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Effect of polar carotenoids on the oxygen diffusion-concentration product in lipid bilayers. An EPR spin label study

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The oxygen diffusion-concentration product was determined in phosphatidylcholine (PC) bilayers from oxygen broadening of the spin label EPR spectra. The use of fatty acid spin labels makes it possible to do structural and oximetric measurements with the same sample. We find that polar carotenoids, zeaxanthin and violaxanthin, increase ordering of hydrocarbon chains in saturated (dimyristoyl-PC) and unsaturated (egg yolk PC) membranes and also significantly decrease the oxygen diffusion-concentration product in the hydrocarbon region of these membranes. At 25 °C in the presence of 10 mol% of carotenoids, the product is about 30% smaller than in pure PC membranes. Intercalation of carotenoids decreases the oxygen diffusion-concentration product in the central part of the bilayer and has little effect on the product in the polar head group region. In contrast, cholesterol molecules significantly reduce the product on and near the membrane surface, and do not change it (saturated PC) or increase it (unsaturated PC) in the middle of the bilayer (Subczynski, W.K., Hyde, J.S. and Kusumi, A. (1989) Proc. Natl. Acad. Sci. USA 86, 4474–4478). The decrease of oxygen diffusion-concentration product may be a mechanism of carotenoid protective activity, which should be effective in plant and animal cells in the light as well as in the dark.

Carotenoids are known to prevent photodynamic destruction of plant and bacterial photosynthetic apparatus [1,2] as well as animal [3] and human [4] cells. It was also found that carotenoids possess anticancerogenic activity [5,6], especially toward skin cancer promoted by UV irradiation [7]. In these activities, carotenoids act as quenchers of triplet excited state of photosensitizers [8–10], quenchers of singlet oxygen [11], free radical scavengers [12,13] and antioxidants [14]. On the other hand, it was found that carotenoids affect the membrane fluidity in *Acholeplasma laidlawii* [15]. Later it was suggested that carotenoids regulate the fluidity of biological membranes, which do not contain cholesterol (*Procaryota*, thylakoids), a function served by cholesterol in *Eucaryota* [16]. Moreover, it appeared that cholesterol modifies not only membrane fluidity but also the product of local oxygen diffusion coefficient, and local oxygen concentration [17], which controls the rate of chemical reactions with oxygen. We thought that the changes of membrane fluidity caused by carotenoids should also be accompanied by changes of oxygen diffusion-concentration product.

In this work, we carried out systematic studies on the influence of the two polar carotenoids zeaxanthin and violaxanthin on the structure of model membranes made of saturated dimyristoylphosphatidylcholine (DMPC) and unsaturated egg yolk phosphatidylcholine (EYPC) lipids. We also examined the oxygen diffusion-concentration product in these membranes. Effect of carotenoids was compared with that of cholesterol. All measurements were carried out using electron paramagnetic resonance (EPR) spin label technique, which allowed us to make structural and oximetric measurements with the same samples.

Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; EPR, electron paramagnetic resonance; SASL, stearic acid spin label; 5-SASL, 5-doxylstearic acid spin label; 16-SASL, 16-doxylstearic acid spin label; T-PC, tempocholine phosphatidic acid.

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Experimental procedures

Chemicals. Phospholipids were purchased from Sigma (St. Louis, MO) and stearic acid spin labels (SASL) were from Molecular Probes (Eugene, Or). Tempocholine phosphatidic acid ester (T-PC) was a generous gift from Dr. S. Ohnishi at Kyoto University. The polar carotenoids, zeaxanthin and violaxanthin were extracted from fresh nettle leaves. The extract was saponified with KOH by the 'cold' method [18]. Carotenoids were separated by thin-layer chromatography on activated Kiselgel plates (Merck, F.R.G.) with solvent system: benzene/ ethyl acetate/ methanol (75 : 20 : 5, v/v) [18]. The narrow strips of the center of zeaxanthin and violaxanthin zones were taken off the TLC plates. The visible absorption spectra of both carotenoids agreed with those known from the literature and did not show any features of the *cis*-isomerization. The concentration of carotenoids has been determined spectrophotometrically using the extinction coefficient given in Ref. 18.

Sample preparation. The membranes used in this work were multilamellar dispersion of phospholipids containing 0 or 10 mol% of carotenoids and 1 mol% of spin label. Briefly, membranes were prepared by the following method [19]: chloroform solutions of the phospholipid, carotenoid and spin label were mixed (containing 10^{-5} moles of total lipids) and chloroform was evaporated with a stream of nitrogen gas and then under reduced pressure (approx. 0.1 mmHg) for at least 12 h. A buffer solution (0.1 ml) was added to the dried lipid at 45 °C and vortexed vigorously. All samples were prepared in 0.1 M borate at pH 9.5 to ensure that all carboxyl groups of SASL were ionized in phosphatidylcholine membranes [20–22]. The lipid dispersion was centrifuged briefly, and loose pellet (approx. 20% lipid w/w) was used for EPR measurements.

Electron paramagnetic resonance. Samples were transferred into a gas-permeable capillary (0.7 mm i.d.) made of the methylpentene polymer TPX [23]. This plastic is permeable to nitrogen, oxygen and carbon dioxide and is substantially impermeable to water. The TPX sample tube was placed inside the EPR dewar, insert and equilibrated with nitrogen or oxygen at 25 °C. EPR spectra were obtained with a Varian E-3 or Varian E-109 X-band spectrometer with Varian temperature control accessories. All preparations and measurements were performed under reduced light.

Line broadening and oxygen diffusion-concentration product. Bimolecular collision of molecular oxygen and nitroxide induces spin exchange which leads to a broadening of EPR lines [24], and this effect is the basis of the method employed here. The line broadening due to oxygen is defined as

$$\Delta H_{pp}(x) = H_{pp}(x, O_2) - H_{pp}(x, N_2) \quad (1)$$

where $H_{pp}(x, O_2)$ and $H_{pp}(x, N_2)$ are the peak-to-peak EPR linewidths of the first derivative spectrum for samples saturated with oxygen and nitrogen, respectively. Since $\Delta H_{pp}(x)$ is proportional to the collision rate of oxygen with the spin label nitroxide group, it is a function of both, the local concentration ($C(x)$) and the local translational diffusion coefficient ($D(x)$) of oxygen at a depth 'x' (distance from the membrane surface at which the nitroxide group of spin label is positioned) in the oxygen equilibrated membrane. On the basis of Smoluchowski equation for isotropic diffusion of oxygen, $\Delta H_{pp}(x)$ can be expressed as [25,26]

$$\Delta H_{pp}(x) = BD(x)C(x) \quad (2)$$

$$B = 8\pi p r_0 / \sqrt{3}\gamma \quad (3)$$

where p is the probability that our observable event occurs when a collision does take place and r_0 is the interaction distance between oxygen and nitroxide group (approx. 4.5 Å) [27,28]. γ is the magnetogyric constant for an electron. It is assumed that the lineshape is Lorentzian, so that EPR linewidth can be expressed in units of frequency as

$$\omega = \gamma H_{pp}\sqrt{3}/2 \quad (4)$$

By taking the ratio:

$$\Delta H_{pp}(x)/\Delta H_{pp}(\text{water}) = D(x)C(x)/D_w C_w(O_2) \quad (5)$$

the effect of uncertainty of p and r_0 (B in Eqn. 2) can be reduced *. Here ΔH_{pp} (water) is the line broadening of EPR spectrum of spin label in water saturated with oxygen, and D_w, C_w (O_2) are oxygen diffusion coefficient and concentration in water. $\Delta H_{pp}(x)$ and ΔH_{pp} (water) are determined for the same class of spin labels (SASL), which at pH 9.5 are soluble in water. Values of oxygen diffusion-concentration product in membrane equilibrated with pure oxygen at 25 °C can be obtained by multiplying $\Delta H_{pp}(x)/\Delta H_{pp}$ (water) by the product $D_w C_w(O_2) = 2.9 \cdot 10^{-11}$ mol cm^{-1} s^{-1} that

* The Smoluchowski equation for the bimolecular collision rate of dissolved oxygen molecules with various spin labels yields values for the diffusion coefficient of oxygen in water that are in agreement with published values obtained by conventional methods [27,28]. The most significant aspect of that agreement is that a Heisenberg exchange at an interaction distance of 4.5 Å occurs with a probability close to one for each encounter. Molin et al. [38] and Salikhov et al. [39] have pointed out that if the translational correlation time, τ_c, is sufficiently short, p will decrease as $T_{1(O_2)}\tau_c/(1 + KT_{1(O_2)}\tau_c)$, where K depends on the exchange integral. Apparently, $KT_{1(O_2)}\tau_c \gg 1$ for oxygen molecules colliding with spin-label molecules even for viscosities as low as 1 cP. Since the method works so well in water with its low viscosity, it is almost certain to work in higher viscosity solvents such as membranes.

was determined by classical diffusion measurements [29] and tables of oxygen solubility in water [30]. Separation of $D(x)$ and $C(x)$ requires an independent experiment, but to our knowledge, only an average oxygen concentration in a pure DMPC bilayer has been determined by two different methods [31,32].

Results and Discussion

As can be seen from Fig. 1, zeaxanthin and violaxanthin increase ordering of hydrocarbon chains in tested membranes. The effect is more pronounced in saturated DMPC membranes than in unsaturated EYPC membranes. From the same figure it follows that the carotenoids increase the apparent order parameter more for 16-SASL (at the center of the membrane) than for 5-SASL (close to the polar head groups). At the same time, the EPR spectra of T-PC suggest that zeaxanthin and violaxanthin increase the mobility of the polar head groups (data not shown). Both carotenoids are rigid rod-shaped molecules with two polar groups at the ends separated by the distance of about 30 Å [33]. These molecules cross the membrane and have their polar groups in the head group regions [34] on both sides of the bilayer. The ordering effect of carotenoids can be explained as a physical interaction

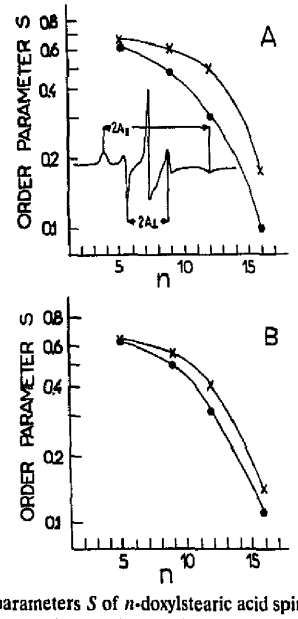

Fig. 1. Order parameters S of n-doxylstearic acid spin label (n-SASL) measured as a function of the doxyl group position (n) along the hydrocarbon chain in DMPC (A) and EYPC (B) bilayers with 0 mol% (●——●) and 10 mol% (×——×) zeaxanthin at 25 °C. S was calculated from EPR spectra using the equation $S = 0.5407 (A_\parallel - A_\perp)/a_0$ where $a_0 = (A_\parallel + 2A_\perp)/3$. $A_\parallel$ and $A_\perp$ were measured directly from the EPR spectra as it is shown in the insert [37]. Similar results were obtained for violaxanthin.

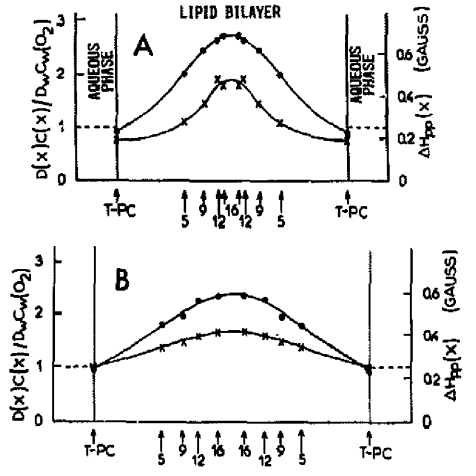

Fig. 2. Profiles of the relative oxygen diffusion-concentration product (or oxygen EPR line broadening) across DMPC (A) and EYPC (B) bilayers with 0 mol% (●——●) and 10 mol% (×——×) zeaxanthin obtained at 25 °C. Approximate location of nitroxide moieties of spin labels are indicated by arrows. ΔH_{pp} (water), indicated in the figure as a broken line, was obtained using SASL spin labels, which at pH 9.5 are soluble in water. Similar results were obtained for violaxanthin.

of rod-like molecules with saturated acyl chains that enhances the extended conformation of lipid hydrocarbon chains (more *trans* bonds) next to the carotenoid. If the lipids contain unsaturated chains, the mismatch between the rigid rod-like structure of carotenoid and the bend of 30 ° at the *cis* double bond diminishes the ordering effect.

Oxygen diffusion-concentration products were measured at five various locations (depths) in the membrane. As a result, we obtained profiles of this product across DMPC and EYPC bilayers. Values of the oxygen diffusion-concentration product in the hydrocarbon region of bilayer are about 30% smaller in the presence of 10 mol% of carotenoids, than that in pure lipid membranes. In the polar head group region, the effect is very weak (see Fig. 2). Comparison of Figs. 2A and 2B leads to the conclusion that carotenoids affect the oxygen diffusion-concentration product in a quite similar way in both types of investigated membranes. So, this effect does not depend on the presence of double bonds as strongly as the ordering effect of carotenoids.

The comparison of the effects of carotenoids on membrane structure and on the oxygen diffusion-concentration product with those of cholesterol can be summarized as follows:

(1) A quantity of 10 mol% of carotenoids exerts similar ordering effect to 15–20 mol% of cholesterol [19].

(2) In the polar head group region, carotenoids do not affect the oxygen diffusion-concentration product

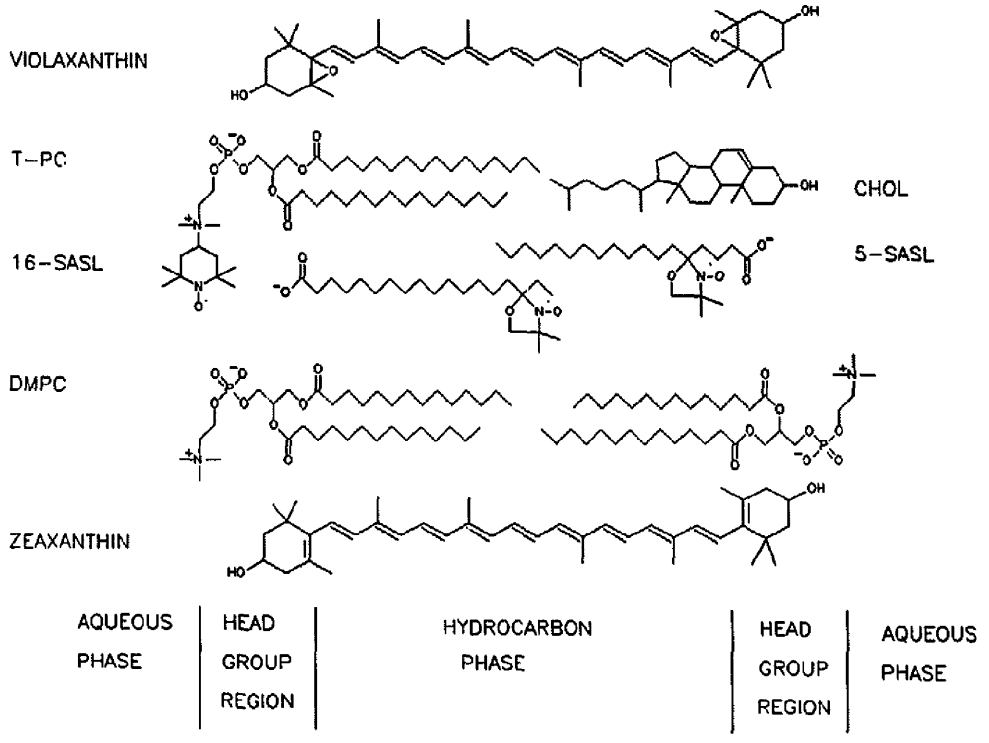

Fig. 3. Drawing of cross-section sketch of DMPC bilayer including spin labels (T-PC, 5-SASL and 16-SASL), polar carotenoids (zeaxanthin and violaxanthin) and cholesterol (CHOL).

while cholesterol significantly decreases that product [17].

(3) In the hydrocarbon region near the polar head groups, the oxygen diffusion-concentration product is decreased to the same extent by 10 mol% of carotenoids or 20–30 mol% cholesterol [17].

(4) In the center of the bilayer carotenoids decrease the oxygen diffusion-concentration product, while cholesterol has no effect in DMPC membranes [17], or strongly increases that product in EYPC membranes (Subczynski, unpublished data).

These differences result from the different structure and the different localization of cholesterol and carotenoids in the membrane (see Fig. 3).The molecule of cholesterol is located in one half of bilayer and its rigid plate-like portion extends to a depth of a 7–10th carbon atoms in lipid alkyl chains [35]. In contrast, one carotenoid molecule influences both halves of lipid bilayer and, with two polar groups interacting with opposite hydrophilic surface of the membrane, it can brace together the two halves of bilayer like a tie-bar. Therefore, the oxygen diffusion and concentration is reduced in those regions of bilayer to which extends the rigid portion of the molecule of modifier (cholesterol or carotenoid).

The rate of all chemical reactions involving molecular oxygen depends on the collision frequency of oxygen with attacked molecules and, thus, on the local oxygen diffusion-concentration product. By lowering this product in hydrophobic region of the membrane, carotenoids should diminish destructive reactions involving oxygen, especially lipid peroxidation. Carotenoids should also lower production of active forms of oxygen (O_2^-, H_2O_2, $OH^\cdot$, $^1O_2$) in that region. In our opinion, the decrease of oxygen diffusion-concentration product induced by carotenoids is large enough to be considered as an effective mechanism of carotenoid protective activity in membranes. This mechanism may help prevent lipid peroxidation, would be effective in plant and animal cells, and in the light as well as in the dark.

It should be pointed out that in some model membranes, molecules of β-carotene are oriented perpendicular to the membrane surface [36]. In these cases,

the effect of β-carotene on membrane structure as well as oxygen diffusion and concentration may be similar to the effect of polar carotenoids.

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