Biochimica et Biophysica Acta, 1067 (1991) 208–212

ADONIS 0005273691003135

BBAMEM 75331

Orientation of carotenoids in the outer membrane of *Synechocystis* PCC 6714 (Cyanobacteria)

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(Received 3 June 1991)

Key words: Carotenoid protein; Glycoside; Cyanobacterium; Linear dichroism; Myxoxanthophyll; Outer membrane; (*Synechocystis*); (*Synechococcus*)

The orientation of outer membrane carotenoids from *Synechocystis* PCC 6714 and *Synechococcus* PCC 6307 was studied by linear dichroism spectrophotometry. Uniaxially oriented, tilted outer membrane films revealed a significant linear dichroism after rotating the polarization vector of the incident light beam, indicating a predominant orientation of the carotenoid transition moments perpendicular to the outer membrane plane. Values for the reduced dichroism at the absorbance maxima presented a linear correlation to a function of the tilt angle ($\sin^2\alpha$).

Introduction

The thylakoid membrane, the cytoplasmic membrane, and the outer membrane (OM) represent three membrane systems with different structures and functions in cyanobacteria. All three membrane types have carotenoids as common membrane constituents, whereas the thylakoid membrane has chlorophyll *a* in addition to carotenoids. A carotenoid-protein (M_r 16000) containing 3'-hydroxyechinenone (stoichiometry of 20 to 40 carotenoid molecules per mol protein) was identified in aqueous extracts from *Spirulina maxima, Anabaena flos-aquae*, and *Microcystis aeruginosa* [1]. A carotenoid-binding protein (M_r 45000) was purified from the cytoplasmic membrane of *Synechocystis* PCC 6714 and was immunologically related to the 46 kDa protein from a carotenoid-containing fraction of *Anacystis nidulans* R2 [2]. The OM of *Synechocystis* PCC 6714 contained echinenone, zeaxanthin, myxoxanthophyll, two related carotenoid glycosides, and an unknown, polar carotenoid, which was absent from the respective cytoplasmic-thylakoid membrane fraction [3,4]. Zeaxanthin, myxoxanthophyll, and the two related carotenoid glycosides accounted for about 75% of the total OM carotenoids [5]. Carotenoids were present in cell walls of a number of unicellular and filamentous cyanobacteria such as *Anacystis nidulans* [6–8], *Synechococcus* UTEX 625, *Synechococcus leopoliensis, Synechococcus* sp. [9] *Synechococcus* PCC 6307 [10], *Microcystis* PCC 7806 [11], *Tolypothrix tenuis, Phormidium foveolarum* [7], and *Anabaena variabilis* [12]. Since carotenoids are unique constituents of the outer membranes of probably all cyanobacteria, their organization, function, and biosynthesis remained to be studied.

This paper describes the orientation of the carotenoids in the outer membranes of *Synechocystis* PCC 6714 and *Synechococcus* PCC 6307 as studied by linear dichroism (LD) spectroscopy of oriented OM films.

Materials and Methods

Strains and culture conditions. Synechocystis PCC 6714 and *Synechococcus* PCC 6307 were obtained from the Pasteur Culture Collection (PCC, Paris, France) and cultivated in BG-11 medium [13], pH 7.5, at 25°C. Mass cultures were grown in a 10 l fermentor (Biostat E, Braun-Diessel Biotech, Melsungen, F.R.G.), gassed

Abbreviations: LD, linear dichroism; M_r, relative molecular mass; OM, outer membrane; PCC, Pasteur Culture Collection; UTEX, Culture Collection of Algae at the University of Texas.

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continuously by a mixture of air and carbon dioxide (99:1, v/v) under stirring and illuminated by white fluorescent light (6000 lx).

Outer membrane isolation. For isolation of outer membranes cells were harvested by centrifugation ($7000 \times g$, 30 min, 4°C) and were broken by using mechanical disruption with glass beads (0.17 to 0.18 mm in diameter) in a vibrogen shaker (Bühler, Tübingen, F.R.G.) at full speed for 20 min. Cell walls were prepared by differential centrifugation ($12000 \times g$, 30 min, 4°C) and discontinuous sucrose density gradient centrifugation as described earlier [4]. Outer membranes were obtained by lysozyme-digestion of gradient-purified cell walls, separated on discontinuous sucrose density gradients and freed from sucrose as described previously [5].

Spectroscopic measurements. Absorbance spectra of aqueous OM suspensions were recorded on a spectrophotometer for highly light-scattering samples built in our laboratory. For preparation of uniaxially oriented OM samples for LD experiments, suspensions of outer membranes from *Synechocystis* PCC 6714 and *Synechococcus* PCC 6307 were pelleted ($12000 \times g$, 15 min, 4°C) and resuspended in a buffer at low ionic strength to avoid salt crystals upon drying. After homogenizing, a few microliters of the concentrated OM suspensions were air-dried on thin microscope cover slides. The resulting thin OM films were sufficiently homogenous and had optical densities at the carotenoid absorbance maxima between 0.1 and 0.3. Polarized absorbance spectra from OM films were recorded using a microspectrophotometer as described previously [14]. Spectra were recorded for tilt angles α in the range from 0° to 40° with respect to the beam axis. For each tilt angle, single-beam spectra were recorded at 0° and 90° polarization on the sample spot (I_0, I_{90}). A micropositioning device was used to move the OM film from the sample spot to the reference spot. Absorbance spectra for 0° and 90° polarization were calculated for each tilt angle. The absorbance at 90° (the tilt axis) should probe the transition moments in the plane of the OM film and thus should be independent of the tilt angle. It reflects, however, the increase of the effective OM layer thickness with increasing tilt angle. This effect was corrected by forming the reduced dichroism $(A_0 - A_{90})/A_{90}$.

Results

Absorbance spectra of outer membrane carotenoids

Absorbance spectra of aqueous OM suspensions from *Synechocystis* PCC 6714 (OM 6714, full line in Fig. 1) and *Synechococcus* PCC 6307 (OM 6307, dashed line in Fig. 1) showed the characteristic absorbance bands at 462, 485, and 525 nm for the carotenoids (myxoxanthophyll and related carotenoid glycosides,

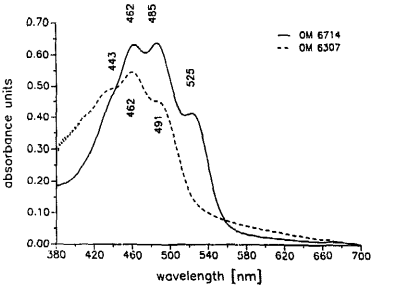

Fig. 1. Absorbance spectra of aqueous outer membrane (OM) suspensions from *Synechocystis* PCC 6714 (full line) and *Synechococcus* PCC 6307 (dashed line), respectively.

zeaxanthin, echinenone, β-carotene) in OM 6714 [5] and at 443, 462, and 491 nm for the carotenoids in OM 6307 [10], respectively.

Geometry of dichroic measurements

A schematic diagram presents the orientation of the OM film axes for the geometry of dichroic measurements (Fig. 2). The light beam (I) is incident upon the OM film in parallel to the direction of z-axis, which is oriented perpendicularly to the plane of the OM film. The x-axis and y-axis correspond to polarizer angles 0° and 90°, respectively. The plane of the OM film

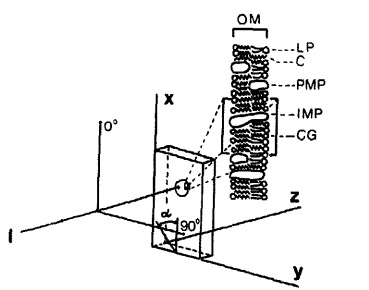

Fig. 2. Geometry of dichroic measurements: OM, outer membrane film on glass cover slide; I, incident light beam; α, tilt angle; x-axis corresponds to 0° polarization; y-axis corresponds to 90° polarization; the untilted sample is placed in the x,y-plane; z-axis corresponds to the direction of beam propagation. Abbreviations for OM components: C, carotenoid; CG, carotenoid-glycoside; IMP, integral membrane protein; LP, lipid; PMP, peripheral membrane protein. Note that symbols for lipopolysaccharide molecules were omitted in the OM scheme. The OM scheme is presented from its side view.

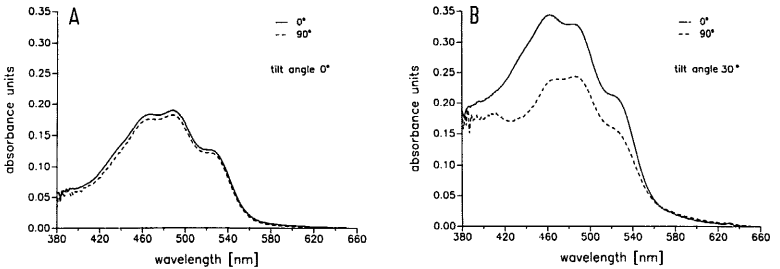

Fig. 3. Polarized absorbance spectra of an oriented OM film from *Synechocystis* PCC 6714 at tilt angles 0° (A) and 30° (B), respectively.

can be tilted with respect to the z-axis by the angle α (0° to 40°). The y-axis was chosen as the tilt axis. The 90° absorbance thus probes the transition moments of the carotenoids in the plane of the OM film. The 0° absorbance (x-axis) probes the projection of the transition moments onto the z-axis.

Linear dichroism of outer membrane carotenoids

The polarized absorbance spectrum of a dried OM 6714 film at 0° tilt angle (Fig. 3A) revealed absorbance maxima of the carotenoid spectrum, which are in good agreement with those of the carotenoid spectrum obtained from an aqueous OM suspension (Fig. 1, full line) from *Synechocystis* PCC 6714. Absence of LD in the x,y-plane was observed, since rotating the polarization vector of the incident light beam obviously had no effect on the absorbance spectrum for the untilted OM film (Fig. 3A).

When the OM film from *Synechocystis* PCC 6714 is tilted by 30° with respect to the beam axis (z), the absorbance spectra at 0° polarization and at 90° polarization differ considerably (Fig. 3B). The strong increase of the absorbance for the electric field vector polarized along the projection of the OM normal strongly indicates a predominant orientation of the carotenoid transition moments perpendicular to the OM x, y-plane.

In order to characterize this orientation, a series of spectra at different tilt angles (0° to 40°) was recorded. Data from three tilt series were combined and plotted against $\sin^2\alpha$. The reduced dichroism $(A_0 - A_{90})/A_{90}$ of these spectra, as a function of the tilt angle α, is shown in Fig. 4. The values calculated for the reduced dichroism at the absorbance maxima (462, 485, and 525 nm) revealed a linear correlation to $\sin^2\alpha$. Careful extrapolation of the reduced dichroism to $\alpha = 90°$ ($\sin^2\alpha = 1$) yields a reduced dichroism $(A_0 - A_{90})/A_{90}$ greater than 1. We thus conclude that the carotenoid transition moments are perfectly aligned along the OM normal. A precise evaluation of this extrapolated value could give the average angle formed between the transition moment and the membrane normal. However, additional information on the refractive index of the OM film (determining the effective tilt angle) is needed.

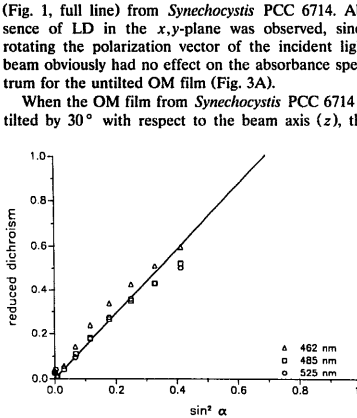

Fig. 4. Plots of values for the reduced dichroism $((A_0 - A_{90})/A_{90})$ as a function of the tilt angle α ($\sin^2\alpha$) at the absorbance (A) maxima (462, 485, 525 nm) of tilted OM films from *Synechocystis* PCC 6714. Tilt series were recorded to 40°.

LD measurements of OM films from *Synechococcus* PCC 6307 as a function of the tilt angle gave comparable results (data not shown) as described for OM carotenoids from *Synechocystis* PCC 6714 (see above). A predominant orientation of the carotenoid transition moments perpendicular to the OM x, y-plane became also evident for *Synechococcus* PCC 6307.

Discussion

The LD measurements indicated a predominant orientation of the carotenoid transition moments perpendicular to the OM x, y-plane of *Synechocystis* PCC 6714. Comparable LD measurements of oriented OM

films from *Synechococcus* PCC 6307 revealed that the orientation of the OM carotenoids was not a feature being restricted to one selected cyanobacterium (e.g. *Synechocystis* PCC 6714) alone. Interestingly, the carotenoids of the photosynthetic membranes of spinach chloroplasts [15,16] and the green alga *Chlorella* [17,18] were found to be lying nearly parallel to the membrane plane. However, carotenoid molecules in isolated chromatophores of *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* were shown to point out of the membrane plane at an angle near 45° [19]. The carotenoids of the B800-850 antenna complex of *Rhodobacter sphaeroides* had a different orientation with repect to the membrane plane, since the carotenoid associated with bacteriochlorophyll (Bchl) 800 was oriented approximately parallel to the membrane, whereas the carotenoid associated with Bchl 850 was more or less perpendicularly oriented to the membrane [20]. The carotenoid of the reaction center of *Rhodobacter sphaeroides* was found to lie in a plane with the two-fold symmetry axis of the reaction center perpendicular to this plane [21].

Calculation of the length of cyanobacterial carotenoid types from their molecular structure gave values of 3.4 to 3.9 nm for one carotenoid molecule in *trans*-conformation. Since the total OM thickness is about 8 nm on ultrathin sections in both investigated unicellular cyanobacteria [4,10], it can be proposed that each carotenoid molecule spans one OM monolayer. We do not yet know whether the carotenoids are equally distributed in both (inner and outer) monolayers of the OM. However, if they are supposed to be distributed so, the carotenoids must have the same orientation in both monolayers as indicated by the LD measurements.

Analysis of the carotenoid types revealed in earlier reports [4,5] that carotenoid glycosides, mainly myxoxanthophyll, were predominant in the OM of *Synechocystis* PCC 6714. The carotenoid-linked neutral sugar in case of myxoxanthophyll is rhamnose [22]. Thus, it seems likely, that the hydrophilic neutral sugar representing the polar head of the perpendicularly oriented hydrophobic carotenoid chain is presumably localized at the (exoplasmic or plasmic) surface of the OM inner or outer monolayer, respectively.

The function of the carotenoids in outer membranes from cyanobacteria is still unclear. We take into consideration that the carotenoids can have at least three possible functions in the outer membranes of cyanobacteria: (i) the carotenoids may act as effective photoprotectors due to their long systems of conjugated bonds in order to withstand high energy irradiation [23–25], (ii) the carotenoids can regulate the rigidity of the OM [26], and (iii) the carotenoids may have a structural function [27,28]. The special organization of the carotenoids in the OM is suggested to be due to their partial association with major OM proteins [29]. However, the carotenoids are also proposed to be integrated in the lipid bilayer and are oriented due to their polar head groups (neutral sugars in carotenoid glycosides and/or hydroxy groups in xanthophylls) as implicated in the OM scheme (see Fig. 2). Further informations are needed about interactions between carotenoids and other OM components such as membrane proteins, lipopolysaccharides, and lipids in cyanobacteria.

Acknowledgement

The authors thank the Deutsche Forschungsgemeinschaft for financial support (grant Dr 29/31-6 K).

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