

**THE ROLE OF THE ORANGE CAROTENOID PROTEIN
AND PsbU PROTEIN
IN PHOTOPROTECTION IN CYANOBACTERIA**

PhD Thesis

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INTRODUCTION

By harvesting solar energy and converting it into chemical energy, plants, algae, and cyanobacteria provide organic carbon molecules and oxygen that are essential for life on earth. This process is called oxygenic photosynthesis. Primary reactions of photosynthesis occur in the photosystems, which are thylakoid membrane embedded pigment-protein complexes. Light in cyanobacteria is captured by phycobilisomes (PB). PBs are located at the outer surface of thylakoid membranes.

Excess light can be lethal for photosynthetic organisms because of production of harmful reactive oxygen species. Cyanobacteria, like plants and algae, have developed several photoprotective mechanisms. Three major physiological processes are known to be involved in adjustment of photosynthetic apparatus under changed light environment: state transitions, D1 protein repair, and non-photochemical PSII fluorescence quenching (NPQ).

OCP protein and photoprotection

Energy dissipation as heat in the antenna is known as non-photochemical quenching (NPQ, qE). In *Synechocystis* sp. PCC6803, saturating blue-green light induces thermal dissipation of the energy absorbed by the PBs which results in the decrease of the energy transfer from the PBs to the reaction centers. This energy dissipation mechanism involves the so-called orange carotenoid protein (OCP). In the absence of OCP, the fluorescence quenching induced by strong white or blue-green light is absent. Under stress conditions such as iron starvation blue-green light induces a very large fluorescence quenching, which is much larger than under complete medium conditions. It was found that the larger fluorescence quenching correlates with a higher OCP concentration.

The function of the OCP in the photoprotective PB-related thermal dissipating mechanism has been examined so far only in *Synechocystis* cells. It is, however, not known if this important mechanism is widespread among cyanobacteria, or not. Screening of the currently available cyanobacterial genomic databases showed that genes encoding homologues of *Synechocystis* OCP are present in most phycobilisome-containing cyanobacteria. Among them only a few strains do not have an OCP gene homologue including *Termostynechococcus*

elongatus, which contain separate but adjacent genes coding for the N- and C-terminal parts of the OCP. In our study, to investigate the role of OCP gene in induction of blue light-induced NPQ under iron-replete and iron-deplete conditions, we used *Arthrospira maxima* (*A. maxima*), containing the OCP gene, *Synechococcus elongatus* PCC 7942 (*S. elongatus*) with no OCP gene and *Termosynechococcus elongatus* (*T. elongatus*) with two separate genes encoding the C- and N-terminal part of the OCP.

The role of the PsbU protein in PSII

The oxygen-evolving complex (OEC) is located on the luminal side of PSII. It consists of a Mn_4Ca cluster and several luminal PSII proteins. The main role of extrinsic proteins is to stabilize Mn cluster and to maintain optimal levels of the calcium and chloride ions which are required as essential cofactors for the water oxidation reactions. Cyanobacterial PSII complexes contain five extrinsic proteins, PsbO, PsbP, PsbQ, PsbU, and PsbV.

The PsbU protein is the 12 kDa extrinsic subunit of PSII. The crystal structure place PsbU between PsbO and PsbV with a majority of its contacts to these two proteins. PsbU protein stabilizes the PSII complex in several ways. The absence of PsbU results in the reduction of oxygen evolution, high sensitivity of cells to photodamage. Additionally, PsbU was found to be required for the stabilization of the Mn cluster. The presence of PsbU contributes to the heat stability of the OEC during the acclimation to high temperature. The removal of PsbU affects not only the luminal side and the core but also the stromal side of PSII. It has been reported that removal of PsbU altered primary photochemical processes and led to the accumulation of uncoupled phycobilisomes decreasing the energy transfer from PBs to PSII. Recently, it was proposed that PsbU provides protection from reactive oxygen species in *Synechococcus* 7942 cells.

In this study, we conducted detailed analysis to clarify the function of PsbU using the deletion mutant of the psbU gene in *Synechococcus* 7942.

AIMS OF THE STUDY

In this work the aim was to obtain an improved understanding concerning the mechanism of the blue light-induced NPQ mechanism as well as the role of the cyanobacterial PsbU subunit in the PSII complex. The particular aims of this work were:

1. To investigate the correlation between the presence of the OCP gene and the occurrence of blue light-induced fluorescence quenching.
2. To compare the effect of iron-starvation on the induction of NPQ and state transitions in cyanobacteria showing distinct OCP gene configurations.
3. To investigate the role and function of the PsbU protein in PSII by using the deletion mutant of the *psbU* gene in *Synechococcus*.

MATERIALS AND METHODS

- Culture growth
- Iron starvation
- Absorbance measurements
- Chlorophyll content determination
- PAM fluorescence measurements
- Fluorescence spectra at 77 K
- Flash-Induced Fluorescence Relaxation Kinetics
- Thermoluminescence measurements
- Oxygen evolution measurements
- Light treatment

RESULTS AND DISCUSSION

OCP and photoprotective mechanisms in various cyanobacteria

In order to test the correlation between the presence of the OCP and the ability of cells to perform blue light-induced fluorescence quenching we compared cyanobacterial species containing OCP gene with species lacking the entire OCP gene. The kinetics of the fluorescence changes were monitored by a PAM fluorometer.

When dark adapted cells were exposed to low intensities of blue-green light, PQ pool became oxidized, and an increase of the maximal fluorescence level (F_m') was observed in all strains. Illumination of cyanobacterial species, which lack the entire OCP gene (*S. elongatus*), or contain only sequences for the C- and N-terminal part of the gene (*T. elongatus*), by strong blue-green light did not induce any decrease of F_m' indicating that in the absence of whole OCP gene this kind of quenching cannot be induced. In contrast, exposure of low blue light-adapted cells to high blue-green light intensities induced a fluorescence quenching in the OCP containing cells (*Synechocystis* and *A. maxima*). In contrast to the lack of blue light-induced NPQ, the absence of OCP did not affect the occurrence of state transitions in all species under normal conditions.

Under iron starvation conditions, illumination of species, which contain OCP gene, with strong blue-green light again induced a reversible decrease of fluorescence intensity. Interestingly, F_0 level increased in iron starved OCP containing cells. The capacity for induction of NPQ increased in prolonged iron starved *Synechocystis* and *A. maxima* cells in comparison to iron containing cells. In contrast, high intensities of blue-green light did not induce any NPQ in iron starved species without OCP gene (*T. elongatus* and *S. elongatus*).

Ability to perform blue light-induced quenching, confirm the idea that the OCP-related photoprotective mechanism is widespread in cyanobacteria. In contrast, in the species, which lack the whole OCP gene the fluorescence quenching mechanism was absent. Moreover, our results also demonstrated that the strains, which are unable to perform blue light-induced, quenching, are more sensitive to photoinhibition.

Absorption spectra of iron replete and iron starved cells with and without OCP gene were compared. *Synechocystis* and *A. maxima* cells after extended iron-starvation have a strong 685 nm fluorescence emission peak in the 77 K fluorescence emission spectrum (excitation at 600 nm) indicating an increase of the quantity of functionally disconnected highly fluorescent

phycobilisomes. In contrast, in the OCP-lacking *T. elongatus* and *S. elongatus* cells, the appearance of functionally disconnected phycobilisomes was not observed due to the quickly decreasing phycobiliprotein content. Thus, when cyanobacteria are unable to reduce the amount of energy transferred to the reaction centers by the blue light-induced NPQ mechanism, they protect themselves by quickly decreasing the cellular content of phycobiliproteins to avoid the accumulation of potentially harmful functionally disconnected phycobilisomes under iron starvation conditions.

The role of PsbU in prevention of photodamage to PSII

To investigate the role of the PsbU subunit of Photosystem II we monitored the electron transport within PSII in WT and Δ PsbU mutant cells of *Synechococcus* 7942. In order to check the forward electron transfer rate at the acceptor side of PSII, as well as charge recombination between the reduced acceptor and oxidized donor side components we used flash-induced Chl fluorescence decay measurements in the absence and presence of the electron transport inhibitor DCMU.

Illumination of dark-adapted samples with a saturating single turnover flash results in the reduction of Q_A in all PSII centers and leads to an increase of variable fluorescence. Thus the amplitude of the fluorescence signal reflects the number of active PSII reaction centers, which was significantly smaller in the PsbU mutant ($\approx 70\%$) than in the WT.

In the absence of DCMU, the time constant of the $S_2Q_B^-$ decay was 1.5 times larger in the Δ PsbU mutant than in the WT. In the presence of DCMU, the time constant of the $S_2Q_A^-$ decay was 2.1 times larger in the Δ PsbU mutant than in the WT. The ratio of the time constants of the $S_2Q_B^-$ and $S_2Q_A^-$ charge recombination processes can give us additional information on the free energy gap between the Q_A and Q_B acceptors. This ratio is different in the WT and mutant strain indicating that the $Q_A \leftrightarrow Q_B$ free energy gap is modified in the mutant. Our calculation resulted in 37 and 28 meV gap between Q_A and Q_B in the WT and Δ PsbU mutant cells, respectively.

In our study, in addition to flash induced chlorophyll fluorescence measurements, thermoluminescence (TL) measurements in the absence and presence of DCMU, were used. In the Δ PsbU mutant the position of the B band, which arises from the $S_2Q_B^-$ recombination (in the

absence of DCMU), as well as the position of the Q band, which arises from the $S_2Q_A^-$ (in the presence of DCMU), were up-shifted to a higher temperatures. Thus, our TL data are in agreement with the flash fluorescence results and confirm the energetic stabilization of both the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs in the mutant.

The B band intensity appears to be 1.7 -fold higher in the Δ PsbU mutant than in the WT on the basis of an equal number of functional PSII reaction centers. There are studies which show that the TL intensity is modulated by the free energy level of the $P_{680}^+Phe^-$ primary charge separated state, which changes the free energy gap between P_{680}^* and $P_{680}^+Phe^-$. The increased TL amplitude of the Δ PsbU mutant, which is accompanied by a slower $S_2Q_A^-$ and $S_2Q_B^-$ recombination rate, is indicative of a decreased free energy level of the $P_{680}^+Phe^-$ primary charge separated state.

The sensitivity of the WT and the Δ PsbU strains to photoinhibition was checked by illuminating cells with strong light ($500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 1.5 hour in the presence and absence of lincomycin, which blocks the synthesis of new D1 protein. Our photoinhibition experiments with lincomycin showed that the *Synechococcus* Δ PsbU mutant has an increased susceptibility to light. Despite of the fact, that the Δ PsbU strain was more strongly inhibited during the high light treatment than the WT, it was still able to recover the PSII activity to a significant extent under growth light conditions in the absence of lincomycin.

There are multiple mechanisms involved in the process of photoinhibition. Reactive oxygen species, which damage the PSII can be produced as a consequence of electron transport events on the acceptor side as well as inactivation of the Mn cluster. Our data suggests that the absence of the PsbU protein induces modifications not only at the donor side but also at the acceptor side of PSII. On the acceptor side, most likely, it modifies not only the environment of the Q_A and Q_B acceptors, but also the hydrogen bonding interactions around Phe, or the P_{D1} Chl of P_{680} , which in turn shifts the free energy level of $P_{680}^+Phe^-$ to more negative values. This in turn leads to charge recombination that forms $^3P_{680}$, which induces the production of harmful singlet oxygen that causes oxidative damage of PSII.

CONCLUSIONS

1. So far, had been not known if the important blue light-induced photoprotective quenching is widespread in cyanobacteria or not. All the studies about cyanobacterial NPQ were done on *Synechocystis* cells.

In our work, for the first time, more cyanobacteria species which contain OCP gene, as well as species which do not have an OCP gene were studied.

It was demonstrated, that only the species which contain the complete OCP gene show a blue light-induced quenching of fluorescence under normal and iron-stress conditions.

In contrast, cyanobacterial species which contain only N-terminal and/or C-terminal OCP-like genes, or do not have OCP-like genes at all, do not show blue light-induced fluorescence quenching under neither iron-replete nor iron-starved conditions. Moreover, in our work, it was found that these cells adopted a different physiological strategy to cope with stressful conditions, such as iron starvation. Under conditions of iron starvation, a large decrease of the phycobiliprotein content was observed in species lacking OCP in comparison with OCP-containing species. This strategy could be useful in avoiding the accumulation of dangerous, functionally uncoupled phycobilisomes.

In the case of the OCP-containing cyanobacteria, they protect themselves against high light, notably under conditions inducing the appearance of disconnected phycobilisomes, via the energy dissipating OCP-phycobilisome mechanism.

2. Because of its fundamental role in photosynthesis the structure of the OEC is of significant interest. Most of the studies on the characterization of PsbU protein of OEC were performed on *Synechocystis* cells.

The role of the PsbU subunit in the electron transport characteristics and light sensitivity of the PSII complex was studied in this work using *Synechococcus* PCC 7942. Experiments were performed, using the PsbU-less mutant of the cyanobacterium *Synechococcus* PCC 7942, which has an enhanced antioxidant capacity.

Flash induced Chl fluorescence measurements in the presence and absence of the electron transport inhibitor DCMU showed that the absence of PsbU slowed down both the $S_2Q_A^-$ and the $S_2Q_B^-$ recombination. Thermoluminescence measurements provided evidence for the increased stability of the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs by increased peak temperature for Q band in the presence and the B band in the absence of DCMU. Moreover, the intensity of the TL bands is also increased in the PsbU mutant (≈ 1.7 times for the B band), as compared to the WT. The mutant cells became more quickly photoinhibited when illuminated with high light in the absence and presence of the protein synthesis inhibitor lincomycin.

Our results indicate that the lack of the PsbU subunit in *Synechococcus* PCC 7942 affects the energetic stability of the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs by modifying both PSII donor and acceptor side components. This effect is most likely caused by structural changes in the vicinity of the Mn cluster and in the inner part of the PSII complex, which are induced by the absence of the PsbU protein from the luminal part of the complex. The photosensitivity of the Δ PsbU *Synechococcus* 7942 cells is likely due to reactive oxygen species, which are produced as a consequence of a disturbed donor side structure and/or due to the modified energetic properties of the primary radical pair.

LIST OF PUBLICATIONS

Publications, which serve as basis for the thesis:

- Boulay, C., **Abasova, L.**, Six, C., Vass, I., Kirilovsky, D., 2008. Occurrence and function of the orange carotenoid protein in photoprotective mechanisms in various cyanobacteria. *Biochim Biophys Acta* 1777, 1344-1354. **IF:4.3**
- **Abasova, L.**, Deák, Z., Schwarz, R., Vass, I., 2011. The role of the PsbU subunit in the light sensitivity of PSII in the cyanobacterium *Synechococcus* 7942. *J Photochem Photobiol B: Biology*. **IF:2.1**

Other publications:

- Bogos, B., Ughy, B., Domonkos, I., Laczko-Dobos, H., Komenda, J., **Abasova, L.**, Cser, K., Vass, I., Sallai, A., Wada, H., Gombos, Z., 2010. Phosphatidylglycerol depletion affects photosystem II activity in *Synechococcus* sp. PCC 7942 cells. *Photosynth Res* 103, 19-30. **IF:2.4**
- Wu, H., **Abasova, L.**, Cheregi, O., Deak, Z., Gao, K., Vass, I., 2011. D1 protein turnover is involved in protection of Photosystem II against UV-B induced damage in the cyanobacterium *Arthrospira* (*Spirulina*) *platensis*. *J Photochem Photobiol B* 104, 320-325. **IF:2.1**
- Funk, C., Hernandez-Prieto, M.A., Tibiletti, T., **Abasova, L.**, Kirilovsky, D., Vass, I., 2011. The small CAB-like proteins of the cyanobacterium *Synechocystis* sp PCC 6803: Their involvement in chlorophyll biogenesis for Photosystem II. *Biochim Biophys Acta* 1807, 1143-1151. **IF:5.1**