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CAROTENOID ORGANIZATION IN MEMBRANES

THERMAL TRANSITION AND SPECTRAL PROPERTIES OF CAROTENOID-CONTAINING LIPOSOMES

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Summary

1. Thermal transition and spectral properties of carotenoid-containing dipalmitoyl-L-α-phosphatidylcholine liposomes were related to the extent of hydrophilic substitution of the pigment.

2. β-Cryptoxanthin ((3R)-β,β-carotene-3-ol) and zeaxanthin ((3R, 3'R)-β,β-carotene-3-3'-diol) depressed and broadened the transition temperature whereas β-carotene (β,β-carotene) had relatively little effect. Below the transition temperature the spectra of all carotenoid preparations were complex. β-Carotene and β-cryptoxanthin preparations were similar, showing several poorly defined bands in the carotenoid region plus a band above 500 nm, in contrast, the spectra of zeaxanthin preparations were degraded with the major band at 402 nm. When warmed through the transition temperature, the spectra of cryptoxanthin and zeaxanthin preparations changed to characteristic carotenoid spectra; under similar conditions β-carotene preparations did not shift.

3. These effects reflect differences in carotenoid organization in liposomes. It is proposed that β-carotene is aggregated in both gel and liquid-crystal states of the lipid bilayer. Xanthophyll organization is phase dependent; both xanthophylls are dispersed in fluid membranes but in the gel state β-crytoxanthin aggregates, whereas zeaxanthin undergoes a conformational change.

4. The possible significance of these models to photoprotection and membrane structure is discussed. The organizational difference between carotene and xanthophylls could be the basis of a functional differentiation.

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Introduction

In photosynthetic membranes carotenoids transfer energy to chlorophyll and act as protective agents against photooxidation [1]. Carotenoids also show absorbance shifts, the so-called 515 change, which reflect changes in membrane potential [2] and, in the case of xanthophylls, undergo a transmembrane epoxide cycle that is sensitive to various photosynthetic activities [3]. Carotenoids may influence membrane fluidity [4] and appear to account for certain Raman bands in red blood cell ghosts [5].

The functional properties of carotenoids in membranes have been investigated in model systems. In bilayer lipid membranes, carotenoids transfer energy to chlorophyll [6] and are required for chlorophyll-sensitized photoconductivity [7]. The ability of β-carotene to protect liposomes from singlet oxygen induced lysis has been reported [8].

The organization of carotenoids in membranes is not clear. In photosynthetic systems, carotenoids appear to be distributed heterogeneously, as are other membrane components [9,10]. Although carotenoids are generally presumed to be localized in the lipid bilayer of membranes there is little direct evidence for this view. In fact the incorporation of carotenoids which are either strictly hydrophobic or hydrophilic at both end groups appear to be problematic considering the relative size of hydrophobic and hydrophilic regions of the membrane. We therefore investigated carotenoid organization utilizing the effects of selected carotenoids on thermal transition and spectral properties of liposomes. β-Carotene, β-cryptoxanthin and zeaxanthin carotenoids were chosen for study since they have identical chromophores and differ only in hydroxyl substitutions.

Materials and Methods

Carotenoids

β-Carotene (β,β-carotene) from Sigma, London and zeaxanthin ((3R, 3'R)-β,β-carotene-3-3'-diol) from Hoffman-La Roche were recrystalized before use. β-Cryptoxanthin ((3R)-β,β-carotene-3-ol), also from Hoffman-La Roche, was used without further treatment. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (dipalmitoyl phosphatidylcholine) was obtained from Koch-Light. Carotenoid concentration was determined in chloroform at 459 nm using an absorbance coefficient of $1.28 \cdot 10^5\ M^{-1} \cdot cm^{-1}$.

Liposomes

Dipalmitoyl phosphatidylcholine (2.5 μmol) and carotenoid (0.1 μmol) in chloroform (0.2 ml) were evaporated to dryness in a glass-stoppered test tube on a rotary evaporator. After the bulk solvent was evaporated, rotation under vacuum was continued for 10 min, during which time the sample was warmed and cooled repeatedly through the transition temperature. The liposomes were swollen in distilled water (5 ml) at about 45°C and under nitrogen with vigorous agitation on a Vortex mixer until the lipid film was completely suspended. The resultant turbid liposome preparations were stored in the dark for 2 h before use. The samples were shielded from light with aluminum foil during preparation and use.

Spectrophotometry

A Perkin-Elmer Model 402 Spectrophotometer was used in all experiments. Transition temperatures were determined with the instrument arrangement described previously [11]. Absorbance and difference spectra of carotenoid-containing liposomes were determined on dilute suspensions with 5-fold expansion of the recorder (0.3 *A* full scale). For difference spectra determinations the sample and reference cuvette holders were arranged with independent temperature controls.

Results

Absorbance spectra

The color of the carotenoid-containing liposome preparations varied from yellow to orange-red and in the case of β-cryptoxanthin and zeaxanthin preparations, but not β-carotene preparations, showed a color change near 40°C. These effects were reflected in the absorbance spectra which varied with the carotenoid and for xanthophyll preparations were temperature dependent.

Fig. 1 shows that β-carotene-containing liposomes had several diffuse bands from about 425 to 525 nm. The spectra at 20 and 43°C were similar, both having broad peaks and shoulders around 510, 470, 450 and 425 nm. These spectra resemble the spectrum of finely dispersed β-carotene crystals in water that Shibata [12] reported over two decades ago. He found that suspensions of α- and β-carotene crystals have an absorbance band above 500 nm which is not present in the spectrum of these pigments in benzene. It is evident that a simi-

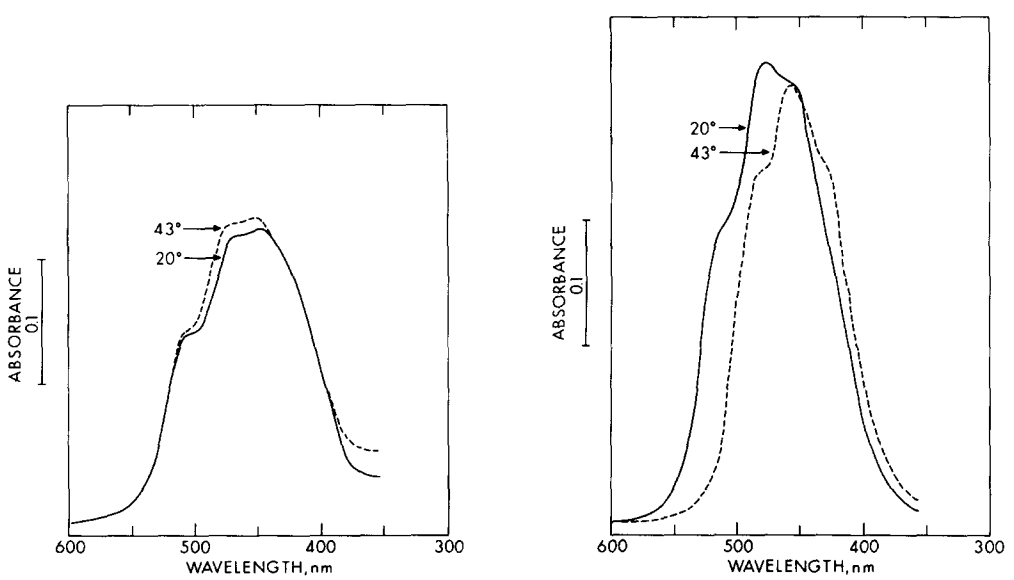

Fig. 1. Absorbance spectra of β-carotene-containing dipalmitoyl phosphatidylcholine liposomes at 20 and 43°C. Final concentrations were 100 μM dipalmitoyl phosphatidylcholine and 4 μM β-carotene.

Fig. 2. Absorbance spectra of β-cryptoxanthin-containing dipalmitoyl phosphatidylcholine liposomes at 20 and 43°C. Final concentrations were 100 μM dipalmitoyl phosphatidylcholine and 4 μM β-cryptoxanthin.

lar band was present in the spectrum of β-carotene-containing liposomes, although the entire spectrum appeared to be blue shifted by about 15 nm.

Fig. 2 shows that at 20°C the absorbance spectrum of β-cryptoxanthin-containing liposomes was similar to the β-carotene preparation with respect to peak positions except that the bands were better defined and the extinctions were higher. However, in contrast to β-carotene preparations, when the β-cryptoxanthin preparation was heated to 43°C the spectrum changed to that closely resembling β-cryptoxanthin in solution. This hypsochromic spectral shift with loss of the band above 500 nm was perceptible as a color change of the suspension from reddish-orange to orange-yellow.

Fig. 3 shows the spectra for zeaxanthin-containing liposomes. Although the absorbance in the 470 nm region was still appreciable and a shoulder near 500 nm evident at 20°C, the spectrum was otherwise markedly different from previous cases. It was considerably degraded, showing a major blue-shifted peak at 402 nm which lacked fine structure. At 43°C the spectrum changed to that of a carotenoid in solution and was similar to the spectrum of β-cryptoxanthin-containing liposomes at 43°C.

The relative changes in the spectra of carotenoid-containing liposomes above and below the transition temperature are more clearly evident in the difference absorbance spectra of samples at 43°C minus 20°C. Fig. 4 shows that for the β-cryptoxanthin preparation the absorbance decrease at 520 and 480 nm was accompanied by an increase of smaller magnitude at 425 nm. The zeaxanthin-containing liposomes showed an absorbance loss at 397 nm with a corresponding increase near 450 nm. Consistent with Fig. 1, β-carotene showed almost no difference. It should be noted that the difference spectra in Fig. 4 do not correspond precisely to the differences which can be derived from Figs. 1—3

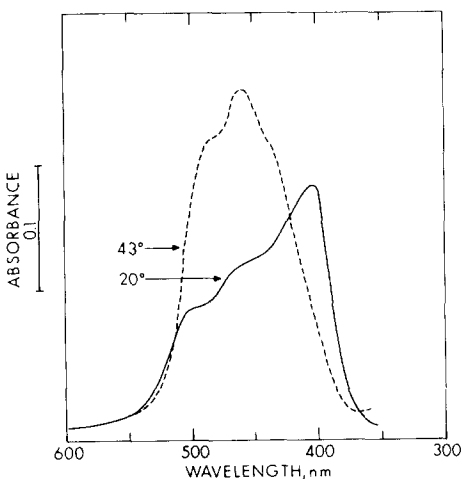

Fig. 3. Absorbance spectra of zeaxanthin-containing dipalmitoyl liposomes at 20 and 43°C. Final concentrations were 100 μM and 4 μM zeaxanthin.

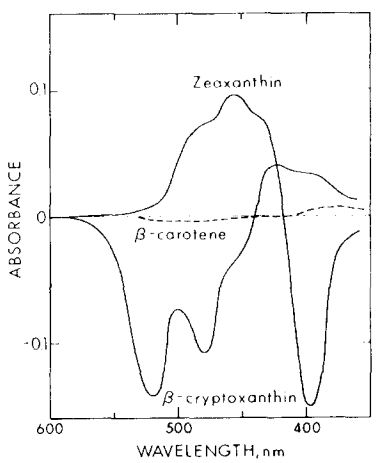

Fig. 4. Difference spectra of carotenoid-containing dipalmitoyl phosphatidylcholine liposomes, 20°C minus 43°C. Final concentrations were 100 μM diplamitoyl phosphatidylcholine and 4 μM carotenoid.

owing to the fact that the former includes light scattering components from thermal transition changes of the bilayer itself.

Carotenoids dispersed without diplamitoyl phosphatidylcholine were also examined. The dispersions were prepared by rapidly diluting concentrated solutions of carotenoids in methanol with water to 5% alcohol concentration. A low final alcohol concentration was sought to minimize potential solvent effects. The spectra were similar to those of carotenoid-containing liposomes at 20°C. Zeaxanthin dispersions showed degraded spectra and both β-carotene and β-cryptoxanthin preparations showed a 500 nm band, although all spectra were generally more diffuse. Significantly, these dispersions did not show temperature-dependent spectral shifts even when carotenoid-free liposomes were added.

Hager [13] found that degraded spectra developed when water was added to various carotenoids in alcohol or acetone. The effects were most apparent with zeaxanthin and lutein but were also seen with lycopene, although not with β-carotene. Interestingly, temperature-dependent absorbance shifts apparently occurred in these systems of relatively high alcohol concentrations.

Temperature transitions of carotenoid-containing liposomes

The effects of the carotenoids on the thermal transition of liposomes are summarized in Fig. 5. Thermal transitions as reflected in light-scattering changes were determined at 600 nm, in the region of minimal carotenoids absorbance (Fig. 5A). In addition absorbance changes were followed at wavelengths corresponding to the maximum absorbance above 500 nm for β-carotene preparations or maximum absorbance difference for β-cryptoxanthin and zeaxanthin preparations (Fig. 5B). Fig. 5A shows that relative to the control the presence of β-carotene resulted in a small depression of the main transition as well as an apparent lowering of the pretransition temperature. In contrast

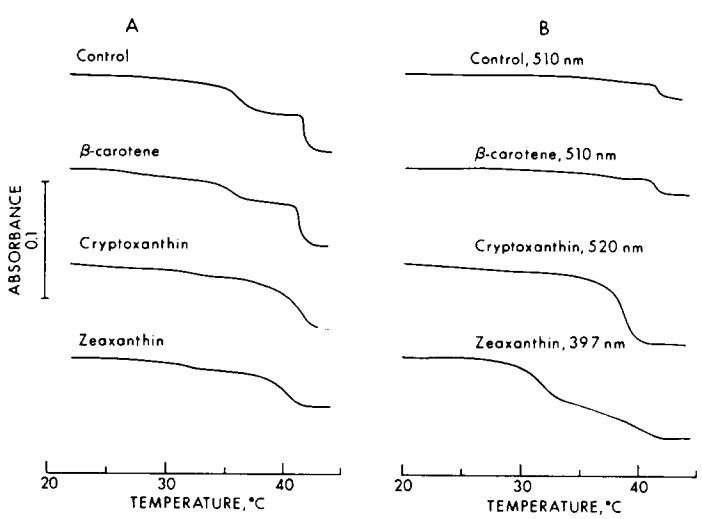

Fig. 5. Effect of temperature on light scattering and absorbance shifts in carotenoid-containing liposomes. Light-scattering changes (A) were monitored at 600 nm and 500 μM dipalmitoyl phosphatidylcholine concentration for all preparations. The changes in B were monitored at the wavelengths indicated and 100 μM dipalmitoyl phosphatidylcholine concentration.

β-cryptoxanthin and zeaxanthin had large effects, namely both the main and pretransitions were depressed and broadened considerably.

Fig. 5B shows that the changes of β-carotene-containing liposomes at 510 nm was similar in character to that observed at 600 nm and about the same magnitude as the control at the lower dipalmitoyl phosphatidylcholine concentration. It is thus apparent that the change at 510 nm was mainly due to light-scattering changes associated with phase transition. As expected the absorbance changes at 520 nm for β-cryptoxanthin and 397 nm for zeaxanthin were large. The absorbance change for β-cryptoxanthin appeared to be associated almost exclusively with the region corresponding to the main transition as seen at 600 nm, whereas for zeaxanthin the changes occurred in both pre- and main transition regions. It is estimated that 60% of the total change at 397 nm occurred during the pre-transition.

The thermal-transition absorbance changes for β-cryptoxanthin and zeaxanthin preparations were reversible. Carotenoid-containing liposomes were also prepared with egg lecithin. Their absorbance spectra resembled those of dipalmitoyl phosphatidylcholine liposomes above the transition temperature and did not show temperature-dependent shifts.

Discussion

The contrasting spectral and thermal transition properties of carotenoid-containing liposomes indicate a marked effect of hydroxyl substituents on carotenoid organization in membranes. A gradation in spectral properties from β-carotene to β-cryptoxanthin to zeaxanthin is evident. Below the transition temperature the spectra of β-carotene and β-cryptoxanthin-containing liposomes are similar, whereas at temperatures above the transition, the similarity is between β-cryptoxanthin and zeaxanthin. In addition, only the hydroxyl-substituted pigments show reversible temperature-dependent spectral shifts and depress the transition temperature significantly.

The spectrum of β-carotene preparations suggests that β-carotene is in a microcrystalline or aggregated state in these systems. These aggregates, either trapped in the bilayer or absorbed on the membrane surface, would have a small effect on the temperature transition, although trapping of hydrophobic aggregates in hydrophobic regions of the bilayer seems to be more favorable than absorption to hydrophilic head-group regions. Yet another possibility, namely, entrapment of free β-carotene aggregates in the interlamella spaces of the liposomes is excluded since free aggregates were not observed in the bulk solution.

If the spectrum of β-carotene-containing liposomes is representative of aggregated pigments it follows that β-cryptoxanthin is also aggregated in liposomes that are in the gel state since the spectra are similar under these conditions. The shift from an aggregated spectrum to that of a carotenoid in solution upon warming then represents deaggregation. This deaggregation appears to be associated primarily with the main thermal transition.

The spectrum of zeaxanthin in liposomes below the transition temperature suggests an organization which differs significantly from either β-carotene or β-cryptoxanthin. The loss of fine structure coupled with a large hypsochromic

shift suggests disruption of the chromophere due to loss of at least part of the planar conformation in the acyclic polyene chain. Degraded spectra are observed in carotenoids with 11-*cis* configurations wherein steric hinderance leads to skewing and a non-planar conformation [14]. In the present case we propose that the non-planar conformation stems from a hydrophobic effect, the skewed conformation apparently being thermodynamically more favorable than a planar conformation in the rigid and highly hydrophobic gel structure of the membrane since skewing would allow both hydroxyl groups of zeaxanthin to reside near the head group region. This view is consistent with the fact that β-carotene and β-cryptoxanthin do not show similar spectral properties.

In the liquid-crystal membrane, deaggregation and restoration of planar conformation results in a spectrum of solubilized zeaxanthin. It may be significant that a major fraction of the absorbance shift appears to be associated with the pretransition. Although the nature of the pretransition is controversial [15,16], it is reasonable to expect that if zeaxanthin resides near the headgroup region, the shift should reflect changes in this region of the membrane.

The proposed models of carotenoid organization in liposomes are summarized in Fig. 6. Below the phase transition (20°C), all carotenoids are in some state of aggregation. Xanthophyll aggregation is reversible and amounts to fractional crystallization of solute upon freezing of the membrane. It is analogous to aggregation or exclusion of chlorophyll [17,18] except that in the case of carotenoids, they remain entirely or substantially within the membrane because of the relatively small size of the hydrophilic group. Although zeaxanthin in the solid membrane is shown with a non-planar conformation at only one point, more such points may exist.

In the liquid crystal membrane (43°C), β-carotene is shown still aggregated

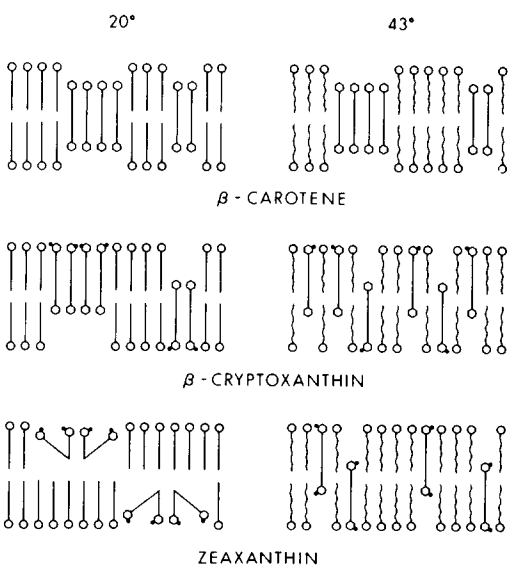

Fig. 6. Proposed models for carotenoid organization in lipid bilayers in the gel and liquid crystal states. Solid circles on carotenoid structures represent hydroxyl groups.

whereas β-cryptoxanthin and zeaxanthin are in membrane solution as monomers. The suggested organization for β-cryptoxanthin is consistent with its amphipatic structure. The organization of zeaxanthin is less apparent because it is symmetrical. Zeaxanthin may, in fact, oscillate across the membrane and on the average reside somewhere between the extremes shown.

Carotenoids in chromoplasts and chloroplasts occur in association with the lipid components of these structures, or as water-soluble carotenoproteins. Fractionation of chloroplast membranes with detergents suggests a heterogeneous distribution of pigments between Photosystems I and II [9]. The complex transmembrane changes of violaxanthin cycle [3] and the results of differential extraction studies [19] also support a heterogeneous distribution of carotenoids in photosynthetic membranes. β-Carotene and violaxanthin-containing carotenoproteins have been isolated [20,21]. The distribution of carotenoids between membrane components and lipid bilayer itself is unknown. When they are components of the bilayer, the present study suggests that in fluid membranes carotenes such as β-carotene are aggregated, whereas xanthophylls are not.

The mechanism by which carotenoids protect membranes from photooxidation has received considerable attention. It is now known that only carotenoids containing nine or more conjugated double bonds have photoprotective activity [22] and that these pigments can interact with the triplet state of the photosensitizer such as chlorophyll [23] or with singlet oxygen [24], the latter resulting from the interaction of the triplet state of the sensitizer and molecular oxygen. The singlet-quenching mechanism has been supported as the biologically important one for photoprotection on the grounds that molecular oxygen is itself a triplet quencher and more effective than carotenoids because of its presence in higher concentration in biological systems. Indeed, quenching of chemically-generated singlet oxygen by carotenoids in model systems parallels their biological activity [24]. On the other hand, if carotenoids are aggregated near the sensitizer there could be a high enough localized concentration for carotenoids to function effectively as triplet quenchers. Close packing of chlorophyll and carotenoids with improved energy transfer from chlorophyll triplet to carotenoid has, in fact, been observed in detergent micelle systems [25,26]. The present results which show that β-carotene could be aggregated in membranes raise the possibility that such aggregates in photosynthetic systems could be clustered near chlorophyll, perhaps around the phytol chain that extends into the membrane [27,28]. The proposed difference in organization of carotenes and xanthophylls, the former aggregated and the latter solubilized, could therefore be the basis for a functional differentiation.

Acknowledgment

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