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Calorimetric studies of the effect of *cis*-carotenoids on the thermotropic phase behavior of phosphatidylcholine bilayers

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ABSTRACT

Carotenoid geometry is a factor that determines their solubility and orientation in the lipid membrane as well as antioxidant capacities and bioavailability. The effects of the *cis*-isomers of carotenoids (zeaxanthin and β-carotene) on the thermotropic properties of lipid membranes formed with dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) were investigated by means of differential scanning calorimetry. The results were compared with the effects caused by the all-*trans*-isomer. Both the *trans* and *cis* isomers of zeaxanthin shifted the main phase transition temperature to lower values and decreased the cooperativity of the phase transition. The effect of all-*trans* zeaxanthin on the physical properties of the lipid bilayers has been shown to strongly depend on the hydrocarbon chain length of the membrane. In the case of *cis*-zeaxanthin this relationship is weaker.

1. Introduction

It has been well-documented that polar carotenoids, in the all-*trans* configuration, are effective modulators of the fluidity of natural and model lipid membranes [1–5]. Carotenoids broaden the main phase transition of membranes formed with phosphatidylcholines [5,6], increase the order [5,7] and decrease the alkyl chain motion in the fluid-phase membranes [4]. Carotenoids also increase the hydrophobicity of the membrane center [5,8]. The effect of carotenoids in the membrane center is probably a result of the introduction of a larger number of conjugated double bonds into that region. Polar carotenoids stabilize both halves of the lipid bilayer acting like a rivet, increasing membrane rigidity by ordering alkyl chains at all depths. The effect of carotenoids on the modulation of the physical properties of lipid membranes is the strongest for dipolar carotenoids, significantly weaker for monopolar, and negligible for non-polar pigments [5]. The presence of polar hydroxyl groups at the ends of the carotenoid molecule, which are anchored at the opposite sides of lipid bilayers, seems to significantly affect the membrane properties. Direct presence of carotenoid pigments in the lipid phase of biomembranes has been documented in the case of membranes of the retina [9–11], bacteria [12–15] and thylakoid membranes of chloroplasts [16,17]. In all those cases the hydrocarbon skeleton of the carotenoid pigments was substituted with polar groups located at the opposite side of the molecule. Our knowledge of where carotenoids actually exist in membranes of different phospholipid composition (phase separation, association with specific lipids) is not well known yet. In the simple model of the photoreceptor outer segment membranes of the retinas the macular xanthophylls are substantially excluded from the raft domains enriched in cholesterol and saturated phospholipids and remain concentrated in the bulk domain, which is enriched in highly unsaturated phospholipids [18].

The role of the all-*trans* carotenoids in the modulation of the physical properties of lipid membranes has been a subject of research for the last three decades. Effects of *trans*-carotenoids on the structural and dynamic properties of lipid bilayers have been studied with application of various techniques, such as differential scanning calorimetry (DSC) [6,19], fluorescence [20], electron spin resonance (ESR) [4,5,7,8] and nuclear magnetic resonance (NMR) [21] spectroscopy, diffractometry [22,23], monolayers technique [24], and others. However, the effect of the *cis*-isomers of carotenoids on the membrane properties is less investigated [25,26].

In nature carotenoids exist primarily in the thermodynamically more stable all-*trans* conformation rather than in *cis* configuration. *Trans–cis* conversion occurs at elevated temperature and/or in the presence of intensive light [27] and triplet sensitizers. The unstable *cis* configurations are naturally present in most fruits and vegetables and

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are also produced during food processing. In living organisms, *cis*-carotenoids have also the specific localization and functions. The natural selection of carotenoid isomers in the photosynthetic system of *Rhodospirillum rubrum S1* bacterium [28] reflects differences in the biological function of these geometrical forms. The all-*trans* isomer of spirilloxanthin is optimized for the excitation energy transfer in the light-harvesting system, whereas the 15-*cis* isomer is usually present in the reaction center as a quencher of the triplet state.

Several research groups have suggested that the *cis* isomers of lycopene are better absorbed from food than the all-*trans* forms [29,30] because of the greater solubility of *cis*-isomers in chylomicrons. *Cis*-lycopene isomers are preferentially absorbed, probably because they do not have a tendency to form large aggregates and crystals [25]. On the other hand, all-*trans* carotenoids may readily organize into aggregates. Several *cis*-isomers of xanthophylls are also present in human plasma and tissues [31,32]. In addition to the all-*trans* isomers, the *cis*-isomers of macular xanthophylls (9-*cis* lutein, 13-*cis* lutein, 9-*cis* zeaxanthin and 13-*cis* zeaxanthin) have also been isolated from human, monkey and bovine eye retina and characterized [32,33]. These *cis*-isomers may be formed in the *macula lutea* as a result of the photo-induced isomerization of the all-*trans*-isomer.

Recently, ESR spin-labeling methods have been applied to study the effect of the all-*trans*, 9-*cis* and 13-*cis* isomers of zeaxanthin on the molecular organization and dynamics of dimyristoylphosphatidylcholine (DMPC) membranes [26]. Effects on membrane fluidity, hydrophobicity, and molecular oxygen penetration have been monitored at the center of the fluid phase DMPC membrane. Unexpectedly, the effects of *cis*-zeaxanthin, observed in the center of the DMPC membrane by the ESR technique, on the oxygen diffusion-concentration product, have been different from those caused by cholesterol, but similar to the all-*trans*-zeaxanthin. In the center of the bilayer, *cis*-zeaxanthin like all-*trans* zeaxanthin decreases the oxygen diffusion-concentration product, while cholesterol has no effect in the DMPC membrane center [7,28,34]. These results suggest that most molecules of *cis*-isomers adopt transmembrane orientation with the polar hydroxyl groups located in the opposite leaflets of the DMPC bilayer. The effects of *cis*-isomers have been even greater than those caused by the *trans*-isomer. This can be explained by the fact that *cis*-isomers are better soluble in the lipid phase (not forming large aggregates).

All carotenoids belong to the group of the most important singlet oxygen quenchers and scavengers of free radicals [35]. The ability of carotenoids to act as antioxidants depends on their structure, physical form and is related to their localization in the lipid membrane. The knowledge regarding carotenoid–lipid interactions in a membrane helps to understand the basic mechanisms of antioxidant activity.

Differential scanning calorimetry has been applied to investigate the problem of interaction of all-*trans* isomers with DPPC and DMPC membranes[6,19], but there are no data about the effects of the *cis*-isomers of carotenoids on the thermotropic properties of lipid membranes yet. In the present work we studied the influence of the *cis*-carotenoids on the thermotropic phase behavior of phosphatidylcholine multilamellar vesicles by means of differential scanning calorimetry. Thanks to this application of the DSC technique we were able to examine the effects of the *cis*-isomers of carotenoid present in the lipid membranes at a relatively small concentration, corresponding to the physiological level. DSC is a well-suited technique to this kind of study because it does not require any external substances as a probe that could influence the structure and dynamics of membranes.

2. Materials and methods

2.1. Chemicals (carotenoids and lipids)

Synthetic crystalline all-*trans* zeaxanthin ((3*R*,3′*R*)-β,β-carotene-3,3′-diol) was a gift from Hoffmann-La Roche, (Basle, Switzerland) or was isolated from the fruit of *Lycium barbarum* [36] and purified chromatographically. 9-*cis* and 13-*cis* isomers of zeaxanthin (see Fig. 1 for molecular structure) were obtained as a product of iodine-catalyzed photo-conversion of the all-*trans* form following a procedure described by Molnar and Szablocs [27]. Isomeric forms of zeaxanthin were separated chromatographically on C-30-coated high-performance liquid chromatography (ProntSIL, length 250 mm, internal diameter: 4.6 mm). A solvent mixture of methyl tertiary butyl ether/methanol (5:95, v/v) was used as a mobile phase. The standard absorption spectra of the isomers of zeaxanthin were recorded directly after purification. The concentration of all-*trans* zeaxanthin was determined spectrophotometrically using the molar extinction coefficients 1.409×10^{-5} mol^{-1} cm^{-1} [36]. The molar extinction coefficients for the isomers of zeaxanthin were assumed to be the same as in the case of the isomeric forms of β-carotene [37]. Zeaxanthin is a derivative of β-carotene (β, β-carotene-3,3′-diol) and in the case of the all-*trans* isomers of both pigments the extinction coefficients are very close to each other (1.409×10^{-5} mol^{-1} cm^{-1} in the case of zeaxanthin and 1.404×10^{-5} mol^{-1} cm^{-1} in the case of β-carotene in the main absorption maximum in ethanol [36]). The relative concentrations

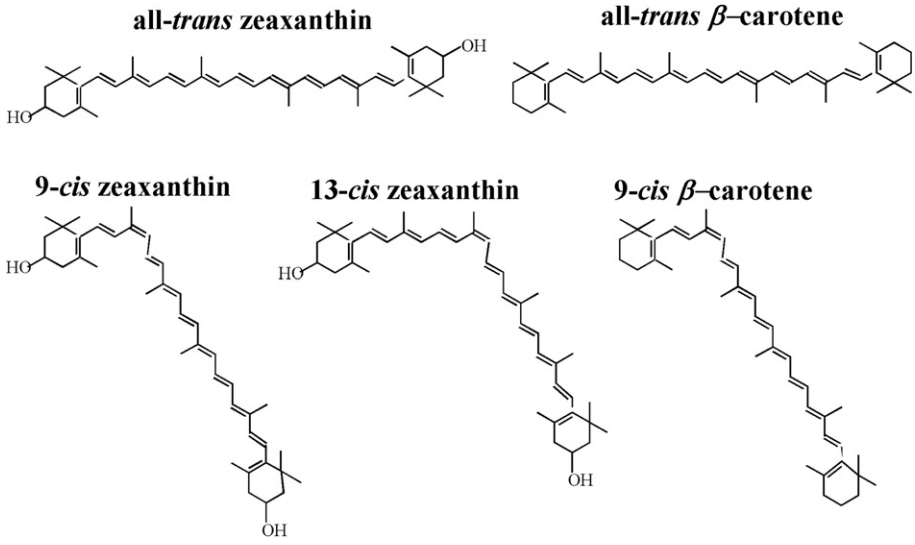

Fig. 1. Chemical structures of all-*trans*, 9-*cis*, and 13-*cis* isomers of zeaxanthin and all-*trans*, 9-*cis* isomers of β-carotene.

of *cis* isomers of zeaxanthin were calculated by multiplication by the following factors: 1.42 for 13-*cis*, 1.16 for 9-*cis*. These factors reflect and combine both the different spectral shifts and the different molar extinction coefficients of individual isomers.

β-Carotene (β,β-carotene-3,3′-diol) was purchased from Sigma Chemical Co. (St. Louis, USA). *Cis*-β-carotene was obtained thermally by keeping a concentrated solution in benzene for 1 h at 70 °C. Isomers of β-carotene were purified and separated chromatographically on C-30-coated high-performance liquid chromatography (YMC, length 250 mm, internal diameter: 4.6 mm). A solvent mixture of methanol: hexane (4:1, v/v) was used as a mobile phase. Carotenoids were dark stored at −35 °C in a nitrogen atmosphere and used shortly after purification. The molar concentration of isomers of β-carotene was evaluated spectrophotometrically using molar extinction coefficients presented in the literature [36,37].

Lipids L-α-dimyristoylphosphatidylcholine (DMPC) and L-α-dipalmitoylphosphatidylcholine (DPPC) were obtained from Sigma Chemical Co. (St. Louis, USA). Both were used without further purification.

2.2. Preparation of liposomes

The membranes used in this work were multilamellar dispersions of DMPC and DPPC lipids containing appropriate amounts of geometrical isomers of zeaxanthin and isomers of β-carotene added to the sample during preparation. Briefly, these liposomes were prepared by the following method: chloroform solutions of lipids and isomers of carotenoids were mixed; the chloroform was evaporated with a stream of nitrogen gas; and the lipid film on the bottom of the test tube thoroughly dried under reduced pressure (about 0.1 mmHg) for 1 h. A buffer solution (20 mM HEPES buffer, pH 7.2) was added to the dried film and vortexed above the main phase transition temperature of the lipid for at least 15 min. The final phospholipid concentration in the buffer was 1 mM.

2.3. Differential scanning calorimetry measurements

The DSC measurements were performed using a Differential Scanning Calorimeter (CSC Model 6100 Nano II, Calorimetry Sciences Corporation, Provo, UT, USA). The heating and cooling rates used were: 1 °C/min and 2 °C/min. The DSC thermograms were analyzed using the CpCalc software (Version 2.1) provided by Applied Thermodynamics Corp., USA.

3. Results

3.1. The effects of isomers of zeaxanthin on thin membranes (DMPC) and thick membranes (DPPC)

All heating thermograms of DMPC multilamellar vesicles containing isomers of zeaxanthin were referred to those made of pure DMPC. The DSC profiles obtained for the pure DMPC and mixtures with 1 mo % of isomers of zeaxanthin are shown in Fig. 2A. All thermal values obtained for the pure lipid multilamellar vesicles are in good agreement with those reported previously [38]. In the absence of carotenoids, the DMPC bilayers exhibit a reversible and highly cooperative phase transition near 24 °C. It can be seen that the incorporation of all isomers of zeaxanthin induced a broadening and a shift of the transition to lower temperature values. The main phase transition peak becomes asymmetric and the pretransition peak becomes flat or vanishes. Fig. 2B represents the shifts of the main phase transition temperature for the DMPC bilayers induced by 1 mol% of zeaxanthin. The effect of the all-*trans* isomer of zeaxanthin is greater than the effect of the *cis*-isomers. However, no significant difference in the effects of the 9-*cis* and 13-*cis* zeaxanthin was observed. The incorporation of all-*trans* zeaxanthin shifts the main phase transition temperature by 2.5 °C, whereas both *cis*-isomers of zeaxanthin shift the main phase transition temperature by about 1.5 °C. The full width at half height (FWHH) of the main phase transition calorimetric peak, as read from the thermograms, is considered to be related to the cooperative nature of the transition. The effect of geometrical isomers of zeaxanthin on the phase transition in the DMPC membranes is stronger for the *trans*-zeaxanthin than for the 9-*cis* and 13-*cis* zeaxanthin (Fig. 2C). The effect of carotenoids on the cooperativity of the phase transition is stronger for membranes formed with DMPC than for the thicker membranes, formed with DPPC (compare Fig. 2C and Fig. 3C). The effect of isomers of zeaxanthin on the main phase transition temperature of the DPPC membranes is different as observed in the case of the membranes formed with DMPC. The effect of *cis*-isomers of zeaxanthin on the main phase transition temperature of DPPC membranes is greater than the effect of the all-*trans* isomer. The difference between the effects of 9-*cis* and 13-*cis* isomers is negligible.

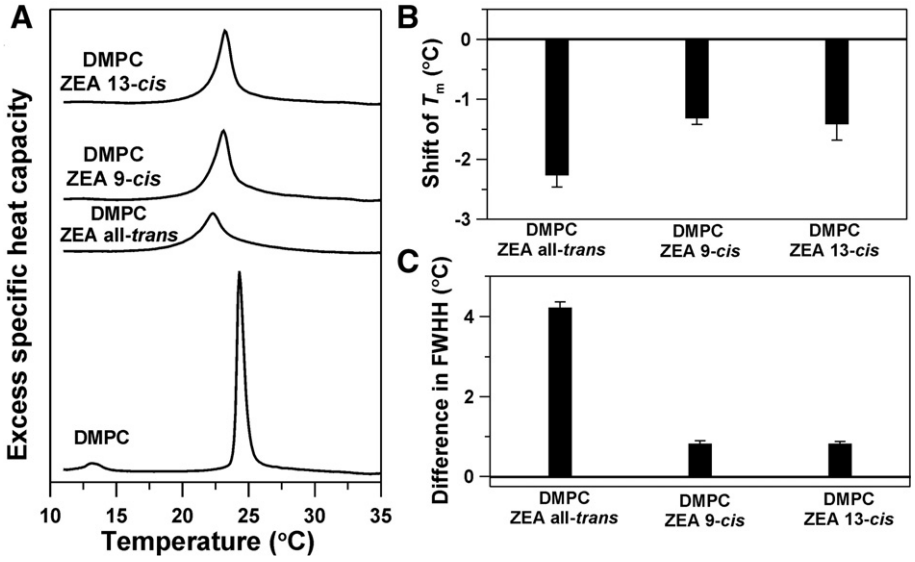

Fig. 2. (A) Representative DSC heating thermograms of DMPC multilamellar vesicles containing 1 mol% of isomers of zeaxanthin in conformation: all-*trans*, 9-*cis*, 13-*cis*. All data shown were acquired at scan rates 1 °C/min. (B) Shifts of the main phase transition temperature, Tm, of DMPC multilamellar vesicles induced by the addition of 1 mol% of isomers of zeaxanthin. Negative values indicate shifts to lower temperatures. (C) Difference in the full width at half height (FWHH) of the main phase-transition of DMPC multilamellar vesicles induced by the addition of 1 mol% of geometrical isomers of zeaxanthin.

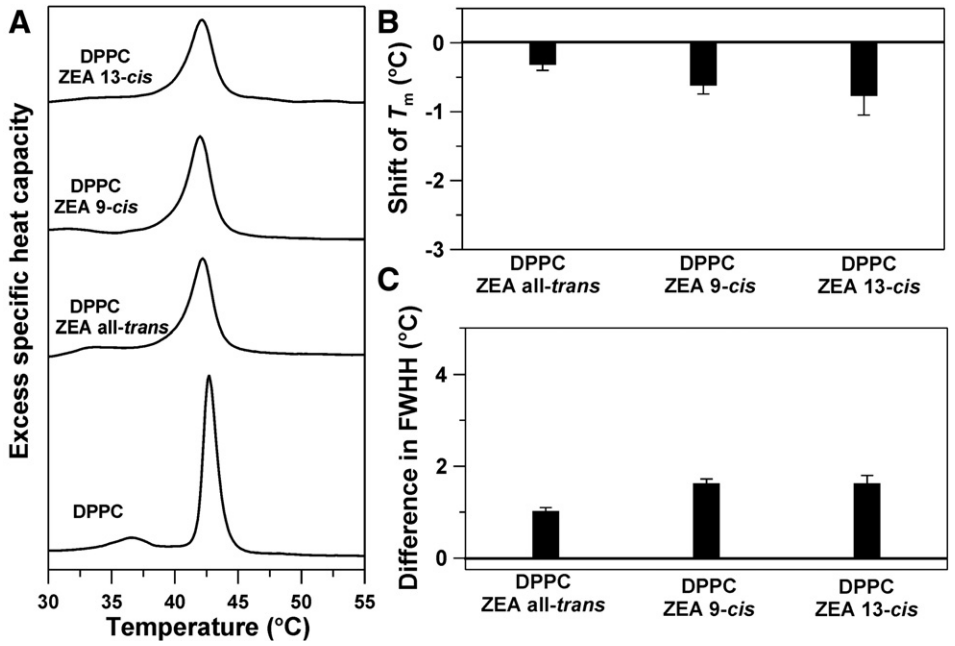

Fig. 3. (A) Representative DSC heating thermograms of DPPC multilamellar vesicles containing 1 mol% of isomers of zeaxanthin in conformation: all-*trans*, 9-*cis*, 13-*cis*. All data shown were acquired at scan rates 2 °C/min. (B) Shifts of the main phase-transition temperature, Tm, of DPPC multilamellar vesicles induced by the addition of 1 mol% of isomers of zeaxanthin. (C) Difference in the full width at half height (FWHH) of the main phase-transition of DPPC multilamellar vesicles induced by the addition of 1 mol% of geometrical isomers of zeaxanthin.

Both the main phase transition and the pretransition peaks in the thermograms decrease in magnitude and shift toward lower temperatures.

The difference between the effects of *cis*-zeaxanthin and *trans*-zeaxanthin is not as pronounced as in the case of the thicker membrane (formed with DPPC). This effect is similar to the effect observed in the case of lutein (dipolar carotenoid) and β-cryptoxanthin (monopolar carotenoid) [5], where the difference in their influence on the membrane structure and dynamics also decreases with an increase in membrane thickness.

3.2. The effects of geometrical isomers of polar and non-polar carotenoids on DPPC membranes

The non-polar carotenoids, (e.g. β-carotene), in contrast to the terminally dihydroxylated carotenoids (e.g. zeaxanthin) do not adopt a transmembrane orientation. Molecules of β-carotene are distributed homogeneously without any well-defined orientation within the membrane [1]. The influence of this nonpolar carotenoid on the membrane properties is negligible and is independent of the thickness of the phospholipid membrane. Such effects can be related to the low solubility of β-carotene in the lipid bilayers [2,39]. In Fig. 4 we present a series of DSC heating thermograms for mixtures of DPPC/β-carotene and DPPC/zeaxanthin. It is clear that carotenoid incorporation progressively decreases the pretransition temperature and cooperativity. The shift of the transition temperature toward lower values is much more pronounced for the pretransition than for the main phase transition. The effect on the conversion of the lamellar gel phase to the rippled gel is greater for the 9-*cis*-isomer than for the all-*trans* form for both membrane modifiers (β-carotene and zeaxanthin) but is clearly much more pronounced for dipolar carotenoids (Fig. 5).

4. Discussion

4.1. Effects of carotenoids on lipid membrane fluidity

The effect of the membrane-bound carotenoid pigments on dynamic properties of the system is based upon van der Waals interaction rigid polyene chain of a pigment and alkyl lipid chains undergoing relatively fast gauche-*trans* isomerization. The decreased cooperativity of the main phase transition, in the presence of carotenoid pigments, manifested by broadening of the transition-related maxima in the thermograms, suggests that carotenoid–lipid interaction results in rigidifying of the fluid phase (L_α) and fluidizing of the ordered phase ($P_{\beta'}$). Similar effect has been observed with application of spin labels [5]. Another interesting observation seems to be related to the temperature range in which the membranes remain in the rippled phase. As can be seen in Fig. 5, the presence of xanthophylls in the DPPC membranes in particular in conformation *cis*, shifts the gel-to-ripple phase transition temperature ($L_{\beta'} \rightarrow P_{\beta'}$) toward lower values. Owing to the fact that the main phase transition ($P_{\beta'} \rightarrow L_\alpha$) is shifted to lower temperatures by much less (Fig. 3B) than the pretransition ($L_{\beta'} \rightarrow P_{\beta'}$) (Fig. 5A), the temperature range in which the modified membrane is present in the $P_{\beta'}$ phase is broader and the energy to transform the membrane from the $L_{\beta'}$ phase to the $P_{\beta'}$ phase is lower.

4.2. Molecular mismatch condition between geometrical isomers of carotenoids and lipid bilayer membranes

Our results clearly show that the presence of two polar groups at the ends of the hydrophobic conjugated hydrocarbon of the carotenoid molecule in the lipid phase markedly alters the calorimetric behavior of phosphatidylcholine bilayers, which reflects a pronounced effect on the membrane's physical properties and dynamics. Dihydroxylated carotenoids modify the thermotropic phase behavior of DPPC membranes more strongly than non-polar carotenoids. The anchoring of carotenoid molecules at opposite membrane surfaces by polar hydroxyl groups is significant in enhancing their effects on membrane properties. However, the *cis*-isomers are different in their influence on the multibilayers. The effect of the all-*trans* zeaxanthin on the physical properties of lipid bilayers strongly depends on the thickness of the hydrophobic core of the membrane. In the case of the *cis*-isomers of zeaxanthin this relationship is weaker.

It seems that the molecular match plays an important role in interaction between the lipid bilayers and individual carotenoids. The tilted orientation observed in the case of zeaxanthin in the lipid

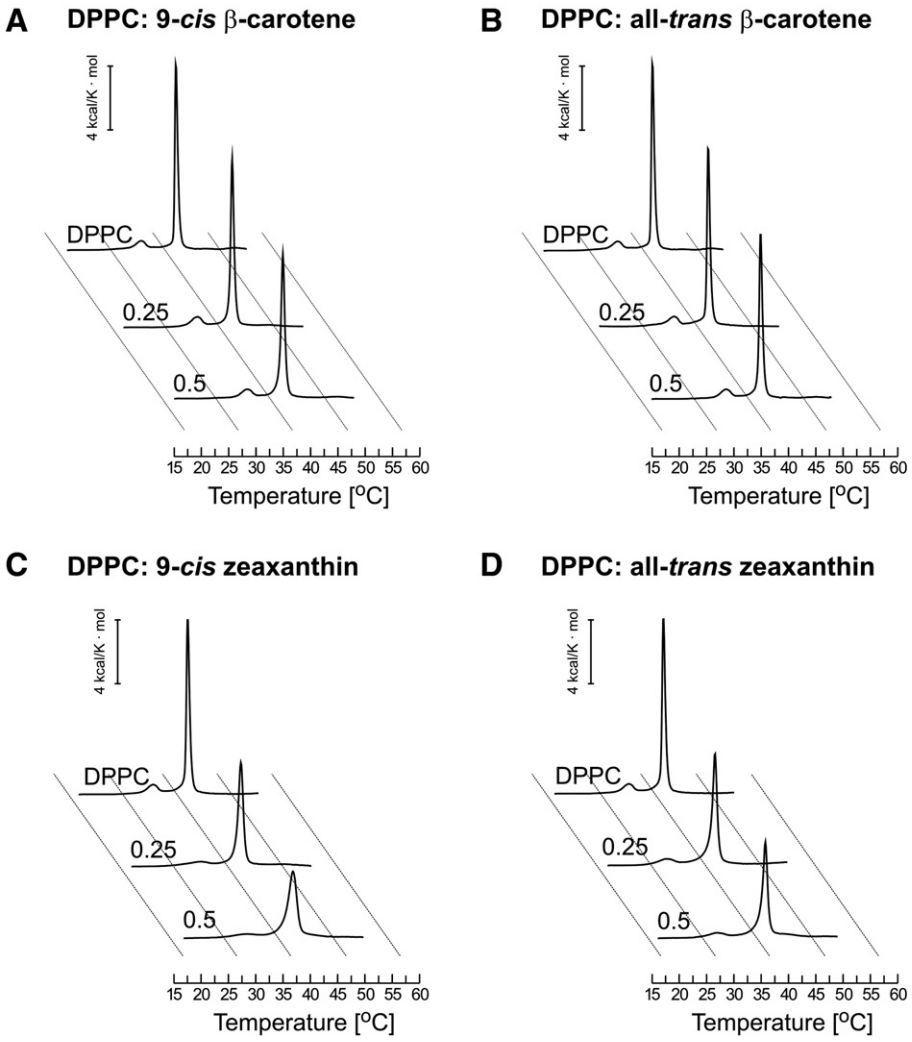

Fig. 4. Representative DSC heating thermograms of DPPC multilamellar vesicles containing various molar concentrations of isomers of all-*trans* zeaxanthin (A); isomers of 9-*cis* zeaxanthin (B); isomers of all-*trans* β-carotene (C) and isomers of 9-*cis* β-carotene (D). All data shown were acquired at scan rates 1 °C/min.

bilayer is known to destabilize the lipid membrane core. In the case of the all-*trans* isomers of zeaxanthin, the molecule tilt depends on the thickness of the lipid membrane (Fig. 6A). The molecules exert a stronger effect on the thin lipid bilayers (e.g. DMPC) than on the thick lipid membranes (e.g. DPPC). The mismatch of the hydrophobic core and the distance between the polar end-groups seem to be the dominant factors causing the tilt. The thickness of the hydrophobic core of the fluid phase of the DMPC bilayer is 24.4 Å [26] whereas the

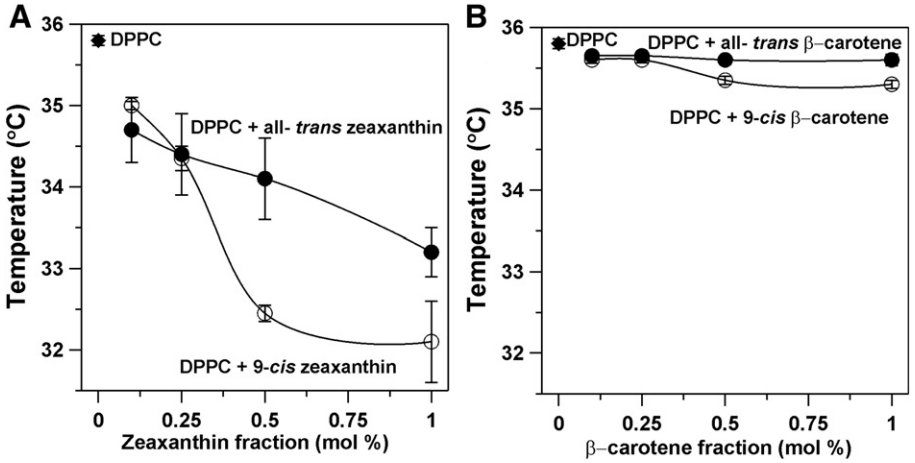

Fig. 5. Pretransition temperature as a function of carotenoid concentration for isomers of zeaxanthin/DPPC mixture (A) and of isomers β-carotene/DPPC mixture (B). Pretransition temperatures were obtained upon heating.

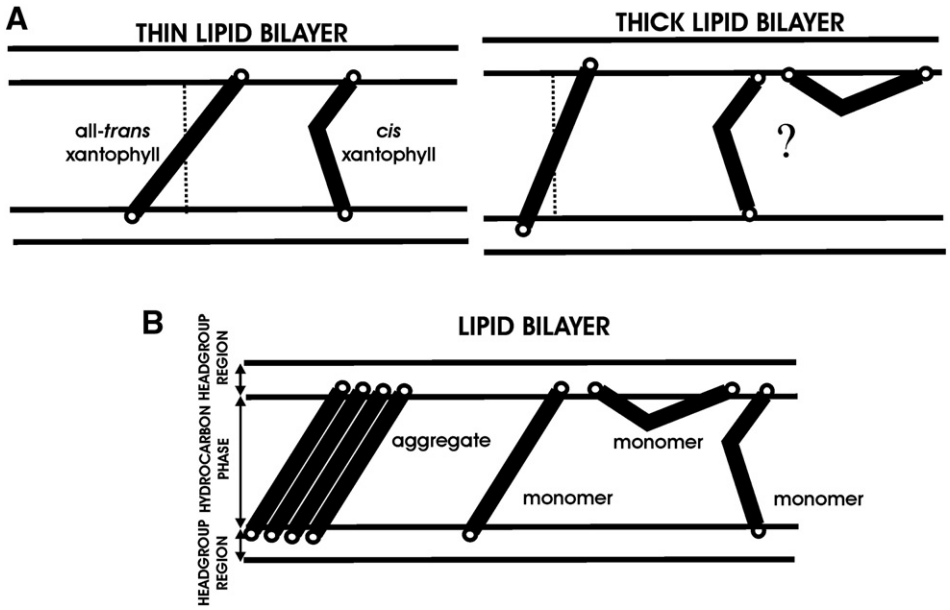

Fig. 6. Schematic drawing of the orientation and organization of different isomers of xanthophylls in the lipid bilayers. (A) Orientation of pigment molecules was drawn as dependent on thickness of the membranes. All isomers are able to span the hydrophobic core of the DMPC membrane (see Ref. [26]). It is not clear for thicker membrane like DPPC. (B) The organization of different isomers of xanthophylls in the lipid bilayer.

distance between polar hydroxyl groups located on the opposite terminal rings in the all-*trans* zeaxanthin is 30.2 Å [40]. Therefore the distance between the opposite polar groups of the all-*trans* zeaxanthin is larger than the thickness of the hydrophobic core of the DMPC membrane and the carotenoid molecules adopt a tilted orientation with respect to the axis normal to the membrane. Simple calculation based on the requirement of match of the hydrophobic membrane core and the nonpolar portion of the carotenoid molecule allows to estimate the orientation angle of a pigment with respect to the axis normal to the plain of the DMPC membranes as 37°. This value is in good agreement with experimental data. The angle between the dipole-transition moment of the *trans*-zeaxanthin molecule and the axis normal to the plane of the membrane, examined by means of linear dichroism technique in ordered lipid-layers sample, is 25° [41]. The angle between the dipole-transition moment and the molecule's axis is about 15° [42]. Consequently, the tilt angle between the conjugated double bond chain of the all-*trans* zeaxanthin and the axis normal to the DMPC membrane can be found as 40°.

The distances between the oxygen atoms in the two hydroxyl groups at the ends of the long hydrocarbon chain in the different geometrical isomers obtained by the molecular modeling technique are as follows: 30.5 Å for the all-*trans*; 26.9 Å for the 9-*cis*; and 24.4 Å for the 13-*cis* [26]. The introduction of a *cis* double bond into the carotenoid molecule reduces its length. The distance between the polar hydroxyl groups in the 13-*cis* isomer of zeaxanthin (24.4 Å) corresponds very well to the hydrophobic core thickness in the fluid phase DMPC bilayer (about 24.4 Å). It is therefore highly probable that the *cis*-isomers of zeaxanthin, similarly to the all-*trans* isomer, adopt a transmembrane orientation with the hydroxyl groups located in the opposite leaflets of the DMPC bilayer. Such an orientation has been concluded previously by Widomska and Subczynski on the bases of the results of the ESR spin-labeling studies [26].

The thickness of the hydrophobic core of the fluid phase DPPC bilayer is 26.1 Å. The distance between the opposite polar groups in the 13-*cis* zeaxanthin molecule is smaller than the thickness of the hydrophobic core of the DPPC bilayer. The difference between effect on lipid bilayers of the all-*trans* isomer and the *cis*-isomer decreases as the membrane becomes thicker (DPPC). Such a decrease is probably due to the interrelation between the thickness of the hydrophobic core of the lipid bilayer and the length of the isomers of the zeaxanthin molecule. The all-*trans* zeaxanthin molecules adopt a less tilted orientation with respect to the axis normal to the membrane in the DPPC bilayers than in the case of DMPC.

4.3. Molecular organization of geometrical isomers of carotenoids in lipid membranes

The antioxidant potential of carotenoids may be related to the specific orientation, localization and organization of different carotenoids in membrane and their biological functions are closely related to the environment they appear in. Non-polar and polar carotenoids exert different effects on the lipid membrane structure and physiological properties. McNulty et al. [43] reported that the antioxidant capacities of various carotenoids were directly correlated with their effects on membrane lipid structure. Astaxanthin (polar carotenoid) reduced lipid peroxidation rate while preserving membrane structure. By contrast, the non-polar carotenoids disturbed the lipid bilayer and showed prooxidant activities. The solubility of xanthophylls in the lipid phase is always higher than in case of carotenes [39]. It seems that this could have an effect on more the efficient antioxidant activity of polar carotenoids too. *Cis*-carotenoids are more bioavailable than *trans* forms, due to a decreased tendency to form aggregates and crystallize [25]. The aggregation level of *trans*-xanthophylls depends strongly on the fluidity of the lipid phase. Aggregate forms were observed even at low concentrations (1 mol%) of *trans*-xanthophyll pigments incorporated into DPPC unilamellar liposomes [24]. The molecular aggregates of pigments in the all-*trans* configuration formed within the lipid bilayers or separated outside, should affect membrane properties to a lesser extent. The *cis*-stereoisomers of polar carotenoids dissolved in the lipid bilayers as monomers seem to be better suited to act as free radical scavengers (Fig. 6B). So far there are no data to confirm this hypothesis. We plan to address the problem of the antioxidant activity of *cis*-xanthophylls in the lipid phase in the near future.

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