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Thesis of the PhD dissertation

The role of lipids and carotenoids in the formation of cyanobacterial photosynthetic macrocomplexes

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Introduction

Cyanobacteria are the only bacteria to perform oxygen-producing photosynthesis. Plants and cyanobacteria share many similarities in both the machinery and mechanisms of photosynthesis. For this reason, cyanobacteria have long been model organisms for the study of oxygen-producing photosynthesis in higher plants. By genetically altering their genome, lipid and carotenoid mutants can be generated which can be used to study the structural and functional role of these molecules in photosynthesis.

Lipids, as important constituents of photosynthetic membranes, are key actors in forming dynamic bilayers. In cyanobacteria thylakoids are dominant membrane structures, therefore their lipid composition is similar to that of the total cellular membranes. Thylakoids are the sites of oxygenic photosynthesis in cyanobacteria and plants and their lipid composition is unique and highly conserved. They include mainly galactolipids, such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), a sulfolipid, sulfoquinovosyldiacylglycerol (SQDG), and the phospholipid phosphatidylglycerol (PG). In cyanobacteria the MGDG biosynthetic precursor monoglucosyldiacylglycerol (MGlcDG) is also present.

The physical properties of different membrane lipid classes is determined by their head group structure. MGDG and DGDG, together with MGlcDG, have neutral head groups, while SQDG and PG are anionic lipids, bearing one negative charge. Interestingly, MGDG, the most abundant galactolipid of thylakoids, and MGlcDG are typical non-bilayer-forming (NBL) lipids. They have a cone-like shape, having small head group and long polyunsaturated tails, which are able to form in aqueous medium an inverted hexagonal structure known as hexagonal II phase. The other lipid classes (DGDG, SQDG and PG) are typical lamellar bilayer-forming (BL) lipids, having bigger head group and more cylindrical shape. A certain ratio of NBL to BL lipids is crucial for functional membranes. Fine tuning of the MGDG/DGDG ratio makes thylakoid membranes extremely dynamic and flexible to cope with various

environmental stress factors. The relatively high NB lipid content in photosynthetic membranes, compared to for example plant mitochondrial membranes, is needed to accommodate their relatively high protein content. The high protein to lipid ratios of thylakoids can be attributed to extremely large protein complexes of the photosynthetic apparatus, which assist in photosynthetic electron transport.

Properties of lipids depend not only on their head groups but also on the saturation level of their fatty acid tails. In cyanobacteria the fatty acyl chain length varies from 14 to 18 carbon atoms (C14–C18), the number of double bonds also varies from one to four, leading to saturated, monounsaturated and polyunsaturated fatty acids.

The level of membrane lipid unsaturation is influenced by changes in the growth temperature, allowing regulation of the fluidity that is necessary for the photosynthetic functions of cyanobacteria. When the fluidity of the membrane is modified by decreased temperature, plants and cyanobacteria maintain membrane homeostasis by increasing the number of double bonds in the glycerolipids. It has been suggested that lipid unsaturation can stabilize heat stess exposed photosynthetic complexes. Polyunsaturated fatty acids are also important in protecting the photosynthetic machinery against salt stress.

Carotenoids (Cars), the other key components of photosynthetic membranes, are also hydrophobic, neutral, lipid-like molecules with multiple conjugated double bonds . In cyanobacteria two main forms are present: carotene (β-carotene) and its oxygenated derivatives, xanthophylls. They are parts of the lipid bilayers, and are also associated with proteins in the main photosynthetic complexes. Despite their hydrophobic character, Cars can form water soluble fractions when associated with the so-called orange carotenoid proteins, or the very recently identified helical carotenoid proteins.

Cars are multi-functional. They take part in the light-harvesting processes and the assembly of the PSII photosynthetic complex, they modulate membrane structures

and protect them from environmental stress factors. They are also required for PSII dimerization and PSI trimerization in *Synechocystis*. Not only Cars but also elevated temperature can stabilize PSI trimers. Whereas in plants, PSI exists only in monomeric form, in cyanobacteria PSI trimers are also present. In some thermophilic cyanobacteria tetramers could be found. A recent study of tetrameric PSI suggests that these supercomplexes may be stabilized by Cars or lipids. Carotenes may also influence the structure and function of phycobilisomes. Cars are also vital for the PSII function.

Aims of the study

Glycerolipids, together with Cars, are present at structurally and functionally important sites of the PSI and PSII, and they have determining roles in these pigment-protein complexes. Therefore, investigating lipid-carotenoid-protein interactions in photosynthetic membranes is an intriguing new field of research. With regard to studying these interactions I aimed to answer the following questions:

1: What impact do(es) Car and/or lipid deficiency have on the morphology of *Synechocystis* cells?

2: In what way does the temperature stress adaptation of *Synechocystis* strains change by lipid-carotenoid cooperation?

2.1: What kind of cooperative and individual stress reducing effects do xanthophylls and polyunsaturated lipids have?

3: Can xanthophyll and/or lipid deficiency cause morphological changes in the photosynthetic complexes?

3.1: How does the temperature stability of PSI oligomers change in certain strains?

4: In what way does the ratio of PSI oligomers differ in these three different cyanobacterial strains: *Synechocystis, Anabaena* and *Spirulina*?

4.1: Which of the following non-invasive techniques [circular dichroism (CD) spectroscopy or low temperature fluorescence emissoin spectroscopy] is more suitable to follow structural changes in PSI?

Materials and methods

The effects of altered lipid and Car composition on cell morphology were studied by TEM and SEM.

I studied the lipid composition of whole cells using a lipidomic approach. The applied tandem mass spectrometric method allowed us to follow the changes in membrane lipid content induced by the absence of xanthophylls and polyunsaturated lipids.

HPLC analysis was used to detect changes in the Car composition resulting from xanthophyll- and polyunsaturated lipid-deficiency.

I performed two *in vitro* (clean native PAGE and FPLC) and two *in vivo* (low temperature fluorescence emission spectroscopy and CD spectroscopy) technique to compare how the level of PSI oligomers are altered in our mutants relative to the WT.

Results and discussion

The protective role of Cars and the importance of lipid unsaturation in photosynthesis are well studied, however cooperative effects of these factors have not been elucidated yet. In the present study we investigated the cooperation between lipids, Cars and proteins in the photosynthetic apparatus. We generated a mutant, *Synechocystis* ROAD, which is xanthophyll- and polyunsaturated lipid-deficient. This strain was used for studying the combined effect of xanthophylls and polyunsaturated lipids on biochemical and physiological processes of photosynthesis in *Synechocystis* cells. In our studies the RO (only xanthophyll-deficient) and AD (only polyunsaturated lipid-deficient) mutants served as references that helped interpreting the mentioned complex cooperative effects.

Xanthophyll and polyunsaturated lipid deficiency resulted in cell enlargement and slight changes in membrane structures of the cell interior. Interestingly, the surface layer, or S-layer of the cellular envelope membranes was missing in the xanthophyll-deficient RO and ROAD mutants. Earlier, it was described the absence of S-layer in a ζ-carotene desaturase-inactivated, therefore carotenoid-less, *Synechocystis* mutant . Our observation that RO and ROAD mutants also lack the S-layer support the conception that xanthophylls can provide a proper environment in the outer membrane for anchoring S-layer proteins to lipopolysaccharides. These morphological results suggest that both polyunsaturated lipids and xanthophylls might have determinant roles in cell and membrane structures, as well as in ensuring the functions of membrane-imbedded proteins.

Mass spectrometry analyses of total lipid extracts revealed that MGDG is the most abundant lipid in WT *Synechocystis* and in all three studied mutants. This is followed as second by DGDG, and then by two anionic lipids, SQDG and PG. Surprisingly, the relative MGDG content in all mutants decreased by about 10% compared to the WT value. This decrease of MGDG in RO and also in the ROAD mutant was counterbalanced by an increase in the amount of other lipid classes. In the RO mutant this was achieved by increasing the DGDG level, whereas in AD and

ROAD not only the DGDG, but also the SQDG and PG contents were substantially enhanced to compensate for the loss of MGDG. These changes in the lipid class distribution suggest that thylakoid membranes are remodeled differentially in response to the loss of xanthophylls and/or polyunsaturated lipids. In addition to the remodeling observed at optimal growth temperature, further fine tuning of lipid classes occurs at ML and MH temperatures . It seems that these conditions, and especially ML temperature, increased the amount of PG and SQDG, which might have crucial functions in the absence of polyunsaturated lipids.

MGDG is the only NBL lipid of the thylakoids, therefore remodeling resulted in a major change in the NBL to BL lipid ratios. In ROAD cells the NBL to BL lipid ratio decreased to about 60% compared to the WT. Similar NBL to BL lipid ratios was observed when only xanthophylls or polyunsaturated lipids were absent. The AD mutant adapts to the ML temperature by a 20–25% decrease of its NBL lipids, relative to those of the WT, at the same temperature. Simultaneously, the level of BL lipids is noticeably increased to compensate for the loss of NBL species. Such compensatory regulations can ensure proper adjustments of the thylakoid membranes to stress conditions of the environment. In the polyunsaturated and xanthophyll-deficient mutants BL lipids can provide protection and stability of the membrane structure, which are required for the maintenance and stress resistance of photosynthetic functions. The adjustment of NBL to BL ratios is a vital adaptive response of the cells. Our results are in agreement with earlier observations that NBL to BL ratios are crucial determinants of membrane functionality.

The effect of low temperatures on the saturation level of glycerolipids is intensely studied in both cyanobacteria and plants. Our results with cells grown at ML and MH temperatures confirm that not only extreme, but also small shifts of the growth temperature can induce rearrengements of the lipid content, especially those of PG and SQDG. Remodeling makes the thylakoid membranes extremely flexible and adaptive to stress conditions. Our remodeling results reveal that lipids and Cars can act cooperatively in this process. These results are in good agreement with earlier observations in higher plants. These revealed that the violaxanthin cycle provides protection against high light exposure-induced toxic processes. It has been shown that

light-induced membrane rigidification is proportional to the amount of zeaxanthin in the membranes. This phenomenon also highlights the strong correlation between membrane structure and xanthophyll content.

Cells compensate for their deficiency in xanthophylls, the main protective agents against reactive oxygen species, by increasing the β-carotene content, consistent with earlier results. In the absence of polyunsaturated lipids not only β-carotene, but also xanthophylls are reorganized in an adaptive response. Cars, being hydrophobic molecules, are often found in the vicinity of fatty acids. Lipid unsaturation and carotenoid content can influence membrane dynamics and mobility of protein complexes, together with other membranous components.Apparently, in the absence of polyunsaturated lipids the cells become sensitive even at optimal growth temperature, therefore they increase their Myx and Ech content.

When exposed to heat stress, ML temperatures seem to have stronger influence on the reorganization of Car content than MH temperatures. In the AD mutant ML temperature caused not only further increase of Myx and Ech content, but also the accumulation of Zea. Our results provide evidence for the interdependence of lipid and Car contents in the thylakoid membranes.

Xanthophyll deficiency resulted in the partial disintegration of PSI trimers, which could be detected by clear-native electrophoresis and FPLC analyses. Similar destabilization of PSI oligomers was observed in the RO mutant by fluorescence methods. For the identification of various protein-pigment complexes FPLC and native electrophoreses are in vitro techniques that require detergent treatments. For studying the aggregation of photosynthetic complexes CD spectroscopy was used as an *in vivo* method. The Car-induced CD signal allows distinguishing between the monomeric and trimeric forms of PSI. With this method I observed a trimer to monomer ratio similar to the one obtained with the in vitro methods. Our findings suggest that xanthophylls are needed for providing optimal environment for the assembly of photosynthetic reaction centers. Interestingly, in the AD mutant all techniques (FPLC, native electrophoresis and CD) indicated an increase in the PSI trimer content. The AD mutation can increase the sensitivity of the cells to light and ML temperature, thus

trimeric PSI may be more advantageous under such stress conditions. It has been shown that among lipids PG has a role in the formation of PSI oligomers, and also in connecting CP43 within the PSII core-complex. PG is a crucial lipid in oligomerization and functionality of PSII both in photosynthetic prokaryotes and plants .

The accumulation of trimeric PSI in the absence of polyunsaturated lipids can be explained by the difference between the spatial requirements of saturated, poly- and monounsaturated lipid. The fatty acyl chains of saturated and monounsaturated lipids are straighter and tighter packed than those of the polyunsaturated ones, which have kinks in the tail with bigger spatial requirement. In the case of ROAD I observed a similar enhancement of PSI monomers as in the RO strain. These results suggest additive cooperation between the lipids and carotenoids, in which xanthophylls have a prevailing impact.

I addressed the question how the PSI structure differs in various cyanobacterial strains. The ratio of PSI tetramers, trimers, and monomers in intact cells was investigated in vivo by CD spectrometry. It was previously observed that PSI trimer has a characteristic fingerprint on the CD spectrum, based on specific pigment–protein interactions within the complex. CD spectroscopy revealed the highest intensity at 515 nm (PSI peak) in *Spirulina platensis* cells, which may originate from PSI multi-oligomerisation. The most sensitive response to heat treatment in this strain was the oligomerisation of PSI RCs. PSI dimers and tetramers in *Anabaena* cells showed smaller changes of the CD signal upon the heat treatment compared to that of *Synechocystis* WT. The lack of γ-linolenic acid affected the filament morphology by the loss of the spiral shape and the PSI monomerisation in *Spirulina I22*.

Conclusions

I: Xanthophyll and polyunsaturated lipid deficiency resulted in cell enlargement and slight changes in membrane structures of the cell interior. Interestingly, the surface layer, or S-layer of the cellular envelope membranes was missing in the xanthophyll-deficient RO and ROAD mutants. These morphological results may suggest that both polyunsaturated lipids and xanthophylls might have determinant roles in cell and membrane structures, as well as in ensuring the functions of membrane-imbedded proteins. (Zakar *et al.*, 2017)

II: We demonstrated that xanthophyll and polyunsaturated lipid deficiency induces lipid remodeling. As a consequence of lipid remodeling, NBL to BL lipid ratios are substantially modified in the membranes. The removal of xanthophylls induces increase mainly in the DGDG level, while polyunsaturated lipid deficiency results in considerable PG and SQDG accumulation. BL lipids are required for stabilizing the unbalanced and unprotected membranes. (Zakar *et al.*, 2017)

III: The removal of polyunsaturated lipids also resulted in the reorganization of the xanthophyll content, increasing the xanthophyll to β-carotene ratio. We demonstrated that lipids and Cars act cooperatively in maintaining and protecting membrane structures. (Zakar *et al.*, 2017)

IV: By using a non-invasive biophysical technique (CD), I demonstrated that deficiencies in both polyunsaturated fatty acids and xanthophylls destabilize PSI trimers. This effect of the xanthophyll deficiency is much more pronounced, as was revealed by a multiple mutant lacking both xanthophylls and polyunsaturated lipids. The exact localization of xanthophylls in the photosynthetic complexes is yet to be determined. (Zakar *et al.*, 2017)

V: I was able to observe and differentiate structural changes of the PSI in several cyanobacterial strains by CD spectroscopy. CD signal revealed the highest intensity at 515 nm in *Spirulina* WT cells. However, PSI dimers and tetramers in *Anabaena* cells did not show significant changes at the 515 nm peak of the CD spectrum as compared

to that of the *Synechocystis* WT.γ-linolenic acid (GLA) deficiency induced PSI monomerisation and the loss of the spiral shape of *Spirulina* WT. Both low temperature fluorescence emission and CD spectroscopy are recommended since these methods are complementary to each other. (Zakar *et al.*, 2018)

Publications

The thesis was based on the following:

Zakar T, Herman E, Vajravel S, *et al.* Lipid and carotenoid cooperation-driven adaptation to light and temperature stress in *Synechocystis* sp. PCC6803. *Biochimica et biophysica acta*. 2017;1858(5):337-350. doi:10.1016/j.bbabio.2017.02.002. **IF:4.280**

Zakar T, Kovács L, Vajravel S, *et al.* Determination of PSI oligomerisation in various cyanobacterial strains and mutants by non-invasive methods. *Photosynthetica*. 2018; 56. 10.1007/s11099-018-0795-7. **IF: 1.740**

Other publications:

Kóbori T O, Uzumaki T, Kis M, Kovács L, Domonkos I, Itoh S, Krynická V, Kuppusamy S GK, **Zakar T**, Dean J, Szilák L, Komenda J, Gombos Z, Ughy B. Phosphatidylglycerol is implicated in divisome formation and metabolic processes of cyanobacteria. Journal of Plant Physiology. 2018; Volume 223.Pages 96-104.ISSN 0176-1617. https://doi.org/10.1016/j.jplph.2018.02.008. **IF:2.833**

Petrova N, Todinova S, Laczko-Dobos H, **Zakar T**, Vajravel S, Taneva S *et al.* Structural integrity of *Synechocystis* sp. PCC 6803 phycobilisomes evaluated by means of differential scanning calorimetry. *Photosynthesis Research*. 2018 Jan 10;1-10. DOI: 10.1007/s11120-018-0481-4 **IF:3.091**

Review: Zakar T, Laczko-Dobos H, Toth TN, Gombos Z. Carotenoids Assist in Cyanobacterial Photosystem II Assembly and Function. *Frontiers in Plant Science*. 2016;7:295. doi:10.3389/fpls.2016.00295. **IF:3.678**