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Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin

Satoru Goto [a], Kentaro Kogure [a,*], Kazutoyo Abe [a], Yukari Kimata [a], Katsuhiro Kitahama [a], Eiji Yamashita [b], Hiroshi Terada [a]

[a] *Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi-1, Tokushima 770-8505, Japan*
[b] *Itano Refrigerated Food Co. Ltd., Seto-Cho, Naruto 772-0060, Japan*

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Abstract

The effects of the carotenoids β-carotene and astaxanthin on the peroxidation of liposomes induced by ADP and Fe^{2+} were examined. Both compounds inhibited production of lipid peroxides, astaxanthin being about 2-fold more effective than β-carotene. The difference in the modes of destruction of the conjugated polyene chain between β-carotene and astaxanthin suggested that the conjugated polyene moiety and terminal ring moieties of the more potent astaxanthin trapped radicals in the membrane and both at the membrane surface and in the membrane, respectively, whereas only the conjugated polyene chain of β-carotene was responsible for radical trapping near the membrane surface and in the interior of the membrane. The efficient antioxidant activity of astaxanthin is suggested to be due to the unique structure of the terminal ring moiety.

Keywords: Astaxanthin; β-Carotene; Lipid peroxidation; Antioxidant; Liposome

1. Introduction

Carotenoids have received considerable attention for their possible clinical use in diseases associated with reactive oxygen species such as cancer [1,2]. Furthermore, procedures for creation of various novel carotenoids having antioxidant activity in bacteria and plants have been developed using new biotechnologies [3–5]. Therefore, carotenoids have been regarded to be of value not only as effective nutrients for the eyes, but also as antioxidants. The antioxidant activities of β-carotene (for chemical structure, see Chart), as a representative carotenoid, in various biological membranes and model membrane systems have been studied extensively [2,6–13]. Other naturally occurring carotenoids such as zeaxanthin (3,3′-dihydoroxyl-β-carotene), canthaxanthin (4,4′-dioxo-β-carotene) and astaxanthin (3,3′-dihydroxyl-4,4′-dioxo-β-carotene; see Chart) are also known to inhibit lipid peroxidation significantly [7,10,13–15].

Abbreviations: BhCL, cardiolipin from bovine heart; EyPC, egg yolk phosphatidylcholine; P_i, inorganic phosphate; TBARS, thiobarbituric acid-reactive substances; MDA, malonedialdehyde; DM, dipole moment; MO, molecular orbital; EHTC, 6-ethenyl-3-hydroxyl-1,1,5-trimethyl-5-cyclohexen-4-one; EHC, 6-ethenyl-3-hydroxyl-5-cyclohexen-4-one; HC, 3-hydroxyl-5-cyclohexen-4-one

* Corresponding author. Fax: +81-88-633-9511;
E-mail: kogure@ph.tokushima-u.ac.jp

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Of these, astaxanthin, which is found as a common pigment in algae, fish and birds [16], is reported to be more effective than β-carotene in preventing lipid peroxidation in solution and various biomembrane systems, such as egg yolk phosphatidylcholine liposomes and rat liver microsomes [7,10,14,15]. However, the reason why the antiperoxidation effect of astaxanthin is more than that of β-carotene is not well understood. Information on the action mechanism of astaxanthin should be useful for development of novel carotenoids having highly efficient antioxidant activities. Accordingly, we examined the effects of astaxanthin and β-carotene on lipid peroxidation of liposomes induced by ADP/Fe^{2+} under various conditions.

2. Materials and methods

2.1. Materials

All-*trans* astaxanthin and β-carotene were prepared as described [17,18]. Cardiolipin from bovine heart (BhCL) was obtained from Sigma Chemical Co. (St. Louis, MO). Egg yolk phosphatidylcholine (EyPC) was purchased from Nihon Seika Co. (Tokyo, Japan). Other reagents were of the highest grade commercially available. The solution of Fe^{2+} was prepared by dissolving $FeSO_4$ in water just before use to avoid precipitation of Fe^{3+} as $Fe(OH)_3$.

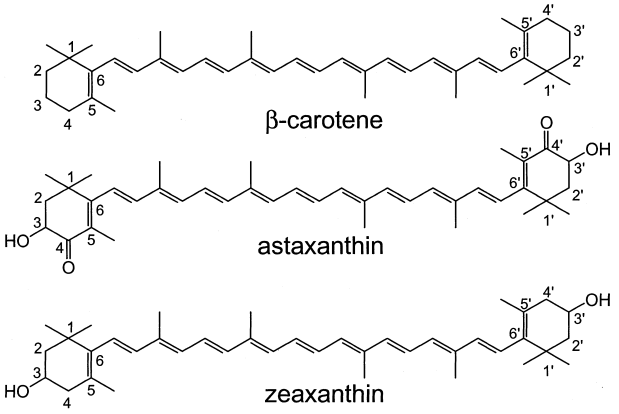

Chart. Chemical structures of β-carotene, astaxanthin and zeaxanthin.

2.2. Preparation of liposomes

Liposomes consisting of EyPC and BhCL in a molar ratio of 9:1 were prepared by reverse-phase evaporation [19] in 175 mM KCl and 10 mM Tris–HCl buffer (pH 7.4) under a stream of nitrogen gas. The amounts of phospholipids were determined in terms of their amounts of P_i as described by Ames [20]. For examination of the effects of carotenoids, they were mixed with phospholipids before preparation of liposomes.

2.3. Assay of lipid peroxidation

Lipid peroxidation was induced by 1 mM ADP and 0.1 mM $FeSO_4$ in 175 mM KCl and 10 mM Tris–HCl buffer (pH 7.4) at 25°C in an airtight cell in a total volume of 2.2 ml. The amount of lipid peroxides were determined as the amount of thiobarbituric acid-reactive substances (TBARS) in terms of malonedialdehyde (MDA) using tetraethoxypropane as a standard [21]. We confirmed that β-carotene and astaxanthin did not affect the formation of TBARS or its absorbance at the λ_{max} of 535 nm under the present experimental conditions. Peroxidation-related oxygen consumption of the suspension of liposomes (0.2 mM phospholipid in terms of P_i) was determined with a Clark-type oxygen probe (Yellow Spring, YSI 5331) in a total volume of 2.2 ml [22,23]. The concentration of saturated O_2 in the solution was assumed to be 258 μM at 25°C.

2.4. Measurements of absorption spectra

Absorption spectra of carotenoids in liposomes suspended in buffer consisting of 175 mM KCl and 10 mM Tris–HCl (pH 7.4) were measured in a dual wavelength/double-beam spectrophotometer, Shimadzu UV-3000, at 25°C with stirring of the solution under air.

2.5. Theoretical studies

Ab initio molecular orbital (MO) computation was performed with a GAUSSIAN 98 program obtained from Gaussian Inc. (Pittsburgh, PA) [24]. The electronic configurations of radicals were determined according to the density functional theory (DFT) cal-

culation, which is suitable for characterization of radical species [25,26]. For the DFT calculation, we used the B3LYP approach based on Beche's three-parameter nonlocal-exchange functional after modification by using the nonlocal correlation of functional of Lee, Yang and Parr (B3LYP) [24]. Molecular geometries were optimized by the DFT calculation with a 6-31G(d) basis set, and then the steric energy and dipole moment (DM) of the optimized structure was computed on a 6-31+G(d,p) basis.

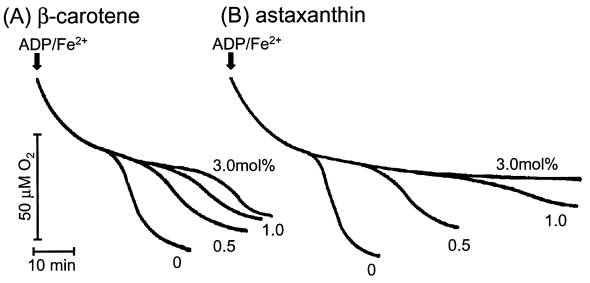

Fig. 2. Changes in O_2 concentration in the presence of β-carotene (A) and astaxanthin (B) during lipid peroxidation of liposomes induced by ADP/Fe^{2+}. Experimental conditions were as for Fig. 1.

3. Results

3.1. Effects of carotenoids on ADP/Fe^{2+}-induced lipid peroxidation in the liposomal membrane

First, we examined the effects of the carotenoids β-carotene and astaxanthin on lipid peroxidation induced by ADP/Fe^{2+} in liposomes. For this, we added a certain amount of carotenoid to an etherial solution of EyPC and BhCL in a molar ratio of 9:1 (EyPC/BhCL liposomes) [23], and then prepared the liposomes by reverse-phase evaporation method [19] at pH 7.4. Lipid peroxidation was initiated with 1 mM ADP and 0.1 mM Fe^{2+}. Progress of lipid peroxidation was determined as the amount of TBARS measured in terms of MDA concentration 45 min after addition of ADP/Fe^{2+}. At this time, peroxidation without carotenoids had completely stopped. As shown in Fig. 1, both β-carotene and astaxanthin inhibited liposomal lipid peroxidation. The production of lipid peroxides was inhibited progressively by these carotenoids with increase in their amounts in the liposomal membrane up to 1 mol%, and their effects almost leveled off at 1 mol%. At 3 mol%, astaxanthin was about two times more effective than β-carotene, being consistent with its relative radical trapping effects in organic solvents [7] and antiperoxidative effects in membrane systems, such as EyPC liposomes and rat liver microsomes [10,15].

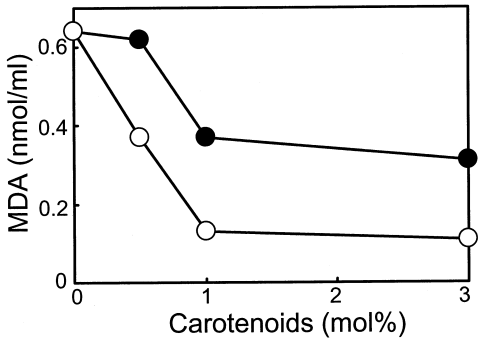

Fig. 1. Effects of β-carotene (●) and astaxanthin (○) on the formation of lipid peroxides in terms of MDA in liposomes. The effects of various concentrations of carotenoids incorporated into liposomes were examined. Formations of lipid peroxides were determined in terms of MDA production in the liposomes 45 min after addition of ADP/Fe^{2+}. Liposomes (0.2 mM phospholipids in terms of P_i) were suspended in 175 mM KCl and 10 mM Tris–HCl buffer (pH 7.4) in an airtight cell at 25°C in a total volume of 2.2 ml.

To investigate the mechanism of the antiperoxidative effects of the carotenoids, we examined their effects on peroxidation-related O_2 consumption of the EyPC/BhCL liposomal suspension induced by 1 mM ADP and 0.1 mM Fe^{2+}. As shown in Fig. 2, decrease in the O_2 concentration was observed immediately after addition of ADP/Fe^{2+} to the suspension of liposomes without carotenoids. The rate of O_2 consumption was first rapid then gradual, and became rapid again 20 min after addition of ADP/Fe^{2+}. This biphasic feature characterizes lipid peroxidation of liposomes and biological membranes [22,23]. The time period before induction of the second rapid O_2 consumption is thought to be due to production and accumulation of active oxygen species derived from O_2 and ADP/Fe^{2+} in the incubation medium, and that of the subsequent rapid O_2 consumption to lipid peroxidation of the membranous phospholipids by a radical chain reaction,

which is initiated after accumulation of enough active oxygen species at the membrane surface [22,23]. In fact, O_2 consumption due to production of active oxygen species took place on addition of ADP/Fe^{2+} to the incubation medium without liposomes, and no MDA production was observed until initiation of the stage of the radical chain reaction after addition of ADP/Fe^{2+} to the phospholipid membrane systems [22]. We refer to the period before initiation of the second rapid O_2 consumption as the lag-time, and the subsequent period of sharp decrease in the O_2 concentration as the stage of the radical chain reaction [22].

Both carotenoids incorporated into the liposomal membrane prolonged the lag-time, and inhibited the radical chain reaction in a manner dependent on their amounts in liposomal membranes (Fig. 2). Astaxanthin prolonged the lag-time and inhibited the radical chain reaction more effectively than β-carotene. The effect of astaxanthin was more significant on the lag-time than on the radical chain reaction, as compared with that of β-carotene.

3.2. Changes in the absorption spectra of carotenoids with lipid peroxidation

In EyPC/BhCL liposomes, β-carotene and astaxanthin showed peaks at 460 nm and 480 nm, respectively, in their absorption spectra due to their conjugated polyene moiety [27,28]. On addition of ADP/Fe^{2+} to the suspension of liposomes with incorporated carotenoids, the absorbances at their λ_{max} decreased without change in their λ_{max} values. The decrease in the absorbance indicated destruction of the conjugated polyene moieties by their antioxidant reactions, as observed with β-carotene in various organic solutions [29,30]. The time-dependent change in the absorbance of carotenoids at their λ_{max} was monitored continuously after addition of ADP/Fe^{2+} to the liposomal suspension. As shown in Fig. 3A, the ADP/Fe^{2+}-induced absorbance change at 460 nm of β-carotene incorporated into the liposomes showed two phases: first a slow but steady decrease in the absorbance, and then a sharp decrease. The period of the first slow decrease was prolonged and the degree of the subsequent sharp decrease of the absorbance was lowered with increase in the amount

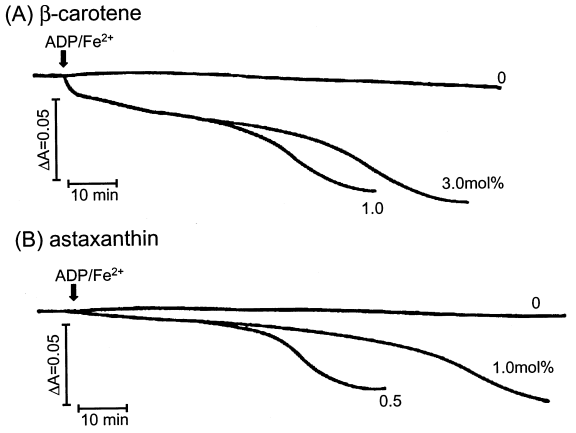

Fig. 3. Changes in absorbances of β-carotene (A) and astaxanthin (B) incorporated into liposomes during lipid peroxidation induced by ADP/Fe^{2+}. Lipid peroxidation was started by addition of ADP/Fe^{2+} to the suspension of liposomes with various amounts of incorporated carotenoids at 25°C. Absorbances at their λ_{max} (at 460 nm for β-carotene and 480 nm for astaxanthin) were monitored continuously. The concentration of phospholipids in liposomes was 0.2 mM in terms of P_i.

of β-carotene. It is noteworthy that the duration of the first period almost corresponded to that of the lag-time in peroxidation-related O_2 consumption (Fig. 2A), showing that destruction of the conjugated polyene moiety was slow in the lag-time, and that it proceeded rapidly in the period of the radical chain reaction as in O_2 consumption.

In contrast, the absorbance at 480 nm of astaxanthin in the liposomes did not decrease appreciably during the period corresponding to the lag-time, and the lag-time was prolonged significantly by a higher amount of astaxanthin (Fig. 3B). Then, the absorbance of astaxanthin decreased rapidly after termination of the lag-time as observed with β-carotene. As the absorbance of astaxanthin did not decrease essentially in the lag-time, the terminal ring moieties, which do not contribute to the absorption, would scavenge active oxygen species generated during the lag-time. It is noteworthy that the rate of rapid absorbance decrease of astaxanthin was almost the same as that of β-carotene, although astaxanthin was more efficient in inhibition in the stage of radical chain reaction than β-carotene (Figs. 2 and 3). Therefore, it is suggested that the two terminal rings of astaxanthin also scavenge radicals in the stage of the radical chain reaction.

3.3. Effects of intramolecular hydrogen bonding on the steric energy and dipole moment of the terminal ring moiety of astaxanthin

In the structure of the terminal ring moiety of astaxanthin, the hydrogen bonding between a hydroxyl group at the C3 position and a carbonyl-oxygen atom at C4 is expected to take place easily, resulting in formation of a five-membered ring. We determined the change in the steric energy of the terminal ring moiety of astaxanthin by molecular orbital (MO) calculations on formation of intramolecular hydrogen bonding. For this, 6-ethenyl-3-hydroxyl-1,1,5-trimethyl-5-cyclohexen-4-one (EHTC) was used as a model compound of the terminal ring moiety of astaxanthin, in which the polyene chain at C6 of the terminal ring moiety of astaxanthin is substituted by the ethylene group, and its 3-hydroxyl-5-cyclohexen-4-one moiety is conserved (for chemical structure, see Fig. 4).

We optimized the conformation of EHTC by MO computation. Its optimized structure was characterized by a dihedral angle at C5=C6–C7=C8 of 45.3° irrespective of hydrogen bonding, showing that the C6 ethylene group is out of plane with the cyclohexenone ring. Therefore, the effect of the ethylene group on the electronic nature of the cyclohexenone ring should be very small. A similar out of plane conformation was observed with retinoids having similar structural features to astaxanthin [31,32]. As shown in Fig. 4, formation of the hydrogen bonding stabilized EHTC by about 7 kcal/mol, suggesting that intramolecular hydrogen bonding is readily formed in astaxanthin.

To examine the effect of the intramolecular hydrogen bonding on the physicochemical properties of EHTC, we determined dipole moment (DM) as an

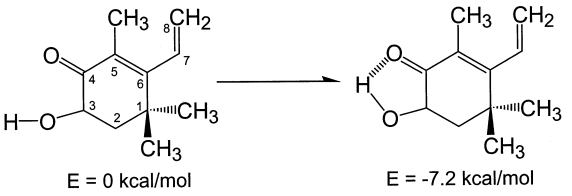

Fig. 4. Change in the steric energy of EHTC as a model compound of the terminal ring moiety of astaxanthin on formation of the intramolecular hydrogen bond. The steric energy of the hydrogen-bonded EHTC relative to that without hydrogen bonding is shown.

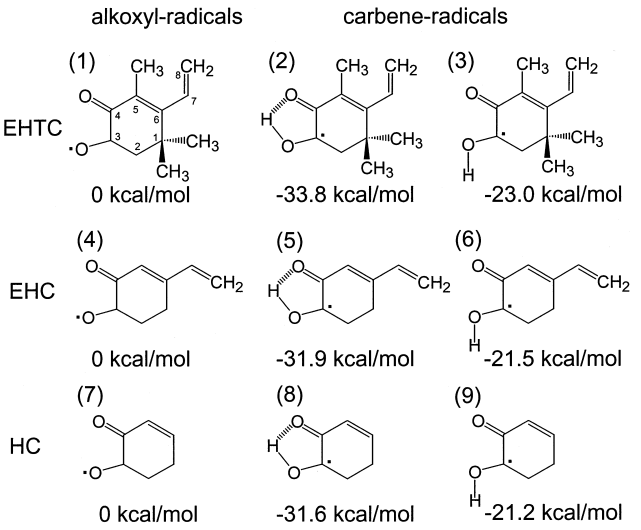

Fig. 5. Steric energies of radical intermediates derived from the model compounds of the terminal ring moiety of astaxanthin EHTC, EHC and HC. Steric energies of the hydrogen-bonded and nonhydrogen-bonded carbene-radical intermediates relative to that of the alkoxyl-radical intermediates of EHTC, EHC and HC are shown under their chemical structures. Radicals were generated by removal of the hydrogen atom attached to the hydroxyl group or methine at the C3 position.

index of polarity. We found that the DM value decreased from 5.3 debye to 4.5 debye on hydrogen bonding, suggesting that hydrogen-bonded ring closure lowered the polarity and increased the hydrophobicity of EHTC. In fact, intramolecular hydrogen-bonded ring formation increased the hydrophobicity more than 4-fold [33,34]. Consequently, the hydrophobicity of the terminal ring moiety of astaxanthin should increase significantly on formation of the intramolecular hydrogen bond.

3.4. Stabilities of possible radical intermediates of the terminal ring moiety of astaxanthin

As described above, the terminal ring moiety of astaxanthin was suggested to react with radicals. Radicals are known to be readily formed by removal of the hydrogen atom from the OH and active methine/methylene CH forming alkoxyl and carbene radicals, respectively [35]. We analyzed the stabilities of these possible radical intermediates derived from the model compound EHTC. Namely, MO computation was performed for the EHTC alkoxyl radical without intramolecular hydrogen bonding (**1**), the EHTC carbene radical with intramolecular hydrogen

bonding (**2**) and the EHTC carbene radical without hydrogen bonding (**3**) (for chemical structures, see Fig. 5). The dihedral angles at C5=C6–C7=C8 of these radical intermediates were all about 45° as in EHTC.

It was found that the hydrogen-bonded carbene radical **2** and nonhydrogen-bonded carbene radical **3** were about 34 kcal/mol and 23 kcal/mol more stable than the alkoxyl radical **1**, respectively (Fig. 5). These results suggest that removal of the hydrogen atom takes place at the C3 methine but not at the hydroxyl group of astaxanthin, and that formation of the intramolecular hydrogen-bonded ring is more favorable for antiperoxidative activity of astaxanthin due to delocalization of an unpaired electron in the hydrogen-bonded five-membered ring.

We further examined the steric energies of similar model radical intermediates derived from 6-ethenyl-3-hydroxyl-5-cyclohexen-4-one (EHC) and 3-hydroxyl-5-cyclohexen-4-one (HC) to determine the effects of the methyl groups at the C1 and C5 positions and the ethylene group at C6 on the stabilities of radicals. In the optimized structure of EHC radicals, the exocyclic ethylene group at C6 and endocyclic ketene (C6=C5–C4=O) of the cyclohexenone ring were on the same plane, unlike the out of plane conformation of the ethylene group of EHTC radicals. As summarized in Fig. 5, the intramolecular hydrogen-bonded carbene-radical intermediates of EHC (**5**) and HC (**8**) were the most stable, as observed with EHTC radical intermediates. Therefore, the stability of radical intermediates derived from the terminal ring moiety of astaxanthin was not affected by the methyl groups and polyene chain attached to the terminal rings, and hence the two terminal rings and polyene chain are independently responsible for the radical scavenging activity of astaxanthin.

4. Discussion

Consistent with previous results on the antiperoxidation activity of astaxanthin in solution and membrane systems [7,10,14,15], astaxanthin was more potent than β-carotene in liposomal lipid peroxidation induced by ADP/Fe^{2+} (Fig. 1). The time-dependent O_2 consumptions showed that both β-carotene and astaxanthin inhibited ADP/Fe^{2+}-induced lipid peroxidation of liposomes in similar manners, i.e., by prolonging the lag-time and inhibiting radical chain reactions (Fig. 2). The effect of astaxanthin on the lag-time was more significant than that on the stage of the radical chain reaction. Namely, astaxanthin preferentially inhibited generation of the active oxygen species at the polar membrane surface.

Although absorbance change due to destruction of the polyene chain has been used as a measure of radical trapping by various carotenoids [7,10,13,36], no clear distinction seems to have been observed in their manners of radical trapping. However, in this study, we observed quite different destructions of the polyene chain in radical trapping of β-carotene and astaxanthin. Namely, destruction of the polyene chain of β-carotene took place both in the lag-time and stage of the radical chain reaction, whereas that of astaxanthin was observed only in the latter stage (Fig. 3). Therefore, the conjugated polyene moiety of β-carotene was associated with quenching of both active oxygen species and lipid peroxyl radicals, but that of astaxanthin was associated only with trapped lipid peroxyl radicals in the stage of the radical chain reaction in the phospholipid membrane, and the terminal ring moiety was associated with quenching of the active oxygen species during the lag-time.

The locations of β-carotene and astaxanthin in the membrane should be related with their effectiveness for antiperoxidative activity. Recently, Gabrielska and Gruszecki [37] proposed that β-carotene is distributed homogeneously in the membrane without taking any preferred orientation. Such a free rotatable nature of the polyene chain of β-carotene should be advantageous for its trapping of active oxygen species at/near the membrane surface and lipid peroxyl radicals in the membrane. On the other hand, as astaxanthin has a polar OH-group in two terminal rings, it is likely to be located in the membrane in such a way that its polar terminal ring is oriented at/near the membrane surface and the polyene chain in the interior of the membrane. Accordingly, it could be effective for the terminal ring of astaxanthin to scavenge reactive oxygen species at/near the membrane surface, and for its polyene chain to inhibit the radical chain reaction in the membrane. Our model is consistent with that of xanthophyll zeaxanthin (see Chart) having a polar OH-group in each terminal ring [37]. Thus the orientation of zeaxanthin

in the membrane is such that its hydrophobic polyene chain is extended through the hydrophobic region of the membrane enabling its two terminal rings to be located at both polar surfaces of the lipid bilayer [37].

In addition, the two terminal rings of astaxanthin are likely to interact with the hydrophilic polar region of membrane phospholipids. Possibly, intermolecular hydrogen bonds are formed between polar ends of the hydroxyl-ketocarotene and polar groups of phospholipids. On the other hand, the hydroxyl and carbonyl groups of astaxanthin readily form intramolecular hydrogen-bonded five-membered ring, increasing the hydrophobicity of astaxanthin advantageous for its location inside the membrane. The energy to form the intramolecular hydrogen bond (about 7 kcal/mol), determined for the model compound EHTC (Fig. 4), corresponds to the 8 kcal/mol required for intermolecular hydrogen bonding of the hydroxyl and carbonyl groups of astaxanthin with the polar head of phospholipids (each 4 kcal/mol [38]).

Therefore, there could be about equivalent amounts of intra- and intermolecular hydrogen-bonded astaxanthin, and interconversion between the two hydrogen bond formations of astaxanthin. Hence the terminal rings of astaxanthin could be located either inside the membrane or at the membrane surface, as shown schematically in Fig. 6. Such a translocation of the terminal rings of astaxanthin should be advantageous for scavenging the lipid peroxyl radicals in the membrane and the reactive oxygen species at the membrane surface. Accordingly, astaxanthin is suggested to scavenge radicals inside the membrane both by the conjugated polyene chain and the terminal ring moiety. In fact, the inhibitory effect of astaxanthin on the radical chain reaction in the membrane was more effective than that of β-carotene (Fig. 2), although the rates of polyene chain destruction of both carotenoids by trapping the peroxyl radicals were almost the same (Fig. 3).

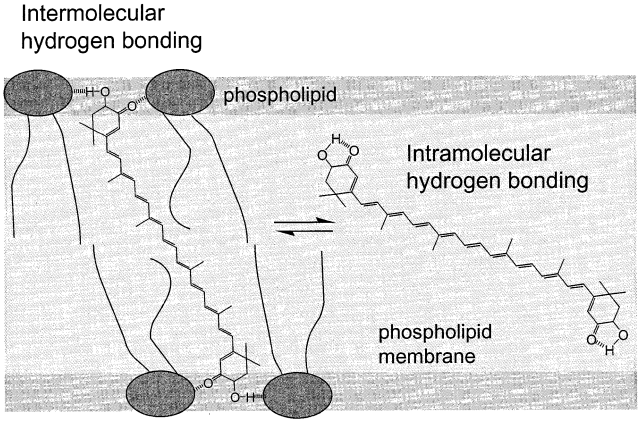

Fig. 6. Schematic representation of the possible locations of astaxanthin molecules having inter- and intramolecular hydrogen bonds in the phospholipid membrane.

Lim et al. [10] proposed, from the effects of xanthophylls on peroxidation of EyPC liposomes and chick plasma phospholipids, that the hydroxyl groups of the two terminal ring moieties of xanthophylls trap free radicals at the membrane surface. On the other hand, Woodall et al. [13,36] assumed that the hydrogen atom at the C4 position of the terminal ring of zeaxanthin is removed by radicals. However, there is no hydrogen atom at the C4 carbon of astaxanthin. According to our MO calculations on the steric energy of the model radicals of EHTC, EHC and HC, the radical scavenging site of the terminal ring moiety of astaxanthin was suggested to be the C3 methine, not the hydroxyl group at the C3 position (Fig. 5).

As the stabilities of carbene radicals of all of these model compounds were almost the same, the radical scavenging of the terminal ring moiety of astaxanthin is likely not to be affected by the methyl groups and polyene chain in the ring. Therefore, the radical scavenge activity of the terminal ring moieties of astaxanthin is suggested to be independent of that of the polyene chain. As the carbene radicals of the hydrogen-bonded radical intermediates were the most stable, five-membered ring formation of astaxanthin is regarded to be favorable for radical trapping as well as gain of hydrophobicity. It is noteworthy that the finding that the active methine group, not the hydroxyl group, was the radical trapping site is important for understanding the radical scavenge mechanism, because versatile radical scavengers having an active methine moiety, such as flavonoids and alkaloids, are known [39–41].

In this study, we found for the first time that the xanthophyll astaxanthin traps radicals not only at the conjugated polyene chain but also in the terminal ring moiety, in which the hydrogen atom at the C3 methine is suggested to be a radical trapping site. Owing to the equivalent amounts of the hydrophobic

intramolecular hydrogen-bonded ring and intermolecular hydrogen bonding with phospholipid polar heads, and the interconversion between the two hydrogen bond formations, the terminal ring of astaxanthin is able to scavenge radicals both at the surface and in the interior of the phospholipid membrane, although its unsaturated polyene chain trapped radicals only in the membrane. These unique properties of astaxanthin should be associated with its potent antiperoxidation activity.

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