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Original Contribution

Antioxidant and redox properties of supramolecular complexes of carotenoids with β-glycyrrhizic acid

N.E. Polyakov [a], T.V. Leshina [a], N.F. Salakhutdinov [b], T.A. Konovalova [c], L.D. Kispert [c,*]

[a] *Institute of Chemical Kinetics and Combustion, Novosibirsk 630090, Russia*
[b] *Novosibirsk Institute of Organic Chemistry, Novosibirsk 630090, Russia*
[c] *Chemistry Department, University of Alabama, Tuscaloosa, AL 35487-0336, USA*

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Abstract

Supramolecular complexes between carotenoids and a triterpene glycoside, β-glycyrrhizic acid (GA), were found to exhibit unusual antioxidant activity. Complexation with GA increases a scavenging rate of canthaxanthin and 7′,7′-dicyano-7′-apo-β-carotene toward OOH radicals more than 10 times, but has no effect on the scavenging rate of zeaxanthin. Scavenging rate constants were measured in DMSO solution of carotenoids using the EPR spin-trapping technique. EPR parameters of spin adducts were determined as a(H) = 2.3 G, a(N) = 13.9 G for PBN (*N-tert*-butyl-α-phenylnitrone)–OOH, and a(H) = 3.4 G, a(N) = 14.9 G for the PBN–CH_3 adduct. Taking into account the previously measured dependence of the scavenging rate constants toward OOH radicals on the oxidation potential of carotenoids, this result can be explained by the hypothesis that the complexation with GA affects the value of oxidation potentials. This hypothesis was confirmed by CV measurements.

Keywords: Carotenoids; Radicals; β-Glycyrrhizic acid; Inclusion complex; Redox potential; Antioxidant activity; Spin trapping

Introduction

Antioxidant activity is known to be one of the most important biological properties of carotenoids, because they react with toxic free radicals and thus prevent damage to living organism [1–6]. One factor contributing to the development of various diseases, including infarction, cerebral thrombosis, and tumors has been attributed to the action of free radicals and the toxic forms of oxygen [7]. Carotenoids are assumed to protect cells by scavenging either free radicals or excited oxygen that have a severe impact on cells. At the same time, wide application of carotenoids as antioxidants has been limited because many factors affecting antioxidant activity are as yet unknown. Complexation is one of these factors. Complex formation is widely used in medicine to improve the solubility of preparations, to deliver drugs, and to decrease their toxicity [8–10]. It is worth noting that progress in the development of novel forms of medicines depends not only on the discovery of new active substances but also on regulating the effect of available preparations; complexation is one of the methods used to regulate this effect.

Many fundamental and applied investigations have recently been devoted to carotenoid inclusion complexes with natural compounds that are thought to possess protective properties and also to decrease the hydrophobic characteristics of the embedded molecules such as the complexes of carotenoids with cyclodextrins [11–18]. Using a series of carotenoids and their structural analogs (retinoids and β-ionone) it has been shown that the terminal cyclohexene ring of carotenoids is included in the cyclodextrin cavity; these complexes are stable in aqueous media [19,20]. However, an increase in stability and solubility of carotenoids in the complexes with cyclodextrins has failed to improve their antioxidant properties. Measuring the scavenging rate of peroxide radicals by carotenoids in homogeneous solutions indicates that the reaction can be almost totally inhibited by a cyclodextrin due to embedding the cyclohexene fragment in

Abbreviations: GA, β-glycyrrhizic acid; DMSO, dimethyl sulfoxide; PBN, *N-tert*-butyl-α-phenylnitrone; SOD, superoxide dismutase.

* Corresponding author.
E-mail address: lkispert@bama.ua.edu (L.D. Kispert).

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the cyclodextrin cavity [18]. We here report some results of a study of antioxidant properties of supramolecular carotenoid complexes with another natural ligand, β-glycyrrhizic acid (GA), a triterpene glycoside (Fig. 1).

This compound is particularly attractive for three main reasons. First, GA in contrast to the cyclodextrins has an open chain structure and thus, for complex formation, there are no rigorous restrictions on the size of a "guest" molecule. Second, authors who measure some parameters of GA complexes with various drugs indicate their unusual stability [21–23]. The stability constant of GA complexes is in the range of 10^5 M^{-1}, which is two orders of magnitude higher than a mean stability constant of cyclodextrin complexes [24]. Third, in vivo experiments indicate a 10-fold increase in the activity of a series of medicines in the complexes with GA [21,25–27] together with a decrease in their toxicity.

In the present work, the influence of complexation with GA on the antioxidant activity of carotenoids **I**–**III** (Fig. 1) was studied using techniques based on EPR and spin-trapping methods [28,29].

Experimental

Glycyrrhizic acid is extracted from the Ural licorice root. For the methods of GA purification see [30]. Carotenoid **I** was purchased from Fluka. Carotenoid **II** was provided by Professor Molnár, the University of Pécs, Hungary, and III was synthesized at the University of Alabama, United States, starting from 8′-apo-β-caroten-8′-al supplied by Roche Vitamins and Fine Chemicals (Nutley, NJ). All carotenoids were kept at −18°C in ampules sealed under vacuum. The purity was checked by ^{1}H NMR spectroscopy (360 MHz, $CDCl_3$) and TLC.

Electrochemical measurements were performed with a BAS-100 B/W electrochemical analyzer. We used a platinum disk electrode as working electrode, a platinum wire as axial electrode, and a calomel electrode for reference. For analysis, we prepared 10 ml of 0.1 mM solution of carotenoid and GA in acetonitrile (Fisher, HPLC grade) with 0.1 M TBAHFP (Fluka) as electrolyte.

The antioxidant activity of carotenoids and their complexes was studied by the previously developed EPR spin-

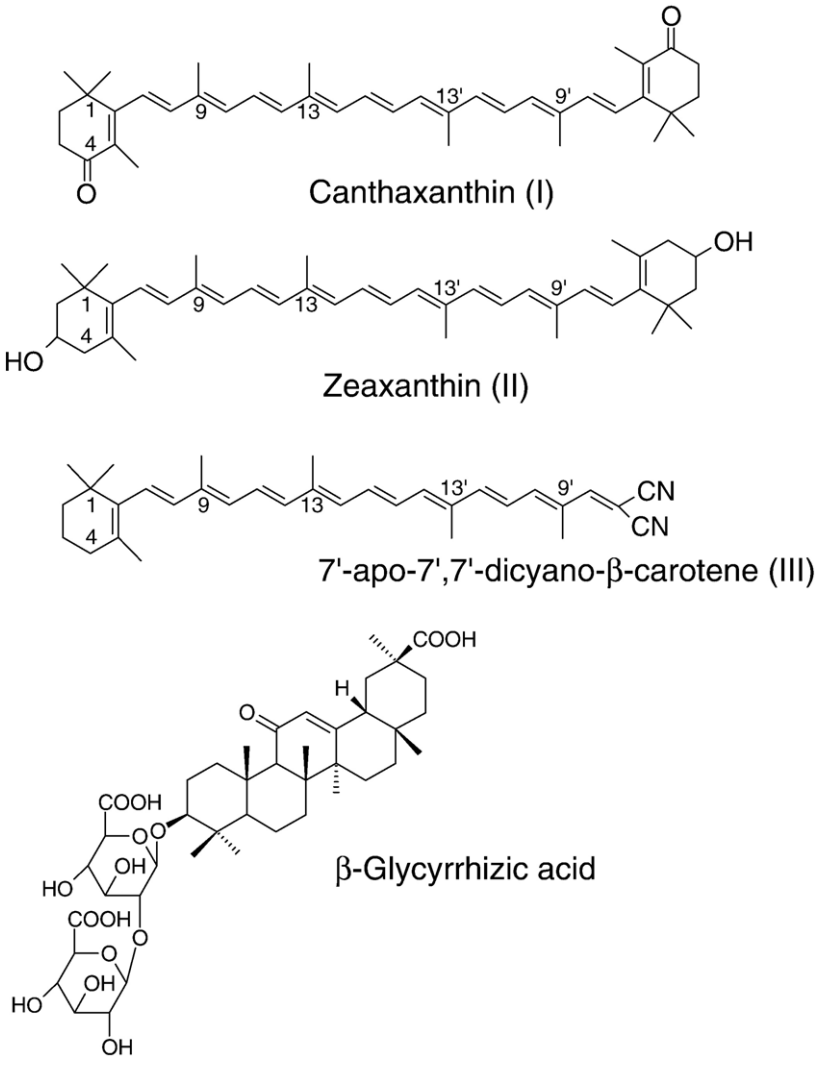

Fig. 1. The structures of carotenoids and glycyrrhizic acid.

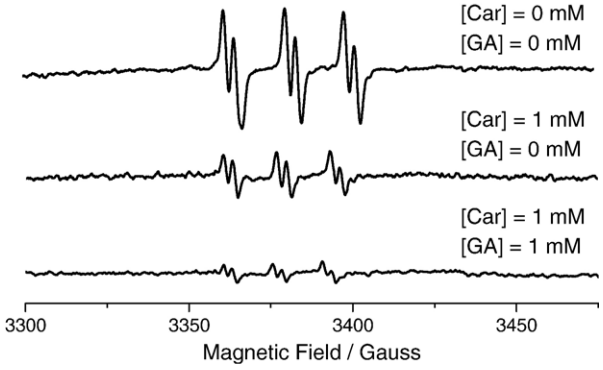

Fig. 2. ESR spectra of PBN–OOH adduct detected without carotenoid (at the top), and in the presence of canthaxanthin or its complex with GA in DMSO. Experimental conditions: [PBN] = 5 mM, [$FeCl_2$] = 1 mM, [H_2O_2] = 0.5 M.

trapping technique. For experimental details see [28,29]. Peroxide radicals were generated by the Fenton reaction with excess hydrogen peroxide in DMSO (99.5%, Aldrich, A.C.S.). The spin adduct of OOH radical with spin-trap *N*-*tert*-butyl-α-phenylnitrone (PBN; Aldrich) was recorded using an ESR Varian E-12 (X-band, 9.5 GHz) spectrometer. The sample of superoxide dismutase was from Sigma (EC1.15.1.1).

Results and discussion

It has been demonstrated [31] that GA can form stable inclusion complexes with carotenoids in various solvents: water, DMSO, alcohols, and acetonitrile. The structure, stability, and reactivity of the resulting host–guest complexes have been determined by HPLC, optical absorption, and fluorescence spectroscopy. It was found that carotenoids form 1:2 complexes with GA and that the structure of the complex is a cyclic-like dimer of GA encapsulating a carotenoid molecule located in the torus of the GA dimer. The stability constants in all solvents are near 10^4 M^{-1}. In addition, GA forms inclusion complexes with carotenoid radical cations, which results in their stabilization. Complex formation (a) decreases the rate of electron transfer from carotenoids to electron acceptors (Fe^{3+} or quinone) and (b) considerably increases the lifetime of the carotenoid–quinone charge transfer complex and the yield of the major product (a carotenoid–quinone adduct). These results are important for understanding both the nature of GA complexes and the influence of GA on the therapeutic activity of some drugs. Furthermore, carotenoid–GA complexes could be used for the design of artificial light-harvesting, photoredox, and catalytic systems.

In the present work, the rate constants of carotenoids reacting with free radicals were determined using the EPR spin-trapping technique [28]. The method involves measuring the yield of the stable spin adducts in the presence of carotenoids. Because of the two competing processes, i.e., reactions of the radical with carotenoid and spin trap, the yield of the spin adduct is proportional to the carotenoid concentration (Fig. 2).

Thus, from the experimental dependence of the spin adduct yield on carotenoid concentration the relative rate of radical scavenging (k_{Car}/k_{ST}) by carotenoid can be assessed:

$$\frac{A_0}{A} = \frac{k_{ST}[ST] + k_{Car}[Car]}{k_{ST}[ST]}.$$

Here, k_{ST} is the rate constant of radical scavenging by the spin trap (ST), A and A_0 are the values of the spin adduct signal intensity with and without carotenoid. The available kinetic database (Spin Trap Data Base: http://epr.niehs.nih.gov) provides the value of the rate constant k_{ST} measured in water ($k_{ST} \le 10^6$ M^{-1} s^{-1} for the PBN spin trap). In the present work, using the same approach, we measured the reaction rates of the OOH radical with carotenoids, glycyrrhizic acid, and their complexes. The PBN–OOH spin adduct was identified using available hyperfine parameters (see Ref. [28] and references within). We also carried out some test experiments to prove our assignment. Under anaerobic conditions, the peroxyl radicals were generated in DMSO via the well-known Fenton reaction [32]:

$$Fe^{2+} + H_2O_2 \leftrightarrows Fe^{3+} {}^{\bullet}OH + OH^-$$

$$^{\bullet}OH + DMSO \rightarrow {}^{\bullet}CH_3 + CH_3(OH)SO$$

$$^{\bullet}CH_3 + H_2O_2 \rightarrow {}^{\bullet}OOH + CH_4$$

At low H_2O_2 concentrations (H_2O_2] ~ [$FeCl_2$] = 1 mM) only one spin adduct PBN–CH_3 was detected with ESR parameters a(H) = 3.4 G and a(N) = 14.9 G [28]. At higher H_2O_2 concentration ([H_2O_2] = 500 mM) the reaction of CH_3 radicals with H_2O_2 become important and this results in the disappearance of the PBN–CH_3 adduct, and the appearance of another adduct with higher yield which was assigned to the PBN–OOH spin adduct (a(H) = 2.3 G and a(N) = 13.9 G)

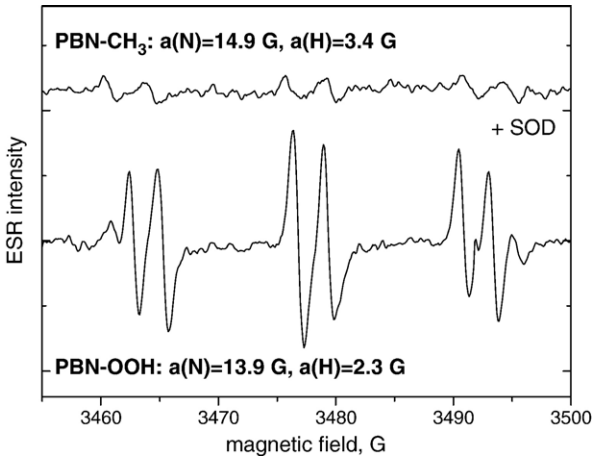

Fig. 3. ESR spectra of PBN–OOH and PBN–CH_3 adducts detected in the presence (at the top), and in the absence of 200 U/ml SOD in DMSO (contained 10% H_2O). Experimental conditions: [PBN] = 5 mM, [$FeSO_4$] = 1 mM, [H_2O_2] = 0.2 M. Experiments with SOD (Sigma, EC 1.15.1.1) were carried out using ER-200D SRC (X-band, 9.5 GHz) ESR spectrometer.

[28]. It is known that the •OOR spin adducts are relatively unstable especially in the presence of transition metal ions which can reduce •OOR radicals yielding the •OR spin adduct [33,34]. However, these facts are mainly related to alkyl peroxyl radicals. Under our experimental conditions with increasing H_2O_2 concentration, the Fe^{2+} ions react primarily with the initial H_2O_2 and consequently the stability of the •OOH spin adduct will increase. Several publications report the observation of the PBN–OOH adducts under similar conditions [35–37].

In the present study, we performed also the superoxide dismutase (SOD) test to confirm the PBN–OOH adduct formation. In the absence of SOD using appropriate concentrations of hydrogen peroxide (200 mM), we observe both the PBN–OOH and the PBN–CH_3 adducts simultaneously (Fig. 3, bottom). The addition of SOD (200 U/ml) completely suppressed the PBN–OOH signal, but had no influence on the PBN–CH_3 adduct (top, Fig. 3).

It was found that GA displays a substantial antioxidant activity with $k/k_{ST} = 12$ (Table 1). Note that the rate constant of the reaction between GA itself and peroxyl radicals is independent of GA concentration. Comparison of the scavenging rates of peroxyl radicals by free carotenoids and their complexes in DMSO shows that the rate constants are quite different depending on the concentration of GA (**I** and **III**) and the carotenoid (**I** and **III** vs **II**).

The k_{Car}/k_{ST} values given in Table 1 were obtained by subtracting the GA contribution from the total rate constant measured in the presence of glycyrrhizic acid. Thus, there is a synergetic GA effect observed by a several-fold increase in the rate of radical scavenging by carotenoids **I** and **III**. Of interest is the absence of this effect for zeaxanthin (**II**). The oxidation potentials of three carotenoids (see Table 1) [38] were compared with their scavenging rate constants [28]. It was found that GA complexation affects the oxidation potential of carotenoids. CV measurement of the oxidation potential of GA complexes of two carotenoids **I** and **II** showed an increase in $E_{1/2}$ by 0.05 V for canthaxanthin (Fig. 4) and by 0.03 V for zeaxanthin.

The results are summarized in the diagram in (Fig. 5) using the data from [28]. As shown in Fig. 5, the dependence of the rate constant of the reaction with peroxyl radicals on the oxidation potential of carotenoids is nonlinear. A change in the oxidation potential for β-carotene and zeaxanthin ($E_{1/2} \sim 0.5$ V) causes no changes in their antioxidant activity. At the same time, the diagram allows us to predict a substantial increase in the reaction rate for carotenoids with $E_{1/2} \sim 0.7$ V when their oxidation potential increases due to complexation.

Table 1
Relative rate constants of OOH radicals scavenging by glycyrrhizic acid, carotenoids, and their complexes (k/k_{ST}) in DMSO

[GA], mM	GA	**I** ($E_{1/2}$ = 0.68 V)	**III** ($E_{1/2}$ = 0.72 V)	**II** ($E_{1/2}$ = 0.56 V)
0		2	7	4
0.5	12	59	133	4
1	12	46	116	4
2	12	6	38	4

Experimental error ~10%.

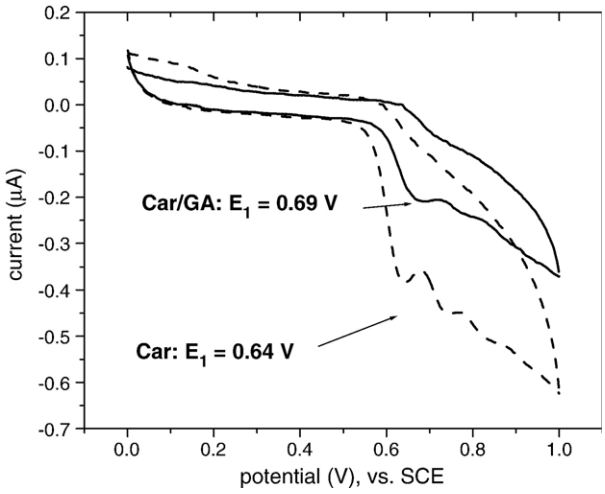

Fig. 4. Cyclic voltammetry (CV) plot of canthaxathin (0.1 mM) and its complex with GA (0.2 mM of GA) in acetonitrile. Scan rate is 10 mV/s.

This prediction was confirmed experimentally and the observed result verifies the hypothesis [28] for the role of electron transfer in the scavenging of free radicals by carotenoids. The most important result of this work is the determination of the effect of GA on the oxidation potential of carotenoids. An increase in the oxidation potential of carotenoids can serve as the main reason for a decrease in their oxidation rate in reactions with electron acceptors. Note, that until now there was no example in the literature of inclusion complexes of GA with unstable radical intermediates. A few examples of the inclusion complexes of cyclodextrins with organic anion and cation radicals exist [39,40]. Most likely the influence of GA on redox properties of the guest molecules and stability of intermediate radical ions is due to the dipole–dipole interaction between the GA and the corresponding guest. In addition, the rate constants of peroxyl radical scavenging by

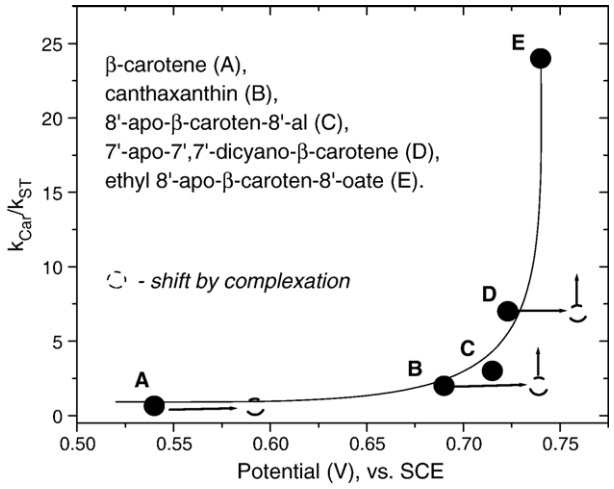

Fig. 5. Diagram of the dependence of the carotenoid scavenging rate toward peroxyl radicals on the oxidation potential of carotenoids. The data are taken from [28]. Arrows denote the shifts in oxidation potentials due to complexation.

carotenoids (Table 1) are different at various GA concentrations. This fact confirms the hypothesis for the dependence of the structure and properties of GA complexes on its concentration. Indeed, the scavenging rates measured at low GA concentrations (0.5 mM) considerably exceed those measured at high concentrations (2 mM). It was assumed [41] that in aqueous solutions, at a concentration of above 1 mM, GA forms micelle-type complexes. This hypothesis for micelle formation has been further developed by the Japanese authors studying the processes of the micelle formation of the water-soluble GA derivatives, in particular, sodium sulfates [42]. However, the data on the micelle formation in other solvents are unavailable in the literature. The stability constant of GA complex with canthaxanthin in DMSO was estimated from the dependence of carotenoid fluorescence intensity on GA concentration to be about 10^4 M^{-1} for low GA concentrations and $<10^2$ M^{-1} for high concentrations. Below GA concentrations of 1 mM, the GA–carotenoid complex stoichiometry was determined to be 2:1. At higher concentrations, micelle-like behavior appears to occur. Thus, the differences in the scavenging rates of peroxyl radicals for various GA concentrations indicate that the properties of 2:1 complexes really differ from those for carotenoids in the assumed micellar solution. The reason for these differences is still unknown.

Conclusions

Thus, the present paper shows that the complexation with glycyrrhizic acid has a noticeable effect on the reactivity of carotenoids. Experimental data on the rate constants of the scavenging of peroxyl radicals by various carotenoids indicate that the effect of GA on the reactivity of carotenoids is, probably, due to a change in their oxidation potentials in a complex. The range within which E_1 increases during complexation amounts to 0.05 V. Such a change in oxidation potential leads to a multiple (10 times) increase in the scavenging rate of peroxyl radicals by carotenoids with a high oxidation potential (~0.7 V) and has no influence on the activity of carotenoids with a lower potential (~0.5–0.6 V). It is assumed then that the structure of complexes changes with increasing GA concentrations greater than 1 mM. It was established that in this case, the stability of the complex decreases and its influence on the reactivity of carotenoids becomes weaker.

The data obtained can be highly useful in practice. It is known that the creation of novel, more effective drugs based on the complexes of tested medicines with natural compounds is now an intensively developed field of medicinal chemistry, cosmetology, and the food industry. Fundamental studies on the nature of the complexation and physicochemical properties of complexes substantially lag behind. At the same time, these studies are highly important from the point of view of their predictive potential. In medicine, screening of new drugs is, as a rule, performed using animals. Therefore, the possibility to control the reactivity of chemical compounds by complexation and, equally important, to predict the range of either an increase or a decrease in therapeutic activity would allow one to substantially reduce the number of in vivo experiments.

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