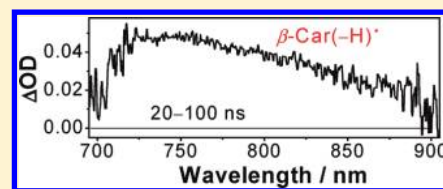


Direct Observation of the  $\beta$ -Carotene Reaction with Hydroxyl RadicalChang-Hui Chen,<sup>†</sup> Rui-Min Han,<sup>†,\*</sup> Ran Liang,<sup>†</sup> Li-Min Fu,<sup>†</sup> Peng Wang,<sup>†</sup> Xi-Cheng Ai,<sup>†</sup> Jian-Ping Zhang,<sup>†</sup> and Leif H. Skibsted<sup>‡</sup><sup>†</sup>Department of Chemistry, Renmin University of China, Beijing, 100872<sup>‡</sup>Food Chemistry, Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

S Supporting Information

**ABSTRACT:** Hydroxyl radical reacts readily with  $\beta$ -carotene following submicrosecond laser photolysis of *N*-hydroxypyridine-2(1*H*)-thione (N-HPT) as a “photo-Fenton” reagent generating hydroxyl and thiyl radicals in acetonitrile:tetrahydrofuran (4:1, v/v) solution. On the basis of spectral evidence, and supported by kinetic considerations and thermodynamic calculations, a short-lived transient species, detected by time-resolved absorption spectroscopy with an absorption maximum at  $\sim 750$  nm and a lifetime of  $\sim 150$  ns at 25 °C under anaerobic conditions, is suggested to be the long-sought neutral  $\beta$ -carotene radical formed by hydrogen-atom abstraction. The transient spectrum is different from the spectra of the  $\beta$ -carotene radical cation ( $\sim 1000$  nm absorption maximum with a millisecond lifetime), the  $\beta$ -carotene radical adducts ( $\sim 520$  nm, several microsecond lifetime), the  $\beta$ -carotene radical cation ion pair ( $\sim 750$  nm, several hundred microsecond lifetime), and the  $\beta$ -carotene radical anion ( $\sim 880$  nm, a few tens of microsecond lifetime). In parallel,  $\beta$ -carotene reacts with the thiyl radical to yield a sulfur radical adduct with absorption maximum at  $\sim 520$  nm with a lifetime of 3.0  $\mu$ s. For astaxanthin and canthaxanthin, the reaction with the thiyl radical dominates and the neutral radical is hardly formed in agreement with the less reducing properties of these 4,4'-diketo carotenoids without the reactive 4,4'-hydrogens.

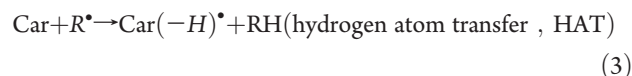


## 1. INTRODUCTION

Carotenoids (Car) are lipophilic and their biochemistry linked to the lipids and to the lipid/water interfaces including membranes. The increasing interest in the chemistry of both carotenoids, such as  $\beta$ -carotene, and xanthophylls, such as astaxanthin, relates to the many functions of these biomolecules with their rich structural diversity.<sup>1–4</sup> Many of these functions are only poorly understood, although carotenoids seem to be an integral part of the inherent defense against oxidative stress in plants and animals as quenchers of singlet oxygen and as radical scavengers.<sup>5,6</sup> As for radical scavenging, it is noteworthy that carotenoid backbones are almost exclusively all-trans conjugate polyenes and accordingly are linear molecules in marked contrast to the polyunsaturated lipids including phospholipids, which are nonconjugated and as cis-isomers have a bend structure. As part of membranes, carotenoids may accordingly scavenge radicals and act as linear transmitters of radicals in functions related to antioxidant protection of sensitive structures.

Theoretical calculations have demonstrated that some carotenoids such as  $\beta$ -carotene may be excellent electron donors, while others such as astaxanthin are good electron acceptors.<sup>7–12</sup> Superoxide radical ( $O_2^{\bullet-}$ ) may be oxidized and scavenged by astaxanthin, while most other reactive oxygen species such as peroxy radicals ( $ROO^{\bullet}$ ), alkoxyl radical ( $RO^{\bullet}$ ), and especially the hydroxyl radical ( $^{\bullet}OH$ ) are oxidizing and scavenged by  $\beta$ -carotene by reduction.<sup>10,13</sup>

As scavengers of oxidizing radicals, mainly three possible reaction pathways have been considered for carotenoids:



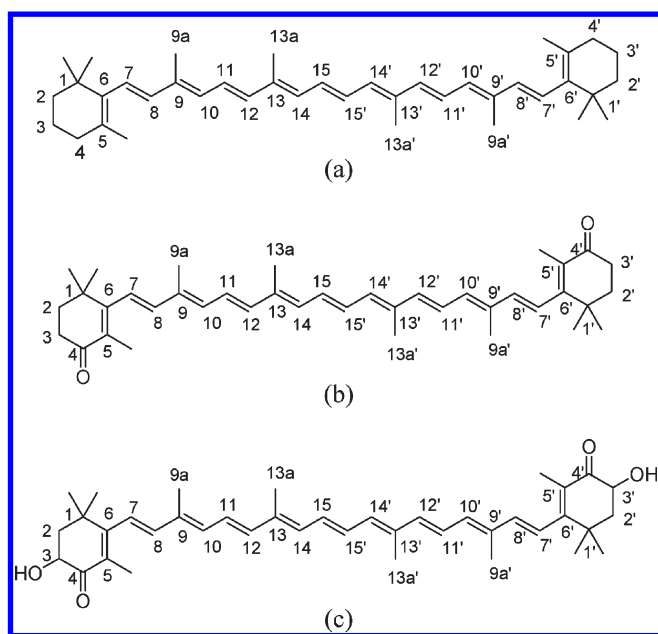
While spectral evidence has been presented for ET reactions leading to the carotenoid radical cation  $Car^{\bullet+}$  and for RAF with oxygen-centered radicals to carotenoid adduct,<sup>14–16</sup> HAT from carotenoids to oxidizing radicals to yield the neutral carotenoid radical  $Car^{\bullet}$  has only been suggested without spectral or any other direct evidence.<sup>16–19</sup> HAT is involved in oxidation of unsaturated lipids,<sup>20</sup> and a demonstration of hydrogen transfer from carotenoids to oxidizing radicals may contribute to a better understanding of carotenoids as antioxidants in membranes especially under extreme oxidative stress.

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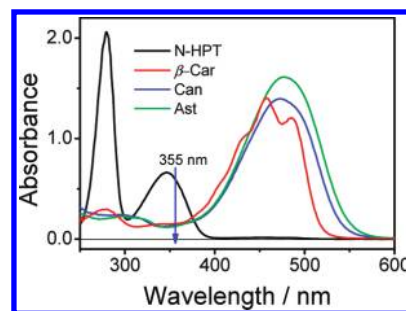
**Scheme 1. Molecular Structures of (a)  $\beta$ -Carotene, (b) Canthaxanthin, and (c) Astaxanthin**



We have undertaken measurement in real time, using laser flash photolysis and transient absorption spectroscopy of the reaction of  $\beta$ -carotene, canthaxanthin, and astaxanthin (the structures are shown in Scheme 1) with  $\cdot\text{OH}$  as generated by the “photo-Fenton” reagent *N*-hydroxypyridine-2(1*H*)-thione (N-HPT). N-HPT is a mild and clean source for hydroxyl radicals and has been used for the study of DNA damage and for induction of intercellular oxidative stress.<sup>21–24</sup> As N-HPT produces thiyl radicals concomitant with hydroxyl radicals, the time-resolved studies will also provide a description of scavenging of thiyl radicals by the three carotenoids. Since a number of thiyl radicals are known to enhance lipid peroxidation initiated by nitrogen dioxide,<sup>25</sup> and also to be involved in oxidation of human low density lipoprotein (LDL) by macrophages, any scavenging of such radicals by carotenoids may be an important antioxidant mechanism.<sup>26</sup> Thiyl radicals have also been found important in cross-linking of the muscle protein myosin.<sup>27</sup>

## 2. MATERIALS AND METHODS

**Sample Preparation.** *N*-hydroxypyridine-2(1*H*)-thione (N-HPT, >99%, Alfa Aesar, Tianjin, China) was recrystallized twice before use in a binary solvent of ethanol/water = 1:1 (v/v). HPLC grade acetonitrile (Caledon Laboratories, Georgetown, Ont., Canada) was used as received. Tetrahydrofuran (THF), purchased from Tianjin Guangfu Fine Chemical Research Institute (>99%, Tianjin, China), was distilled with tetrahydroaluminum lithium and dried by the use of 3 Å molecular sieves (Kishida Chemicals, Osaka, Japan) before use. Ethanol purchased from Beijing Chemical Plant (>99%, Beijing, China) was used as received. *all-trans*- $\beta$ -Carotene ( $\beta$ -carotene), canthaxanthin, and astaxanthin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Methyl methacrylate (MMA, 99%) and *tert*-butyl alcohol (*t*-BuOH, 99%) were purchased from Alfa Aesar Co., Ltd. (Tianjin, China).



**Figure 1.** UV–visible absorption spectra in acetonitrile:THF = 4:1 (v/v) binary solvent of N-HPT ( $1.3 \times 10^{-3}$  M),  $\beta$ -carotene ( $\beta$ -Car,  $1.0 \times 10^{-4}$  M), canthaxanthin (Can,  $1.0 \times 10^{-4}$  M), and astaxanthin (Ast,  $1.2 \times 10^{-4}$  M). Arrow indicates the excitation wavelength ( $\lambda_{\text{exc}} = 355$  nm).

**Laser Flash Photolysis.** The laser flash photolysis apparatus has been described elsewhere.<sup>28</sup> Briefly, the excitation laser pulses at 355 nm (3–5 mJ/pulse, 7 ns, 10 Hz) were supplied by a Nd<sup>3+</sup>:YAG laser (Quanta-Ray PRO-230; Spectra Physics), and the probe light was provided by a xenon-flash lamp synchronized to the electric gate of the ICCD detector (L7685, Hamamatsu Photonics, Hamamatsu City, Japan). The optical path length of the flow cuvette ( $\sim 20$  mL) used for laser flash photolysis was 1 mm. The anaerobic condition was achieved by bubbling the solution with high-purity argon for  $\sim 30$  min. The absorbance of N-HPT was adjusted to 0.6 at the excitation wavelength. The concentration of  $\beta$ -carotene, canthaxanthin, and astaxanthin was  $1.0 \times 10^{-4}$  M,  $1.0 \times 10^{-4}$  M, and  $1.2 \times 10^{-4}$  M, respectively. Solution samples were used immediately after preparation. By applying focused (diameter of laser spot = 2 mm) and unfocused (diameter of laser spot = 5 mm) conditions, thermal effects induced by the laser on the near-infrared (NIR) spectra were taken into account in data analysis. All of the measurements were carried out at room temperature (25 °C).

## 3. RESULTS

**3.1. Steady-State Absorption Spectroscopy.** Figure 1 shows the steady-state absorption spectra in acetonitrile:THF = 4:1 (v/v) binary solvent of N-HPT ( $1.3 \times 10^{-3}$  M),  $\beta$ -carotene ( $1.0 \times 10^{-4}$  M), canthaxanthin ( $1.0 \times 10^{-4}$  M), and astaxanthin ( $1.2 \times 10^{-4}$  M). The binary solvent was used to increase the solubility of carotenoids. At the excitation wavelength of 355 nm, N-HPT was excited predominantly ( $\sim 80\%$ ) in the mixed solution of N-HPT and carotenoids.

**3.2. Laser Flash Photolysis.** **3.2.1. Photochemistry of N-HPT.** It is well established that photoexcitation of N-HPT homolytically cleaves the N–O bond and gives rise to the 2-pyridylthiyl radical (PyS $\cdot$ ) and the hydroxyl radical ( $\cdot\text{OH}$ ) as shown below.<sup>29</sup>

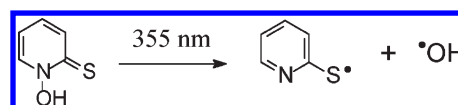


Figure 2a and 2b show the transient absorption spectra and the corresponding time evolution profiles for the N-HPT solution, respectively. No spectral features were observed in the NIR region. Previous flash photolysis studies with the same excitation wavelength of aqueous solutions of protonated N-HPT have ascribed a broad-band transient (400–550 nm,  $\Delta t < 100$  ns) to

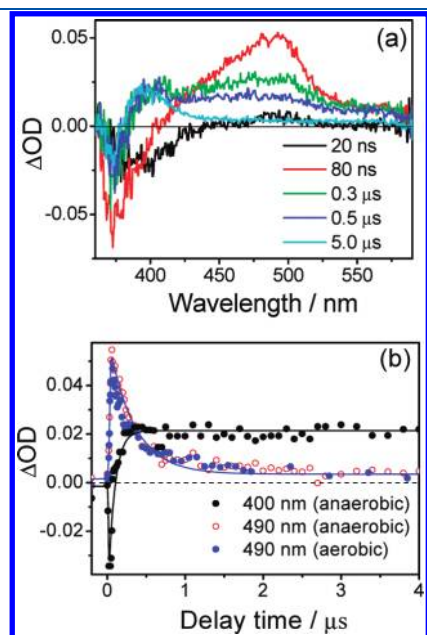
the superposition of N-HPT triplet excited-state  $^3(\text{N-HPT})^*$  and  $\text{PyS}^*$  absorption, and the formation and decay of  $\text{PyS}^*$  were found to be unaffected by oxygen.<sup>29,30</sup> In Figure 2b, the small reduction of the absorption amplitude at 490 nm in the presence of oxygen indicates the involvement of a small quantity of  $^3(\text{N-HPT})^*$ . The species absorbing at  $\sim 400$  nm observed at a later delay time had been identified as a stable product formed from  $\text{PyS}^*$  dimerization.<sup>24,29</sup> The time constants listed in Table 1, i.e. less than 100 ns rise and  $\sim 300$  ns decay, respectively, are obtained by fitting the kinetics at 400 and 490 nm with a monoexponential model function.

**3.2.2. Photochemistry of  $\beta$ -Carotene.** The transient spectra of  $\beta$ -carotene ( $1.0 \times 10^{-4}$  M) in acetonitrile:THF = 4:1 binary solvent in the visible (vis) and NIR regions recorded under anaerobic conditions are shown in Figure 3a and 3b. The negative feature at 450–520 nm in Figure 3a is apparently due to photochemical bleaching of  $\beta$ -carotene. The decay of the absorption band with a maximum at 516 nm is found sensitive to oxygen; therefore, this band is ascribed to the transient absorption of triplet excited-state  $\beta$ -carotene ( $^3\beta\text{-Car}^*$ ).<sup>31,32</sup> Note that the decay of the  $^3\beta\text{-Car}^*$  is not concomitant with the recovery of the ground-state bleaching. Kinetic analysis of the time evolution

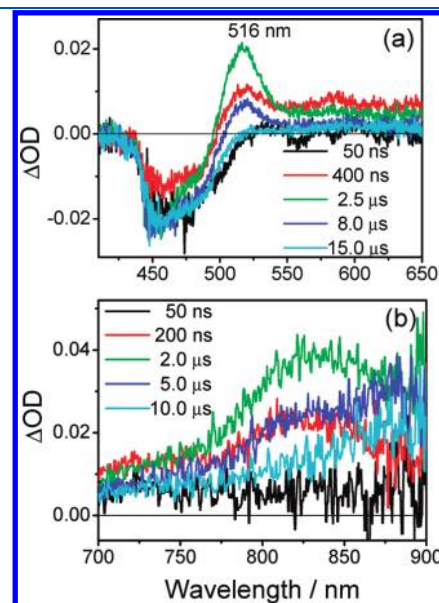
profile at 516 nm shows a  $\sim 1.5 \mu\text{s}$  rise time constant for  $^3\beta\text{-Car}^*$ , which is comparable with the  $\sim 5 \mu\text{s}$  slow formation known for the triplet excited state of zeaxanthin.<sup>31</sup> The presence of these triplet species for carotenoids with their low intersystem crossing yields under direct excitation have been explained by a stationary population of excited states created by the excitation pulses longer than their own lifetimes. Excited molecules may within their lifetimes subsequently interact with photons in the longer excitation pulses, and this kind of multiphoton excitation may lead to a more efficient triplet formation.<sup>31,33</sup>

In the NIR region (Figure 3b), the  $2.0 \mu\text{s}$  transient species with maximum absorption at  $\sim 830$  nm is assigned to the carotenoid/solvent complex,<sup>34,35</sup> which transforms into another species at  $10 \mu\text{s}$  with a characteristic absorption maximum at longer wavelengths ( $>900$  nm). This long-lived transient species absorbing in the NIR region and the unrecovered bleaching in the vis region at longer delay time were also found in previous investigations of both hydrocarbon carotenoids and xanthophylls in organic solvents.<sup>32,34,36</sup> This transient is assigned to the carotenoid radical cation ( $\beta\text{-Car}^{+\bullet}$ ) as a relatively stable intermediate oxidation product of  $\beta$ -carotene absorbing in the NIR region transformed from the initially formed  $\beta$ -carotene/solvent complex.<sup>35</sup>

**3.2.3. Reaction of  $\beta$ -Carotene with Excited N-HPT.** The vis and NIR transient spectra of N-HPT ( $1.3 \times 10^{-3}$  M) and  $\beta$ -carotene ( $1.0 \times 10^{-4}$  M) in the acetonitrile/THF (4:1, v/v)



**Figure 2.** (a) Transient absorption spectra of N-HPT ( $1.3 \times 10^{-3}$  M) in acetonitrile:THF = 4:1 (v/v) binary solvent following photoexcitation at 355 nm (3 mJ/pulse) under anaerobic conditions, and (b) corresponding time evolution profiles probed at 400 and 490 nm under aerobic or anaerobic conditions.



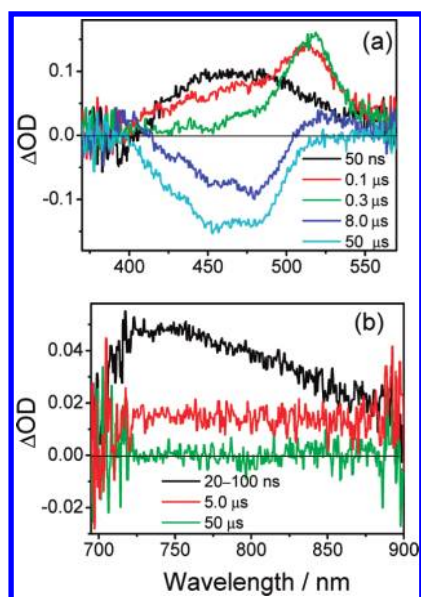
**Figure 3.** Transient absorption spectra of  $\beta$ -carotene ( $1.0 \times 10^{-4}$  M) in acetonitrile:THF = 4:1 (v/v) binary solvent following photoexcitation at 355 nm (4 mJ/pulse) in the (a) visible and (b) near-infrared spectral regions under anaerobic conditions.

**Table 1.** Rise and Decay Time Constants Derived from Exponential Fitting of Kinetics Traces in Figure 2b, Figure 5b, and Figure 7 at Selected Probing Wavelengths<sup>a</sup>

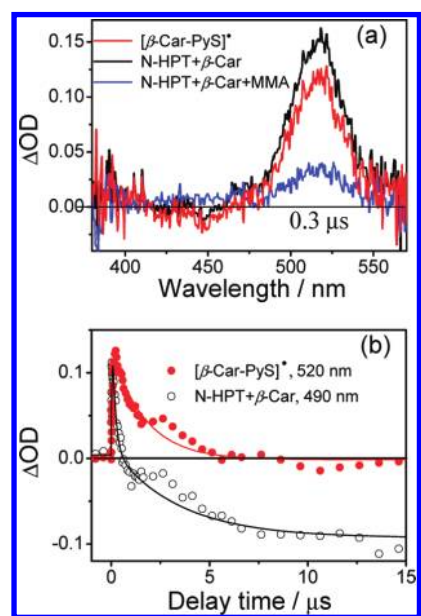
	400 nm (A)	490 nm (A)	490 nm (B)	520 nm (B)	750 nm (C)
N-HPT	$0.09 \pm 0.01 \mu\text{s}$ (f)	$0.30 \pm 0.01 \mu\text{s}$ (d)			
$\beta\text{-Car} + \text{N-HPT}$			$0.18 \pm 0.02 \mu\text{s}$ (d) $3.0 \pm 0.2 \mu\text{s}$ (f)	$0.06 \pm 0.02 \mu\text{s}$ (f) $3.0 \pm 0.2 \mu\text{s}$ (d)	$0.15 \pm 0.06 \mu\text{s}$ (d)

<sup>a</sup> Letters A, B, and C within parentheses refer to Figure 2b, Figure 5b, and Figure 7, respectively. Letters f and d within parentheses indicate “formation” and “decay” of absorption, respectively.





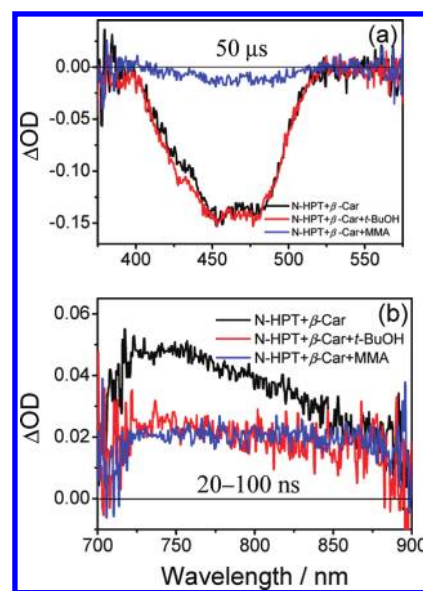
**Figure 4.** Transient absorption spectra in the (a) visible and (b) near-infrared spectral regions for  $\beta$ -carotene ( $1.0 \times 10^{-4}$  M) and N-HPT ( $1.3 \times 10^{-3}$  M) in acetonitrile:THF = 4:1 (v/v) binary solvent under anaerobic conditions. Unfocused laser beam was used for excitation ( $\lambda_{\text{exc}} = 355$  nm, 5 mJ/pulse).



**Figure 5.** (a) Transient absorption spectra at 0.3  $\mu$ s of solutions of  $\beta$ -carotene ( $\beta$ -Car,  $1.0 \times 10^{-4}$  M) and N-HPT ( $1.3 \times 10^{-3}$  M) in the absence or presence of methyl methacrylate (MMA, 2.0 M) and the spectrum of  $[\beta\text{-Car-PyS}]^*$  obtained as the difference between the spectrum recorded in the absence and in the presence of MMA, and (b) the corresponding time evolution profiles of  $[\beta\text{-Car-PyS}]^*$  at 520 nm and the bleaching at 490 nm in the absence of MMA following 355 nm excitation (5 mJ/pulse, unfocused) in acetonitrile:THF = 4:1 (v/v) binary solvent under anaerobic conditions.

binary solvent following 355 nm excitation under anaerobic conditions are shown in Figure 4a and 4b, respectively.

In the vis region, a broad transient absorption in the region 420–550 nm emerging immediately after the laser excitation

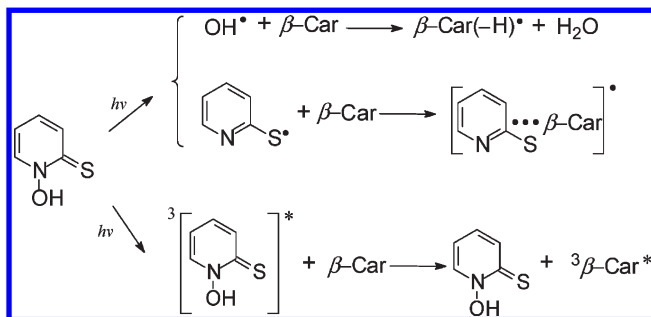


**Figure 6.** Transient absorption spectra at 50  $\mu$ s in the (a) visible and (b) near-infrared spectral regions of  $\beta$ -carotene ( $\beta$ -Car,  $1.0 \times 10^{-4}$  M) and N-HPT ( $1.3 \times 10^{-3}$  M) in acetonitrile:THF = 4:1 (v/v) binary solvent following 355 nm excitation (5 mJ/pulse, unfocused) in the absence or presence of MMA (2.0 M) or *t*-BuOH (2.0 M) under anaerobic conditions.

(50 ns-species in Figure 4a) can be ascribed as a superposed transient absorption of  $^3(\text{N-HPT})^*$  and  $\text{PyS}^*$ . The intensity of the absorption spectra at 500–550 nm is remarkably higher in the presence than in the absence of N-HPT and reached the maximum value around 0.3  $\mu$ s. Compared with the transient spectra of  $\beta$ -carotene in the absence of N-HPT shown in Figure 3a, a larger ground-state bleaching of  $\beta$ -carotene at 400–520 nm is observed at  $\sim 8$   $\mu$ s in the presence of N-HPT, which implies that  $\beta$ -carotene does interact with the reactive radicals generated from the photoexcitation of N-HPT.

A well-known thiyl radical scavenger, MMA,<sup>37,38</sup> was used to characterize the new transient species. The intensity of the 500–550 nm positive absorption at 0.3  $\mu$ s and the bleaching at 400–520 nm for 50  $\mu$ s are both found to be recovered to a high degree in the presence of MMA as seen in Figure 5a and Figure 6a, suggesting that the reaction of  $\beta$ -carotene with the reactive radicals generated from the photoexcitation of N-HPT is inhibited by MMA. Since it has been reported that  $^3\text{N-HPT}^*$  and  $^3\beta\text{-Car}^*$  are not quenched by MMA,<sup>29,37,38</sup> the observed component at 0.3  $\mu$ s in the presence of MMA (Figure 5a) may thus rather be attributed to the  $^3\beta\text{-Car}^*$  formed from  $^3(\text{N-HPT})^*$  sensitization<sup>29</sup> and/or from direct photoexcitation (Figure 3). The difference between the vis-spectra of  $\beta$ -carotene/N-HPT solution recorded in the absence and presence of MMA, as shown in Figure 5a, is assigned to absorption of a radical adduct  $[\beta\text{-Car-PyS}]^*$  formed by the addition of radical  $\text{PyS}^*$  to  $\beta$ -carotene. A similar adduct radical  $[\beta\text{-Car-PhS}]^*$  has also been reported by Ali et al. as a product of the reaction of the phenylthiyl radical ( $\text{PhS}^*$ ) with  $\beta$ -carotene.<sup>15</sup> Kinetic analyses at 520 nm as shown in Figure 5b indicate that the formation and decay time constants of the adduct radical product  $[\beta\text{-Car-PyS}]^*$  are  $\sim 60$  ns and  $\sim 3.0$   $\mu$ s, respectively; see Table 1. In addition, the decay of  $[\beta\text{-Car-PyS}]^*$  is also found to correlate with the slow formation of the 490-nm bleaching ( $\sim 3.0$   $\mu$ s) as seen in Figure 5b and Table 1, which indicates that the initially generated

**Scheme 2.** Proposed Reactions of  $\beta$ -Carotene with Hydroxyl Radical, Thiyl Radical, and the Triplet Excited State Generated from Photoexcitation of N-HPT

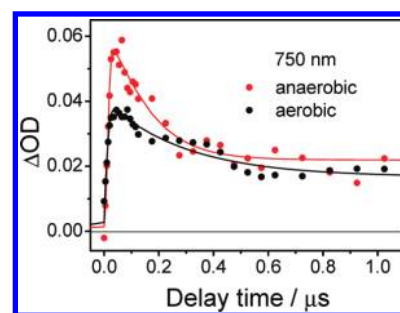


thiyl radicals product  $[\beta\text{-Car-PyS}]^\bullet$  further react with the ground-state  $\beta$ -carotene in a secondary reaction.

In the NIR spectral region, a short-lived species with maximum absorption at  $\sim 750$  nm is generated instantaneously after excitation (the species at 20–100 ns in Figure 4b). A new featureless absorption band covering the spectral region 700–900 nm appears within a few microseconds and decays out at  $\sim 50$   $\mu\text{s}$  as further seen in Figure 4b. The featureless spectra are merely due to thermal effects as concluded from a comparison of absorption obtained with the laser focused for varying degrees and for varying excitation energy in the presence or absence of radical scavengers (cf. Supporting Information and the following descriptions). Kinetic analysis for 750 nm as seen in Figure 7 indicates that the short-lived component decayed with a time constant  $150 \pm 60$  ns (Table 1). By comparison with the results shown in Figure 3b for excitation of  $\beta$ -carotene solution, it is evident that no carotenoid radical cation or carotenoid/solvent complex is observed for the  $\beta$ -carotene/NHPT solution (Figure 7), which further implies that electron transfer does not occur for  $\beta$ -carotene reacting with the radicals  $\text{PyS}^\bullet$  and  $^\bullet\text{OH}$ .

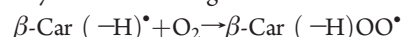
In order to identify the short-lived species with maximum absorption at 750 nm as seen in Figure 4b, *t*-BuOH, an efficient hydroxyl radical scavenger,<sup>29</sup> was used in addition to MMA. The transient absorption spectra at 50  $\mu\text{s}$  in the vis region and at 20–100 ns in the NIR region recorded in the absence and presence of either MMA or *t*-BuOH are presented in Figure 6a and 6b. Figure 6a shows that the presence of *t*-BuOH has no obvious influence on the bleaching of ground-state  $\beta$ -carotene in the vis region, but it leads to the disappearance of the 750-nm short-lived species (Figure 6b). Since *t*-BuOH cannot react with  $\text{PyS}^\bullet$  but does scavenge  $^\bullet\text{OH}$ , the 750-nm short-lived species accordingly can be ascribed to the product of  $^\bullet\text{OH}$  reacting with  $\beta$ -carotene. The fast formation ( $<10$  ns) and fast decay (150 ns) of this component absorbing at 750-nm (Figure 7) excludes that this species is produced in a reaction of  $\beta$ -carotene with  $\text{PyS}^\bullet$  ( $\sim 60$  ns time constant for the formation of  $[\beta\text{-Car-PyS}]^\bullet$ ). The short-lived 750-nm species is accordingly concluded to arise from the reaction of  $\beta$ -carotene with  $^\bullet\text{OH}$ . Aside from scavenging  $\text{PyS}^\bullet$  as described above, MMA is also an efficient  $^\bullet\text{OH}$  radical scavenger.<sup>39</sup> MMA is seen to quench the 750-nm species as was *t*-BuOH as shown in Figure 6b for the NIR absorption. The mechanism for the reaction of  $\beta$ -carotene with the excited N-HPT is summarized in Scheme 2.

A more detailed analysis indicates that the lifetime and spectral characteristics of the 750-nm species are clearly different from other



**Figure 7.** Time evolution profiles of absorption at 750 nm of  $\beta$ -carotene ( $1.0 \times 10^{-4}$  M) and N-HPT ( $1.3 \times 10^{-3}$  M) in acetonitrile:THF = 4:1 (v/v) binary solvent following 355 nm excitation under anaerobic and aerobic conditions (5 mJ/pulse, unfocused).

well-characterized oxidized form of  $\beta$ -carotene, i.e., the radical cation  $\beta\text{-Car}^{*\bullet}$  ( $\sim 1000$  nm, millisecond lifetime),<sup>35,40</sup> the radical adduct  $[\beta\text{-Car-R}]^\bullet$  ( $\sim 520$  nm, microsecond lifetime),<sup>16,25</sup> the radical cation ion pair  $[\beta\text{-Car}^{*\bullet}\text{-R}^-]$  ( $\sim 750$  nm, microsecond lifetime),<sup>16</sup> and the one-electron reduced radical anion,  $\beta\text{-Car}^{\bullet-}$  ( $\sim 880$  nm, microsecond lifetime).<sup>41</sup> The 750-nm component shown in Figure 6b is accordingly suggested to be the long-sought  $\beta$ -carotene neutral radical  $\beta\text{-Car}(-\text{H})^\bullet$  formed by rapid hydrogen atom abstraction (150 ns lifetime). A comparison of the time evolution profiles at 750 nm under anaerobic and aerobic conditions as shown in Figure 7 also agrees with the character of a neutral radical  $\beta\text{-Car}^\bullet$ , the decay of which is accelerated by the presence of oxygen probably via the following reaction:<sup>15</sup>

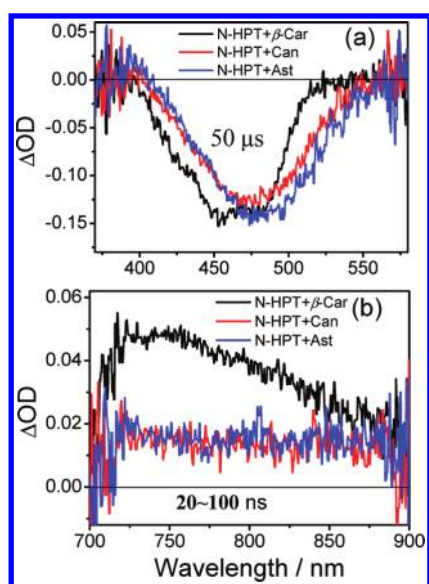


In addition, an indistinguishable difference in the bleaching at 50  $\mu\text{s}$  between  $\beta$ -carotene/N-HPT in the absence and in the presence of *t*-BuOH is found in Figure 6a. Excessive  $\text{PyS}^\bullet/^\bullet\text{OH}$  is seen to deplete almost completely the equivalent concentration of ground-state  $\beta$ -carotene. (Detailed calculation of the molecular proportion of  $\beta$ -carotene and  $\text{PyS}^\bullet/^\bullet\text{OH}$  is presented in Supporting Information). In the presence of *t*-BuOH, although most of  $^\bullet\text{OH}$  is scavenged, the longer lived  $\text{PyS}^\bullet$  radicals not being scavenged by *t*-BuOH react with approximately the equivalent amount of  $\beta$ -carotene, which in the absence of *t*-BuOH otherwise reacts with both  $\text{PyS}^\bullet$  and  $^\bullet\text{OH}$ .

**3.2.4. Reaction of Canthaxanthin and Astaxanthin with Excited-State N-HPT.** The reactions between carotenoids and  $^\bullet\text{OH}$  were further investigated by replacing  $\beta$ -carotene with canthaxanthin or astaxanthin. As seen in Figure 8a, the bleaching of the two xanthophylls canthaxanthin and astaxanthin in the vis region is similar, but a somewhat larger bleaching of  $\beta$ -carotene is obtained under the same experimental conditions.

The hydroxyl radical only reacts to a very small degree with canthaxanthin and astaxanthin compared to  $\beta$ -carotene as is evident from the NIR spectra shown in Figure 8b. This difference in reactivity between  $\beta$ -carotene and the two 4,4'-diketo xanthophylls further supports the assignment of the 750 nm short-lived species as the neutral  $\beta$ -carotene radical, since the 4,4'-hydrogen atoms, not present in the two xanthophylls, are the more reactive among the hydrogen atoms in  $\beta$ -carotene with the smallest BDE as shown by quantum mechanical calculations.<sup>9</sup>

Based on the extinction coefficients of the steady state and the bleaching seen in Figure 8, the relative efficiency of three carotenoids reacting with  $\text{PyS}^\bullet$  is  $\beta$ -carotene:canthaxanthin:astaxanthin = 1.15:1.02:1.00. This result agrees well with the

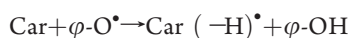


**Figure 8.** Transient absorption spectra in acetonitrile:THF = 4:1 (v/v) binary solvent of  $\beta$ -carotene ( $\beta$ -Car,  $1.0 \times 10^{-4}$  M), canthaxanthin (Can,  $1.0 \times 10^{-4}$  M), and astaxanthin (Ast,  $1.2 \times 10^{-4}$  M) each in the presence of N-HPT ( $1.3 \times 10^{-3}$  M) in the (a) visible and (b) near-infrared spectral regions following 355 nm excitation (5 mJ/pulse, unfocused) under anaerobic conditions.

reported less reactivity for astaxanthin and canthaxanthin to form adduct with  $\text{PyS}^\bullet$  as compared to  $\beta$ -carotene and zeaxanthin, when reacting through the RAF mechanism.<sup>42</sup>

#### 4. DISCUSSION

Among the three reaction pathways available for radical scavenging of  $\beta$ -carotene and other carotenoids, the electron transfer leading to the carotenoid radical cation appears to be the most well-documented. The NIR absorption around 1000 nm provides firm evidence for this long-lived and moderately oxidizing radical species which may be generated chemically, by radiolysis or photochemically in electron-accepting solvents such as chloroform.<sup>6,32,34,35,40</sup> The radical cation seems to be formed through a precursor absorbing with a maximum at 920 nm for  $\beta$ -carotene and in the same spectral region for other carotenoids.<sup>35</sup> Reaction of  $\beta$ -carotene with radicals such as trichloromethylperoxyl and phenoxyl radicals ( $\varphi\text{-O}^\bullet$ ) leads to a radical adduct absorbing at wavelength slightly shorter than the radical cation,<sup>40,43</sup> although it has been argued that this absorption is rather due to the neutral radical formed by hydrogen atom abstraction:



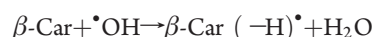
The evidence now presented from time-resolved studies of the hydroxyl radical reaction with  $\beta$ -carotene to yield a very short-lived species absorbing with a maximum at  $\sim 750$  nm, however, supports the suggestion of adduct formation (920 nm) upon reaction of  $\beta$ -carotene with phenoxyl radicals, since the  $\beta$ -carotene neutral radical clearly absorbs at lower wavelengths (750 nm). Adduct formation is also suggested for reaction of  $\beta$ -carotene with peroxy radicals of linoleic acid, as formed in lipid autoxidation,<sup>2</sup> and in reaction with the simplest peroxy radical,  $^\bullet\text{OOH}$ .<sup>8</sup> A comparison between the thermodynamics of the reaction of  $^\bullet\text{OH}$  and  $^\bullet\text{OOH}$  with  $\beta$ -carotene may be useful for a further discussion.

**Table 2.** Gibbs Free Energies ( $\Delta G$ , kJ/mol) for the HAT, ET, and RAF Reaction of  $\beta$ -Carotene with  $^\bullet\text{OH}$  and  $^\bullet\text{OOH}$

reaction mechanism		$\Delta G$ (kJ/mol)	
		$R = ^\bullet\text{OH}$	$R = ^\bullet\text{OOH}$
HAT	$\beta\text{-Car} + \text{R}^\bullet \rightarrow \beta\text{-Car}(-\text{H})^\bullet + \text{HR}$	$-214^a$	$-52^b$
ET	$\beta\text{-Car} + \text{R}^\bullet \rightarrow \beta\text{-Car}^{\bullet+} + \text{R}^-$	$-71^c/-69^d$	$-17^c/19.7^d$
RAF	$\beta\text{-Car} + \text{R}^\bullet \rightarrow [5\text{-}\beta\text{-Car}\cdots\text{R}]^\bullet$	$-142^e$	$-38^b$

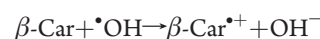
<sup>a</sup> Calculated from bond dissociation energy (BDE) reported in refs 2 and 44. <sup>b</sup> Reference 8. <sup>c</sup> Calculated from standard reduction potentials reported in refs 45, 46, and 48. <sup>d</sup> Reference 47. <sup>e</sup> Reference 42.

Reaction Gibbs free energies for reaction of  $\beta$ -carotene with  $^\bullet\text{OH}$  and  $^\bullet\text{OOH}$  for the HAT, ET, and RAF reaction channels are summarized and listed in Table 2. For the HAT reaction of  $\beta$ -carotene with  $^\bullet\text{OH}$ ,



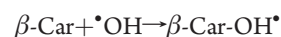
$\Delta G = -214 \text{ kJ}\cdot\text{mol}^{-1}$  is calculated from BDE ( $285 \text{ kJ}\cdot\text{mol}^{-1}$  for C–H in  $\beta$ -carotene at C-4 and C-4'<sup>2</sup> and  $499 \text{ kJ}\cdot\text{mol}^{-1}$  for H–O in  $\text{H}_2\text{O}^{44}$ ).

For the ET reaction of  $\beta$ -carotene with  $^\bullet\text{OH}$ ,



$\Delta G = -71 \text{ kJ}\cdot\text{mol}^{-1}$  is calculated from the standard reduction potentials  $E^\circ = 1.06 \text{ V}$  for  $\beta\text{-carotene}^{\bullet+}/\beta\text{-carotene}^{45}$  and  $E^\circ = 1.8 \text{ V}$  for  $^\bullet\text{OH}/\text{OH}^-$ ,<sup>46</sup> which is quite similar to  $-69 \text{ kJ}\cdot\text{mol}^{-1}$  as obtained by theoretical calculations.<sup>47</sup>

For the RAF reaction of  $\beta$ -carotene with  $^\bullet\text{OH}$ ,



$\Delta G \approx -142 \text{ kJ}\cdot\text{mol}^{-1}$  was obtained from quantum mechanical calculations for addition to C-5, identified as the most reactive site.<sup>42</sup>

On the basis of above descriptions, the driving force for HAT reaction is evidently larger than for ET and RAF. A similar difference is also seen for the reaction of  $\beta$ -carotene with  $^\bullet\text{OOH}$  radical as shown in Table 2.

For the HAT reaction of  $\beta$ -carotene with  $^\bullet\text{OOH}$ , a value of  $\Delta G \approx -52 \text{ kJ}\cdot\text{mol}^{-1}$  is obtained from quantum mechanical calculations.<sup>8</sup> For the ET reaction,  $\Delta G \approx -17 \text{ kJ}\cdot\text{mol}^{-1}$  is similarly obtained from the standard reduction potentials  $E^\circ = 1.06 \text{ V}$  for  $\beta\text{-carotene}^{\bullet+}/\beta\text{-carotene}$  and  $E^\circ = 1.24 \text{ V}$  for  $^\bullet\text{OOH}/\text{H}_2\text{O}_2$ ,<sup>48</sup> while  $\Delta G \approx 19.7 \text{ kJ}\cdot\text{mol}^{-1}$  in Table 2 is obtained from quantum mechanical calculations.<sup>47</sup> RAF as the third reaction channel seems, however, also important with  $\Delta G \approx -38 \text{ kJ}\cdot\text{mol}^{-1}$ , as based on quantum mechanical calculations.<sup>8</sup> Considering the results of these calculations for reactions of  $\beta$ -carotene with  $^\bullet\text{OH}$  and  $^\bullet\text{OOH}$ , the driving forces for HAT and RAF reactions are significantly larger than for ET reaction. The HAT and RAF reactions are accordingly more thermodynamically favorable than the ET reaction. The ET reaction of  $\beta$ -carotene with peroxy radicals is even found endergonic ( $\Delta G > 0$ ).<sup>7,47</sup>

Although the driving force for the HAT reaction of  $\beta$ -carotene with  $^\bullet\text{OOH}$  is larger than for the RAF reaction as seen in Table 2, a further transformation from the adduct  $[\beta\text{-Car-OOH}]^\bullet$  to endoperoxides is possible, in effect favoring this type of reaction over the HAT reaction.<sup>2,42</sup> Rate constants for  $\beta$ -carotene reaction with  $^\bullet\text{OOH}$  calculated using Transition State Theory demonstrated a reaction nearly 2 orders of magnitude faster for the



RAF reaction than for the HAT reaction.<sup>8</sup> RAF is accordingly expected to make the main contribution to the overall process for reaction between  $\cdot\text{OOH}$  and  $\beta$ -carotene. Notably, adduct formation between  $\cdot\text{OH}$  and  $\beta$ -carotene will not be favored through a subsequent reaction as seen for the  $\cdot\text{OOH}$  adduct formation. During the course of hydrogen abstraction by  $\cdot\text{OH}$ , the water molecule formed as the leaving group may, however, coordinate transiently to the carbon radical of  $\beta$ -carotene and may reduce the activation energy of the transition state. Larger driving force and favored dynamics may accordingly together lead to oxidation of  $\beta$ -carotene preferably through a HAT mechanism.

The lack of reaction between  $\cdot\text{OH}$  and canthaxanthin or astaxanthin to form the neutral carotenoid radical is understood by the absence of the reactive 4,4'-hydrogen atoms, which in these xanthophylls are substituted with keto groups. This substitution of keto groups for the labile hydrogens in canthaxanthin and astaxanthin may have two interrelated effects: (i) the hydrogen atom with the lowest BDE in  $\beta$ -carotene are not available for reaction in the 4,4'-ketoxanthophylls, and (ii) due to the presence of the two electron-withdrawing keto groups in conjugation with the carotenoid polyene backbone, canthaxanthin and astaxanthin are electron acceptors rather than electron donors in contrast to  $\beta$ -carotene and accordingly both less reducing.<sup>11</sup>

The  $\beta$ -carotene neutral radical is rather short-lived, but the fate of the molecule is not clear and cannot simply be deduced from the spectral changes, since the bleaching of  $\beta$ -carotene, astaxanthin, and canthaxanthin to a larger degree is due to the nonreversible reaction with the  $\text{PyS}^\bullet$  radical. It may be speculated whether the neutral radical is abstracting a hydrogen atom from a solvent molecule to reform  $\beta$ -carotene or combine with another  $\beta$ -carotene radical. Provided the short lifetime of the  $\beta$ -carotene neutral radical, any dimerization reactions, however, seem unlikely because of the bimolecular nature of such a reaction between two radicals.

The  $\beta$ -carotene neutral radical has been long-sought. The evidence now presented based on transient absorption spectroscopy of the reaction product between  $\beta$ -carotene and photochemically generated hydroxyl radical further allows reassignment of spectrally characterized intermediates in  $\beta$ -carotene radical reactions as adducts between  $\beta$ -carotene and radicals rather than the neutral radical of  $\beta$ -carotene.<sup>16–18</sup> Neutral carotenoid radicals are apparently only formed when the most reducing carotenoids are exposed to the most oxidizing radicals, such as the hydroxyl radical, while for other combinations of carotenoids and radicals, other reaction pathways become dominating. The neutral radical of  $\beta$ -carotene and other more reducing carotenoids such as lycopene may, however, be important, short-lived intermediates in radical communication reactions under oxidative stress in membrane structures and should be examined in future investigations of the role of carotenoids as protectors against extreme oxidative stress.

## ■ ASSOCIATED CONTENT

**S Supporting Information.** The comparisons of the long-lived and structureless absorption in the NIR region (thermal effect) under the laser focused for varying degrees and for varying excitation energy. Calculated molecular ratio of the ground-state  $\beta$ -carotene and the reactive radicals ( $\text{PyS}^\bullet$  and  $\cdot\text{OH}$ ) under present experimental conditions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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