Integration of β-carotene molecules in small liposomes

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Abstract. The most typical feature of carotenoids is the long polyene chain with conjugated double bonds suggesting that they can serve as conductors of electrons, acting as ‘‘molecular wires’’, important elements in the molecular electronic devices. Carotenoids are essential components of photosynthetic systems, performing different functions as light harvesting, photoprotection and electron transfer. They act also as natural antioxidants. In addition they perform structural role stabilizing the three-dimensional organization of photosynthetic membranes. Carotenoids contribute to the stability of the lipid phase, preserving the membrane integrity under potentially harmful environmental conditions. Carotenoids can be easily integrated into model membranes, facilitating the investigation of their functional roles. In carotenoid-egg phosphatidylcholine (EPC) liposomes β-carotene is randomly distributed in the hydrocarbon interior of the bilayer, without any preferred, well defined orientation and retains a substantial degree of mobility. Here we investigate the degree of integration of β-carotene in small unilamellar EPC liposomes and the changes in β-carotene absorption and Raman spectra due to the lipid-pigment interaction. All observed changes in β-carotene absorption and Raman spectra may be regarded as a result of the lipid-pigment interactions leading to the polyene geometry distortion and increasing of the environment heterogenety in the liposomes as compared to the solutions.

1. Introduction

Carotenoids are widespread natural molecules, which play multiple important physiological functions. The most typical feature of carotenoids is the long polyene chain with conjugated double bonds [1]. The long chain of conjugated bonds acts like a wire, allowing the electrical energy to move from one side of the molecule to the other, so called “molecular wire” [2]. Molecular wires, which would allow electron flow to take place between different components, are important elements in the design of molecular devices. Carotenoids are essential components of biological membranes performing multiple functions. In photosynthetic membranes they play role in light-collecting, photoprotection and electron transfer as well as stabilizing the three-dimensional integrity of bacterial and plant antenna complexes and for the assembly of functional photosystem II [3,4]. The structural role of carotenoids is probably not restricted only to the photosynthetic membranes but can be

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extended to the stability of the lipid phase, preserving the membrane integrity under potentially harmful environmental conditions [5].

To unravel the carotenoid contribution to the stability of the lipid phase, small unilamellar lipid vesicles containing pigment molecules have been used as a model system. Carotenoids can be easily integrated into model membranes thus making easier the investigation of the membrane thermodynamic and mechanical properties. The orientation of pigments within bilayer is dependent on the structure of the particular carotenoid and lipid bilayer composition. \( \beta \)-carotene molecules are randomly distributed in the hydrocarbon interior of carotenoid-egg phosphatidylcholine (EPC) liposomes without any preferred, well defined orientation and retains a substantial degree of mobility [6,7]. \( \beta \)-carotene, when integrated into EPC liposomes, was shown to increase motional freedom of both lipid headgroup and alkyl chains in liquid crystalline state [6].

Here we investigate the degree of integration of \( \beta \)-carotene in small unilamellar EPC liposomes and the changes in \( \beta \)-carotene absorption and Raman spectra due to the lipid-pigment interactions.

2. Materials and methods

2.1. Materials

\( \beta \)-carotene, TRIS (Tris-hydroxymethylaminomethane) and pyrene were obtained from Sigma, EPC from Avanti Polar Lipids and used as purchased.

2.2. Liposome formation

Lipids equivalent to 1mM (0.75mg/ml) dissolved in chlorophorm, pure or mixed with 1, 2.5 or 5 mol % \( \beta \)-carotene (1 mM in chlorophorm), were dried from the solvent under a gentle steam of \( \text{N}_2 \) to obtain a thin lipid layer on the bottom of a glass test tube. Final traces of solvent were removed form the lipid film under deep vacuum for at least 4 h followed by hydration with 50 mM TRIS buffer (pH 7.4). Small unilamellar liposomes were formed by sonication for 20 sec using ultrasound generator system. The residual, not integrated into liposomes \( \beta \)-carotene was removed by two steps centrifugation at 15 000xg. Supernatant contained only \( \beta \)-carotene-doped-EPC liposomes [8].

2.3. Determination of \( \beta \)-carotene concentration

Concentration of \( \beta \)-carotene incorporated into liposomes was evaluated from absorbance spectra (350-600 nm) of ethanol extract, using molar extinction coefficient of \( \beta \)-carotene in ethanol at 453 nm, 141x10³ M⁻¹ cm⁻¹ [9]. The results were used to calculate the \( \beta \)-carotene incorporation yield (IY) as the ratio between concentrations of integrated and applied \( \beta \)-carotene.

2.4. Steady-state fluorescence measurements

Membrane fluidity of pure EPC liposomes and of those containing different concentrations \( \beta \)-carotene was determined using the fluorescent probe pyrene that forms excimers (E) from monomers (M) by a diffusion-controlled process in fluid membranes that is directly related to membrane fluidity [10]. Pyrene was added to liposomes at concentration 4 mol % in respect to lipids from a stock ethanol solution (2 mM). Care was taken the amount of added ethanol not to exceed 0.5%. Liposome-pyrene mixture was incubated at 25°C for 30 min and pyrene fluorescence was recorded with a Jobin-Ivon spectrofluorimeter. Fluorescence was excited at 332 nm and registered in the region 350-550 nm. Slit widths were 4 nm. Pyrene, being apolar molecule, is completely buried within the hydrophobic region of the membrane [10]. In a monomeric form pyrene gives a rise to a fluorescent peak at 393 nm (F393) and a broad one at 470 nm, coming from the excimers (F470).

2.5. Resonance Raman spectra measurements

Room temperature resonance Raman (RR) spectra were measured using a microRaman spectrometer (Jobin-Ivon, HR 800) with a grating 1800 gr/mm. The excitation was provided by an argon ion laser (Innova 307, Coherent) at 488 nm. The laser intensity was 4 mW, the spectral resolution – 0.5 cm⁻¹.
3. Results and discussion
In figure 1 are presented the chemical structures of the predominant lipid class of EPC (64% POPC – palmitoyl oleoyl phosphatidyl choline) (a) and of β-carotene (b). The relative length of both molecules is comparable.

![Chemical structure of predominant lipid species of EPC (a) and of β-carotene (b).](image)

Liposomes were formed either from pure EPC or in the presence of β-carotene different concentrations - 1, 2.5 and 5 mol % in respect to lipids denoted as epc1, epc2 and 3, respectively. The absorbance spectra of β-carotene-doped liposomes, in TRIS buffer (figure 2a), are compared to the spectra of ethanol extracts from EPC liposomes, formed in the presence of different concentrations of β-carotene (figure 2b). The spectra of ethanol extracts show a typical carotene contour, with two maxima, at 454 and 476 and a shoulder at 422nm (figure 2b). In EPC liposomes spectra an additional absorbance peak is observed at 518 nm (see arrows in figure 2a).

![Absorbance spectra of β-carotene, integrated into liposomes dissolved in TRIS buffer (a) and extracted from liposomes with ethanol (b): 1 – epc1, 2 – epc2, 3 – epc3. For comparison are included the curves 4: of 22 μM β-carotene in TRIS buffer (a) and of 5 μM β-carotene in ethanol (b).](image)

The peak at 454 nm is used for determining the concentration of β-carotene, integrated into small unilamellar EPC liposomes using the molar extinction coefficient of carotene in ethanol (figure 3a). As expected the concentration of β-carotene integrated into liposomes is linearly dependent on the concentration of applied pigment. The values of integrated and applied β-carotene concentrations were used to calculate the integration yield of the pigment (IY) (figure 3b). The obtained data are in a good agreement with the values obtained for IY of β-carotene in dipalmitoylphosphatidylcholine (DPPC) liposomes [8].
As the observed in EPC liposomes additional absorbance peak at 518 nm is missing in the spectra of pure liposomes of the same concentration and changes in height with increase of pigment concentration, nevertheless that the concentration of liposomes is the same for all the samples (1 mM lipids), it can supposed to be due to lipid-pigment interactions. To further verify this possibility we calculated two types of ratio from the \( \beta \)-carotene spectra – A454/A476 and A454/A516, for both ethanol extract of carotene from different liposomes and of the same vesicle, dissolved in buffer. The ratio A454/A476 for the pure \( \beta \)-carotene and the ethanol extracts with different pigment concentration, dissolved in ethanol, being 1.15±0.005, 1.18±0.02, 1.17±0.01, and 1.16±0.01, respectively, is very similar, nevertheless that the calculated from the spectra concentrations of \( \beta \)-carotene are different. Both calculated ratios, for liposomes containing different concentrations of \( \beta \)-carotene and dissolved in buffer, are decreasing in value: 1.44±0, 1.39±0 and 1.04±0.05 for A454/A476 and 1.44±0.04, 1.39±0.04 and 1.04±0.05 for A454/A516 with increase of pigment concentration. This indicates that the observed peak at 518 nm in \( \beta \)-carotene-EPC liposomes spectra is due to the lipid-pigment interactions.

The changes in pyrene fluorescence in \( \beta \)-carotene-EPC liposomes are presented in figure 4.

With increase of the concentration of integrated pigment the overall pyrene fluorescence is decreased. The observed decrease supposes that the pyrene fluorescence is quenched by \( \beta \)-carotene, due to an effective energy transfer from pyrene excimers to \( \beta \)-carotene. The quenching of pyrene
fluorescence indicates that the pigment molecules are localized in the hydrophobic environment of the bilayer, in the vicinity of pyrene molecule, and the distance between them is not higher than 7 Å in order the supposed energy transfer to take place.

To further analyse the pigment-lipid interactions in EPC liposomes we used RR scattering. The RR spectra of β-carotene-EPC liposomes excited at 488 nm at room temperature are compared to the spectrum of β-carotene dissolved in buffer in figure 5. They manifest the characteristic for carotenoids’ Raman spectrum four main frequency bands (called from ν1 to ν4). The main carotenoid bands have been assigned as follows: ν1 to in phase stretching vibrations of the C=C bonds, ν2 to C-C stretching coupled to C15-H in plane (ip) bending, ν3 to methyl CH3 ip rocking vibrations, ; ν4 – to C-H out of plane bending modes coupled with C7=C8 torsion [11-13].

![Figure 5. Resonance Raman spectra excited at 488 nm: (a) 1 – epc1, 2 – epc2, 3 – epc3, and 4 – β-carotene in buffer. For comparison are included spectra: (a) 5 – pure buffer and (b) 6 – β-carotene in ethanol, 7 – pure ethanol. Differences are mainly observed in the regions of ν4 and ν2 bands. This becomes particularly clear from figure 6 where the bands were normalized in (figure 6a) to the intensity of ν3 band and in (figure 6b) to the intensity of the main ν2 band. The transitions in ν4 region are forbidden for fully planar molecules and become significant under conditions in which carotenoid undergoes molecular distortion due to the interaction with its environment [11,14]. Structured ν4 bands, indicative of out-of-plane distortions of conjugated backbone of the β-carotene molecule [11,13,14], were only observed in EPC liposomes with incorporated β-carotene in them. They exhibit two transitions at 954 and 964 cm⁻¹. Their intensity increases weakly with the pigment concentration, thus indicating that the polyene geometry is distorted under the pigment inclusion in liposomes. Our experimental data (figure 6b) in the cis-isomerization fingerprint region ν2 (1100-1300 cm⁻¹) [11,12,14] did not show any transition around 1134 cm⁻¹. It leads us to the conclusion that the presence of isomers in liposomes is not likely. We observed characteristic frequencies at 1175, 1192 and 1211 cm⁻¹. The 1175 cm⁻¹ line could be assigned to methyl rocking, where the others two to localized C-C stretching modes, according to the recently published theoretical results for β-carotene Raman-active modes [15].
Figure 6. Two regions of the β-carotene resonance Raman spectra (shown in figure 5), where the differences are clearly seen: the ν₄ region (a) and ν₂ region (b). Original spectra were normalized in (a) to the intensity of ν₃ band and in (b) to the intensity of ν₂ band. Normalized spectra are upshifted for better visualization.

All observed changes in β-carotene absorption and Raman spectra may be regarded as a result of the lipid-pigment interactions leading to the polyene geometry distortion and increasing of the environment heterogeneity in the liposomes as compared to the solutions.

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References
[1] Fraser P D and Bramley P M 2004 Progress in Lipid Research 43 228
[2] Visoly-Fisher I, Daie K, Terazono Y, Herrero C, Fungo F, Otero L, Durantini E, Silber J J, Ereno L, Gust D, Moore T A, Moore A L and Lindsay S M 2006 PNAS 103 8686
[3] Moskalenko A A and Karapetyan N V 1996 Z. Naturforsch. 51c 763
[4] Frank H and Cogdell R J 1993 Carotenoids in photosynthesis, ed A Young and G Britton (London: Chapman&Hall) pp. 253-326
[5] Havaux M 1998 Trends Plant Sci. 30 147
[6] Gabrielska J and Gruszecki W I 1996 Biochim. Biophys. Acta 1285 165
[7] Gruszczf P I and Strzała K 2005 Biochim. Biophys. Acta 1740 108
[8] Socaciu C, Lausch C and Diehl H A 1999 Spectrochim. Acta A 55 2289
[9] http://epic.awi.de/publications/jef1997f.pdf
[10] Gallah J H and Sackmann E 1974 Biochim. Biophys. Acta 339 103
[11] Robert B 2009 Photosynth. Res. 10 147
[12] Koyama Y and Fujii R 1999 The Photochemistry of Carotenoids ed H Frank, A Young , G Britton and R Cogdell (Dordrecht: Kluwer Acad. Publ.) pp. 161-188.
[13] Andreeva A and Velitchkova M 2005 Biophys. Chem. 114 129.
[14] Koyama Y, Takatsuka I, Nakata M and Tasumi M 1988 J. Raman Spectrosc. 19 37
[15] Tschirner N, Schenderlein M, Brose K, Schloder E, Mroginski M A, Thomsen C and Hildebrandt P 2009 Phys. Chem. Chem. Phys. 11 11471