

Summary of Ph.D. thesis

**CAROTENOIDS ASSIST IN THE ASSEMBLY AND THE FUNCTIONS OF
PHOTOSYNTHETIC COMPLEXES IN CYANOBACTERIA**

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INTRODUCTION

Photosynthesis is a vital process by which the energy of sunlight is converted into chemical energy needed for life. Cyanobacteria are the oldest known oxygenic photosynthetic prokaryotic organisms regarded as ancestors of plant chloroplast according to the endosymbiosis theory.

Cyanobacteria are remarkably resistant organisms to changes in environmental conditions and very well protected against photooxidative damage generated by reactive oxygen species (ROS) whose attacks might result in the decline of the photosynthetic activity (photoinhibition). Carotenoids are indispensable components in the protection. Their photoprotective role is related to their ability to quench excited singlet-state and triplet-state chlorophyll, and scavenge the singlet-state oxygen, those are highly destructive ROS. Besides this very essential protective role, carotenoids act as accessory light harvesting pigments and regulate the membrane organization.

Phytoene synthase encoded by the *crtB* gene, which is the first committed step in carotenoid biosynthesis pathway, produces phytoene which is converted to various carotenes and xanthophylls. In *Synechocystis* sp. PCC 6803, one of the most studied cyanobacteria, the major carotenoids are as follows; β -carotene; its hydroxyl derivative, zeaxanthin; its keto derivative, echinenone and 3'-hydroxyechinenone and the carotenoid glycoside, myxoxanthophyll. Recent X-ray structural analysis revealed that β -carotene is the only carotenoid identified in the photosynthetic complexes of cyanobacteria. Besides β -carotene, all other carotenoids are found in the thylakoid membrane, cytoplasmic membrane and cytosol in cyanobacteria. The increase in their level is detected under various stress conditions. The substitutions of the β -carotene molecule determine the antioxidative properties against photooxidative stress. In general, hydroxy carotenoids are good in inactivating peroxy radicals whereas keto derivatives are more efficient in quenching of reactive singlet oxygen and possess the best stability against peroxy radicals and photooxidation.

Twelve carotenoids were found in monomeric PSII complex of *Thermosynechococcus elongatus* (*T. elongatus*) by X-ray crystallography. PSII is believed to be the most sensitive photosynthetic complex to photooxidative stress which is likely due to the absence of central antenna domain in PSII. Earlier studies suggested that β -carotene is vital for accumulation of D1 in the green alga *Chlamydomonas reinhardtii* as well as in *Synechocystis* PCC 6803. Therefore, carotenoid availability seems to be a major factor in the assembly of functional PSII.

Twenty two carotenoids have been identified in the structure of monomeric PSI from *T. elongatus*. PSI is generally less sensitive to light and its carotenoid-triggered photoprotection is

less known. Norflurazon (inhibitor of phytoene synthase)-treated *Scenedesmus obliquus* cells lacking carotenoids still contain some PSI and charge separation can be detected. Samples in which all carotenoids are extracted with organic solvents lack the quinones but are still able to perform electron transfer from P700 to A0.

Photosynthetic processes occur in PSI and PSII RC composed of pigment-protein complexes embedded in the thylakoid membrane. Lipid-protein and lipid-pigment interactions play an important role in the regulation of photosynthesis. Thylakoid membranes of cyanobacteria possess a characteristic membrane lipid composition that consists of glycolipids and phospholipids. In cyanobacteria and in the thylakoid membrane of higher plants, phosphatidylglycerol (PG) is the only phospholipid. The recent X-ray crystallographic structure of *T. elongatus* showed that there are PG molecules in the PSII complex localized between CP43 and D1 as well as in PSI. PG depletion results in a decrease of the Chl content of cells and in a general slowdown of photosynthetic processes. A suppression of electron transport from Q_A to Q_B was detected, which may lead to over-reduction of Q_A and to the generation of Q_A^{2-} . PG plays an essential against low-temperature stress and high light stress.

AIMS

Changes in global environment might lead to stress conditions in photosynthetic organisms and limit the efficiency of photosynthesis. Understanding photosynthesis and its protective mechanisms might lead to the development of plant, algae and cyanobacteria with improved growth characteristics even under stress conditions. Cyanobacteria, our model organisms, are regarded as an origin of the plant chloroplast; therefore they are excellent to study higher plant photosynthesis. Carotenoids are very important protective agents in photosynthesis due to their anti-oxidative properties against photooxidative damage. Their structural and functional features in protection mechanisms are not completely elucidated yet. Complete genome sequences and the transformability of several cyanobacterial strains allow us to generate various mutants and study the importance of carotenoids in photosynthetic processes. Therefore, the aims of my thesis are:

I. Generation of a viable oxygenic photosynthetic prokaryotic mutant in cyanobacterium *Synechocystis* that is completely deficient in carotenoid synthesis. The aim of creating this mutant is to study (i) the structural and (ii) the functional consequences of carotenoid deficiency on photosynthesis for the first time, *in vivo*.

II. Studying how carotenoids respond to stress conditions other than light stress; to this end PG depletion was induced in the *Synechocystis* sp. PCC 6803 *pgsA* strain.

MATERIALS & METHODS

Construction of *Synechocystis* Δ *crtH/B* mutant

Restriction enzymes *Bgl*III and *Not*I were used to cut the cosmid clone cs0798 (<http://genome.kazusa.or.jp/cyanobase/Synechocystis/map/Chr/orf16>) provided by S. Tabata. A 7.7 kb *Bgl*III fragment containing the phytoene synthase encoding *crtB* gene of *Synechocystis* was cloned into the *Bam*HI site of pMPMA2. A part of the *crtB* gene coding region was removed by *Apa*I-*Hind*III digestion and replaced with an omega cassette. This construct was used to transform Δ *crtH* mutant cells of *Synechocystis*. Transformants were selected under LAHG conditions on BG11 agar plates supplemented with glucose and increasing amount of spectinomycin by several restreaking of single colonies. Complete segregation of Δ *crtH/B* cells was confirmed by PCR using the primers crtBup (5'-CGGTGCCCAACTTTTACCTA-3'), and crtBdown (5'-TCACCTAAGGGGAAACATCG-3').

Organisms and growth conditions

Cells of *Synechocystis* Δ *crtH*, and Δ *crtH/B* strains were grown at 30 °C in BG11 medium supplemented with 5 mM HEPES buffer (pH 7.5), and 10 mM glucose under light-activated heterotrophic growth (LAHG) conditions which was carried out in the dark with 10 min daily illumination of 35 μ mol photons $m^{-2}s^{-1}$ with white fluorescent lamps. Twenty μ g ml^{-1} kanamycin and 40 μ g ml^{-1} spectinomycin, and 20 μ g ml^{-1} kanamycin were added to the medium of Δ *crtH/B* and Δ *crtH* cells, respectively. Cultures were aerated on a gyratory shaker at 100 rpm.

Cells of *Synechocystis* *pgsA* strain were grown photoautotrophically in BG11 medium supplemented with 5 mM HEPES-NaOH (pH 7.5), 20 μ g ml^{-1} kanamycin and 20 μ M dioleoyl-PG (18:1/18:1 PG) (P-9664, Sigma, St Louis, MO, USA) at 30°C under continuous illumination at a light intensity of 30 μ mol photons $m^{-2}s^{-1}$. PG depletion was achieved by washing the cells twice with PG-free medium and keeping them afterwards in PG-free medium. Cultures were aerated on a gyratory shaker at 100 rpm.

Pigment analysis

Synechocystis cells and subcellular fractions isolated from them were extracted with acetone:methanol (7:2 v/v) and centrifuged for 3 min at 4°C in a Sigma K-18 centrifuge (20,000 \times g). The supernatant fluid was evaporated under nitrogen gas and the extracted pigments were dissolved in HPLC-grade ethanol. Pigments were separated on a Prostar HPLC system (Varian, Miami, FL, USA), equipped with a photodiode array spectrophotometric detector Tidas I (World Precision Instruments, Sarasota, FL, USA) and a Nucleosil 100 C18 reversed phase column, 5 μ m particle size (Technokroma, Barcelona, Spain), using the solvent system described

by Lagarde and coworkers (Lagarde et al. 2000). Samples (100 μ l) were filtered through a stainless steel filter ($\phi = 0.22 \mu\text{m}$) and loaded on the column equilibrated with Solvent A (acetonitril:water:triethylamine, 9:1:0.01 by vol.). The column was eluted with Solvent B (ethyl acetate 100%), by a three-step gradient (0-40% B for 10 min, 40-60% B for 10 min, 60-100% B for 3 min) followed by an isocratic hold (2 min) at 100% B. During separation, a constant flow rate of 1.5 ml min^{-1} was ensured. The absorption spectra of the elutes (380–800 nm) was recorded every 0.2 s.

Carotenoid derivatives were identified on the basis of both their absorption spectra and their retention times. The relative content of each pigment was estimated by a comparison of peak areas on chromatograms recorded at 440 nm. The concentrations of carotenoid and Chl species were calculated from Beer–Lambert's law using their specific extinction coefficients at 440 nm and at 665 nm, respectively. (The echinenone content was calculated at 460 nm). Values are means \pm SD from at least three individual experiments.

Thylakoid membrane isolation

Thylakoid and cytoplasmic membranes were isolated with some modifications according to Murata and Omata (1988, *Methods Enzymol.* 167: 245-251). The 0.2% lysozyme-treated (37°C, 2 h) and pelleted cells were disrupted with 0.1 mm glass beads in a Bead Beater homogenizer (Biospec Products, Bartlesville, OK, USA) in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor. The disrupted cells were treated with 0.1% DNase for 15 min, and the unbroken cells were removed by centrifugation (10 min, 7,000 \times g). The membrane vesiculi were ultracentrifuged in a discontinuous sucrose density gradient (130,000 \times g, 16 h, 4°C). After flotation centrifugation, the cytoplasmic membranes formed a yellow band in the 30% sucrose layer, and the thylakoid membranes formed a green band at the interface between the 39 and 50% sucrose layers.

Cytosolic fractions were isolated from PG-supplemented and PG-depleted cells that were suspended in a buffer containing 50 mM MES (pH 6.5), 2 mM ϵ -amino-caproic acid, 5 mM EDTA, 1 mM PMSF and 1 mM benzamidine. Cells were disrupted with 0.1 mm glass beads in a Bead Beater homogenizer. Intact cells, cell walls and all membranes were removed by ultracentrifugation (45 min, 145,000 \times g, 4°C). Pigments were extracted from lyophilized supernatants.

Protein analysis

Radioactive labeling of the cells; Cells containing 75 μg of Chl were resuspended in 250 μl of BG11 in a microcentrifuge tube, shaken at 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for 15 min and then a mixture of [^{35}S]Met and [^{35}S]Cys (Trans-label, MP Biochemicals, Irvine, USA) was added (final specific activity 400 $\mu\text{Ci ml}^{-1}$). The suspension was exposed to 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ white light for 15 min (pulse), then chloramphenicol (1 mg ml^{-1} final concentration) and a mixture of cold Met and Cys (5 mM final concentration) were added and incubation continued for an additional 15 min (pulse-chase). Afterwards the cells were frozen in liquid nitrogen and used for isolation of thylakoid membranes.

Thylakoid membrane isolation; Thylakoid membranes were prepared by breaking the cells using glass beads according to Komenda and Barber (1995, Biochemistry 34: 9625-9631) with the following modifications: the cells were washed, broken and resuspended in 25 mM MES/NaOH, pH 6.5, containing 10 mM CaCl_2 , 10 mM MgCl_2 and 25% glycerol. Glass beads were subsequently removed by filtering and thylakoid membranes were obtained by differential centrifugation

2D gel electrophoresis -BN/SDS-PAGE; For the analysis of protein complexes, the isolated membranes were solubilized with dodecyl- β -D-maltoside (DM/Chl = 40:1 w/w) and analyzed by blue-native electrophoresis at 4 $^\circ\text{C}$ in a 5 to 14% polyacrylamide gel. Samples with 6 μg Chl content were loaded onto the gel. The protein composition of the complexes was assessed by a 2nd electrophoresis in a denaturing 12 to 20% linear gradient polyacrylamide gel containing 7 M urea. The lanes from the native gel were excised along their entire length, incubated for 30 min in 25 mM Tris/HCl, pH 7.5 containing 1% SDS (w/v) and placed on top of the denaturing gel. Proteins separated in the gel were stained by Coomassie Blue. For autoradiography, the gel or the membrane with labeled proteins was visualized on X-ray films exposed at room temperature for 2 to 3 days or on Phosphorimager plates (GE Healthcare, Vienna, Austria) overnight. Quantitation of bands was done using ImageQuant 5.2 software (GE Healthcare, Vienna, Austria).

Western blot analysis- Samples containing 1 μg Chl were loaded onto the denaturing gel (described above) and the separated proteins were transferred onto a PVDF membrane. Membranes were incubated with specific primary antibodies and then with a secondary antibody-horseradish peroxidase conjugate (Sigma, St. Louis, USA).

Oxygen-evolving activity measurements

Oxygen-evolving activity in whole cells was measured with a Clark-type oxygen electrode (Hansatech Instruments, Kings Lynn, U.K.) PSII oxygen-evolving activity was

measured from H₂O to parabenzoquinone (artificial acceptor), at a concentration of 500 μM. The cells were washed with BG11 medium and re-suspended in fresh BG11 medium for the measurement of oxygen evolution. An incandescent lamp equipped with a red optical filter was the light source. This arrangement was used for all the oxygen evolution measurements at a saturating light intensity of 500 μmol photons m⁻² s⁻¹. The Chl concentration of the cells was adjusted to 5 μg ml⁻¹.

Chlorophyll *a* fluorescence measurements (Fv/Fm) and the changes in P700 signal (Pm-Po), and oxidation-reduction measurements of P700 kinetics

Redox changes of P700 and Chl *a* fluorescence were measured by a Dual-PAM-100 Measuring System (Heinz Walz GmbH, Germany) equipped with DUAL-E Measuring Head (difference of intensities of 830 nm and 875 nm) with P700 Near Infra Red Emitter (720 nm) and DUAL-DR (red) Measuring Head (620 nm). The same amount of sample (equivalent to 20 μg Chl concentration) was filtered onto a Whatman GF/C glass-fiber disc. Three independent repetitions were made for each type of measurement.

Maximum PSII yield, (Fv/Fm) was determined after 20 min dark-adaptation to allow relaxation of the photosynthetic electron transport and determination of the fluorescence yield of open PSII RCs (Fo). Maximal fluorescence yield of close PSII RCs in dark adaptate state (Fm) is detected during a 2000 μmol photons m⁻² s⁻¹ saturation pulse. Using these parameters, the following ratios were calculated. Fv (Fm-Fo), variable fluorescence, indicates the maximum fluorescence change in dark-adapted cells. Fv/Fm=(Fm-Fo)/Fm is a sensitive indicator of maximum photosynthetic efficiency of PSII in the dark-adapted state.

Maximum changes of P700 signals (Pm-Po) were determined after 20 min dark adaptation that allows reduction of P700 (P₀). Then, P700 was oxidized by far red light pre-illumination for 10 seconds and then maximum signal level was induced by 20000 μmol photons m⁻² s⁻¹ actinic red light in the presence of far red light. Maximum changes of P700 signal levels between P700 fully reduced (P₀) and P700 fully oxidized (Pm) were recorded on a millisecond time scale.

Oxidation-Reduction Kinetics of P700 was determined by 53 μmol photons m⁻² s⁻¹ continuous actinic red light illumination after 20 min dark adaptation at room temperature. P700 absorbance changes were recorded on a millisecond time scale. Linear electron transport was inhibited by the addition of 100 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

RESULTS

The *crtB* gene encoding phytoene synthase was inactivated in the partially carotenoid-less mutant $\Delta crtH$. Thus, a carotenoid-less double mutant, $\Delta crtH/B$, was produced. $\Delta crtH/B$ cells are extremely light sensitive and only grown in light activated heterotrophic growth LAHG conditions (in the presence of 5-10 mM glucose and in the dark with an exposure of 5 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ white light for 10 min/day). HPLC analysis demonstrated that the $\Delta crtH/B$ cells contained no carotenoid derivatives. The bluish color of the $\Delta crtH/B$ cells indicated that phycobiliproteins were the most dominant pigments and their synthesis and accumulation was not significantly suppressed. On the other hand, chlorophyll synthesis and accumulation was suppressed by carotenoid deficiency. The mutant cells gradually bleached and died in the light indicating that carotenoids are indispensable components in photoprotection mechanism against light stress.

In the carotenoid-less $\Delta crtH/B$ mutant levels of the large protein subunits of PSII were strongly suppressed, however, not those of PSI and Cyt *b₆f* as detected by western blotting. D1 and D2 core-complex protein subunits were present at lower level than those of the WT under LAHG conditions. After the cells were exposed to light, even lower level of D1 and D2 protein subunits could be detected indicating photooxidation of those proteins in the absence of carotenoids. The level of CP47 and especially CP43 Chl-binding antenna protein subunits were the most affected. This is in agreement with the latest X-ray crystallographic model of PSII structure, which shows that most of the β -carotene molecules are located in the vicinity of the transmembrane α -helices of CP47 and CP43. 2D gel analyses of protein subunits of $\Delta crtH/B$ in combination with their radioactive pulse-chase labeling provided clues how to explain why no active PSII complexes were assembled. The results show that, dimeric PSII complex could not be formed in the absence of β -carotene, even though very unstable monomeric PSII complex could be detected only by radioactive labeling. CP47 and especially CP43 protein subunits were not stable in the PSII complex. Therefore, only the intermediate PSII complex, RC47 lacking CP43 was able to accumulate to the level detectable by gel electrophoresis. The D1 protein subunit was the most labeled protein in $\Delta crtH/B$ indicating that the steady state D1 turnover was functioning in the carotenoid deficient mutant. D1 labeling could be detected mostly in RC47 as well as in monomeric PSII complex and RCa, intermediate PSII complex lacking both CP43 and CP47 protein subunits. After chase experiment together with chloramphenicol treatment which is a protein synthesis inhibitor, monomeric PSII complex disappeared indicating instability and light susceptibility of the complex. Moreover, small protein subunits binding to the unassembled PSII large protein subunits were not stable without β -carotene. In contrast to PSII, PSI and Cyt *b₆f*

could be assembled, however the ratio of trimeric/monomeric PSI complexes was significantly decreased in the carotenoid deficiency.

Definitely, among photosynthetic membrane complexes in the $\Delta crtH/B$ mutant, PSII was the most severely affected complex by the absence of carotenoids. Indeed, PSII-dependent oxygen-evolving activity was not detected in the carotenoid-less $\Delta crtH/B$ cells under all conditions demonstrating the absence of functional PSII complexes. Moreover, measurements of PSII Chl *a* fluorescence and transient reduction kinetics of P700 confirmed that in $\Delta crtH/B$ cells no active PSII centers are formed. In contrast, PSI charge separation and cyclic electron transport could be efficiently performed in $\Delta crtH/B$ cells grown under LAHG conditions. However, changes in the level of active PSI centers significantly decreased after cells were exposed to low light illumination. To understand the reason of PSI photoinhibition in carotenoid-less and active PSII-less $\Delta crtH/B$, we made use of PSII-less mutant strain (psbDI, psbDII and psbC genes encoding D2 and CP43 proteins, respectively were blocked). Nevertheless, PSII-less mutants, containing carotenoids showed a constant PSI activity even if the cells were grown in the constant light. These results suggested that not the inefficient linear electron transport between PSII and PSI but the carotenoid deficiency was the reason for the photoinhibition of PSI.

Phosphatidylglycerol (PG) depletion suppressed the PSII activity in *Synechocystis* PCC6803 *pgsA* mutant cells most likely caused by elevated ROS production. Therefore, the changes in carotenoid content and composition during longer-term PG depletion were measured. In order to analyze and localize the carotenoid accumulation in PG-deficient mutant we isolated thylakoid and cytoplasmic membrane fractions either from PG-supplemented or from PG-depleted cells. The separation of cytoplasmic and thylakoid membrane layers by ultracentrifugation on a stepwise sucrose density gradient revealed that the PG-depleted cells (–PG14day and –PG21day) contained higher amount of carotenoids than the PG-supplemented (+PG) cells, especially in the upper part of the gradient, which contained a water-soluble non-membrane fraction of the cells. We analyzed the carotenoid species extracted from *pgsA* cells and from isolated membrane fractions by HPLC. In *pgsA* cells, myxoxanthophyll, zeaxanthin, echinenone and β -carotene were identified on the basis of their absorption spectra and their retention times. The amounts of β -carotene and zeaxanthin decreased in the PG-depleted samples compared to those of the PG-supplemented samples. The amount of myxoxanthophyll increased approximately 3-fold in all PG-depleted samples, and the amount of echinenone was twice as high in the PG-depleted thylakoid membranes and to a lesser extent in whole cells than in PG-supplemented cells. Relative amount of the individual carotenoid species was also estimated from their peak areas from the HPLC chromatograms. The proportion of myxoxanthophyll and

echinenone in the carotenoids of PG-depleted whole cells, isolated membranes and cytosol proved to be higher than in the corresponding PG-supplemented controls. These results suggested that photooxidative damage mediated by PG depletion induced the level of specific carotenoids. This is most likely the result of differently regulated biosynthetic steps of carotenoids under stress conditions.

We can conclude that carotenoids are indispensable components for the assembly and the function of photosynthetic complexes especially that of PSII shown for the first time, *in vivo*. In the absence of carotenoids cells were extremely light sensitive and exposure to the low light resulted in strong photoinhibition. On the other hand we demonstrate that carotenoid accumulation is a response to different stress conditions other than high light stress as well, however, the increase in the level of specific carotenoids induced by PG-depleted stress conditions are regulated differently.

PUBLICATION LIST

Source of the publication of the thesis

Domonkos I, Malec P, Laczko-Dobos H, **Sozer O**, Klodawska K, Wada H, Strzalka K, Gombos Z. Phosphatidylglycerol depletion induces an increase in myxoxanthophyll biosynthetic activity in *Synechocystis* PCC6803 cells **Plant Cell Physiol**. 2009 Feb; **50**(2):374-82

Sozer O, Komenda J, Ughy B, Domonkos I, Laczkó-Dobos H, Malec P, Gombos Z, Kis M. Involvement of carotenoids in the synthesis and assembly of protein subunits of photosynthetic reaction centers of *Synechocystis* sp. PCC 6803 **Plant Cell Physiol**. 2010 May; **51**(5):823-35

Sozer O, Kovacs L, Kis M, Gombos Z

Involvement of carotenoids in the activity of photosynthetic reaction centers of *Synechocystis* sp. PCC 6803 (**manucript**)

Other publications

Sozer O, Kis M, Gombos Z, Ughy B

Proteins, glycerolipids and carotenoids in the functional photosystem II architecture **Frontiers in Bioscience** 2011 Jan; **16**: 619-643

Laczko-Dobos H, Todinova S.J., **Sozer O.**, Komenda J., Kis M, Sallai A., Dobrikova A.G., Ughy B., Debreczeny M., Gombos Z., Apostolova E.L., Domonkos I. Identification of thylakoid membrane thermal transitions in *Synechocystis* sp. PCC6803 photosynthetic mutants (**submitted**)

A szerzőtársak nevében, mint „corresponding author” kijelentem, hogy Ózge Sözer a dolgozatában felhasznált következő cikkben:

„Domonkos I, Malec P, Laczko-Dobos H, **Sozer O**, Klodawska K, Wada H, Strzalka K, Gombos Z.

Phosphatidylglycerol depletion induces an increase in myxoxanthophyll biosynthetic activity in *Synechocystis* PCC 6803 cells.

Plant Cell Physiol. (2009) 50: 374-82.”

Meghatározó jelentőségű önálló munkát végzett valamint azt, hogy ezt a munkát a szerzőtársak közül senki sem használja fel egy másik doktori dolgozat elkészítéséhez.

Szeged, 2011 január 3.

Gombos Zoltán
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