Occurrence of Secondary Carotenoids in PS I Complexes Isolated from Eremosphaera viridis De Bary (Chlorophyceae)

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Photosystem I complexes of Eremosphaera viridis De Bary (Chlorophyceae, Chlorococcales) were isolated and partially characterized. In the isolated PS I complexes, peptides of 64–60, 26, 23, 20, 15, 11 and 8.5 kDa could be detected. When Eremosphaera was grown under regular conditions the pigment composition of the isolated PS I complexes was similar to that found in PS I complexes from other green algae. However, when Eremosphaera was grown under nitrogen deficient conditions, PS I complexes contained the secondary carotenoids canthaxanthin and traces of astaxanthin and echinenone in addition to β-carotene, violaxanthin and lutein. The results presented indicate that the secondary carotenoids are associated with the LHC I of PS I. To our knowledge this represents the first report about the association of secondary carotenoids with light harvesting pigment complexes of green algae.

Introduction

Photosynthetic pigments in plants are most, if not all, located in pigment protein complexes in thylakoid membranes [1]. Among these pigments carotenoids have two major functions in photosynthesis. Firstly they protect the chlorophylls and membranes from photooxidative damage and secondly they act as accessory pigments in light harvesting pigment protein complexes. Synthesis of carotenoids seems to be closely restricted to chloroplasts [2] and the biosynthetic enzymes are thylakoid bound or associated with the inner envelope [3, 4]. Several authors have shown that under conditions of stress green algae synthesize additional pigments, the so-called secondary carotenoids (SC) [5, 6, 7].

Previously, we have shown that Eremosphaera viridis De Bary, grown in nitrogen-deficient medium, synthesizes SC and accumulates them in lipid bodies formed within the plastids during stress situations [10]. In this paper we have examined the location of secondary carotenoids in chloroplasts, and we show that they are associated with pigment protein complexes in PS I of Eremosphaera viridis.

Materials and Methods

Eremosphaera viridis De Bary (Algal Coll. Göttingen, F.R.G., SAG-LB 221) was grown as described previously on nitrate as N-source [11]. Under nitrogen free conditions, Na-nitrate was omitted from the medium.

Pigment analysis

Pigments were extracted with acetone and, if necessary, saponified with KOH according to Czygan [6, 7]. Primary and secondary carotenoids were separated and identified by TLC according to Hager and Meyer-Bertenrath [12], Czygan [6, 7] and Vechtel et al. [8]. HPLC was carried out as described by Wright and Shearer [13] using an RP-18 column (125 × 4 mm, 5 μm particle-size) and a Merck-Hitachi HPLC-system. Pigments were eluted with a linear gradient from 100% acetonitrile/H₂O (9:1, v/v) to 75% ethylacetate over 20 min. Thereafter the gradient was returned over 3 min and equilibrated for 5 min before injection of the next sample. Carotenoids were detected at 440 nm, chlorophylls at 660 nm. Their spectra were recorded using a diode array detector. The pigments were
during thylakoid damage, are transported into lipid bodies formed within the plastids during stress situations [10].

Abbreviations: PS I, Photosystem I; LHC I, light-harvesting-complex of PS I; PS II, photosystem II; SC, secondary carotenoids; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol indophenol; Chl, chlorophyll.

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identified by their retention times, their absorption spectra and their “hypsochromic shift” [14], or by co-chromatography with pigments isolated by TLC or commercial pigments (Sigma, F.R.G.). Chlorophyll content was determined as described by Schmid [15].

Isolation of chloroplasts

Cells of *Eremosphaera* were harvested and washed once with isolation buffer (35 mM Hepes-KOH, 550 mM sorbitol, 5 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, pH 7.7). The cells were resuspended in isolation buffer and broken up with a glass homogenizer and a teflon plunger connected with a power drill. The homogenate was filtered through a 4-fold nylon cloth (40 μm mesh) and centrifuged for 1 min at 200 x g to remove starch and cell debris. The supernatant was then centrifuged for 2 min at 1200 x g, and the pelleted chloroplasts were washed twice with the isolation buffer (95% intact chloroplasts estimated by phase contrast microscopy).

Isolation of thylakoids

Chloroplasts of *Eremosphaera* were broken up by incubation for 15 min on ice with gentle stirring in a medium containing 15 mM Hepes-KOH, 10 mM NaCl, pH 7.5. Thylakoids were then collected by centrifugation for 15 min at 20,000 x g, washed with the above buffer and once with 5 mM EDTA-KOH, pH 7.5. After resuspending in H₂O, thylakoids were kept on ice for 5 min, centrifuged for 30 min at 42,000 x g and, if necessary, stored at -20 °C.

Isolation of pigment-protein-complexes

a) SDS-PAGE

Thylakoids were resuspended in buffer (20 mM Tris-HCl, 1% SDS, pH 8.6) at a detergent/chlorophyll ratio of 1:1 (w/w), diluted with H₂O to 1 mg Chl/ml, stirred for 10 min and centrifuged for 5 min at 4300 x g to remove insoluble material. 1 ml of supernatant was applied immediately to slab gels (8-12% acrylamide in resolving and 4.5% in stacking gel in 375 mM Tris-HCl, 0.1% SDS, pH 8.8). Gels were run at 100 V for 1 h in 50 mM Tris/384 mM glycine, 0.1% SDS, pH 8.5. All steps were carried out at 4 °C in the dark. After electrophoresis green gel zones were cut off and homogenized. Thereafter, pigments were extracted with petrol ether, dried under N₂ and dissolved in chloroform for DC or in 90% acetonitrile for HPLC.

b) Sucrose density gradient centrifugation

Thylakoids were resuspended in 20 mM Hepes-KOH, 10 mM NaCl, 1% Triton X-100, pH 7.5 at detergent/Chl ratio of 1.5:1 (w/w), diluted with H₂O to 1.2 mg Chl/ml, stirred for 30 min at RT and centrifuged for 30 min at 42,000 x g. 4 ml of supernatant were loaded onto sucrose gradient (7 ml) made up of 0.3, 0.5, 0.8, 1.1 and 1.3 M sucrose in 15 mM Hepes-KOH, 0.02% Triton X-100, pH 7.5. After centrifugation for 17 h at 150,000 x g the green zone above the 1.3 M sucrose was collected and dialyzed for 6 h against 15 mM Hepes-KOH, pH 7.5, to remove sucrose and detergent. Thereafter, PS I particles were concentrated by centrifugation for 30 min at 100,000 x g and stored at -20 °C. All steps were carried out at 4 °C in the dark.

Polypeptide analysis

PS I particles, isolated by sucrose density centrifugation, were incubated in buffer (30 mM Tris-HCl, 4% SDS, 10% glycerol, 5% β-mercaptoethanol, pH 8.6) for 2 h at 40 °C and centrifuged for 5 min at 4300 x g. Higher temperatures were not effective and lead to aggregation of reaction center proteins preventing them from correct separation [16-18]. Proteins were separated by SDS-PAGE and stained either with Coomassie brilliant blue or silver stain [19].

PS I activity was measured with a Gilson oxygraph (model IC-OXY) fitted with a Clarke type electrode. The solution was saturated with air, and illumination was performed with a halogen lamp (24 V, 240 W) from Spindler and Hoyer, Göttingen. The light was filtered through a 2% CuSO₄ solution and a red glass filter RG 1 from Schott, Mainz. PS I mediated electron flow from reduced DCMU (6.3 μM with 0.26 mM Na-ascorbate to reduce DCMU) to methylviologen (13 μM) in 0.53 mM Hepes-KOH, pH 7.0, was measured in presence of 0.8 μM DCMU and 32 μM KCN by recording the O₂ uptake. The reaction mixture with a final volume of 1.9 ml and about 50 μg chlorophyll was stirred at 20 °C.
Results

Thylakoid membranes were isolated from chloroplasts of *Eremosphaera viridis* grown in complete or in N-deficient medium. The thylakoid membranes obtained from *Eremosphaera* grown in complete medium contained in addition to chlorophyll *a* and *b*, β-carotene, lutein, zeaxanthin, neoxanthin and violaxanthin (Fig. 1 A, Table I). Lutein-5,6-epoxid, antheraxanthin and α-carotene were only found in minor amounts. In isolated thylakoids from algae grown under N-deficiency for 25 days, the secondary carotenoids (SC) astaxanthin, in two esterified forms, and canthaxanthin could be detected by TLC in addition to normal carotenoids. Those three are the major SC in *Eremosphaera* [8]. Pigment extracts of algae grown in N-deficient medium were saponified prior to the determination of the pigment composition with the HPLC system (Fig. 1B), since presence of chlorophylls prevents separation of SC, especially of the astaxanthin esters.

For investigation of the exact localization of SC in thylakoids, pigment protein complexes from *Eremosphaera* grown in normal and N-deficient media were solubilized. Separation by SDS-PAGE revealed six pigment protein complexes (Fig. 2) classified according to Anderson *et al.* [20] and

Fig. 1. A and B. HPLC-elution profiles of acetone extracts obtained from thylakoid membranes of *Eremosphaera viridis*. A: grown in complete medium; B: grown in N-deficient medium for 25 days. The acetone extract was saponified (B) or not (A). The numbers represent the various carotenoids as shown in Table I.

Table I. Pigments of *Eremosphaera viridis* grown in complete or N-deficient medium separated by HPLC. Lutein/zeaxanthin, antheraxanthin/lutein-5,6-epoxid/astaxanthin and α-/β-carotene could be separated by co-chromatography with TLC.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time [min]</th>
<th>Pigment</th>
<th>Absorption maxima (in eluent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.10</td>
<td>neoxanthin</td>
<td>412 437 462</td>
</tr>
<tr>
<td>2</td>
<td>8.05</td>
<td>violaxanthin</td>
<td>415 440 470</td>
</tr>
<tr>
<td>3</td>
<td>11.10</td>
<td>antheraxanthin + lutein-5,6-epoxid</td>
<td>446 479</td>
</tr>
<tr>
<td>4</td>
<td>12.00</td>
<td>lutein + zeaxanthin</td>
<td>448 478</td>
</tr>
<tr>
<td>5</td>
<td>13.70</td>
<td>chlorophyll <em>b</em></td>
<td>643</td>
</tr>
<tr>
<td>5'</td>
<td>14.05</td>
<td>chlorophyll <em>b</em> epimer?</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>15.00</td>
<td>chlorophyll <em>a</em></td>
<td>660</td>
</tr>
<tr>
<td>6'</td>
<td>15.25</td>
<td>chlorophyll <em>a</em> epimer?</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>19.00</td>
<td>α + β-carotene</td>
<td>419 449 478</td>
</tr>
<tr>
<td>8</td>
<td>12.50</td>
<td>canthaxanthin</td>
<td>478</td>
</tr>
<tr>
<td>10</td>
<td>17.30</td>
<td>phaeophytin</td>
<td>655</td>
</tr>
<tr>
<td>11</td>
<td>16.90</td>
<td>echinenone</td>
<td>460</td>
</tr>
<tr>
<td>12</td>
<td>11.30</td>
<td>astaxanthin</td>
<td>478</td>
</tr>
</tbody>
</table>
Nakayama et al. [21]. These pigment protein complexes were extracted with acetone and the obtained pigments were separated by TLC or HPLC.

CP1a, the PS I macrocomplex consisting of reaction center and LHC I, contained Chl a and b (a/b ratio of 3:1), β-carotene, lutein and violaxanthin. In contrast to complete thylakoids only traces of neoxanthin could be found (Fig. 3A). CP1, the reaction center complex of PS I, contained Chl a, little Chl b (a/b ratio of 12:1), β-carotene and minor amounts of xanthophylls (Fig. 3B). LHCP3 and its oligomeric form LHCP1, the LHCs of PS II, consisted of Chl a and b (a/b ratio of 1.5:1), β-carotene, lutein, neoxanthin and of a minor amount of violaxanthin (Fig. 3C, D).

In our gel system, CPa, the reaction center of PS II, and LHCP2, supposedly an oligomer of LHCP3, could not be obtained in sufficient amounts to allow pigment analysis.

In LHCP1 and LHCP3 of thylakoids from algae grown in N-free medium for 25 days, no changes in pigment composition could be demonstrated when compared to the corresponding complexes obtained from algae grown under regular conditions. However, when the macro PS I complex (CP1a) was examined, in addition to the major carotenoids canthaxanthin and traces of astaxanthin and echinenon could be shown to be present (Fig. 4). When the reaction center complex CP1...
was isolated from the macro PS I complex (CP 1a) containing the SC, it could be shown that the CP I did not contain these SC indicating that the SC were associated with the LHC I in PS I.

Since in addition to the regular carotenoids some SC are present in PS I complexes grown in N-deficient medium, it was interesting to investigate whether differences could be detected in the polypeptide composition of PS I complexes obtained from normally grown cells and cells kept under N-free conditions. PS I complexes from *Eremosphaera*, solubilized from the membranes and isolated by sucrose density gradient centrifugation, contained 7 polypeptides with an apparent molecular mass of 55–50, 26, 23, 20, 15, 11 and 8.5 kDa (Fig. 5). The upper band showed a broad diffuse band in the range of 55–50 kDa, which disappeared after heating of samples leading to the occurrence of some material at the interface of running and stacking gel. When the samples were extracted with acetone, the resulting pellet could be solubilized again by mild sonication in SDS containing buffer. Thereafter, these bands have an apparent molecular mass of 64 and 60 kDa on SDS gels. These proteins seemed to be associated with lipids leading to an unusual behavior during SDS-PAGE and an incorrect molecular weight (MW).

In contrast to the RCI proteins, the other polypeptides in PS I complexes showed no changes in their molecular mass after acetone extraction of the samples. As the results in Fig. 5 show, no significant differences could be demonstrated between the polypeptide composition of PS I complexes of *Eremosphaera* grown in complete or in N-depleted medium indicating that the additional SC seem to be associated with the regular antenna proteins. Unfortunately, the activity of isolated PS I complexes from *Eremosphaera* decreased rapidly when detergents like Triton X-100 were used. Therefore, no great increases in specific activity in the isolated PS I complexes could be achieved. The rate of PS I-mediated electron transport of isolated thylakoids was 400, and that of solubilized thylakoids 90 μmol O₂ taken up/mg Chl × h. Isolated PS I complexes from sucrose density gradient centrifugation showed a rate of 120 μmol O₂ taken up/mg Chl × h.

**Discussion**

Whereas our knowledge about pigment composition of PS I remained remarkable poor, polypeptide compositions of PS I complexes have been examined in great extent [1, 22–25]. As the results represented here show, the polypeptide composition of PS I complexes from *Eremosphaera* agrees well with that of other green algae or higher plants, since we could show the presence of peptides of 64, 60, 26, 23, 20, 15, 11, 8.5 kDa. In agreement with information in the literature, we suggest that the peptides of 64 and 60 kDa represent the reaction center of PS I carrying P₇₀₀ and the electron acceptors A₀, A₁, and Fₓ (for review see 24), and that the peptides in the range of 25 to 20 kDa MW are most likely LHC I proteins [23, 26, 27]. The 20 kDa protein might be a ferredoxin binding peptide as e.g. suggested for cucumber [28], and the 15 kDa polypeptide could be the plastocyanin docking protein as e.g. shown for spinach PS I complexes [29], while the 8.5 kDa most likely carries the iron sulfur centers Fₐ and Fₐ. Since no significant differences could be detected in polypeptide composition of PS I complexes from algae grown in complete or N-free medium, it seems that the SC present in PS I complexes from algae kept under N-deficient conditions, are associated with the regular LHC apoproteins.
In recent years, evidence is presented that pigments play an important role during assembly of pigment protein complexes of PS I and PS II. Humbeck et al. [30] and Paulsen et al. [31] showed that pigments in distinct quantities and relations are necessary for self assembly of LHCs suggesting a strong correlation between carotenoid synthesis in thylakoid membranes [3, 32] and protein synthesis. Secondary carotenoids (SC) are commonly shown to be located in cytosolic lipid bodies of green algae [5–8], whereas occurrence of SC in pigment protein complexes has not been reported to our knowledge with one exception. Echinonene, a SC in green algae, seems to be a constituent of the reaction center of PS I in the blue-green bacteria *Anabaena* [33]. Whereas higher plants contain one distinct pigment system, algae living in light-limited environment show a great variety of light-harvesting pigment systems [34]. Therefore, it is not surprisingly that *Eremosphaera* can synthesize additional SC under conditions of stress and that some of these SC are associated with pigment protein complexes in *Eremosphaera*. Why these SC in thylakoids of *Eremosphaera* are mainly located in PS I can not be answered at the present time. However, PS I seems more stable under conditions of stress than PS II, and SC might become incorporated into PS I complexes during their turn-over and self assembly during stress periods. On the other hand, a relationships between LHC apoproteins and early light-inducible proteins (ELIPs) has been suggested, and it is assumed that those proteins might be involved in carotenoid biosynthesis and in assembly of the thylakoid membranes [35]. Therefore, it might be possible to speculate that those LHC apoproteins could regain their catalytic activities under special stress conditions leading to a modification of carotenoids within the antenna complexes, and that enzymatic activities leading to formation of SC are preferentially associated with PS I complexes.

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