become increasingly difficult towards the center due to decreasing bond alternancy [19]. The preferential formation of terminal peroxides 2–5 and the lack of more centrally oxygenated products are then in line with the more ready formation of s-*cis*-dienes near the end of the conjugated system. The energetic barrier of adopting an s-*cis* conformation also increases in *cis*-isomers of Crts [19] and therefore the 2:4 cycloaddition of singlet oxygen would be inhibited in such pigments. Indeed, in our system no oxygenation products were found which would originate from *cis*-isomers of β-carotene. *cis*-Crts are relatively rare in nature, but are often found at specific sites and presumed to have specific functions. An example is the occurrence of 15-*cis*-Crts in purple bacterial photosynthetic reaction centers [20,21], which are involved in quenching triplets of the primary donor. 15-*cis* and/or other *cis*-Crt-isomers have also been found in reaction centers and antenna complexes of oxygenic organisms [12,22–27]. (B) None of the products showed indications of being a mixture of diastereomers, as judged from the chromatographic behavior (single, homogeneous peak) and,

in particular, from the NMR spectra (lack of satellite signals). Previously, to account for the formation of endoperoxides of β-carotene, a primary attack of peroxyl radicals to C-7 had been suggested [11], which would be favored by the relatively high electrophilicity of this site. The ensuing radical of β-carotene, which is stabilized by charge delocalization, was proposed to react with molecular (triplet) oxygen to produce the cyclic endoperoxide [28]. This mechanism would lead to several products starting from a single, delocalized cation radical [29], and should yield several stereoisomers. As β-carotene does not contain chiral centers, and two new ones are generated in the formation of the monoendoperoxides 2 and 3, two pairs of diastereoisomers are expected for each (see the Supplementary material in Appendix A). Since they are pairs of enantiomers, two sets of NMR spectra should be expected to appear in an achiral environment. However, only one set is observed, that corresponds to the relative stereochemistry shown in Fig. 2. For each of the diendoperoxides 4 and 5, a total of eight endoperoxides are expected, since the two ends of the molecule are expected to react independently of each other. However, by the same token it is unlikely that the stereochemistry at one end will reflect on the NMR signals arising from nuclei at the other end of the molecule. Therefore, for each of the endoperoxide fragments again two sets of signals are expected theoretically, while only one such set is observed. This stereoselective course of the reaction then also supports the concerted mechanism.

The reactions of β-carotene with singlet oxygen to produce the peroxides described here may be relevant to the in vivo antioxidant function of Crts. First of all, they would explain the auto-oxygenation of β-carotene in the present system as well as the unexpected pro-oxidant activity of Crt reported under certain conditions [6,11]. The cyclic endoperoxides are candidates for this surprising feature of Crt (photo)chemistry. Being readily formed from β-carotene and
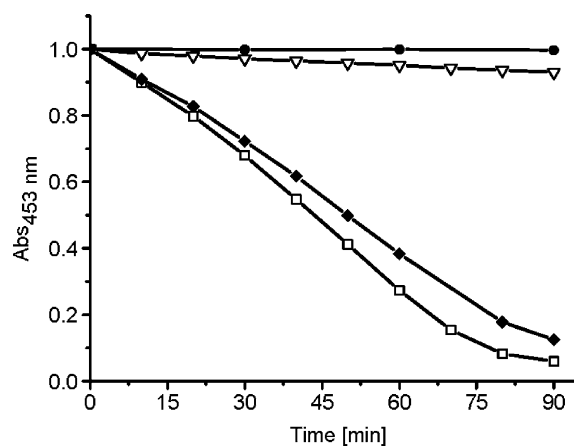

Fig. 4. Stability of β-carotene in acetone towards singlet oxygen generated by photosensitization with bacteriopheophytin and red light ($\lambda > 630$ nm). Absorption changes at 453 nm under illumination without any further addition ($-\square-$), in the presence of DABCO (0.01 M, $-\triangledown-$), in the presence of BHT (0.01 M, $-\blacklozenge-$), and dark control ($-\bullet-$).

singlet oxygen, yet of sufficient stability, they can promote autooxidation, and possibly oxidations of other species. Such reactions would be of critical importance in living cells, where harmful lipid peroxidation may take place in spite of, or in some cases even due to the presence of Crts.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2005.05.008.

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