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Original Contribution

INCLUSION COMPLEXES OF CAROTENOIDS WITH CYCLODEXTRINS: ^{1}H NMR, EPR, AND OPTICAL STUDIES

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Abstract—Direct evidence of carotenoid/cyclodextrin inclusion complex formation was obtained for the water-soluble sodium salt of β-caroten-8′-oic acid (**IV**) by using ^{1}H NMR and UV-Vis absorption spectroscopy. It was shown that this carotenoid forms a stable 1:1 inclusion complex with β-cyclodextrin (stability constant $K_{11} \approx 1500\ M^{-1}$). All other carotenoids under study in the presence of cyclodextrins (CDs) form large aggregates in aqueous solution as demonstrated by very broad absorption spectra and considerable change in color. By using the EPR spin trapping technique, the scavenging ability of **IV** toward OOH radicals was compared in DMSO and in the aqueous CD solution. A considerable decrease in PBN/OOH spin adduct yield was detected in the presence of uncomplexed **IV** because of a competing reaction of the carotenoid with OOH radical. No such decrease occurred in the presence of the **IV**/CD complex. Moreover, a small increase in spin adduct yield (pro-oxidant effect) is most likely due to the reaction of the carotenoid with Fe^{3+} to regenerate Fe^{2+}, which in turn regenerates the OOH radical. Our data show that CD protects the carotenoid from reactive oxygen species. On the other hand, complexation with CD results in considerable decrease in antioxidant ability of the carotenoid.

Keywords—Cyclodextrin, Hydroxypropyl cyclodextrin, Inclusion complex, Carotenoid, Antioxidant, Spin trap, Fenton reaction, Pro-oxidant effect, Free radicals

INTRODUCTION

Cyclodextrins (CDs) are natural macrocyclic oligosaccharides, formed by six, seven, or eight glucopyranose units called α-, β-, or γ-CD, respectively. They have torus-shaped structures with rigid lipophilic cavities. CDs are able to form host–guest inclusion complexes with suitably sized hydrophobic molecules. The guest molecules encapsulated by CDs may change their physical, chemical, and biological properties (for details, see reviews [1–3]). In the pharmaceutical, cosmetics, and food industries, cyclodextrins have been used primarily as complexing agents to increase the water solubility of various compounds, such as drugs, vitamins, and food colorants (for reviews see [4–6]). It was demonstrated that complexation can considerably increase the stability and bioavailability of the guest molecules. Cyclodextrins can be used to reduce gastrointestinal and ocular irritation, to eliminate unpleasant odors or taste, and to prevent drug-additive interactions. The capability of CDs for molecular recognition and separation of enantiomers is of fundamental importance for designing artificial enzymes [7,8].

Because carotenoids, essential for human life, are natural, highly hydrophobic, air- and light-sensitive compounds, developing methods for increasing their bioavailability and stability toward irradiation, reactive oxygen, and other radical species is essential. Despite wide application of carotenoid/CD complexes in the food, cosmetics, and pharmaceutical industries [9–11], there still is no strong evidence of real inclusion

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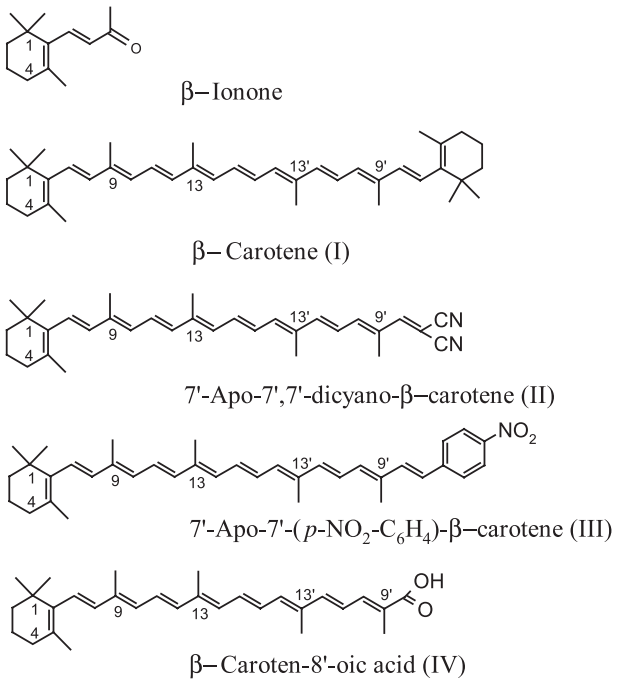
complex formation, and only a few attempts at structural studies of such complexes have been reported [12,13]. Previous studies of short-chain analogues of carotenoids, β-ionone [14], and retinoids [15] demonstrated the formation of stable inclusion complexes of these substrates with different cyclodextrins (β-CD), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), and 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD). It was shown that the terminal cyclohexene fragment of β-ionone, which is present in most carotenoids (see Scheme 1), has the requisite size for incorporation into the CD cavity. It seemed likely that carotenoids, which are even more hydrophobic, should also form inclusion complexes with CDs.

This paper focuses on two aspects. Our first goal was to obtain direct evidence of inclusion complex formation. For this purpose complexes of various carotenoids with different CDs were investigated by ^{1}H NMR and UV-Vis absorption spectroscopy. The second aim was the investigation of the reactivity of carotenoid/CD inclusion complexes toward peroxyl radicals (antioxidant activity). It has been reported that β-carotene and other carotenoids react with peroxyl radicals primarily at the 4-C position of the cyclohexene ring (see Scheme 1) [16–18]. One can expect that the reactivity of carotenoids toward free radicals may decrease if the cyclohexene ring is embedded in the CD cavity. This investigation will be beneficial for application of the carotenoid/CD complexes to protect carotenoids against damage caused by O_2 and free radicals.



Scheme 1. The structures of β-ionone and carotenoids **I-IV**.

MATERIALS AND METHODS

Chemicals

β-Carotene (**I**) was supplied by Sigma, 7′-apo-7′,7′-dicyano-β-carotene (**II**), 7′-apo-7′-(*p*-NO_2-C_6H_4)-β-carotene (**III**), and β-caroten-8′-oic acid (**IV**) were synthesized as previously described [19,20]. Purity of the carotenoids was checked by ^{1}H NMR (360 MHz, $CDCl_3$) spectroscopy. The compounds were stored at −16 °C in the dark in a desiccator containing Drierite or in ampoules sealed in vacuo. The solvents CH_2Cl_2, MeOH (Fisher, ACS), and dimethyl sulfoxide (DMSO) (99.5%, Aldrich, ACS) were used as received. Hydrogen peroxide (30% aqueous solution; Fisher, ACS) and $FeCl_2$ (Aldrich) were used to prepare the Fenton reagent. The spin trap *N-tert*-butyl-α-phenylnitrone (PBN) (98%) was obtained from Aldrich.

Deuterated solvents CH_3OD, $CDCl_3$, and D_2O (Aldrich) were used as supplied. β-Cyclodextrin, 2-hydroxypropyl-β-cyclodextrin (DS 4.6), and 2-hydroxypropyl-γ-cyclodextrin (MS) were supplied by CarboMed, Inc.

Radical generation

We generated OOH radicals by means of the well-known Fenton reaction [21–25]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + HO^{-}.$$

Depending on experimental conditions, different radical species can be generated through the Fenton process [16]. Thus, when hydrogen peroxide is in excess, the initial $^{\bullet}$OH radicals will produce secondary $^{\bullet}$OOH radicals. Identification of spin adducts was made by using the Spin Trap Database [26].

Apparatus

EPR measurements were carried out with an X-band (9.5 GHz) Varian E-12 EPR spectrometer, equipped with a rectangular cavity. The magnetic field was measured with an ER 035M gaussmeter, and the microwave frequency was measured with a Hewlett–Packard 5245M frequency counter. UV-Vis absorption spectra were recorded with a Shimadzu UV-1610 spectrophotometer. ^{1}H NMR spectra of CDs and their complexes were detected using Bruker NMR spectrometers AM-360 and AM-500 (360 and 500 MHz ^{1}H operating frequency).

EPR sample preparation

Solutions of all reagents in CH_2Cl_2 or DMSO were freshly prepared and purged with N_2. Complex concentrations of about 1 mM were used for EPR experiments.

Solutions of carotenoid, PBN, and H_2O_2, prepared separately, were mixed just before measurement. The concentration of H_2O_2 was 500 mM. The reaction was started by addition of $FeCl_2$ solution (1 mM in CH_2Cl_2). The final mixture was then transferred to the stop-flow EPR tube by means of a Hamilton syringe, and the spectra were recorded several times within 1–6 min after mixing.

Preparation of inclusion complexes

Two methods of complex preparation, described in the literature, were used in the present work [9–12]. In the first method, "solid mixture" (SM), solid carotenoid and requisite amounts of CD (1 or 2 equivalents) were ground until a homogeneous powder was obtained. Grinding was continued after a small amount of deionized water was added to give a paste, which was then stored overnight under nitrogen, treated with water to obtain a final carotenoid concentration of 1 mM, and stirred for several hours. In the second method, "liquid mixture," the solution of carotenoid in methanol (or other organic solvent) was added to the aqueous CD solution. This procedure has been described in detail in a number of patents [9–11].

Calculation of association constants and stoichiometry of inclusion complexes

The variation in NMR chemical shifts of CD molecules as a function of complex is widely used to calculate inclusion complex stoichiometry and association constants [27,28]. The equilibrium formation of the *n*:*m* complex (C_{nm}) between cyclodextrin (CD) and guest (G) molecules is represented by

$$m\mathrm{G}+n\mathrm{CD} \leftrightarrows C_{nm}. \quad (1)$$

The association constant of this complex is described by

$$K_{nm} = \frac{[C_{nm}]}{[\mathrm{G}]^m[\mathrm{CD}]^n}. \quad (2)$$

In the case of a 1:1 complex the value K_{11} can be obtained using Eq. (3) [27] from the dependence of cyclodextrin chemical shift $\Delta\delta_{obs}$(CD) on the G_0 concentration:

$$\Delta\delta_{\mathrm{obs}} = \frac{\Delta\delta_C}{2[\mathrm{CD}]_0}\left\{[\mathrm{G}]_0 + [\mathrm{CD}]_0 + \frac{1}{K_{11}} - \left(\left([\mathrm{G}]_0 + [\mathrm{CD}]_0 + \frac{1}{K_{11}}\right)^2 - 4[\mathrm{G}]_0[\mathrm{CD}]_0\right)^{1/2}\right\}. \quad (3)$$

Here, $\Delta\delta_C = \delta_C - \delta_{CD}$ is the change in chemical shift of fully complexed CD relative to the free CD molecule. Experimental data, $\Delta\delta_{obs}$(CD), were measured at varying carotenoid (Car) concentrations and a constant concentration of CD_0. A small amount of CH_3OH (~1 mM) was added to all samples as reference. It has been shown [29] that methanol is a good internal reference due to its low association constant with cyclodextrins [30]. The stoichiometry of the carotenoid/CD inclusion complex was obtained by the continuous variation technique (Job's plot) [31–33]. For this purpose, solutions with different CD/Car molar ratios were prepared. The total molar concentration was kept constant for all samples.

RESULTS AND DISCUSSION

UV-Vis absorption study

The solutions of all carotenoid/CD complexes, prepared in H_2O by the SM method (see Materials and Methods), show a considerable change in color compared to carotenoid solutions in organic solvents (with the exception of **IV**, all carotenoids are completely insoluble in water). For example, the β-carotene/CD complex aqueous solution is an intense opalescent pink-orange. The **II**/CD complex is black, whereas the MeOH and CH_2Cl_2 solutions of **II** are violet. All complexes have a very broad absorption band up to 1100 nm with reduced intensity (about 1 order of magnitude). We suggest that the broadening of the absorption band is due to aggregation of complexes in aqueous solution. Indeed, such aggregate formation was previously detected by light-scattering spectroscopy [12,13]. Assuming that only a cyclohexene ring of the carotenoid can be embedded in the CD cavity, we suggest that aggregates of the complexes have a micelle-like structure in the aqueous media.

Aggregate formation could be detected from the UV-Vis spectral changes when separately prepared solutions of CD in H_2O and carotenoid in EtOH were mixed. Similar effects were observed for the highly water soluble HP-β-CD and HP-γ-CD used in UV-Vis experiments. The maximum absorption of Car/CD complexes in water shifts to shorter wavelength was compared to that of the pure carotenoids in ethanol. The most significant blue shifts were observed for carotenoids with polar terminal groups: CN, Ph-NO_2, COOH. For example, carotenoid **III** shows a blue shift of the absorption maximum from 485 nm in ethanol to 315 nm in water with simultaneous considerable decrease of extinction coefficient. The spectrum of the Car/CD mixture gradually changed, attributable to aggregate formation. When a large excess (50 eq.) of HP-β-CD was used, the changes with time were significantly attenuated (see Fig. 1). This is consistent with the

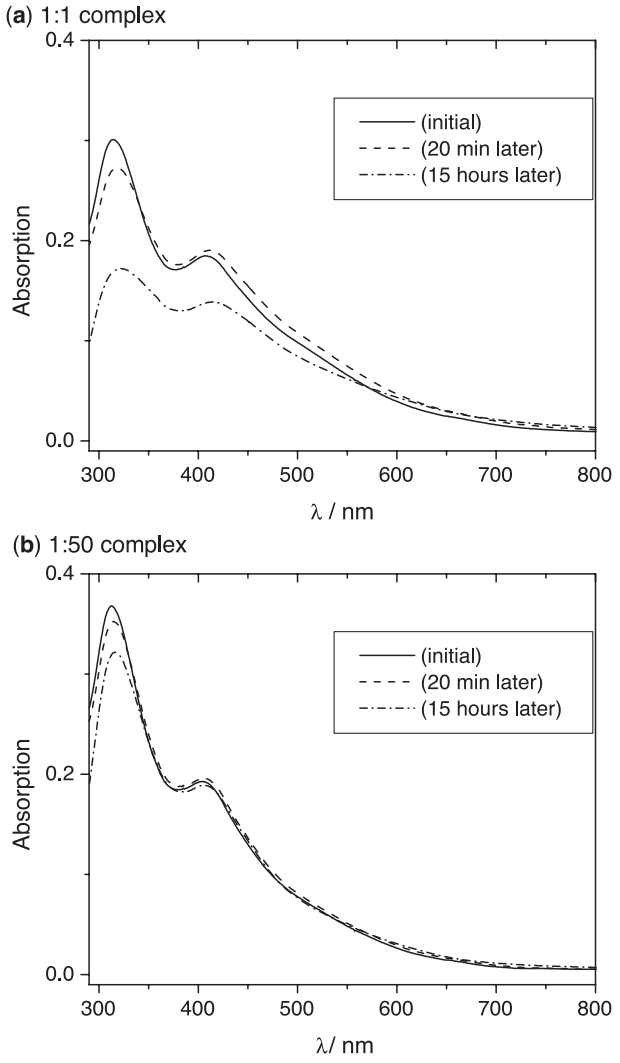


Fig. 1. Changes in UV-Vis absorption spectra of **III**/HP-β-CD (a) 1:1 and (b) 1:50 in water:ethanol mixture as a function of time.

existence of an equilibrium between individual complexes and aggregates of the complexes. Compounds with other polar groups (**II** and **IV**) showed similar behavior, an increase in complex solubility with increase in CD concentration.

However, nonpolar β-carotene (**I**) displayed divergent spectral changes (Fig. 2). No large blue shift was observed in this case (from 450 nm in EtOH to 440 nm in 1:1 H_2O:EtOH), and the presumed equilibrium favors the formation of complex aggregates. According to published phase-solubility diagrams [34], there are two types of complexes. The first type demonstrates the increase in solubility with increased CD concentration; the second type shows the opposite effect. Figures 1 and 2 show that both types of complexes take place for the carotenoids under study. Different behaviors of various carotenoids could be explained by the difference in their structure, namely the structure of terminal groups. In other words, different types of complexes predominate for polar and nonpolar carotenoids. Assuming that the Car/CD complex has a hydrophilic CD head and hydrophobic carotenoid tail, we propose formation of more stable aggregates for complexes with nonpolar carotenoids.

The aggregation process and low complex solubility precluded elucidation of the structure of these Car/CD complexes. However, it is known that the solubility of CD complexes with organic acids can be increased dramatically by changing the pH of the solution [35]. In this work the partly water-soluble acidic carotenoid (**IV**) was used. Indeed, the presence of 4 mM NaOH in 2 mM aqueous complex solution resulted in sufficiently increased complex solubility so that the monomers of the **IV**/CD complex could be observed by the UV-Vis as well

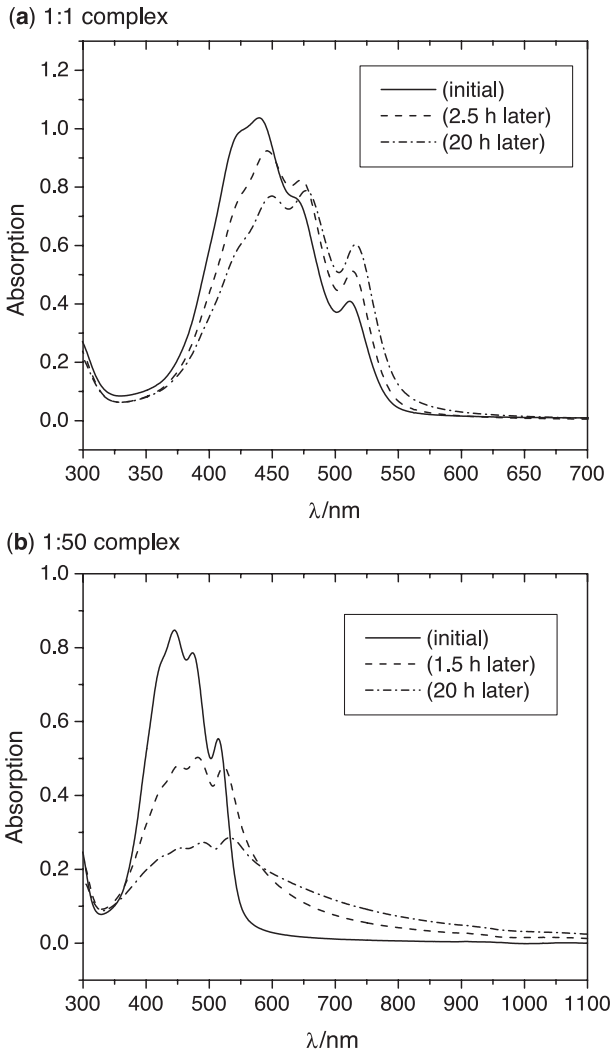


Fig. 2. Changes in UV-Vis absorption spectra of β-carotene/HP-β-CD (a) 1:1 and (b) 1:50 in water:ethanol mixture as a function of time.

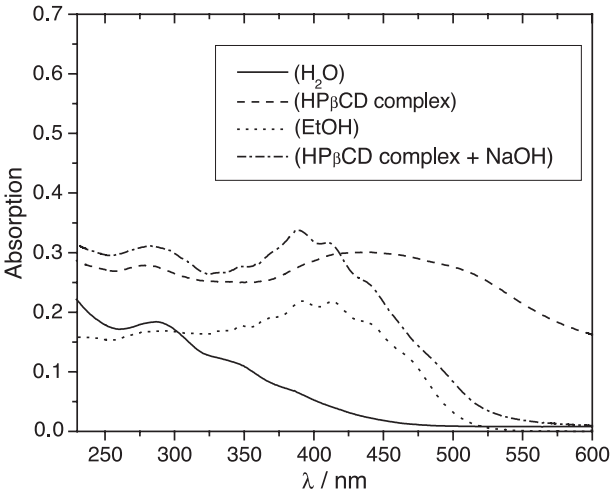


Fig. 3. UV-Vis absorption spectra of **IV** in different solvents. The concentrations of carotenoid were different in each case.

as the NMR technique. The absorption maximum of **IV** in water shows a strong blue shift compared to that in ethanol: from λ_{max} = 400 to 285 nm. The UV-Vis spectrum of the **IV**/CD complex in the presence of NaOH is nearly identical to that of **IV** in ethanol (see Fig. 3). Because it is known that the polarity of the CD interior is similar to that of an ethanolic solution [2], this observation might be considered proof of carotenoid incorporation within the CD cavity. In a control experiment, no changes were observed in the UV-Vis absorption spectrum of **IV** in H_2O in the absence of CD after addition of NaOH.

^{1}H NMR study

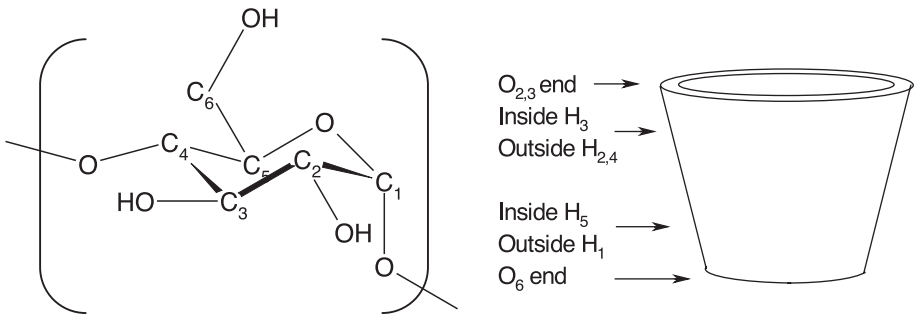
Direct evidence of inclusion complex formation can be obtained from ^{1}H NMR experiments, which can provide information about the stoichiometry, stability, and structure of CD complexes [1–3,31]. In particular, Job's plot, which correlates the chemical shift and the guest/CD ratio, has been widely used to determine complex stoichiometry [32,33]. If a guest molecule is incorporated into the CD cavity, the screening constants of the CD protons inside the cavity (H_3 and H_5) should be sensitive to the changed environment, but that of the outside protons (H_1, H_2, and H_4) should not. This should result in chemical shift changes of the inside protons (see Scheme 2).

We did not observe changes in the NMR spectrum of CD complexes with carotenoids **I**–**III** probably due to the very low solubility of these complexes in water even for the highly soluble HP-β-CD and HP-γ-CD. Attempts to use other organic solvents, DMSO or methanol, were also unsuccessful. Our previous experiments with β-ionone complexes showed that organic solvents destroy the complex. As an example, in 50% aqueous methanol the stability constant of the β-ionone complex with HP-β-CD is decreased by almost 2 orders of magnitude. As stated above, sufficiently high concentrations of carotenoid/CD complex (1 mM) could be obtained only for the acid **IV** in the presence of NaOH. Also, for NMR experiments only β-CD was used for carotenoid/CD complex preparation. NMR studies showed the shift of the internal protons of the CD cavity in the presence of **IV** (Fig. 4 and Scheme 2).

Job's plot (Fig. 5) shows that **IV** forms a 1:1 complex with β-CD. This was concluded from the position of the maximum at R = 0.5, which corresponds to the 1:1 complex stoichiometry. Figure 6 shows the dependence of CD chemical shift on carotenoid concentration at constant CD concentration from which the value of the association constant K_{11} = 1536 ± 75 M^{-1} was extracted using Eq. (3). This is the first direct evidence of inclusion complex formation of a carotenoid.

EPR study of scavenging ability of carotenoids toward peroxyl radicals

The scavenging ability of carotenoid **IV** toward OOH radicals in the absence and presence of CDs was also investigated. In previous studies, the EPR spin trapping technique was applied to measure the scavenging ability of a set of carotenoids toward free radicals [16,36]. The



Scheme 2. A macrocyclic oligosaccharide, cyclodextrin, forming a torus-shaped structure of cyclodextrins with rigid lipophilic cavities.

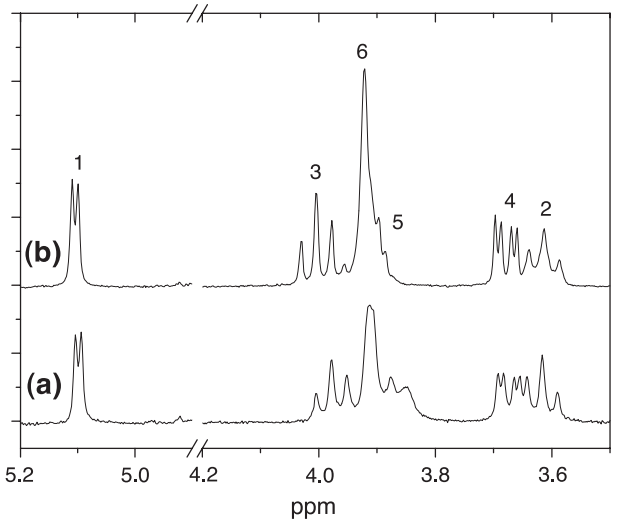


Fig. 4. ^{1}H NMR (360 MHz) spectra of aqueous solution of β-CD with addition of 4 mM NaOH in the presence (spectrum a) and absence (spectrum b) of **IV**. See Scheme 2 for identification of CD protons. Concentrations of CD and carotenoid are 2 mM.

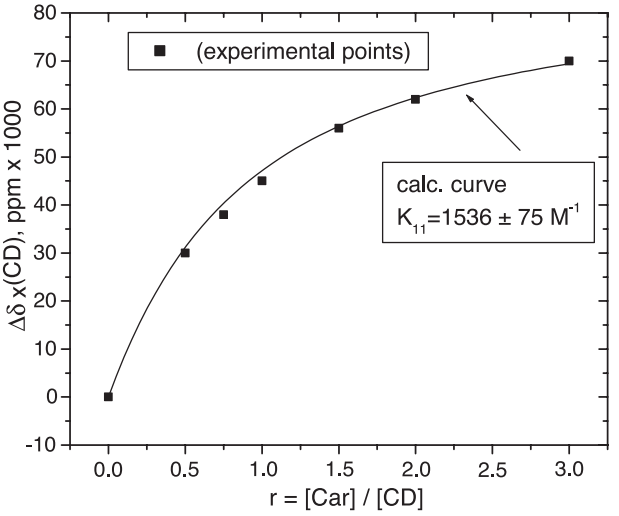


Fig. 6. Dependence of 3-H β-CD protons chemical shift on carotenoid **IV**/CD ratio in D_2O. Experimental points and calculated curves for association constant K_{11} = 1536 M^{-1}. [β-CD] = 2 mM and [NaOH] = 4 mM.

scavenging ability was measured as a relative scavenging rate of Car and spin trap (ST). Such values were determined for the set of carotenoids from concentration dependence of spin adduct yield (A) by using the equation

$$A/A_0 = k_{ST}[ST]/(k_{ST}[ST] + k_{Car}[Car]),$$

where k_{Car} and k_{ST} are the reaction rate constants of carotenoid and spin trap with a free radical and A_0 is spin adduct yield at zero carotenoid concentration. It was demonstrated that k_{Car} depends on the redox properties of carotenoid and increases with an increase in their oxidation potential [16]. The k_{Car}/k_{ST} ratio changes from 0.6 for **I** to 24 for **II** [16]. The absolute values of the k_{Car}(OOH) can be estimated assuming that k_{ST}(OOH) ~ 10^6 M^{-1} s^{-1} [26]. In the present study the k_{Car}/k_{ST} ratio was measured for carotenoid **IV** according to the procedure described in [16]. Figure 7 depicts a decrease in PBN/OOH spin adduct yield with an increase in **IV** concentration as a result of the scavenging process.

The ratio k_{Car}/k_{ST} = 40 calculated for carotenoid **IV** (Fig. 8) was greater than those obtained for all previously studied carotenoids [16]. The scavenging ability of the inclusion complex of **IV** in water was examined using the EPR technique. The highly water soluble hydroxypropyl-substituted CD was used for this experiment. No decrease in spin adduct yield was observed in this case (see Fig. 9). Moreover, a small pro-oxidant effect (increase in

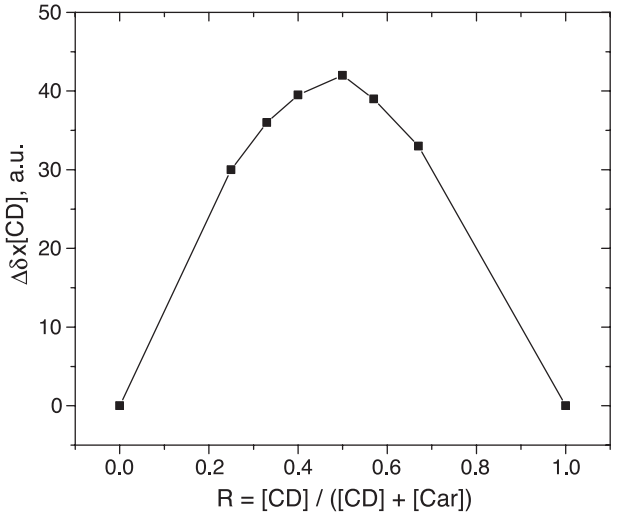


Fig. 5. Job's plot corresponding to the chemical shift displacement of 3-H protons of β-CD in the presence of **IV**. The total concentration of CD and carotenoid was 2 mM in all experiments.

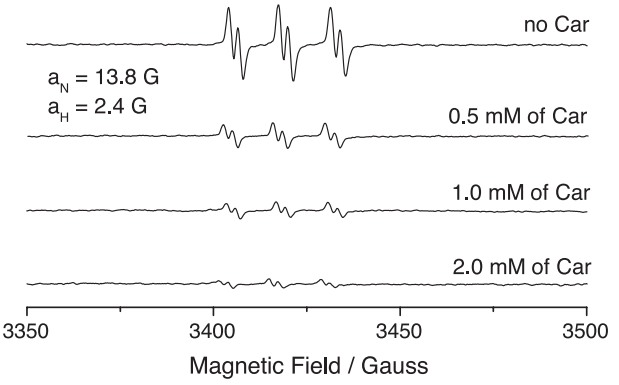


Fig. 7. Variation of PBN/OOH spin adduct EPR spectrum in the presence of **IV**. Concentration of PBN was 10 mM, Fe^{2+} was 1 mM, H_2O_2 was 500 mM; solvent was DMSO.

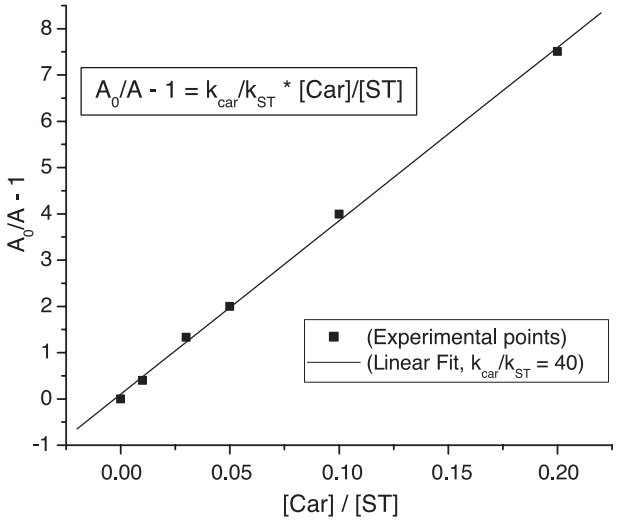


Fig. 8. Dependence of PBN/OOH spin adduct yield on carotenoid **IV** concentration in DMSO. Concentration of PBN was 10 mM, Fe^{2+} was 1 mM, H_2O_2 was 500 mM.

spin adduct yield in the presence of carotenoid) was detected.

This effect is not due to encapsulation of the spin trap by CD. The test experiment in the absence of carotenoid showed no change in spin adduct yield. The absence of the antioxidant effect in CDs (decrease in spin adduct yield in the presence of carotenoid) might be due to protection of the radical sensitive sites of the carotenoid (cyclohexene ring) by the CD. This also means that the OOH radical attacks only the cyclohexene ring of the carotenoid. Observation of the pro-oxidant effect for the Car/CD complex can be explained by the reaction of carotenoid with Fe^{3+} [36]. Fe^{3+} ions, present in solution as a product of the Fenton reaction (see Materials and Methods), oxidize the carotenoid and regenerate Fe^{2+}:

$$\mathrm{Car} + \mathrm{Fe}^{3+} \rightarrow \mathrm{Car}^{+\bullet} + \mathrm{Fe}^{2+}.$$

When an excess of H_2O_2 is present, this reaction can also lead to repetition of the redox cycle of the Fenton process (see Materials and Methods) and can result in an additional portion of free radicals. Our previous UV-Vis and EPR studies of carotenoid oxidation by Fe^{3+} [36–39] confirmed the formation of the carotenoid radical cation, $Car^{+\bullet}$, as a product, providing evidence for this electron transfer reaction. It was also shown that complexation between Fe^{3+} ions and carotenoids containing cyano-groups greatly facilitates the electron transfer reaction [40].

In addition to Fe^{3+} ions, the electron transfer reactions of carotenoids with Al^{3+}, Ni^{2+}, Cu^{2+}, and Ti^{4+} ions have been studied [41–43]. For instance, when carotenoids are embedded in Al-MCM-41 molecular sieves, the $Car^{+\bullet}$ photo yield increases compared to that in siliceous MCM-41. This is a result of complexation between carotenoid and Al^{3+} confirmed by ENDOR (electron nuclear double resonance) spectroscopy [41]. EPR, ENDOR, and UV-Vis studies indicate that a carotenoid–Ti^{4+} complex, formed in Ti-MCM-41 molecular sieves, enhances the photo-induced electron transfer efficiency [42]. EPR measurements demonstrated that Cu^{2+} also forms complexes with β-carotene and permits reversible electron transfer upon temperature cycling [43].

Our interest in the reactions of carotenoids with redox-active metal ions is due to their biological importance. The role of the Fenton-like processes in the formation of toxic free radicals in vivo has been widely discussed [24,25,44,45]. In biological systems, transition metal ions such as Fe(II), Cu(II), Cr(VI), Cd(II), Co(II), Mn(II), V(V), and Ni(II) have been shown to catalyze the formation of active oxygen species by the reaction with H_2O_2 and promote metal-mediated oxidative damage [46–49]. Carotenoids can function as antioxidants in these systems. Our previous studies showed that carotenoid antioxidant abilities depend on carotenoid structure and oxidation potential. The scavenging ability of the carotenoids increases with their oxidation potential [16]. As the oxidation potential increases from that of β-carotene (0.6 V vs. SCE) to that of the dicyano compound (**II**) (0.8 V vs. SCE) the rate constant increases by 25-fold. The rate constant of the reaction of carotenoid with oxygen-centered radical ($^{\bullet}OH$) was reported to be approximately $1 \times 10^9\ M^{-1}\ s^{-1}$ [16]. This is 3 orders of magnitude greater than with carbon-centered radicals ($^{\bullet}CH_3$).

The results obtained have shown that in order to be an effective antioxidant, the carotenoid should be extracted from the CD cavity after delivery to the cell membrane. It is an important consideration in using carotenoid/CD complexes in medical practice. Similar conclusions can be made from recent in vivo and in

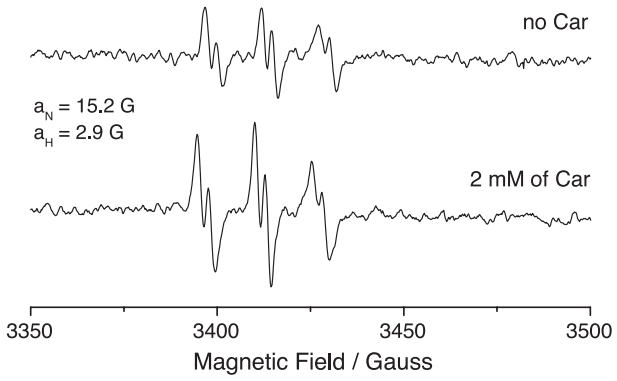


Fig. 9. Variation of PBN/OOH spin adduct EPR spectrum in the presence of **IV**/HP-β-CD complex. Concentration of PBN was 10 mM, Fe^{2+} was 1 mM, H_2O_2 was 500 mM, HP-β-CD was 4 mM in H_2O.

vitro experimental data [50–53]. It was demonstrated that cyclodextrins can be used as carriers for the incorporation of dietary carotenoids into plasma and mitochondrial and microsomal cell membranes. Cyclodextrins, in contrast to dimethyl sulfoxide, stabilize carotenoids and allow efficient cellular uptake. At the same time, carotenoids encapsulated in the CD cavity show no photoprotection of human skin fibroblasts against UV irradiation [52].

CONCLUSION

This paper presents the first direct evidence of carotenoid:β-cyclodextrin 1:1 inclusion complex formation. The structure and stability of the complex were investigated by ^{1}H NMR and UV-Vis absorption spectroscopy for a carotenoid with a terminal carboxylic acid group (β-caroten-8′-oic acid). We cannot exclude the possibility of similar complex formation for other carotenoids containing the same structural fragment (cyclohexene ring, see Scheme 1). Our results show that cyclodextrin does not prevent the reaction of carotenoids with Fe^{3+} ions, but reduces their scavenging rate toward OOH radicals. This means that different sites are responsible for interaction of carotenoids with free radicals and Fe^{3+} ions. Because cyclodextrins are widely used as carriers and stabilizers of dietary carotenoids, the demonstration that CDs protect the carotenoids from reactive oxygen species and provide their safe delivery to the cell membrane is of importance.

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