Summary of Ph.D. thesis

Role of phosphatydilglycerol in cellular functions of photosynthetic organisms

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INTRODUCTION

Photosynthetic organisms capture solar energy and store it to be used in a series of biochemical reactions that convert pure energy of light into biochemical energy needed for life. Cyanobacteria are among the oldest prokaryotic photosynthetic organisms. According to the endosymbiotic theory they are the ancestors of chloroplasts.

Cyanobacteria are ideal model organisms for the investigation of oxygenic photosynthetic processes. Their simple cell structure, easy culturing potential, and the natural transforming competence of some strains make them attractive subjects for photosynthesis research. Photosynthetic reactions occur in pigment-protein complexes embedded in a lipid bilayer, the thylakoid membrane. This shows that lipid-protein interactions play an important role in the regulation of photosynthetic processes. Thylakoid membranes of cyanobacteria possess a characteristic membrane lipid composition that consists of neutral glycolipids, as major components, and anionic phospholipids, such as phosphatidylglycerol (PG), as minor component. In cyanobacteria and in thylakoids of higher plants, however, PG is the only phospholipid. The most recent X-ray cristallography structure of *Thermosynechococcus elongatus* showed the presence of two PG molecules between protein CP43 and protein D1 in PSII.

The availability of the complete genome sequence of the cyanobacterium *Synechocystis* PCC6803 opened the way for studying the structural and functional role of PG *in vivo*: by creating PG-deficient mutants, Δ*pgsA* and Δ*cdsA*, molecular genetic approaches could be employed. These mutants are not able to synthesize PG because the genes encoding certain key enzymes of PG biosynthesis have been

inactivated. Genes *pgsA* and *cdsA* encode PGP synthase and CDP-DG synthase, respectively. Cyanobacteria provide a unique system for studying the role and remodeling of this lipid since, unlike higher plants, they can take up PG from the medium by passive transport. Using these mutants it has been shown that PG plays an important role in PSII dimmer and PSI trimer formation, and in the regulation of electron transport processes on the acceptor side of PSII, in the vicinity of Q_B.

The glycerol backbone of PG is esterified with saturated or unsaturated fatty acids whose proportion depends on the strain and on the growing temperature. Both prokaryotes and eukaryotes respond to a variety of environmental stress by altering the fatty acid composition of their membrane lipids. However this metabolic process, called remodeling of PG has not been well understood.

GOALS

The availability of the complete genome sequence of the cyanobacterium *Synechocystis* sp. PCC6803 opened the way for studying the structural and functional role of PG *in vivo*, by creating PG-deficient mutants: Δ*pgsA* and Δ*cdsA*. Using these mutants it has been shown that PG plays an important role in PSII dimer and PSI trimer formation, and in the regulation of electron transport processes on the acceptor side of PSII. A variety of specific roles of PG has not been revealed yet.

My aims were:

I. To create a PG-deficient mutant which has a lot of advantage compared to the existing ones, in studying the role of PG.

II: To study the role of PG in PSII structure and function.

III. To reveal the enzymatic processes, which can lead to the remodeling of PG in a prokaryote organism.

METHODS

Organisms and growth condition

Cells of *Synechocystis* PCC6803 PAL/Δ*cdsA* and Δ*pgsA* mutant strains were grown photoautotrophically in BG11 medium supplemented with 5 mM HEPES–NaOH (pH 7.5), 20 μg/ ml kanamycin and in the presence of 20 μM dioleoyl–PG (18:1/18:1 PG; P-9664; Sigma, St Louis). Cells were grown at 30 °C, under continuous illumination at the intensity of 30 μmol photons m^{-2} s^{-1}. Cultures were aerated on a gyratory shaker operating at 100 rpm. PG depletion was achieved by washing the cells twice with PG-free medium, followed by cultivation in PG-free medium.

Construction of PAL/Δ*cdsA* mutant strain of *Synechocystis* PCC6803

A 1.4-kb genomic fragment of *Synechocystis* PCC6803 containing *cdsA* was amplified using PGF (5′ AGGTCCGCAACGTGGAGGTG-3′) and PGR (5′-CTGGAACGACTTGGGAAGTG-3′) primers. The primers were designed on the basis of the published complete genome sequence of *Synechocystis* PCC6803 (http://www.kazusa.or.jp/). The kanamycin resistance cassette from pUC4K was inserted between the MscI and NsiI sites of the cloned *cdsA* gene, yielding the inactivated construct. This *cdsA*-inactivated construct was used to transform the

Synechocystis PCC6803 wild-type and PAL mutant. In the inactivated strain the *aphI* gene cassette replaces a 543-bp-long *cdsA* gene fragment. Transformants were selected on BG11 agar plates containing 20 μM PG and 50 μg/ml kanamycin.. Complete segregation of the mutants was confirmed by PCR and by the fact that PG was required for their growth.

Measurement of cell density and pigment concentration

Absorption spectra of cell suspensions were recorded in a Shimadzu UV-1601 spectrophotometer. Cell density in the cultures was determined by measuring OD750. The chlorophyll concentration was measured by absorbance at 665 nm, using a 90% methanol extract. The A665 was multiplied by an extinction coefficient of 78.74 to calculate Chl a concentration (μg Chl a/ml).

Electron microscopic measurements

The harvested cells were fixed in 1% formaldehyde and 1% glutaraldehyde for 4 h at 4 °C and post-fixed in 1% osmium tetroxide. The samples were dehydrated in aqueous solutions of increasing ethanol concentrations, and then embedded in resin. Following polymerization, 85-90 nm ultra thin sections were cut out by a Reichert Ultracut E ultramicrotome. The sections were treated with uranyl acetate and lead citrate and subjected to electron microscopy in a Zeiss EM 902 electron microscope.

Two dimensional gelelectrophoresis

Cells were labeled with [^{35}S] methionine (at >1000 Ci mmol $^{-1}$, Isotope Institute Ltd, Hungary) under irradiance of 60 μmol photons m^{-1} s $^{-1}$ at 29 °C. Cyanobacterial membranes were prepared by breaking the cells with glass beads (150–200 μm diameter) at 4 °C followed by differential centrifugation as described by Komenda and Barber [Biochemistry 34 (1995) 9625–9631]. Breaking of cells and final resuspension were done in 25 mM Mes-NaOH, pH 6.5 containing 10 mM CaCl2, 10 mM MgCl2 and 25% glycerol. The isolated membranes were solubilized with dodecyl-β-D-maltoside (at a dodecylmaltoside/chlorophyll ratio of 20:1, w/w) and analyzed by BN/SDS-PAGE at 4 °C in a 5–14% polyacrylamide gel, according to Schägger and von Jagow [Anal. Biochem 199 (1991) 223–231]. The whole lane from the gel was excised, incubated for 30 min in 25 mM Tris–HCl buffer (pH 7.5) containing 1% SDS and then layered onto the top of a denaturing gel. The protein composition of the complexes was then assayed by a 2nd electrophoresis in a denaturing 12–20% linear gradient polyacrylamide gel containing 7M urea. In the gel the protein bands were stained by Coomassie blue.

77K fluorescence emission spectra

Low temperature steady-state fluorescence emission spectra (600–780 nm) were recorded using an excitation wavelength of 437 nm. A spectrofluorimeter (Fluorolog-3/Jobin Yvon-Spex Instrument S.A., Inc.) equipped with an adapter for measurement in liquid nitrogen was applied. The emission spectra were corrected for photomultiplier sensitivity and normalized to the fluorescence intensity at 700 nm.

Measurement of photosynthetic oxygen-evolving activity

Photosynthetic oxygen evolution in intact cells was measured with a Clark-type oxygen electrode (Hansatech Instruments, Kings Lynn, U. K.). PSII oxygen evolving activity was measured from H_2O to exogenously added artificial quinones (500 μM p-benzoquinone). Light from an incandescent lamp equipped with a red optical filter was used for all the oxygen evolution measurements at a saturating light intensity of 500 μmol photons m^{-2} s^{-1}.

Thermoluminescence (TL) measurement

Intact cells were centrifuged and resuspended in fresh BG11 medium. The measurement of glow curve was carried out in a temperature range from −10 °C to +80 °C. Light emission of the samples was measured by a red-sensitive photomultiplier (Hamamatsu R/2228) and the signal was amplified through a home made differential amplifier and fed to PC. The temperature of the sample holder was monitored using a platinum resistor thermometer placed under the samples, and cooling was achieved by liquid nitrogen. Samples were illuminated with 2 flashes (5 μs) at 5 °C before freezing.

The fast Chl a fluorescence (OJIP transient)

Samples were dark-adapted for about 2 h before the start of the measurements. Fluorescence measurements were carried out by a Handy-PEA instrument (Hansatech Instruments Ltd, UK). The samples were illuminated with continuous red light (650 nm peak wavelength) at the light intensity of 3500 μmol photons m^{-2} s^{-1}. The light was provided by three LEDs focused on a circle of 5 mm diameter. The first reliable measurable point of the fluorescence transient was at 20 μs. This intensity was taken as the F0 value for dark-adapted cells. The length of the measurements was 3 s.

Lipid isolation and gas chromatography

For gas chromatographic analysis, lipids were extracted from the collected cells (around OD_{750}=50) by the method of Bligh and Dyer [J. Biochem. Physiol. 37 （1959）911-917]. The analysis of lipids was carried out according to the method of Sato and Murata [Methods Enzymol. 167 （1988） 251-259]. For mass spectrometry analyses 20 μg PG(14:0/14:0) (Avanti Polar Lipids, Alabaster, USA) was added to each sample as internal standard before lipid extraction. Lipids were fractionated on precoated thin layer chromatography (TLC) plates (Merck 5721) developed with $CHC1_3$/CH_30H/28% NH_40H (65:35:5, v/v). The fatty acid methyl esters of PG were analyzed with a Hewlett Packard (Palo Alto, California, USA) HP6890 gas chromatograph equipped with a hydrogen flame-ionization detector. Fatty acid methyl esters were separated on a 30 m × 0.25 mm i.d. SP-2330 capillary column (Supelco, Bellefonte, Pennsylvania, USA). Temperatures of the column and the flame-ionization detector were 180 and 260 °C, respectively.

Mass spectrometry

The extracts of the biological material or calibration solutions were directly infused at a flow rate of 5 μl/min into a hybrid linear ion trap–Fourier transform ion cyclotron resonance (FTICR) mass spectrometer with a 7-Tesla

superconducting magnet (LTQ-FT, Thermo Fisher, San Jose, CA) and equipped with an ESI source operated in the negative ionization mode. The spray voltage was set to 5 kV, sheath gas flow rate to 10 units, capillary temperature to 275 °C and the ion optics elements voltages were set by the built-in automatic tuning procedure using a solution of the PG(18:1/18:1) standard.

Singly negatively charged phospholipid $[M-H]^-$ molecular ions were detected by full-scan FTICR acquisition in the 150 to 2000 Th mass-to-charge ratio (*m/z*) range. Mass resolution (m/Δm) was set to 50000 at *m/z* of 400 Th. Approximately 80 mass spectra were averaged per sample. For further structural characterization, MS/MS experiments were performed. The $[M-H]^-$ ions were isolated and subjected to collision-induced dissociation (CID) in the linear ion trap that was also used to detect the product ions (MS/MS spectra). The isolation width was set to 1.0 Th and the collision energy to 15%, respectively, with helium as target gas.

To estimate the concentration of PG species in the biological extracts, the following simple equation was used: $c_A / c_{IS} = k_A . (I_A / I_{IS})$, where c_A is the concentration of analyte, c_{IS} is the concentration of internal standard [PG(14:0/14:0)] and I_A and I_{IS} are the ion intensities of analyte and the internal standard, respectively.

RESULTS

I. For studying the role of PG in cellular functions we created a *Synechocystis* sp. PCC6803 PAL/Δ*cdsA* mutant. The PAL/Δ*cdsA* is a mutant that does not have phycobilisomes and cannot synthesize PG. This strain allows quite an easy interpretation of PSII fluorescence emission spectra and decay kinetics without the contribution of PBS.

The PAL/Δ*cdsA* mutant required supplementation of PG in the culture medium for growth. By PG depletion we were able to follow the changes caused by a gradual depletion of PG.

In the PG-depleted medium the growth rate decreased gradually from the 4th day, and almost stopped on the 14th day of the PG depletion period. Morphological investigations by electron microscopy revealed a deformation of the PG-depleted cells. The PG-depleted cells had a unstructured cell interior with deformed thylakoid membranes. The volume of the cells increased. This latter finding suggests that the cells were ready for division which, however, did not take place. We think that PG might have the same role in cell division in *Synechocystis* as cardiolipin has in other bacteria, in which lipid accumulates in the mid-cell membrane and takes part in the formation of the Z-ring of cell division.

II. Using two dimensional gelelectrophoresis (2D-BN-PAGE) we demonstrated a new aspect of PG depletion, which was not identified before, namely, detachment/destabilization of binding of CP43 to PSII Reaction Center Core (RCC). PG served like as a glue required for the binding of CP43 to RCII.

By using [^{35}S]-labeled methionine we confirmed that PG depletion does not inhibit the synthesis of the large PSII subunits. Low temperature fluorescence data also confirmed the detachment of CP43 from RCII upon PG depletion.

PG depletion severely affected the electron transport processes and a damage occurred mainly on the acceptor side. This was confirmed by inactivation of oxygen-evolution, and by a slowing down of electron transport from the Q_A to the Q_B site. Due to PG depletion a shift of the B TL band toward the Q band has been demonstrated by thermoluminescence measurements. This underlined inhibition on the acceptor side. Changes in OJIP fluorescence curve kinetics also revealed suppression of electron transport processes between Q_A and Q_B.

III. Gas chromatographic analyses showed that the exogenously added artificial PG(18:1/18:1) was remodeled, fatty acid content was modified in the PAL/Δ*cdsA* and *Synechocystis* PCC6803/Δ*pgsA* mutant cells.

We induced PG depletion of the cells, then we re-added the PG to the medium and we followed the remodeling process. We confirmed the formation of new, retailored PG species by using negative-ion electrospray ionization–Fourier-transform ion cyclotron resonance and ion trap tandem mass spectrometry. Our data suggest that the remodeling of diacylphosphatidylglycerol likely involve reactions catalyzed by phospholipase A_1 and A_2 or acyl-hydrolase, lysophosphatidylglycerol acyltransferase and acyl-lipid desaturases for remodeling of diacylphosphatidylglycerol molecules. Our data provide evidence that cyanobacteria possess the enzymatic machinery capable of PG remodeling.

LIST OF PUBLICATIONS

Source publications of the thesis

Laczko-Dobos H, Ughy B, Toth SZ, Komenda J, Zsiros O, Domonkos I, Parducz A, Bogos B, Komura M, Itoh S, Gombos Z (2008) Role of phosphatidylglycerol in the function and assembly of Photosystem II reaction center, studied in a *cdsA*-inactivated PAL mutant strain of *Synechocystis* sp. PCC6803 that lacks phycobilisomes. **Biochim Biophys Acta 1777**: 1184-1194. IF: 3.835

Laczko-Dobos H, Fryčák P, Ughy B, Domonkos I, Wada H, Prokai L, Gombos Z (2009). Remodeling of Phosphatidylglycerol in *Synechocystis* PCC6803/Δ*pgsA*. BBA Molecular and Cell Biology of Lipids (submitted).

Domonkos I, **Laczkó-Dobos H,** Gombos Z (2008) Lipid-assisted protein-protein interactions that support photosynthetic and other cellular activities. **Prog Lipid Res 47**(6)**:** 422-435. IF: 11.194

Publications related to the thesis

Domonkos I, Malec P**, Laczko-Dobos H,** Sozer O, Klodawska K, Wada H, Strzalka K, Gombos Z (2009) Phosphatidylglycerol depletion induces an increase in myxoxanthophyll biosynthetic activity in *Synechocystis* PCC6803 cells. **Plant Cell Physiol**. 50(2): 1-9. IF: 3.31

Bogos B, Ughy B, Domonkos I, **Laczkó-Dobos H**, Komenda J, Abasova L, Cser K, Vass I, Sallai A, Wada H, Gombos Z (2009). Phosphatidylglycerol depletion affects photosystem II activity in *Synechococcus* sp. PCC 7942 cells. Photosynth Res (in press).

Laczko-Dobos H, Szalontai B (2009). Lipids, proteins, and their interplay in mambrane dynamics. Biochemistry (in press).

Sozer O, Komenda J, Ughy B, Domonkos I, **Laczkó-Dobos H**, Malec P, Gombos Z and Kis M (2009). Involvement of Carotenoids in the Synthesis and in the Assembly of Protein Subunits of Photosynthetic Reaction Centers of *Synechocystis* sp. PCC 6803. Plant Cell Physiol (submitted).

Abstract

Gombos Z, Toth S, **Laczko-Dobos H**, Ughy B (2007) Involvement of phosphatidylglycerol in the assembly and function of photosystem II. Photosynth Res 91: 141