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Review

Carotenoids as modulators of lipid membrane physical properties

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Abstract

Carotenoids are a group of pigments present both in the plant and animal kingdoms, which play several important physiological functions. The protection against active oxygen species, realised via the quenching of excited states of photosensitising molecules, quenching of singlet oxygen and scavenging of free radicals, is one of the main biological functions of carotenoids. Several recent research indicate that the protection of biomembranes against oxidative damage can be also realised via the modification of the physical properties of the lipid phase of the membranes. This work presents an overview of research on an effect of carotenoids on the structural and dynamic properties of lipid membranes carried out with the application of different techniques such as Electron Paramagnetic Resonance, Nuclear Magnetic Resonance, Differential Scanning Calorimetry, X-ray diffractometry, monomolecular layer technique and other techniques. It appears that, in most cases, polar carotenoids span lipid bilayer and have their polar groups anchored in the opposite polar zones of the membrane. Owing to the van der Waals interactions of rigid rod-like molecules of carotenoid and acyl chains of lipids, pigment molecules rigidify the fluid phase of the membranes and limit oxygen penetration to the hydrophobic membrane core susceptible to oxidative degradation.

Keywords: Carotenoid; Membrane

1. Introduction

Carotenoids are widespread yellow and orange pigments of bacteria, algae, plants and animals. Until the present, almost 750 naturally-occurring carotenoid pigments have been identified [1]. Humans and animals are not capable of carotenoid biosynthesis, and therefore, the presence of this group of pigments in their organisms is totally dependent upon diet. Carotenoids are recognized to play several important physiological roles, including antenna function and photoprotection in photosynthetic apparatus [2], scavenging active oxygen species and filtering out the short-wavelength radiation in the retina of the vision apparatus and, in particular, in the macula lutea of primates [3–10] and the regulation of physical properties of biomembranes [11–13]. According to a general view, carotenoid photoprotection in all environments is realised via quenching of singlet oxygen, scavenging free radicals and the quenching of excited triplet state of molecules of photosensitiser [14]. The most recent findings show that carotenoid pigments can quench directly the lowest singlet excited state of photosensitiser via the singlet–singlet excitation energy transfer, leading to population of the low-lying singlet energy level of polyenes (S1, $2A_g^-$) [15,16]. The hydrophobic core of biomembranes composed of polyunsaturated fatty acids is a potential target of attack of active oxygen species, which may directly lead to the membrane degradation. Besides all the physical mechanisms involved in carotenoid photoprotection, referred to above, a direct effect of carotenoid pigments on lipid membranes, in particular the effect on structural and dynamic properties, seems to decrease the lipid membrane susceptibility to oxidative

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degradation. For example, the presence of polar carotenoids in the lipid phase has an impact on the membrane physical properties modulating membrane fluidity and changing penetration barrier of small molecules, including oxygen [17,18]. In this paper, some aspects of modulation of lipid membrane physical properties by carotenoid pigments are addressed, and recent publications on this problem are summarized.

2. Chemical structure and some physical properties of carotenoids

Most naturally occurring carotenoid pigments are tetraterpens; some of them ended with cyclic jonone rings at one or at two sides (see Fig. 1). In several cases, the hydrocarbon skeletons of carotenes are modified with oxygen functional groups such as hydroxy, keto or epoxy groups. In such a case, the carotenoids are called xanthophylls. A very important property of carotenoids, both from spectroscopic and structural points of view, is the presence of double bonds in a conjugated system. A conjugated double bond system of a polyene longer than 9 is responsible for the pigment properties of carotenoids. Namely, the energy of the strongly allowed electronic transition from the ground energy level ($1A_g^-$) to the S2 state ($1B_u^+$) appears on the energy scale below 3 eV and therefore corresponds to the absorption of electromagnetic radiation from the visual region (see Fig. 2). From the structural point of view, the conjugated double bond system constitutes a rigid, rod-like skeleton of carotenoid molecules. This feature seems to play a key role in the stabilization function of carotenoids, both with respect to lipid membranes and proteins.

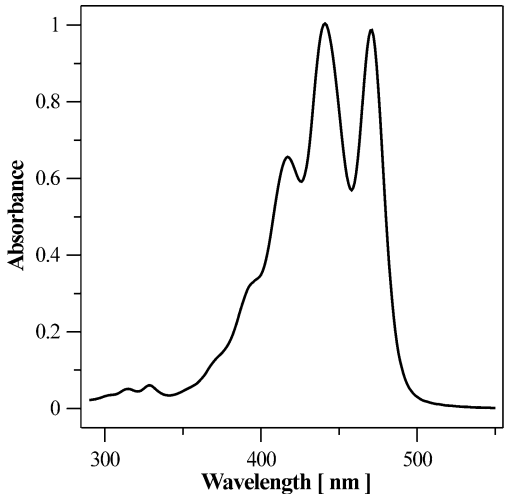

Fig. 2. Absorption spectrum of violaxanthin in the organic solvent mixture acetonitrile:methanol:water (72:8:3, by volume) in the UV–Vis region. The molar extinction coefficient of most carotenoids in the main absorption maximum (0–1 vibrational transition) varies between 123,000 and 153,000 M^{-1} cm^{-1} in organic solvents. For example, the molar extinction coefficient of all-*trans* violaxanthin in ethanol at 440 nm is 153,000 M^{-1} cm^{-1} [64].

3. Localization and orientation of carotenoid pigments in lipid membranes

Carotenes are hydrophobic molecules; therefore, their localization within the hydrophobic core of the lipid

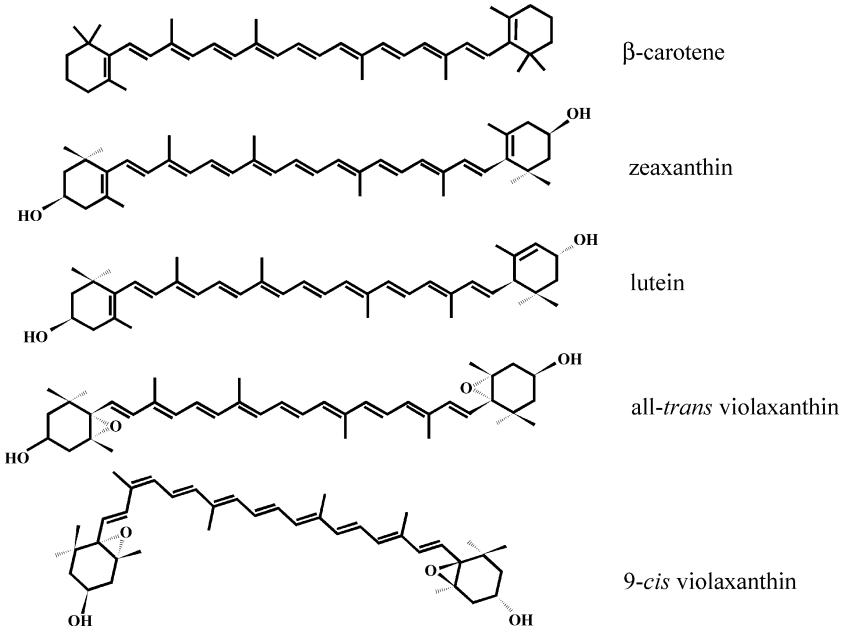

Fig. 1. Chemical formulas of selected carotenoid pigments: β-carotene, zeaxanthin, lutein, violaxanthin all-*trans* and violaxanthin 9-*cis*.

membrane can be predicted. In fact, the analysis of the position of the absorption maxima in the UV–Vis spectral region of carotenes incorporated into lipid membranes indicate that chromophores (the conjugated double bond system of a molecule) are located in the environment characterized by the dielectric properties typical of the hydrocarbon lipid chains [12,13,19–23]. In most cases, polar carotenoids have located their hydrophilic groups at two opposite sides of a long-shaped molecule, and therefore, the absorption spectra of xanthophyll pigments incorporated to lipid membranes indicate the same localization of molecular chromophores also in this case [12,13,19–23]. In order to minimize the energy of the system, xanthophyll pigments have to adopt localization in the lipid membranes, such that the hydrophilic groups remain in contact with the polar head-groups of the lipid bilayer. Two possible orientations of polar carotenoids have been discussed, on the basis of the linear dichroism measurements (see Fig. 3) [12,13]. In one case, polar carotenoids span the membrane, and the hydrophilic groups located at the opposite ends of the molecule are anchored in the two opposite polar zones of the membrane (for example, zeaxanthin anchored with two hydroxy groups located at the 3 and 3′ positions). It is also possible that all polar groups of a xanthophyll molecule remain in contact with the same polar zone of the membrane. Such orientation has been proposed not only for pigments in a conformation *cis* [24] but also in the case of lutein in the conformation all-*trans* [25–28]. In terms of chemical structure, lutein is very close to zeaxanthin, except that one double bond in the end ring of lutein (ε ring) is located between the carbon atoms 4′ and 5′, different to that in zeaxanthin (between the carbon atoms 5′ and 6′, respectively). Due to that fact, the conjugated double bond system of lutein does not extend to the ring, and the entire ε ring possesses relative rotational freedom around the 6′–7′ single bond. A natural consequence of such a rotational freedom is an ability to "tune" the orientation of the hydroxy group located at the 3′ carbon atom in dependence of the actual localization of the molecule. Owing to this ability, lutein was proposed to adopt two orthogonal orientations with respect to the lipid bilayer: one roughly vertical and one horizontal [25–29]. In the case of carotenes lacking polar groups, such as β-carotene and lycopene, possible orientation in the lipid membrane environment seems to be exclusively governed by van der Waals interactions with the hydrocarbon acyl chains of lipid molecules, forming the hydrophobic core of the membrane. Resonance-raman spectroscopy studies revealed that the orientation of β-carotene with respect to the lipid bilayer is not as well defined as xanthophyll pigments [30]. The homogeneous orientational distribution of β-carotene in the membrane system formed with egg yolk phosphatidylcholine has been concluded, based on the linear dichroism, determined orientation angle 55°, exceptionally close to the magic angle (54.7°) [12,13].

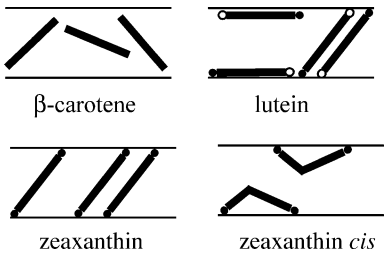

Fig. 3. Schematic representation of the main patterns of localization and orientation of carotenoid pigments in the hydrophobic core of lipid membranes. The following carotenoid pigments were used as examples: β-carotene, zeaxanthin all-*trans*, zeaxanthin 13-*cis* and lutein.

4. Effect of carotenoids on the physical properties of biomembranes as revealed by different experimental methods

4.1. Electron Paramagnetic Resonance (EPR) experiments

EPR combined with the spin label technique, provides several important information regarding the effect of carotenoids on both the structural and dynamic properties of lipid membranes, owing to the fact that the shape of an EPR spectrum highly depends on the motional freedom of a free radical segment of the spin label molecule embedded to the membranes. In particular, the application of specific spin label molecules, which tend to localize in well defined membrane localizations, such as head-group region or hydrophobic core at its different depth, let gain precise "microscopic" information on molecular mechanisms of carotenoid–membrane interaction. Some parameters that can be obtained from the analysis of an EPR spectrum provide information on the effect of carotenoids on the structural properties of the membranes (for example, order parameter *S*) and also on the dynamic properties of the membranes (for example, correlation time τ). According to the original approach introduced by Subczyński et al. [17], the analysis of EPR spectrum is able to provide also information regarding an effect of carotenoids on oxygen penetration to the membrane (oxygen diffusion–concentration product). EPR studies have demonstrated that:

1) Polar carotenoids (zeaxanthin, violaxanthin, lutein) increase the membrane fluidity in the ordered phase of the membrane and decrease fluidity in the liquid crystalline phase of the membranes formed with phosphatidylocholines [31–33]. This effect has been shown to be concentration dependent and the complete removal of the main thermotropic phase transition ($P'_\beta \rightarrow L_\alpha$) has been observed at 10 mol% carotenoid with respect to lipid. The incorporation of carotenoids

at lower concentration decreased the cooperativity of the phase transition [31,32].
2) The incorporation of polar carotenoids to the lipid membrane increases the order parameter across the bilayer formed with egg yolk phosphatidylcholine and dimyristoyl phosphatidylcholine, in particular in the central region of the hydrophobic core [31,32,34].
3) Xanthophyll pigments incorporated to the lipid membranes increase the penetration barrier to molecular oxygen into the hydrophobic membrane interior [17].
4) The effect of nonpolar β-carotene on the membrane was considerably lower compared to the effect of polar carotenoids and was pronounced mainly in the fluidization of the well-ordered phase of phosphatidylcholine membranes [33].
5) β-Carotene has been also demonstrated to decrease penetration barrier to small molecules to the membrane head-group region [33].

4.2. Nuclear Magnetic Resonance (NMR) experiments

Similarly to the EPR spectra, also the NMR spectra recorded from the samples containing lipid dispersions are sensitive to the physical state of the membranes. In particular, the rate of different kinds of molecular motion, including the rotation of entire molecule and gauche-trans isomerization of alkyl chains of lipids, influences the parameters of the NMR spectra. This dependence has been extensively applied to examine the effect of carotenoid pigments on the dynamic properties of lipid molecules forming a membrane. The application of ^{31}P NMR, ^{13}C NMR and ^{1}H NMR has been reported [25,35,36]. NMR studies have demonstrated that:

1) Polar carotenoids (lutein, zeaxanthin) restrict molecular motions of both CH_2 and terminal CH_3 groups of alkyl chains of lipid membranes [25,36] in contrast to β-carotene, whose orientation in the membrane is not as much restricted and defined [35].
2) β-Carotene increases the motional freedom of lipid molecules in the head-group region of the membranes formed with phosphatidylcholines [35], in contrast to its polar derivative (zeaxanthin) [36].
3) Both β-carotene and zeaxanthin (to a lesser extent) increase the penetration ability into the membrane polar zone of small charged molecules, as demonstrated with the application of praseodymium ion assay [36].

4.3. Differential Scanning Calorimetry (DSC) measurements

DSC has been also successfully applied to follow the effect of carotenoid pigment on structural and dynamic properties of lipid membranes, especially on the thermotropic phase transitions. Several combinations of carotenoids and model lipid membrane constituents have been studied, such as lutein in DMPC and in multicomponent lecithin membranes [37], canthaxanthin and astaxanthin in DMPC [38,39], and various carotenoids in DPPC such as β-carotene [40,41], zeaxanthin [40,41], lutein [41], lycopene [41] and violaxanthin [41]. In general, the effect of carotenoids on the thermotropic phase transitions of lipid membranes, as revealed by means of the DSC technique, may be summarized as follow:

1) Polar carotenoids shift the main phase transition temperature ($P'_\beta \rightarrow L_\alpha$) towards lower values by ca. 1° or less, depending on concentration [41].
2) Carotenoids shift the phase pretransition temperature ($L'_\beta \rightarrow P'_\beta$) towards lower values by values from the range 0.5° in the case of lycopene to 3.2° in the case of violaxanthin, at 1 mol% carotenoid in the lipid phase [41].
3) Polar carotenoids decrease cooperativity and the molar heat capacity of the main phase transition [41].
4) Comparison of the effects of structurally different carotenoids and perhydro-β-carotene (a β-carotene derivative) on membrane thermotropic properties revealed that the most important structural feature of carotenoids, altering the thermotropic properties of membranes, is the presence of the rigid polyisoprenoid chain [41].
5) Carotenoid with polar groups attached to their rings alter the thermotropic behaviours of DPPC membranes stronger than carotenes [41].

4.4. X-ray diffractometric measurements

Self-organization of lipid molecules in a hydrated system leads to the formation of bilayer lipid membranes characterized by a well-defined thickness. The preparation of the samples composed of a certain number of lipid bilayers, deposited one to each other (multibilayers), have opened a possibility to determine the thickness of a single bilayer by means of the diffractometric techniques, including X-ray diffractometry [28,42–44]. The diffractometric measurements demonstrate that the physical state of hydrocarbon acyl chains, which constitute the hydrophobic core of the membrane, and, in particular, the rate of the gauche-trans isomerization are the main determinants of the thickness of lipid bilayers. The effect of carotenoid pigments on the thickness of lipid membranes has been also studied in order to gain information regarding the effect of the pigments on structural properties of lipid bilayers, also those determined by dynamic alkyl chain isomerization [28,42–45]. It has been reported that:

1) Xanthophyll pigments (in particular lutein) force acyl chains of lipids to adopt extended conformation via the van der Waals interactions, which is demonstrated by the increase in the thickness of lipid membranes formed with DMPC and DPPC [28,42–44].

2) Lycopene was found to disorganize the hexagonal packing of the fatty acid hydrocarbon chains of the DPPC bilayer, while its effect on the polar head-group region was negligible [45].
3) Xanthophylls and especially violaxanthin exerted a strong, disturbing effect to the polar region of DPPC as compared with carotenes [45].

4.5. Fluorescence measurements

Although carotenoids may emit weak fluorescence [46,47], its intensity is far too low to be directly used for carotenoid–membrane lipid interaction studies. Instead, various fluorescence probes have been applied to monitor the effect of carotenoids on lipid membrane physical properties. Using pyrene as the fluorescence probe, Socaciu et al. [48,49] reported on changes in the micropolarity of the probe environment after the incorporation of carotenoids into phospholipid model membranes. However, in the case of microsomes, the incorporated carotenoids did not modify significantly the polar environment of pyrene molecules [50].

The same group measured also the effect of various carotenoids on fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in phospholipid liposomes and microsomal membranes. It was found that the observed effect depends both on the type of the membrane, as well as on the carotenoid species [48–50]. Still other fluorescence probes were used for measuring the effect of carotenoids on such lipid membrane physical properties, as ordering, hydrophobicity and permeability to water molecules. [51]. Again, the observed effects varied for different types of carotenoids showing a dependence on their incorporation degree and location in the bilayer.

In general, the results of the experiments carried out with the application of fluorescence probes corroborate with the conclusions based on the EPR technique. In particular, these results show that the effect of polar carotenoids on the physical properties of the membranes is little in the ordered phase and more pronounced in the fluid membrane phase.

4.6. Monomolecular layer technique

Some carotenoids, such as xanthophylls in the conformations *cis* but also lutein in the conformation all-*trans*, are postulated to adopt the horizontal orientation with respect to the lipid membrane plane. In such an orientation, the carotenoid remains in contact with a single lipid layer from the bilayer. The monomolecular layer technique has been applied to study the details of lipid–carotenoid interaction in the two-component films [24,26,29,52]. The deposition of mixed lipid–carotenoid monolayers to solid support, by means of the Langmuir–Blodgett technique, made it possible to perform spectroscopic analysis of carotenoid–lipid interaction, carried out with the application of electronic absorption spectroscopy and FTIR technique [24,26,29,46]. Monomolecular layer technique studies:

1) confirm the ability of the *cis* xanthophylls and all-*trans* lutein to adopt horizontal orientation at the polar–nonpolar interface in the two-component system with lipids;
2) indicate that polar groups of xanthophylls (in particular, hydroxyl groups located at the 3 and 3′ positions) are involved in the interaction to lipids and the stabilization of the carotenoid orientation in a lipid phase.

4.7. Permeability experiments

Permeability experiments for small ions and other solutes have been performed in the lipid membrane systems (liposomes) modified with the carotenoid pigments in order to analyse directly the effect of carotenoids on the transport properties of biomembranes, but also to analyse the influence of carotenoids on the mechanical properties of lipid membranes [18,53].

1) Polar carotenoids, such as zeaxanthin and thermozeaxanthin (zeaxanthin glucose ester), were shown to increase significantly the permeability barrier of the lipid membranes for protons and water-soluble fluorescent dye calcein, respectively [18,53].
2) The effect of thermozeaxanthin has been observed in the case of the membranes formed with egg yolk phosphatidylcholine but not in the case of the membrane system characterized by bigger thickness (DMPC, DPPC and POPC [53]). Such an effect has been interpreted in terms of the structural effect of the xanthophyll on physical membrane properties as strictly dependent on the thickness of the hydrophobic core of the membrane and the distance between the polar groups of the carotenoid.
3) β-carotene (<1%) and especially zeaxanthin (2%) increased the permeability of digalactosyldiacylglycerol vesicles for glucose [18].

4.8. Computer simulation of molecular dynamics

A powerful technique which permits to obtain data not available from experiments is the computer simulation of the molecular dynamics of lipids in bilayer [54]. Using this approach, we studied the orientation of β-carotene in 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) membrane [55]. Obtained results show that both β-carotene rings are localized in the region occupied by carbonyl groups of POPC γ-chain. The ordering effect of β-carotene on both the β- as well as the γ-chain was observed. Interestingly, the low value of the order parameter and a high tilt angle were found for those segments of the β-carotene molecule where methyl groups are present. Our data suggest an existence of

two pools of β-carotene in the POPC membrane, differing in its preferential orientation.

5. Conclusions

Carotenoid pigments incorporate to the lipid bilayer system in such a way that the chromophore is entirely embedded in the hydrophobic core of the membrane. Most xanthophylls (in particular in the conformation all-*trans*), containing polar groups located at two opposite sides of the molecule, orient in the membrane in such a way that these groups remain anchored in two opposite polar zones of the bilayer, owing to the hydrogen bonds formation with the hydrophilic groups of lipid molecules. Such a pigment localization and orientation provides favourable conditions for carotenoid interaction with alkyl chains of lipids via van der Waals interactions. These interactions modify significantly physical properties of the lipid bilayer and of the hydrophobic membrane core in particular. This modification is pronounced, among others, in the rigidifying and stabilizing effect of carotenoids with respect to the membrane and in the modification of the diffusion barrier to and across the membrane to ions, molecular oxygen and other small ions.

It should be mentioned that the effect of polar carotenoids on phospholipid membrane physical properties resembles, in many cases, that of cholesterol. Using different experimental techniques and also molecular dynamics simulation approach, it has been demonstrated that cholesterol increases the order of saturated alkyl chains of phospholipids [54,56,57] and membrane surface density [58–60] at temperatures above the main phase transition. Also, a decrease in permeability [61] and increase in the mechanical strength of the bilayer [62] has been reported.

The majority of the available data concerning the effect of carotenoids on membrane physical properties have been obtained for model lipid membranes. However, the results from such simplified systems may be extrapolated to the natural membranes. Carotenoids may play a role of modulators of physical properties of the natural membranes which do not contain cholesterol. We have already reported that the changes in the carotenoid pigments composition in the thylakoid membranes as an effect of the activity of the xanthophyll cycle or due to incorporation of exogenous pigments result in distinct modification of the fluidity of these membranes [11,63].

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