THE ROLE OF THE ORANGE CAROTENOID PROTEIN

AND PsbU PROTEIN

IN PHOTOPROTECTION IN CYANOBACTERIA

PhD Thesis

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Preface

This thesis represents a part of work that has taken place over a period of five years (2006-2011) in the laboratories of Dr. Imre Vass (Szeged, Hungary) and Dr. Diana Kirilovsky (Saclay, France). Except the research topics described in this thesis, during my PhD studies I have also been involved in a number of interesting scientific projects and collaborative work.

My research work had its ups and downs. But I am grateful to my supervisors for their patience, support and ideas. I am very grateful to the ITC (International Training Course) program which gave me the opportunity to be involved in a high level research. I would like to say a world of thank to my research group in Azerbaijan, especially to Dr. Ralphreed Gasanov and Dr. Yagut Allahverdieva without whom I would never come to Szeged.

I am very grateful to my family for their motivational support. I appreciate very much Julian, a very important person of my life, for his optimism, encouragement and love during the final stages of my PhD.

CONTENTS

ABBREVIATIONS

APC - allophycocyanin

ATP - Adenosine-5'-triphosphate

Chl - chlorophyll

CP43, CP47 - chlorophyll protein 43, chlorophyll protein 47

Cyt-b_6f - cytochrome b_6f complex

D1, D2 - proteins of the PSII reaction center

DBMIB - 2,5-*dibromo*-3-methyl-6-isopropyl-p-benzoquinone

DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea

DMBQ - 2,5-dimethyl-p-benzoquinone

E_m - midpoint redox potential

F_0 - minimum fluorescence

Fe-S - iron-sulfur protein

F_m - maximum fluorescence in dark

F_m' - maximum fluorescence at light

F_s - steady-state fluorescence

hECN - 3' -hydroxyechinenone

IsiA – iron-stress-induced protein A

L_{cm} - phycobilisome core-membrane linker polypeptide

LHCI, LHCII - light-harvesting complex I, light-harvesting complex II

NADP - nicotinamide adenine dinucleotide phosphate

NDH - NAD(P)H dehydrogenase

NPQ - non-photochemical quenching

OCP - orange carotenoid protein

OD - optical density

OEC - oxygen evolving complex

P_{680} - chlorophyll of PSII reaction center with a light absorption peak of 680 nm

P_{700} - chlorophyll of PSI reaction center with a light absorption peak of 700 nm

PB - phycobilisome

PC - phycocyanin

PE - phycoerythrin

PEC - phycoerythrocyanin

Phe - pheophytin

PQ - plastoquinone

PsbO, PsbV, PsbU, PsbP, PsbQ, PsbR - lumenal extrinsic proteins of photosystem II

PSI - photosystem I

PSII - photosystem II

Q_A, Q_B - primary and secondary quinone molecules involved in the electron transport chain

qE - energy-dependent quenching of fluorescence

qI – photoinhibition-dependent quenching of fluorescence

qP - photochemical quenching of fluorescence

qT – state transition-dependent quenching of fluorescence

S_0, S_1, S_2, S_3, S_4 - oxidation states of the oxygen evolving complex

TL - thermoluminescence

Tyr_D or Y_D - tyrosine-160 of the D2 protein

Tyr_Z or Y_Z - tyrosine-161 of the D1 protein

WOC - water oxidizing complex

1. INTRODUCTION

1.1. The photosynthetic light reactions

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. The light reactions take place in the thylakoid membrane. In eukaryotes thylakoids are located inside of the chloroplasts. Light-independent reactions (CO_2 fixation) take place in the stroma of chloroplast. Prokaryotes such as cyanobacteria do not have chloroplasts but they have an internal system of thylakoid membranes where the fully functional electron transfer chains of photosynthesis and respiration are located. Thylakoids contain specialized light-absorbing pigments - chlorophylls (Chl). Chlorophylls absorb mainly in the red and blue parts of the spectrum. When chlorophyll absorbs a photon it makes transition from its ground state to excited state (Chl*). The exited chlorophyll has four different pathways to return to its ground state:

1. Fluorescence – when a chlorophyll molecule re-emits a photon and thereby returns to its ground state.
2. Dissipation as a heat – when a chlorophyll molecule converts its excitation energy into heat.
3. Energy transfer – when excited chlorophyll molecule transfers its energy to another pigment molecule.
4. Photochemistry – when energy of excited chlorophyll induces a stable charge separation.

Primary reactions of photosynthesis occur in the photosystems, which are thylakoid membrane embedded pigment-protein complexes containing light harvesting, as well as photochemically active pigments and redox-active cofactors that mediate electron transport reactions. The purpose of these photosystems is to collect light energy over a broad range of wavelengths and concentrate it in a reaction center which uses the energy to initiate charge separation and a subsequent series of electron transport reactions. Electron flow proceeds from photosystem II (PSII) through cytochrome b_6f (Cyt-b_6f) to photosystem I (PSI). During light reactions of photosynthesis PSII uses light energy to oxidize two molecules of water into one

molecule of molecular oxygen. Electrons extracted from the water molecules are transferred by an electron transport chain via Cyt-b_6f and PSI to $NADP^+$. During the electron transport process a proton gradient is generated across the thylakoid membrane. This proton motive force is then used to drive the synthesis of ATP. NADPH and ATP formed during the light reactions drive the reduction of CO_2 to organic compounds such as glucose (Fig.1.1.).
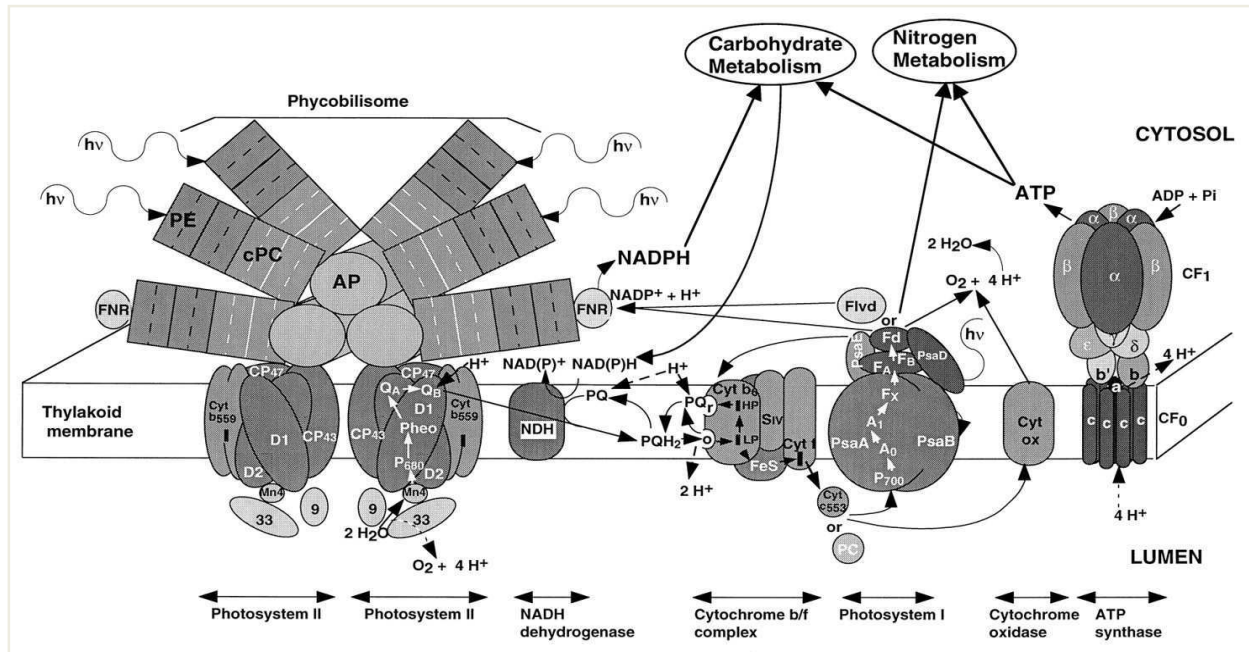
Figure 1.1. Photosynthetic apparatus in cyanobacteria (Donald A Bryant 1994).

Light energy absorbed by the phycobilisomes can be transferred to either PSII or PSI. There are different pathways of electron flow including linear flow from water to $NADP^+$, several possible cyclic pathways and respiratory flow of electrons through the plastoquinone pool. Possible pathways are indicated by black arrows. Proton uptake and transmembrane transport are indicated by dashed arrows. The plastoquinone pool can be reduced by electrons from PSII, from an NAD(P)H dehydrogenase(s) (NDH) and from the Cyt-b_6f complex. Plastoquinone reduction by NDH is the entry point for electrons derived from respiration. The various plastoquinone reduction pathways involve proton uptake from the stroma, while oxidation of a plastoquinol releases two protons into the lumen. During ATP synthesis, protons enter ATP-synthase from the lumen, and exit to the cytosol.

1.2. Phototsystem II. Mechanisms of electron transport

PSII is an integral pigment-protein complex embedded in the thylakoid membrane. The core of this membrane protein is formed by two proteins D1 and D2. PSII has a pair of two chlorophyll *a* molecules that absorb light at wavelength of 680 nm. This pair is called P_{680}. Excitation energy that reaches P_{680} drives a rapid transfer of an electron to a nearby pheophytin *a* (Phe). The electron is then transferred to a tightly bound plastoquinone (PQ) at the Q_A site. From Q_A the electron is transferred to an exchangeable plastoquinone located at the Q_B site. Q_B plastoquinone is a two-electron acceptor. On the arrival of a second electron Q_B becomes double reduced Q_B^{2-} , which is followed by the uptake of two protons from the stroma to form plastoquinol, PQH_2. PQH_2 is then replaced by an oxidized PQ from the PQ pool. PQH_2 diffuses in the lipid phase of the membrane to the Cyt-b_6f complex, which reoxidises it and passes the electrons to plastocyanin (PC). PC transports the electron to PSI and reduces oxidized P_{700} (chlorophyll pair bound to PSI). PSI in turn, reduces $NADP^+$ to NADPH. When the electron is rapidly transferred from P_{680}* to pheophytin *a*, a positive charge is formed on P_{680}. P_{680}^+ is an incredibly strong oxidant which extracts electrons from the Mn cluster. The oxidation of H_2O follows the extraction of 4 electrons from the Mn cluster. The Mn cluster and its protein environment are often called OEC (oxygen evolving complex) or WOC (water oxidizing complex). The electrons extracted from the Mn cluster are delivered to P_{680} via a redox-active tyrosine residue, which is called Y_z or Tyr_z. According to the model proposed by Kok et al., the OEC cycles through 5 redox states designated S_0, S_1, S_2, S_3, S_4, where the subscript is the number of positive charges (Kok *et al.*, 1970). The S_4 state spontaneously reacts with water generating the S_0 state and oxygen is released. Only S_0 and S_1 are stable in the dark. S_2 is unstable and decays back to S_1 via a recombination between acceptor side and the Mn cluster. The S_3 state decays via S_2 to S_1. It is assumed that under continuous illumination the S-states (S_0 to S_3) are scrambled (each 25%), but after a few minutes of dark adaptation only S_0 and S_1 states are present in proportions of 25:75 %.

1.3. Organization of light-absorbing antenna systems

Sunlight is absorbed by the photosynthetic pigments. Photosynthetic organisms contain a mixture of more than one kind of pigment. Different types of chlorophylls, carotenoids and bilin pigments are found in photosynthetic organisms. The majority of the pigments serve as an antenna complex collecting light and transferring the energy to the reaction centers of the photosystems where the chemical oxidation and reduction reactions occur. In contrast to the reaction centers, which are strongly conserved, the antenna systems of different classes of photosynthetic organisms vary considerably. In eukaryotes there is a pigment-protein antenna complex associated with PSII which is called light harvesting complex II (LHCII) and an antenna complex associated with PSI called light harvesting complex I (LHCI). In addition to the outer antenna, PSII of plants and cyanobacteria contains two chlorophyll *a* containing core antennas: CP43 and CP47. LHCII and LHCI consist of a complex of several hydrophobic membrane-intrinsic polypeptides non-covalently binding chlorophyll *a* and *b* and carotenoid molecules.

Cyanobacteria do not have Chl-containing LHC. Light in cyanobacteria is captured by phycobilisomes (PB). PBs are located at the outer surface of thylakoid membranes. They contain phycobiliproteins which covalently bind bilin pigments and linker peptides which are required for the organization of the phycobilisome (Glazer, 1984; Grossman *et al.*, 1993; Tandeau de Marsac, 2003; Adir, 2005). The PBs consist of an allophycocyanin core (APC) from which rods radiate. The subunits of rod segments are phycocyanin (PC), phycoerythrin (PE) or phycoerythrocyanin (PEC). In freshwater cyanobacteria the rods contain only phycocyanin, while marine cyanobacteria are usually rich in phycoerythrin (MacColl, 1998). These rods are associated with the APC core, which in turn is connected to a linker protein. Linker proteins connect the PBs with the thylakoid membranes. The wavelength of the absorption maxima of the pigments increases from the outer segment towards the core and linker peptide. This leads to a directed energy transfer. Harvested light energy is transferred from the linker polypeptide to the chlorophylls of PSII and PSI (Mullineaux, 1992; Rakhimberdieva *et al.*, 2001).
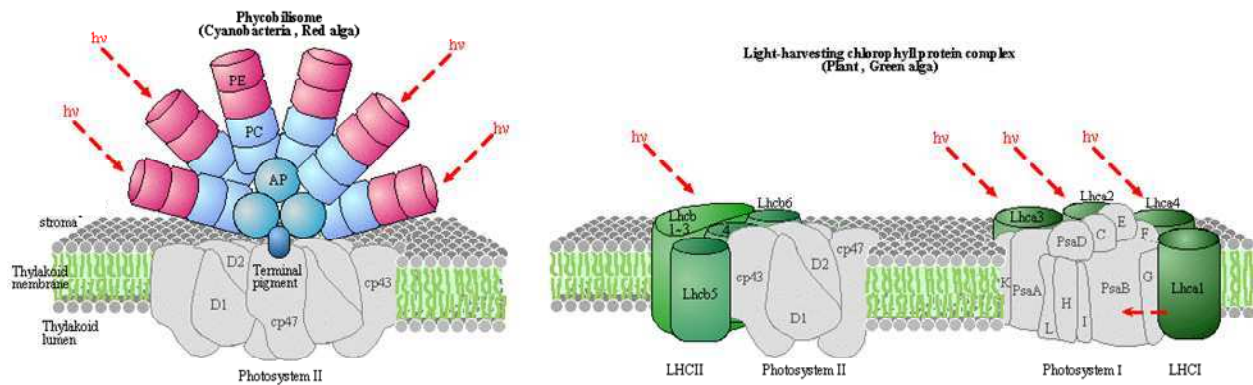
Figure 1.2. PSII-antenna complex from different photosynthetic organisms (Kanehisa Laboratories)

1.4. Excess light. Regulation of the photosynthetic machinery

1.4.1. Energy distribution between the photosystems

High efficiency of photochemical reactions in limiting light results in a quenching of fluorescence which is called photochemical quenching (qP).

Under high light conditions, when absorbed energy exceeds the rate of carbon fixation, excess light can be lethal for photosynthetic organisms because of production of harmful reactive oxygen species (Aro *et al.*, 1993; Melis, 1999). Cyanobacteria, like plants and algae, have developed several photoprotective mechanisms. Three major physiological processes are known to be involved in adjustment of photosynthetic apparatus to the different light environment: state transitions, D1 protein repair and non-photochemical PSII fluorescence quenching.

State transitions (qT) regulate the energy distribution between the two photosystems (Williams and Allen, 1987; van Thor *et al.*, 1998; Wollman, 2001). This process depends on redox state of the PQ pool. In green algae and plants, the mechanism of state transitions involves redox-dependent phosphorylation-dephosphorylation reactions of LHCII, inducing a movement of LHCII between the PSII and the PSI complexes (Williams and Allen, 1987; Allen, 1992; Wollman, 2001). In cyanobacteria exposure of cells to light absorbed predominantly by phycobilisomes (orange light) causes the reduction of the PQ pool due to increased energy pressure on PSII from PBs. This induces energy transfer to PSI and leads to the relative decrease

of the PSII fluorescence yield (State 2). Conversely, illumination with light (blue-green) absorbed by PSI - which are abundant in cyanobacteria - causes the oxidation of the PQ pool. This induces energy transfer to PSII and lead to the increase of the PSII fluorescence yield (State 1). In PB-containing cyanobacteria, two theories have been proposed to explain the state transition mechanism. The first one is based on the fact that PBs can migrate along the surface of the thylakoid membrane. It proposes that the PBs are the mobile elements which, by changing their association with PSII and PSI, deliver energy preferentially to one or the other photosystems (Mullineaux and Holzwarth, 1990; Mullineaux *et al.*, 1997; Sarcina *et al.*, 2001; Joshua and Mullineaux, 2004). The second theory based on results suggesting that the mobile elements are the photosystems (Biggins and Bruce, 1985; Schluchter *et al.*, 1996; El Bissati *et al.*, 2000). The decrease of fluorescence (State 2 transition) in cyanobacteria can be induced by a preferential illumination of cells by orange light principally absorbed by the PBs, which leads to the reduction of the PQ pool. In contrast, blue-green light, principally absorbed by PSI, induces the oxidation of the PQ pool and an increase of fluorescence associated with State 1 transition.

1.4.2. Repair of PSII

Exposure of oxygenic photosynthetic organisms to strong light can damage their photosynthetic apparatus. Due to this damage a decrease of PSII fluorescence can be observed. This phenomenon is called **photoinhibition** (qI). It happens due to oxidative damage induced by excessive excitation (Aro *et al.*, 1993; Vass *et al.*, 2007; Tyystjarvi, 2008). The main target of this damage is the D1 protein. The decrease of PSII fluorescence is associated with damage to the D1 protein. This process is reversible in intact systems, which are capable of protein synthesis. This means that when D1 is damaged by high light, it will be removed from the membrane and replaced by newly synthesized D1 protein. PSII activity and fluorescence recovery occur after replacement of the damaged D1 with a new copy. The process of recovery does not occur in the presence of inhibitors of prokaryotic protein synthesis, which helps to dissect the damaging and repair processes during photoinhibition. Both, acceptor and donor side electron transport inactivation can result in D1 protein damage. During acceptor side photoinhibition the PQ pool becomes fully reduced and Q_A stays reduced due to a block of the forward electron transfer (so-called closed state of the reaction center). In the case when the

forward electron transport cannot proceed, charge recombination reactions can occur. Reduced Q_A molecules recombine with P_{680} and form triplet P_{680} ($^3P_{680}{}^*$) which in the presence of oxygen produce singlet oxygen ($^1O_2{}^*$) (Vass *et al.*, 1992b; Hideg *et al.*, 1994).

$^3P_{680}{}^*$ also can be formed via recombination of reduced Q_A molecules with the S_2 or S_3 states of the water oxidizing complex even under low light excitation (Keren *et al.*, 1997).

There are also studies which propose that oxidative stress due to H_2O_2 and singlet oxygen does not inactivate PSII directly but inhibit the repair of photoinactivated PSII (Nishiyama *et al.*, 2005).

Another hypothesis for the mechanism of photoinhibition of PSII was proposed, namely, that photoinhibition is caused by a process that is independent of electron transport. It was postulated that the earliest step in photoinhibition is damage induced by the direct excitation of Mn atoms of the Mn cluster leading to the release of a manganese ion from PSII (Hakala *et al.*, 2005).

The extent of PSII photodamage can be influenced by the redox potential of Q_A. It has been shown that DCMU shifts the redox potential of Q_A by +50mV and this lead to decreased singlet oxygen production and photodamage. In contrast, bromoxynil shifts redox potential of Q_A by -50mV which induces enhanced photodamage due to singlet oxygen production (Rutherford and Krieger-Liszkay, 2001; Fufezan *et al.*, 2002). These results indicate that the decrease in the Q_A redox potential decreases the Phe $\leftrightarrow$ Q_A redox gap, which in turn leads to the increased recombination of $P_{680}{}^+Q_A{}^-$ with production of $^3P_{680}{}^*$ which in turn increases a singlet oxygen production. In contrast, the increase in Q_A redox potential increases the Phe $\leftrightarrow$ Q_A redox gap, which decreases recombination and singlet oxygen production .

The shift in the redox potential of Phe can modify the recombination pathways of $P_{680}{}^+Phe^-$. The direct recombination pathway increased when the redox potential of Phe was shifted by +33mV and therefore this mutant appeared to be more phototolerant. In contrast, in mutant with the redox potential of Phe shifted by -50mV, the indirect recombination pathway of $P_{680}{}^+Phe^-$ increased which led to increased photodamage (Cser and Vass, 2007a).

1.4.3. Non-photochemical quenching (qE)

Energy dissipation as a heat is a process that helps to protect the reaction centers. This phenomenon is also accompanied by a decrease of PSII related fluorescence emission and is known as **non-photochemical quenching (NPQ or qE)**.

NPQ prevents photoinhibition and the decrease of PSII fluorescence emission is due to energy dissipation. In plants, this mechanism involves LHCII (Horton *et al.*, 1996; Niyogi, 1999; Muller *et al.*, 2001).

Except chlorophyll pigments, the LHCII complex contains a significant amount of carotenoids. Carotenoids have several essential functions in photosynthetic systems. They have an important role in light harvesting and the transfer of energy to chlorophylls. At the same time carotenoids have a photoprotective role. Carotenoids quench triplet excited states of chlorophylls before they can react with oxygen to form damaging excited singlet state oxygen.

In plants and green algae carotenoids are involved in NPQ mainly via the xantophyll cycle (Gilmore and Yamamoto, 1993). Violaxantin and zeaxantin are xantophyll carotenoids of LHCII. During the xantophyll cycle epoxide-containing carotenoid violaxantin is converted in two steps to the non-epoxide-containing carotenoid zeaxantin (Demmig *et al.*, 1987; Demmig-Adams and Adams, 1993). Under saturating light conditions, the decrease of the lumen pH activates the xanthophyll cycle and promotes the protonation of the PsbS subunit of PSII (Li *et al.*, 2000). This induces conformational changes in LHCII, modifying the interaction between chlorophylls and carotenoids increasing the rate constant for thermal dissipation (Ruban *et al.*, 1993; Horton *et al.*, 2000; Horton *et al.*, 2005). Energy dissipation is accompanied by a diminution of PSII related fluorescence emission.

It has been recently demonstrated that in cyanobacteria there also exists an energy dissipating mechanism accompanied by a diminution of PSII fluorescence emission (El Bissati *et al.*, 2000; Rakhimberdieva *et al.*, 2004; Scott *et al.*, 2006; Wilson *et al.*, 2006). It was demonstrated that exposure of cells of the cyanobacterium sp. *Synechocystis* PCC 6803 (hereafter called *Synechocystis*) to strong intensities of blue-green light induces PSII fluorescence quenching under conditions in which the PQ pool was largely oxidized. The fluorescence quenching affects all fluorescence levels (F_m', F_s and F_o), and is reversible in the dark, or under dim illumination after the first minutes of illumination (Fig.1.3.).
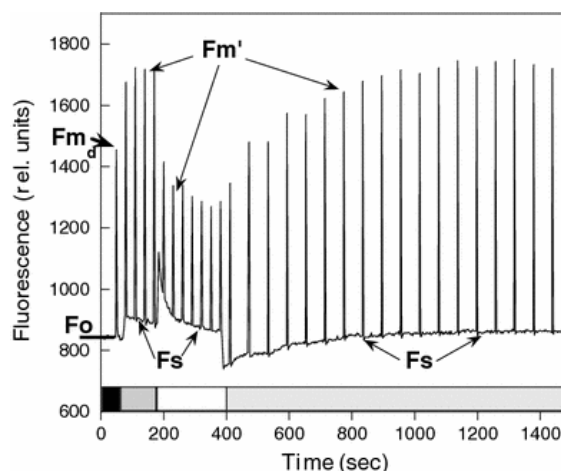
Figure 1.3. Changes in fluorescence levels induced by different intensities of blue-light measured in a PAM fluorometer. Dark adapted WT Synechocystis cells (Chl 3 μg/ml) were successively illuminated with low blue–green light (400–550 nm) at 80 μmol photons $m^{-2} s^{-1}$ followed by blue–green light at 740 μmol photons $m^{-2} s^{-1}$ and finally again with low blue–green light at 80 μmol photons $m^{-2} s^{-1}$. Saturating pulses (1 s duration, 2000 μmol photons $m^{-2} s^{-1}$) were applied to measure Fm_d and Fm' levels in darkness and in light, respectively. Chloramphenicol (34 μg ml^{-1}) was present throughout the experiment (Kirilovsky, 2007).

Blue light-induced fluorescence quenching is not inhibited in the presence of DCMU which prevents the reduction of the PQ pool by inhibition of the Q_A to Q_B electron transport (Scott *et al.*, 2006; Wilson *et al.*, 2006). In addition, recovery of the fluorescence yield occurs in the presence of inhibitors of protein synthesis (Fig.1.3) (El Bissati *et al.*, 2000; Wilson *et al.*, 2006). These results demonstrated that the blue light-induced quenching was not related to process of state transitions or photoinhibition. This quenching is not affected by the transthylakoidal pH gradient (Wilson *et al.*, 2006). Moreover, cyanobacterial cells do not have a PsbS protein and do not show xanthophyll cycle activity. It was shown that in a *Synechocystis* mutant, lacking reaction center II, blue-green light induced a reversible quenching of the phycobilisome emission (Rakhimberdieva *et al.*, 2004; Scott *et al.*, 2006; Wilson *et al.*, 2006). In contrast, blue-green light was unable to induce fluorescence quenching in a phycobilisome-deficient mutant or in a mutant containing only the phycocyanin rods of the phycobilisome while the presence of only the core of the phycobilisome is sufficient to induce the quenching (Wilson *et al.*, 2006). Thus, phycobilisomes form the location of the blue light-induced NPQ mechanism.

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Saturating blue-green or white light induces thermal dissipation of the energy absorbed by the PBs which results in the decrease of the PB fluorescence emission and of the energy transfer from the PBs to the reaction centers. This quenching is only light quality dependent, because only high intensity blue or green light can induce it; orange or red light do not (Wilson *et al.*, 2006). Moreover, Wilson *et al*. demonstrated that in *Synechocystis* this energy dissipation mechanism involves the so-called orange carotenoid protein (OCP).

1.5. OCP protein and photoprotection

1.5.1. Structure and the function of the OCP protein

Water-soluble OCP is a 35kD protein containing a single non-covalently bound carotenoid (Holt and Krogmann, 1981; Wu and Krogmann, 1997; Kerfeld, 2004a, b), which in *Arthrospira maxima* and *Synechocystis* is a 3'-hydroxyechinenone (hECN) (Kerfeld *et al.*, 2003; Wilson *et al.*, 2008). OCP contains an α-helical N-terminal domain and an α-helical/β-sheet C-terminal domain. The hECN molecule spans both N- and C-terminal domains (Fig.1.4) (Kerfeld *et al.*, 2003).
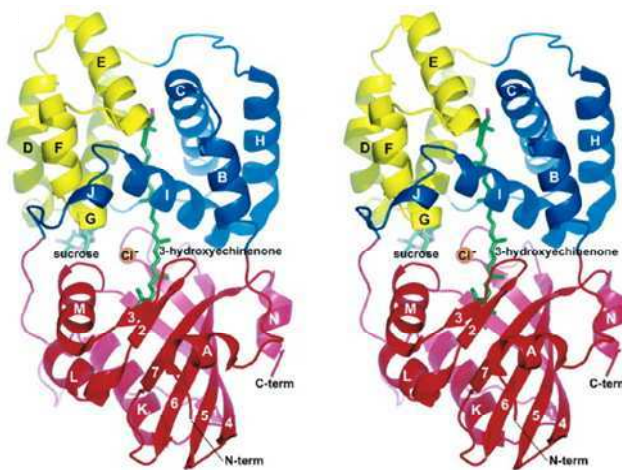
Figure 1.4. Structure of the Orange Carotenoid Protein (OCP) from the Arthrospira maxima (Kerfeld et al., 2003).

It has been shown that OCP transcript levels increase upon transfer to high-light intensities (Hihara *et al.*, 2001). The fact that the action spectrum of the PB fluorescence

quenching exactly matches the absorption spectrum of the carotenoid hECN in the OCP (Rakhimberdieva *et al.*, 2004; Polivka *et al.*, 2005) allowed Wilson *et al.* to propose that absorption of light by the carotenoid of the OCP induces changes in the carotenoid and the protein leading to energy dissipation (and fluorescence quenching) through interaction with the PB core (Wilson *et al.*, 2006).

Studies using immunogold labeling and analysis by electron microscopy showed that OCP is present in the inter-thylakoid cytoplasmic region, on the phycobilisome side of the membrane (Wilson *et al.* 2006). The existence of an interaction between OCP and phycobilisomes was supported by the co-isolation of OCP with the phycobilisome-associated membrane fraction (Wilson *et al.*, 2006; Wilson *et al.*, 2007; Boulay *et al.*, 2008).

In the absence of OCP, the fluorescence quenching induced by strong white or blue-green light is absent (Fig.1.5.) and *Synechocystis* cells are more sensitive to high light (Wilson *et al.*, 2006).
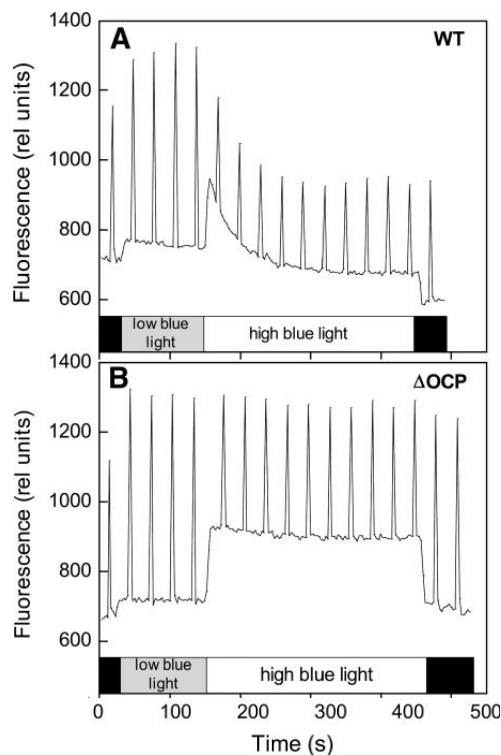
Figure 1.5. Effect of the OCP on the development of blue light-induced NPQ. Measurements of fluorescence yield by a PAM fluorometer in dark-adapted wild-type (A) and ΔOCP (B) cells illuminated with low intensity blue-green light and high intensity blue-green light (Wilson et al., 2006).

It has been recently demonstrated that the OCP is a photoactive protein with an "active" red form (Kirilovsky *et al.*, 2011). The absorbance of light by the OCP, which appears orange under resting conditions, induces structural changes in the carotenoid and its apoprotein, which

in turn converts the dark stable orange form into a relatively unstable active red form (Wilson *et al.*, 2008). The red form accumulates under conditions in which cyanobacteria need photoprotection. It was also demonstrated that, in the mutant which lacks hydroxyechinenone and echinenone, OCP binds zeaxanthin. The stability of the binding in this case is low and light is unable to photoconvert the dark form into a red active form. Strains containing zeaxanthin-OCP, were not able to perform blue light-induced photoprotective quenching. In contrast, in mutants, in which OCP binds echinenone, the protein is photoactivated and photoprotection is induced (Kirilovsky *et al.*, 2009).

The mechanism of this energy dissipation is still uknown. There are at least two possibilities. One possibility is that the OCP is the light sensor and by absorption of blue-green light OCP turns into its active form. By interaction with the phycobilisome core OCP could mediate the conformational changes in the phycobilisome leading to the energy quenching.

It was proposed by Kirilovsky D. (2007) that OCP acts not only as sensor of high light, but also as the energy and fluorescence quencher, capable to receive the energy absorbed by the phycobilisome and to convert it into heat (Fig.1.6.) (Kirilovsky, 2007).

Figure 1.6. Model for the role of OCP in NPQ formation. The absorption of blue-green light by the hECN of OCP induces confo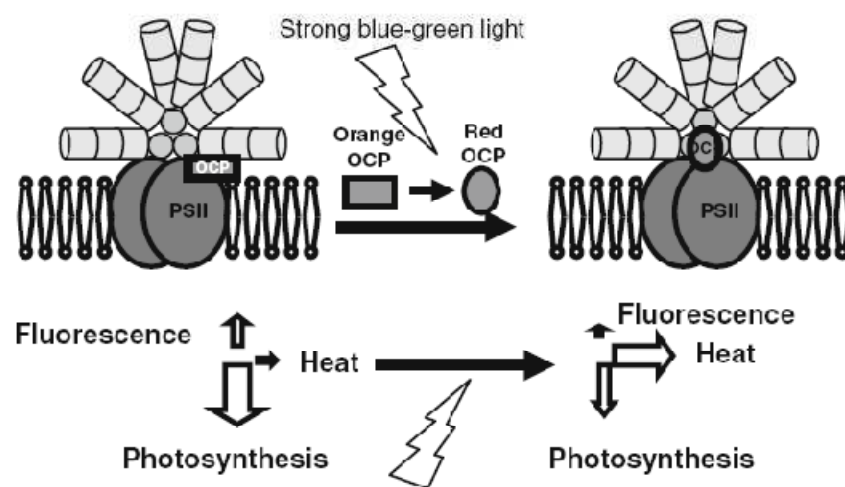rmational changes in the carotenoid and the protein that binds it converting the orange OCP form into an active red form which lead to the energy transfer from the phycobilisome to the OCP in which the energy is thermally dissipated (Kirilovsky, 2010).

1.5.2. The OCP protein and iron starvation conditions

Photosynthetic reactions rely on a number of iron-containing proteins and iron starvation stress induces the reorganization of the regulation of light utilization. For instance, in many cyanobacteria it induces the synthesis of the chlorophyll-binding protein IsiA (iron-stress-induced protein) (Laudenbach and Straus, 1988; Burnap *et al.*, 1993). Under iron starvation conditions (Cadoret *et al.*, 2004; Rakhimberdieva *et al.*, 2007; Wilson *et al.*, 2007) blue-green light induces a very large fluorescence quenching, which is much larger than under complete medium conditions. It was suggested that IsiA protein induce this NPQ. But, using ΔIsiA and ΔOCP *Synechocystis* mutants it has been recently demonstrated that blue light-induced quenching is associated with OCP and not with IsiA protein (Wilson *et al.*, 2007).

It was found that the larger fluorescence quenching correlates with a higher OCP concentration (Wilson *et al.*, 2007; Boulay *et al.*, 2008) showing the essential role of OCP in the acclimation of cyanobacteria to environmental changes (Fig.1.7.). The relationship between the concentration of OCP and energy dissipation was later confirmed by using an OCP overexpressing *Synechocystis* mutant, in which large quantities of OCP were present and a very large fluorescence quenching was observed (Wilson *et al.*, 2008).

In mutants lacking the OCP protein, blue light was unable to induce fluorescence quenching even after long periods of iron starvation (Fig.1.8.) (Wilson *et al.*, 2007).
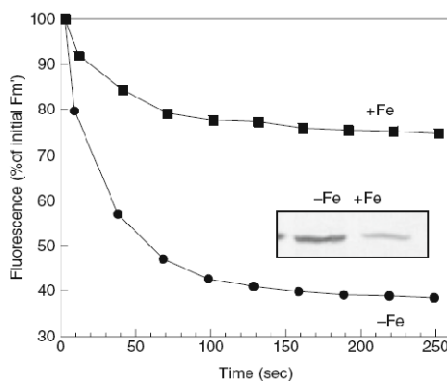
Figure 1.7. Blue light-induced fluorescence quenching in iron-replete and iron-starved Synechocystis cells. Blue light-induced decrease of the maximum fluorescence level (Fm') measured by saturating flashes with a PAM fluorometer in iron-containing (+Fe) and 10-day ironstarved (–Fe) Synechocystis cells. Insert: OCP immunoblot of MP fractions from these cells (each lane contained 1 µg of Chl) (Kirilovsky, 2010).
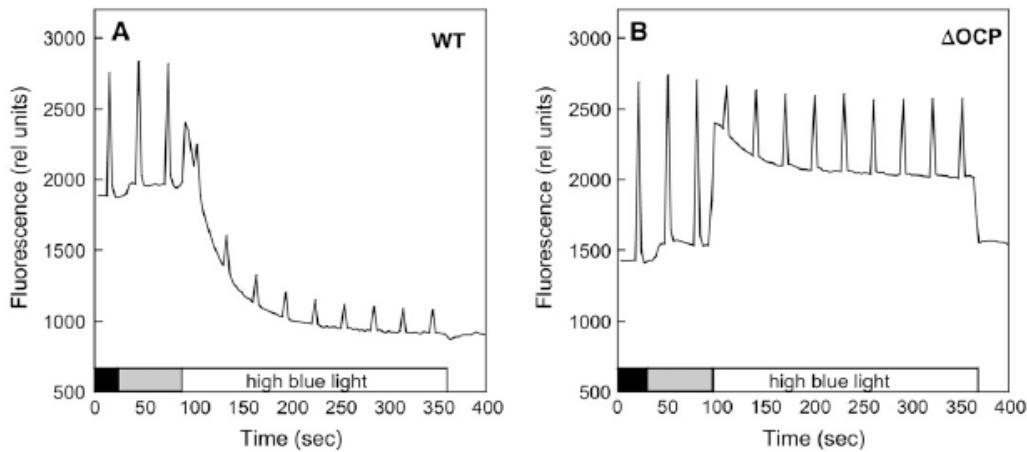
*Figure 1.8. Changes in the fluorescence yield induced by strong blue-green light in iron starved Synechocystis WT and OCP mutant cells. Measurements of the fluorescence intensity by a PAM fluorometer of dark-adapted 14 d iron-starved wild-type **(A)** and ΔOCP **(B)** cells illuminated with low intensity blue-green light and high intensity blue-green light (Wilson et al., 2007).*

1.5.3. Presence of the OCP gene in diferent cyanobacteria

The function of the OCP in the photoprotective PB-related thermal dissipating mechanism has been examined so far only in *Synechocystis* cells (Wilson *et al.*, 2006; Rakhimberdieva *et al.*, 2007; Wilson *et al.*, 2007). 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. Their sequence is highly conserved. OCP sequences from freshwater cyanobacteria present a higher similarity to *Synechocystis* OCP (66–82%) than those present in marine cyanobacteria (62–64%) (Boulay *et al.*, 2008). The marine *Synechococcus* OCP sequences are very similar to each other, with identities ranging from 77 to 95%. The expression of full-length OCP was confirmed in seven strains where OCP was constitutively present (Boulay *et al.*, 2008). Among PB-containing cyanobacteria, only a few strains do not have an OCP gene homologue, including the freshwater *Synechococcus elongatus* PCC 7942 and PCC 6301; the thermophile *Thermosynechococcus elongatus*, *Synechococcus* sp. A, and *Synechococcus* sp. B'; the nitrogen fixing strains *Cyanothece* sp. PCC 7822, PCC 8801, and PCC z8802, and *Nostoc* sp. PCC 7425; and the

marine *Synechococcus* sp. CC9605, *Cyanobium* PCC 7001, and *Crocosphaera watsonii* WH8501. However, *Thermosynechococcus elongatus, Cyanothece* sp. PCC 7822, PCC 8801, and PCC 8802, and *Nostoc* sp. PCC 7425 contain separate but adjacent genes coding for the N- and C-terminal parts of the OCP (Boulay *et al.*, 2008).

In this work the correlation between the presence of the OCP gene and the occurrence of blue light-induced fluorescence quenching was investigated using the *Thermosynechococcus elongatus* (hereafter *T.elongatus*) cells without whole OCP gene, *Synechococcus elongatus* PCC7942 (hereafter *S. elongatus*) with no OCP gene, and *Arthrospira maxima* (hereafter *A. maxima*), containing the OCP gene.

1.6. The role of PsbU protein in photoprotection of PSII

1.6.1. Occurrence and function of OEC extrinsic proteins

The OEC is located on the lumenal side of PSII. It consists of a Mn_4Ca cluster and several lumenal PSII proteins. Because of its fundamental role in photosynthesis the structure of the OEC is of significant interest. Recent crystallographic data on the cyanobacterial PSII complex at a resolution of 3.8, 3.5, 3.0, 2.9 and 1.9 Å allowed a detailed characterization of the Mn cluster. It was shown which amino acid residues of the PSII intrinsic proteins are required for the ligation of the Mn cluster (Zouni *et al.*, 2001; Ferreira *et al.*, 2004; Loll *et al.*, 2005; Guskov *et al.*, 2009; Umena *et al.*, 2011). The mechanism of water oxidation is conserved from cyanobacteria to higher plants, but the extrinsic protein composition of the OEC is varies among the photosynthetic organisms. The largest extrinsic subunit, PsbO, is the only one that is always associated with the OEC (Fig.1.9.). The main role of the extrinsic proteins is to stabilize the Mn cluster and to maintain optimal levels of bound calcium and chloride ions which are required as essential cofactors for the water oxidation reactions.
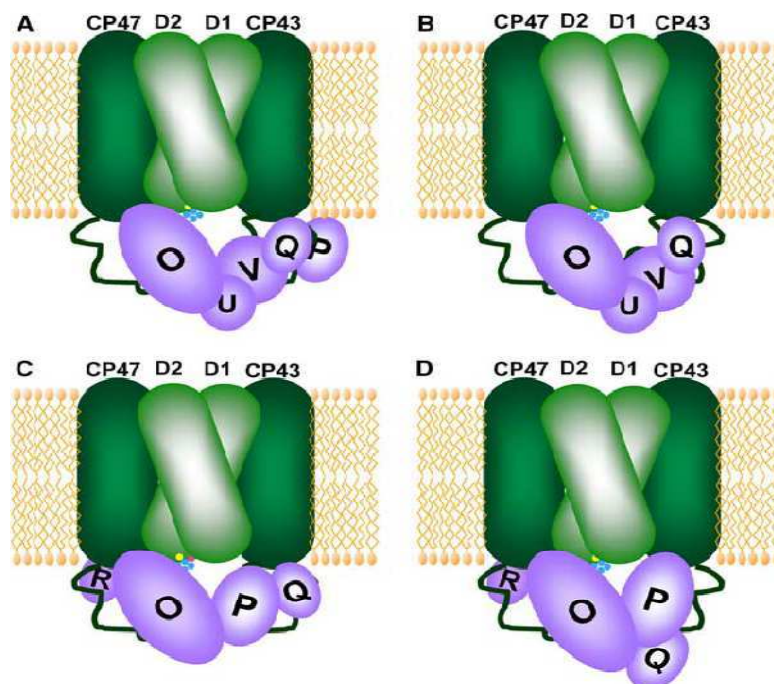
Figure 1.9. Models for the OECs in different organisms: in cyanobacteria (A), red algae (B), green algae (C), and plants (D). The extrinsic proteins are labeled as O (PsbO), P (PsbP), Q (PsbQ), R (PsbR), U (PsbU), and V (PsbV) (Pakrasi et al., 2007).

Cyanobacterial PSII complexes contain five extrinsic proteins, PsbO, PsbP, PsbQ, PsbU, and PsbV. PsbP and PsbQ are not present in the crystal structures and have not been studied well due to their only recent discovery in cyanobacteria (Thornton et al., 2004). Structural studies of PSII from the cyanobacterium *Thermosynechococcus elongatus* and *Thermosynechococcus vulcanus* have confirmed the association of PsbO (33 kDA), PsbU (12 kDA) and PsbV (Cyt. c_{550}) with the oxygen evolving complex (Ferreira *et al.*, 2004; Guskov *et al.*, 2009; Umena *et al.*, 2011) (Fig.1.10.).
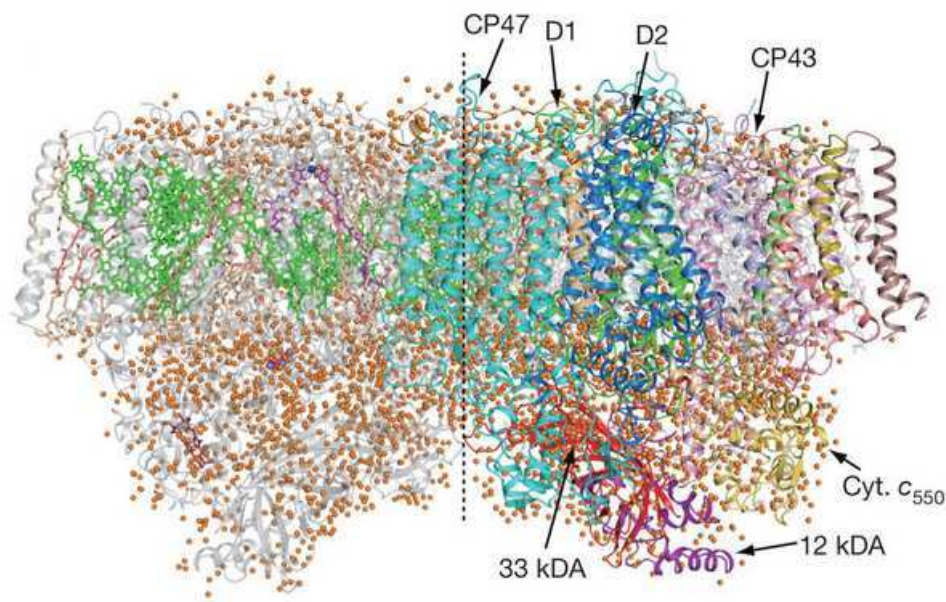
Fig. 1.10.   Crystal structure of PSII from Thermosynechococcus vulcanus. View from the direction perpendicular to the membrane normal (Umena et al., 2011).

Biochemical and genetic studies have shown that these proteins are important for the stabilization of the manganese cluster and minimization of the Ca^{2+} and Cl^- requirements for oxygen evolution (Burnap *et al.*, 1992; Shen *et al.*, 1998; Eaton-Rye *et al.*, 2003). The binding order of these proteins is well established. It is known that PsbO can bind to PSII in the absence of other proteins. PsbV can also bind independently, but it is stabilized by presence of PsbO and PsbU. In contrast, PsbU cannot bind in the absence of PsbO or PsbV (Shen and Inoue, 1993; Shen *et al.*, 1997; Eaton-Rye *et al.*, 2003). Thus it is thought that PsbO binds first, then PsbV, and PsbU is the last to bind.

1.6.2. The effect of PsbU at the donor and acceptor side of PSII

The PsbU protein is a 12 kDa extrinsic subunit of PSII. The PSII crystal structure places PsbU between PsbO and PsbV with a majority of its contacts to these two proteins (Kamiya and Shen, 2003; Ferreira *et al.*, 2004; Loll *et al.*, 2005). While there is a significant distance between PsbU and the membrane, it interacts with CP47 and CP43 (Eaton-Rye, 2005). The function of PsbU is to shield the Mn cluster from the aqueous phase and to provide structural stability to PSII. The PsbU protein stabilizes the PSII complex in several ways. The absence of PsbU results

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in the reduction of the rate of oxygen evolution (Shen *et al.*, 1997; Inoue-Kashino *et al.*, 2005) and a high sensitivity of cells to photodamage, resulting in rapid degradation of the D1 protein (Inoue-Kashino *et al.*, 2005; Balint *et al.*, 2006). Additionally, PsbU was found to be required for the stabilization of the Mn cluster. A PsbU lacking mutant of *Synechocystis* showed decreased growth in medium lacking Ca^{2+} and Cl^- (Shen *et al.*, 1997). Shen et al. (1997) demonstrated that the peak temperatures of both the thermoluminescence Q and B bands were increased by 4 ^{o}C in ΔPsbU *Synechocystis* mutant cells compared to wild type cells. The presence of PsbU contributes to the heat stability of the oxygen-evolving machinery during the acclimation to high temperature (Nishiyama *et al.*, 1997; Nishiyama *et al.*, 1999). The removal of PsbU affects not only the lumenal 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 (Veerman *et al.*, 2005). Recently, flash-induced Chl fluorescence decay and thermoluminescence (TL) measurements performed with a PsbU mutant of *Synechococcus* PCC 7942 showed that the rate of charge recombination from the $S_2Q_A^-$ state is slowed down, as shown by the retarded rate of fluorescence decay and the upshifted peak position of the Q band in the presence of the electron transport inhibitor DCMU (Balint *et al.*, 2006). Balint et al. also proposed that PsbU provides protection from reactive oxygen species because PsbU mutants have exhibited higher resistance to oxidative stress compared with the wild type strain.

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

2. 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*.

3. MATERIALS AND METHODS

3.1. Propagation of cells

Cells of *Synechococcus* sp. PCC 7942 and its PsbU deletion mutant were obtained from the Bar-Ilan University in Israel. *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942 and its ΔPsbU mutant were grown in BG-11 medium in a rotary shaker at 30 °C under air enriched with 5% CO_2. The intensity of irradiation during growth was 40 μmol photons m^{-2} s^{-1}. The *Synechococcus* PsbU deletion mutant was grown in the presence of 50 μg/mL spectinomycin. Thermophilic *Thermosynechococcus elongatus* cells were grown under similar conditions but in a DTN medium at 45 °C. The cells were maintained in the logarithmic growth phase and were collected at OD_{800} = 0.6-1.2. As a starter culture 1.5 ml culture that had been kept at -80 °C was used. Cyanobacterial cells were grown in 500 ml flasks containing 200 ml of growth medium.

3.2. Iron starvation

Synechocystis sp. PCC 6803 and *Thermosynechococcus elongatus* cyanobacterial cells were collected in the logarithmic growth phase, precipitated, resuspended (OD_{800} = 0.6) in the modified Fe-free BG11 or DTN medium, respectively. The cells were diluted every 3 days.

3.3. Absorbance measurements

Cell absorbance was monitored with an $UVIKON_{XL}$ spectrophotometer (SECOMAN, Alès), UV-1601 (SHIMADZU).

3.4 Chlorophyll content determination

Chlorophyll content was determined using the methanol extraction method. Cells were centrifuged and then diluted in 100% methanol. A second centrifugation step needed to precipitate phycobilisomes. The Chl concentration was determined at 665 nm after extracting the

pigments with methanol (Bennett and Bogorad, 1973). Each value for the chlorophyll content was the average of three independent determinations.

3.5. PAM fluorescence measurements

Changes in the fluorescence kinetics of *Synechocystis* and *T. elongatus* cells were monitored with a pulse amplitude modulated fluorometer (101/102/103-PAM; Walz, Effelrich, Germany). The fluorometer detects the fluorescence emitted in response to a weak measuring beam consisting of a train of pulses at a frequency of 1.6 kHz. Changes in the fluorescence intensity reflect changes in the fluorescence yield due to various quenching processes. Since fluorescence emitted in response to other sources of light is ignored by the detection system, the measurements can be made using different intensities and qualities of actinic light or changing the actinic light during the measurement without the need for normalization of the fluorescence intensity. The minimal fluorescence level (F_0) is the fluorescence intensity emitted in response to the measuring beam. Under these conditions, all PSII reaction centers are assumed to be open. The maximum fluorescence intensity (F_m and F_m') is induced by applying pulses of saturating white light (2000 µmol photons m^{-2} s^{-1}, 1 s), which are assumed to close all PSII reaction centers. Under continuous illumination the steady-state fluorescence level (F_s) can be measured. F_s depends on the redox state of Q_A.

All measurements of the induction of NPQ and state transitions induction measurements were carried out in a stirred cuvette of 1 cm diameter kept at the growth temperature at a concentration of 3 µg Chl/ml. The minimal fluorescence level in dark-adapted sample (F_0) was determined by illuminating dark-adapted cells with a weak, modulated light that preferentially excited the phycobiliproteins (1.6 kHz, 0.024 µmol photons m^{-2}s^{-1}, 650 nm) (El Bissati and Kirilovsky, 2001).

In order to measure NPQ the following protocol was used:

1. illuminating dark-adapted cells with low irradiance of blue-green light (400–550 nm, 80 µmol photons m^{-2}s^{-1}) for about 1 min

2. illuminating cells with 740 µmol photons m^{-2}s^{-1} of blue-green light for about 2 min to induce fluorescence quenching

3. shifting cells back to low intensity of blue-green light

For state transition measurements the following protocol was used:

1. illuminating cells with low intensity blue-green light (400–550 nm, 80 µmol photons $m^{-2}s^{-1}$) to induce State 1
2. illuminating cells by orange light (600–650 nm) at 20 µmol photons $m^{-2}s^{-1}$ of light intensity to induce State 2
3. re-illuminating cells with low blue-green light

3.6. Fluorescence spectra at 77 K

Fluorescence emission spectra at 77K were measured in a CARY Eclipse fluorometer (Varian). All samples were at a concentration of 3 µg Chl/ml. Samples in nuclear magnetic resonance tubes (5 mm diameter) were quickly frozen by immersion in a mixture of ice, CO_2, and ethanol and then in liquid nitrogen. Cells were excited at 430 nm to monitor the chlorophyll emission spectra and at 600 nm to detect the emission spectra from both phycobiliprotein and chlorophyll.

3.7. Flash-Induced Fluorescence Relaxation Kinetics

The flash-induced fluorescence increase and the subsequent decay of Chl fluorescence intensity were measured in cells of *Synechococcus* WT and the PsbU deletion mutant using a double-modulation fluorometer (PSI Instruments, Brno) in the 150 µs–100 s time range as described earlier (Vass *et al.*, 1999). Charge separation between Q_A and the PSII donor side was induced by a single saturating flash (sourse: the red LED of the PSI machine). For most experiments the measuring light was also red. However, for some experiments blue excitation light was used from an external Xenon lamp equipped with a blue filter, as well as blue measuring flashes provided by the PSI machine, in order to ensure that only the Chls, and not the phycobilisomes were excited. The sample concentration was 5 µg Chl/ml. A multicomponent deconvolution of the measured curves was made using a fitting function consisting of three components. The fast and middle phases were fitted with exponential functions. However, slow recoxidation of Q_A^- via charge recombination has been shown to obey hyperbolic kinetics

corresponding to an apparently second-order process (Bennoun, 1994). Therefore, the slow phase was fitted with a hyperbolic function:

$$F(t) - F_o = A_1 exp(-t/T_1) + A_2 exp(-t/T_2) + A_3/(1 + t/T_3) + A_0 \qquad (1)$$

where $F(t)$ is the fluorescence intensity at time t, F_0 is the minimum fluorescence level before the flash, A_1–A_3 are the amplitudes and T_1–T_3 are the time constants of the decay phases, and A_0 is the fluorescence amplitude which does not decay within the monitored time domain after the flash. The nonlinear correlation between the fluorescence intensity and the redox state of Q_A was corrected by using the Joliot model with a value of 0.5 for the energy-transfer parameter between the PSII units.

3.8. Thermoluminescence measurements

TL glow curves were measured with a home-built apparatus as described earlier (Vass *et al.*, 1992a). Cells were harvested and 50 µg Chl were brought on a filter paper disc. Samples were dark adapted for 3 min at 0 °C. The sample was excited either in the absence or in the presence of the electron transport inhibitor DCMU. In the absence of DCMU dark-adapted samples were excited with a saturating single turnover flash at +5 ºC followed by a TL measurement from 0 to +80 ºC. In other experiments 10 µM DCMU was added to the sample in the dark, and after excitation of the sample at -2 ºC, TL was detected during heating from -4 to +80 ºC.

3.9. Oxygen evolution measurements

Oxygen evolution was measured with a Hansatech DW2 O_2 electrode at saturating light intensity in the presence of 0.5 mM artificial electron acceptor DMBQ. 1 ml of cells at 7 µg Chl/ml was used in each measurement, and three replicates were measured. After the 1.5 hour of the light treatment in the absence of protein synthesis inhibitor lincomycin, cells were transferred back to 40 µmol photons $m^{-2}s^{-1}$ to monitor their ability for repairing PSII activity.

3.10. Light treatment

Cells of *Synechococcus* WT and the PsbU deletion mutant cells were harvested by centrifugation at 8000 g for 5 min and resuspended in 100 ml of fresh BG-11 medium at concentration of 7 μg of Chl/ml. Before starting the high light treatment cells were left for one hour under 40 μmol photons $m^{-2}s^{-1}$ while being stirred continuously followed by a measurement of the control value of oxygen evolution, which was used as the activity at the zero time point for the high light treatment. For the photoinhibitory treatment cells were illuminated with 500 μmol photons $m^{-2}s^{-1}$ in the presence and absence of the protein synthesis inhibitor lincomycin.

4. RESULTS AND DISCUSSION

4.1. Occurrence and role of the Orange Carotenoid Protein in different cyanobacteria (Boulay *et al*., 2008)

4.1.1. Blue light-induced NPQ and state transitions in the presence and absence of OCP gene in iron-containing cells

It was recently demonstrated that the OCP is essential for the buildup of blue light-induced NPQ in *Synechocystis* cells (Wilson *et al.*, 2006). In this work we extended our observation to other cyanobacterial strains. 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, first of all, compared the *Synechocystis* strain containing OCP gene and *T. elongatus* strain lacking the entire OCP gene.

The kinetics of the fluorescence changes were monitored by a PAM fluorometer. The low intensity measuring light was used to determine the minimal fluorescence yield (F_0) in dark adapted cells. Under these conditions all centers are assumed to be open. The low intensity measuring light has a maximum of excitation at 650 nm. In cyanobacteria the 650 nm measuring light is absorbed mostly by phycobilisomes and therefore the F_0 level depends on the cellular phycobiliprotein content. This was confirmed by the low level of fluorescence emission from mutants which do not have phycobilisomes (El Bissati and Kirilovsky, 2001). The maximal fluorescence level (F_m and F_m') were measured by high intensity light pulses which reduce Q_A and close all PSII centers. In general, the fluorescence (F_0, F_s and F_m') detected by the PAM fluoremeter could be result both from chlorophyll and phycobilisomes (Campbell *et al.*, 1998). Thus the changes in the fluorescence levels observed in a PAM fluorometer could be the result of changes of either the phycobilisome emission, or the chlorophyll emission or caused by a decrease (increase) of the energy transfer from the phycobilisome to PSII.

Figure 4.1. demonstrates the effects of different intensities of blue-green light on the fluorescence intensity in *T. elongatus* and *Synechocystis* cells. Dark adapted cyanobacteria cells are always in State 2 which is characterized by low F_m. This is due to rather reduced plastoquinone pool in dark adapted cyanobacterial cells (Dominy and Williams, 1987;

Mullineaux and Allen, 1990). 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 both strains. In cyanobacterial cells the number of PSI is much higher than PSII and absorption of low intensity blue-green light by PS I leads to the oxidation of PQ pool which in turn induce a redistribution of PB between PSII and PSI with a subsequent increased energy transfer to PSII. This process is called State 1 transition.

After reaching the maximal fluorescence level in State 1, *T. elongatus* cells were illuminated by strong intensity blue-green light. Illumination of *T. elongatus,* which lacks the full OCP gene, and contains only sequences for the C- and N-terminal part of the gene, 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 (Fig.4.1.).

In contrast, exposure of low blue light–adapted cells to high blue-green light intensities induced a fluorescence quenching in the OCP containing *Synechocystis* strain. All fluorescence levels (F_0, Fs and F_m′) in this case were decreased (Fig.4.1.). When cells were transferred back to low intensities of blue-green light, fluorescence again reached its maximum, indicating that full recovery occurred (Fig.4.1.) (Wilson *et al.*, 2007).

These results suggest that in *T. elongatus*, products of the C- or the N-terminal part of the OCP, if they are translated, do not lead to NPQ induction.
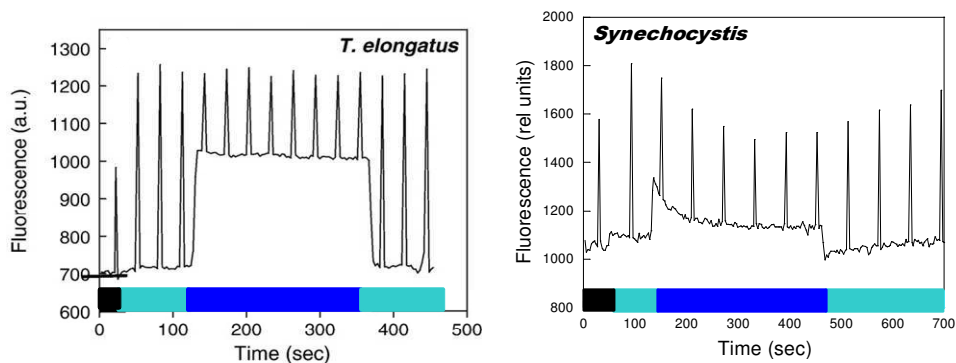
Figure 4.1. Strong blue-green light effect in T. elongatus and Synechocystis cells. T. elongatus and Synechocystis cells (at 3 µg Chl/ml) were dark-adapted, illuminated with low intensity blue-green light (400-550 nm, 80 µmol photons $m^{-2}s^{-1}$) (light blue bar) and high intensity blue-green light (740 µmol photons $m^{-2}s^{-1}$) (dark blue bar); and then illuminated by low intensity blue-green light. In case of Synechocystis cells longer measurement was done in order to look at the recovery of fluorescence intensity. Fluorescence yield changes were detected using a PAM fluorometer and saturating pulses were applied to measure the maximum fluorescence levels.

In contrast to the lack of blue light-induced NPQ the absence of OCP did not affect the occurrence of state transitions.

Figure 4.2. represents state transition process in *T. elongatus* strain. After three minutes of dark adaptation, cells of *T. elongatus* are in State 2 (due to dark reduction of the PQ pool). During the next few minutes, cells were illuminated by low intensity blue-green light to induce a transition to State 1. As discussed above, blue-green light, depending on its intensity, may result in state transitions or in the induction of NPQ. Only high intensities of blue-green light are able to induce NPQ. Upon illumination by low intensities of blue-green light, absorbed mostly by PSI, a maximum level of F_m' was reached (State 1). Then, cells were illuminated by orange light, which is absorbed mostly by the phycobilisomes and transferred mainly to PSII. Orange light induces a reduction of the PQ pool and thus a decrease of the F_m' level, which is characteristic to State 2 (Fig.4.2.A).

We were also able to induce state transition in *T. elongatus* cells by artificial oxidation or reduction of the plastoquione pool (Fig.4.2.B and C). DCMU inhibits the reduction of the

plastoquinone pool by binding to the Q_B site of the reaction center II and thereby blocking electron transport beyond Q_A. In contrast, 2,5-Dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB) inhibits the reoxidation of the plastoquinone pool. It binds to the Cyt- b_6f complex and blocks the transfer of electrons from the plastoquinone pool to the Cyt-b_6f complex.

Addition of DCMU to our cells immediately induced the closure of all PSII centres, and high F_m' level (similar to State 1) remained even under orange excitation (Fig.4.2.B).

DBMIB was also added to the cells after the blue-green illumination when cells were in State 1. After its addition, F_m' quenching was observed (similar to State 2) even under low intensity blue-green light, due to the reduction of plastoquinone pool (Fig.4.2.C).
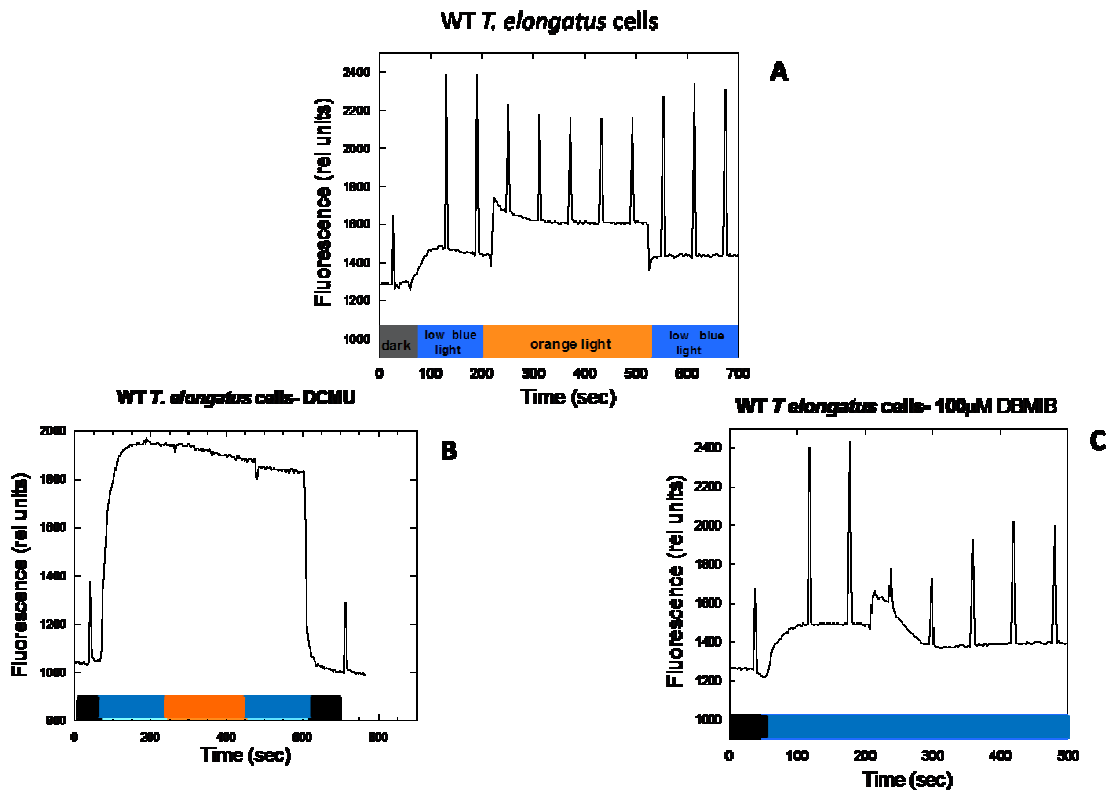
*Figure 4.2. State transitions induced in T. elongatus cells. Dark-adapted cells were illuminated with low intensity blue-green light (400–550 nm, 80 μmol photons $m^{-2}s^{-1}$) (blue bar), low intensity orange light (20 μmol photons $m^{-2}s^{-1}$) (orange bar), and then again with low intensity blue-green light (control cells) (**A**), in the presence of DCMU (10 μM) (**B**), and dark-adapted cells illuminated only with low intensity blue-green light in the presence of DBMIB (10 μM) (**C**). Fluorescence intensity changes were detected with a PAM fluorometer and saturating pulses were applied to measure maximum fluorescence levels.*

4.1.2. Blue light-induced NPQ and state transitions in the presence and absence of the OCP gene in iron-starved cells

Under iron-starvation conditions high intensities of blue-green light also induce a large reversible quenching (Cadoret *et al.*, 2004; Bailey *et al.*, 2005; Mullineaux *et al.*, 2005). It has been recently demonstrated that in iron-starved *Synechocystis* cells, as well as in iron-containing cells, the blue light-induced fluorescence quenching is associated with the OCP protein. Moreover, in iron-starved cells the fluorescence quenching was stronger than in iron-containing cells (Wilson *et al.*, 2007). The increased NPQ was associated with a higher content of OCP in iron-starved cells compared with nonstarved cells.

We tested the possible relationship between iron starvation and the blue light-induced NPQ in two cyanobacterial species that differed in their OCP gene configuration: *T. elongatus,* lacking a complete OCP gene and *Synechocystis*, containing a functional OCP gene. To monitor fluorescence changes under conditions of iron starvation in *T. elongatus* cells we again used a PAM fluorometer. Fluorescence kinetics of iron-starved *T. elongatus* cells were compared with iron-starved *Synechocystis* fluorescence data from Wilson et al. (2007).

Interestingly, illumination of dark adapted *T. elongatus* cells iron-starved for 7 days by low intensity blue-green light did not induce similar increase of F_m' as earlier observed in iron containing cells. The same effect was observed in iron-starved *Synechocystis* cells. F_m' remained at the level of F_m in prolonged iron starved cells from both strains (Fig.4.3.).

Further, we illuminated *T. elongatus* cells with strong blue-green light and found that exposure of 7 days (and 14 days) iron-starved *T. elongatus* cells with high intensities of blue-green light did not induce any NPQ. In contrast, illumination of *Synechocystis* cells by high intensities of blue-green light induced a reversible decrease of fluorescence intensity. The capacity for induction of NPQ increased in prolonged iron starved *Synechocystis* cells in comparison to iron containing cells (Fig.4.3.). Previously Wilson et al. (2007) showed that iron starvation in *Synechocystis* cells leads to an increase of the OCP to Chl ratio with an increase of blue light-induced NPQ.

Another difference that we observed between the two species was that in iron-starved *Synechocystis* cells the F_0 level increased in comparison to iron replete cells (Fig.4.3.), while in prolonged iron-starved *T. elongatus* cells the F_0 level slightly decreased (data not shown). The F_0

level also depends on the coupling of phycobilisomes: energetically coupled phycobilisomes show low-yield fluorescence emission, while uncoupled phycobilisomes show high-yield fluorescence emission. In *Synechocystis* cells the increase of F_0 is related to a rapid increase of a population of energetically uncoupled phycobilisomes (Wilson *et al*., 2007).
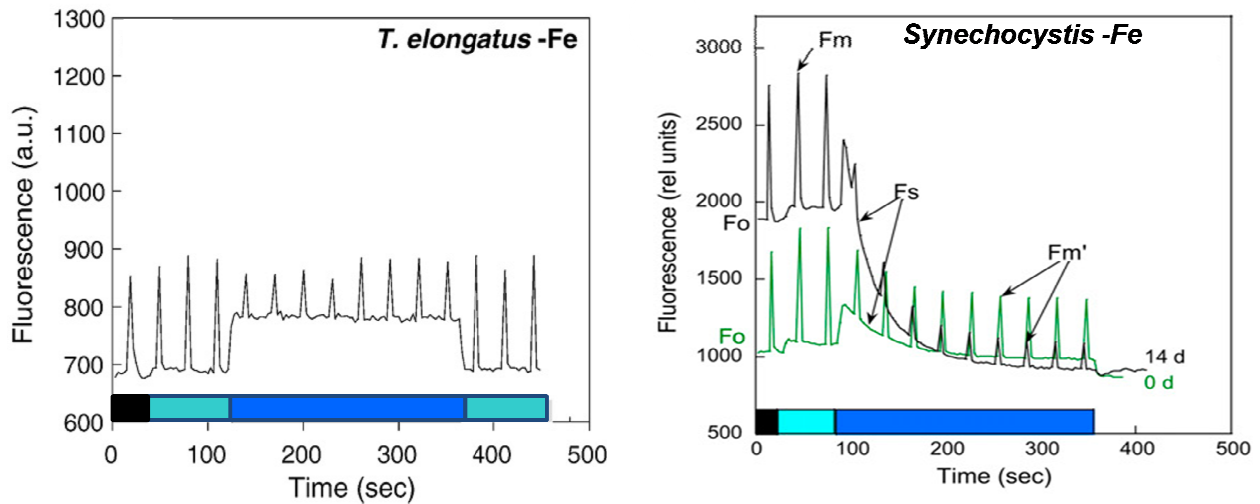
Fig.4.3. Effect of strong blue-green light on iron-starved T. elongatus and Synechocystis cells. Iron-starved T. elongatus and Synechocystis cells (at 3 µg Chl/ml) were dark-adapted and then illuminated successively with low intensity blue-green light (400-550 nm, 80 µmol photons $m^{-2}s^{-1}$) (light blue bar) and high intensity blue-green light (740 µmol photons $m^{-2}s^{-1}$) (dark blue bar). Fluorescence intensity changes were monitored with a PAM fluorometer; saturating pulses were applied to determine the maximum fluorescence levels.

It was also interesting to compare the occurrence of state transitions during iron starvation in *T. elongatus* and *Synechocystis* cells (Fig.4.4.).

Low intensity blue-green light illumination was used to induce the State 1 transition (high fluorescence state), and orange illumination to induce of the State 2 transition (low fluorescence state). State transitions were inhibited in iron-starved cells from both *T. elongatus* (Fig.4.4.) and *Synechocystis* (not shown) cells. F_m' remained almost at the dark F_m level during the blue-green and orange illuminations, which means that cells were locked in one state.

Usually, under normal conditions, in dark-adapted cyanobacterial cells the plastoquinone pool is reduced by redox components of the respiratory electron transfer chain and cells are in State 2. Since the NAD(P)H dehydrogenase is one of the most iron-abundant complexes within the cyanobacterial thylakoid membranes, its down-regulation would result in a minimal contribution of stromal reductants to the intersystem electron flow in iron-stressed cells. This would minimize dark reduction of the PQ pool and maintain iron-stressed cells in State 1 (Ivanov *et al.*, 2006). However, this cannot explain why orange illumination failed to induce State 2 in
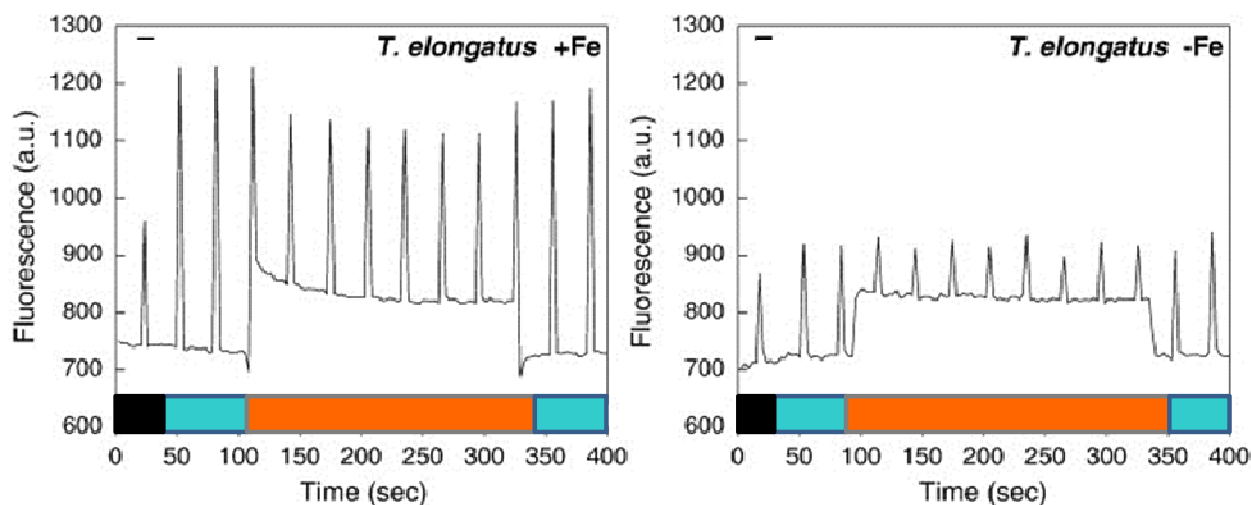
Fig.4.4. State transitions in iron-replete and iron-starved cells of T. elongatus. Dark-adapted cells were illuminated successively with low-intensity blue-green light (400 to 550 nm, 80 µmol·m^{-2}·s^{-1}) (light blue bar) and orange light (600 to 650 nm, 20 µmol·m^{-2}·s^{-1}) (orange bar). Fluorescence intensity changes were monitored with a PAM fluorometer; saturating pulses were applied every 30 s to assess changes in the Fm' intensity.

iron-starved cells. Our results also showed, that when blue light acclimated, iron-starved cells are transferred to orange light, the concentration of closed centers increased, as shown by the increase of the F_s level. It was also proposed that the balance between the monomeric and trimeric forms of PSI plays a role in the regulation of state transitions (Schluchter *et al.*, 1996), with more PSI trimers in State 2 than in State 1. While in normal growth conditions the trimeric form of PSI is predominant, there is a dramatic shift to the monomeric form in iron stress conditions (Sandstrom *et al.*, 2002; Ivanov *et al.*, 2006) that may favor State 1.

Before moving to the next step of the characterization of iron-starved cells that differ in their OCP configuration, it is worth mentioning that after these interesting results it was decided to monitor a few other cyanobacteria species that had or lacked the OCP gene.

Other OCP containing species and species which do not contain an OCP gene homologue confirmed the relation between the presence of this protein and blue light-induced fluorescence quenching.

The expression of full length OCP was confirmed in seven species in which OCP was present. All of these strains were able to perform blue light-induced quenching, confirming the idea that the OCP-related photoprotective mechanism is widespread in cyanobacteria. Four out of seven strains were marine *Synechococcus* strains with lower OCP gene similarity to *Synechocystis* and differerent PB structure and composition. But even in this case high intensities of blue-green light induced NPQ.

In contrast, in the *S. elongatus* which lacks the whole OCP gene, the fluorescence quenching mechanism was absent. Moreover, these results also demonstrated that the strains, which are unable to perform blue light-induced quenching, are more sensitive to photoinhibition (Boulay *et al.,* 2008).

For iron starvation experiments two strains were chosen, one with and another without the OCP gene: *A. maxima*, containing the OCP protein and *S. elongatus* with no OCP protein. Obtained results confirmed our data by demonstrating that blue light-induced fluorescence quenching increases in iron-starved *A. maxima* cells accompanied by an increased F_0 level, while in *S. elongatus* cells iron starvation doesn't induce any quenching. The larger fluorescence quenching corresponded to a higher OCP to Chl ratio (Boulay *et al.,* 2008).

State transitions in both strains under normal and iron starvation conditions followed the same scenario previously observed in *Synechocysti*s and *T. elongatus* (Boulay *et al.,* 2008).

4.1.3. Absorbance and fluorescence spectra of iron-replete and iron-starved cells of
T. elongatus **and** *Synechocystis*

Absorption spectra of iron-replete and iron-starved *T. elongatus* and *Synechocystis* cells were compared. Under normal conditions, the peak at 680 nm is related to Chl *a,* while the peak observed at 630 nm is related to phycobiliproteins (Fig.4.5.).

Iron starvation induces the synthesis of the "chlorophyll-binding-iron-stress induced protein", IsiA, in both species. The presence of IsiA causes a blue-shift in the Chl *a* absorbance peak (680-673 nm) (Laudenbach and Straus, 1988; Burnap *et al.*, 1993). Under iron stress conditions both chlorophyll and phycobilisome level decreased in all cells. Changes induced by iron starvation in the photosynthetic apparatus were described in previous studies (Guikema and Sherman, 1983a; Sandmann, 1985). IsiA can be present in iron-starved cells in two forms. In one case it encircles the PSI reaction center and forms complexes consisting of a trimeric PSI surrounded with 18 IsiA molecules (Bibby *et al.*, 2001; Boekema *et al.*, 2001). By doing this, IsiA increases the absorption cross-section of PSI and acts as an additional antenna complex for PSI (Andrizhiyevskaya *et al.*, 2002; Melkozernov *et al.*, 2003). In the second case, IsiA is present in iron starved cells as empty rings (without PSI). Empty rings of IsiA aggregates usually can be found in prolonged iron starved cells (Yeremenko *et al.*, 2004). These IsiA aggregates, in vitro, are in a strongly quenched state, suggesting that they are responsible for thermal dissipation of absorbed energy (Ihalainen *et al.*, 2005). In cells grown under high light conditions, IsiA is also synthesized to protect the cyanobacterial cells from photodestruction (Havaux *et al.*, 2005). In previous studies it has been proposed that IsiA is involved in the generation of the large blue light-induced NPQ observed in *Synechocystis* under iron starvation (Cadoret *et al.*, 2004). But later, it was shown that this iron-stress induced NPQ was OCP- but not IsiA-dependent (Rakhimberdieva *et al.*, 2007; Wilson *et al.*, 2007).

In iron-starved *T. elongatus* cells, the phycobiliprotein content determined by the absorption at 630 nm, decreased faster than the chlorophyll content at 672 nm. In contrast, iron-starved *Synechocystis* cells show a different reorganisation of the pigments compared to *T. elongatus*: chlorophyll content decreased faster than phycobiliprotein content (Fig.4.5.).
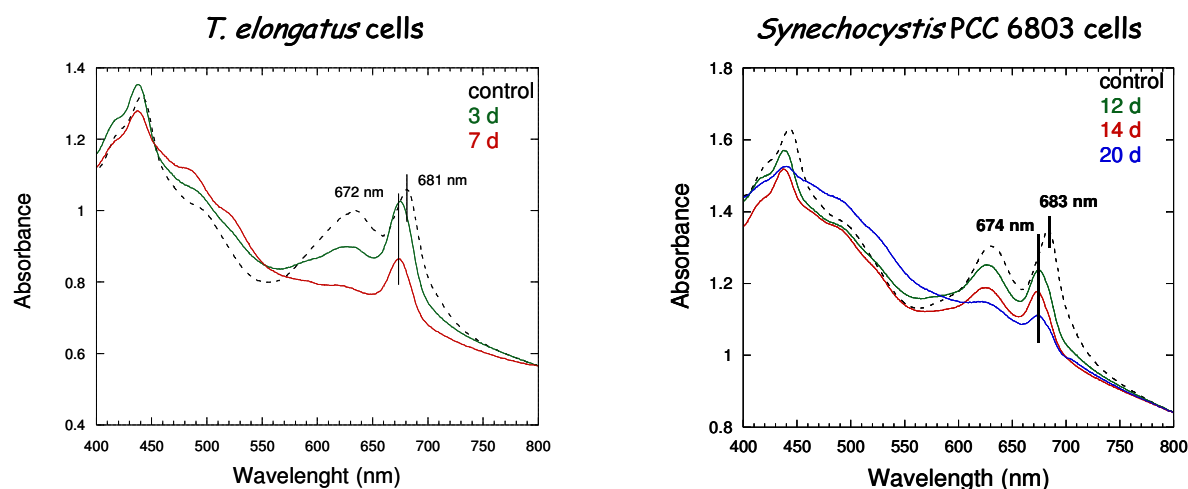
Fig.4.5. Changes in absorption spectra induced by iron starvation in T. elongatus and Synechocystis cells

To further characterize iron starved cells we used 77K fluorescence emission spectra with excitation light at 430 nm, preferentially absorbed by chlorophylls, and at 600 nm, predominantly absorbed by the phycobilisomes.

The 77K fluorescence spectra of *T. elongatus* cells at 430 nm, have bands at 685 nm and 695 nm related to the inner antennae of PSII, CP43 and CP47 respectively (Vandorssen *et al.*, 1987) and a larger PSI related band at 730 nm (Fig.4.6.A).

In the fluorescence spectra of *T. elongatus* cells at 430 nm the first manifestation of iron starvation was an increase in the PSII (685 and 695 nm peaks) to PSI (730 nm peak) ratio. The origin of this increase is related to the fact that the content of PSI which contains 4 Fe-S centers, decreased faster than that of PSII (Guikema and Sherman, 1983b; Sandmann, 1985; Falk *et al.*, 1995). Another possible reason for this can be that the increase of the PSII to PSI fluorescence ratio reflects that the dark-adapted iron-replete cells are in State 2 while the dark-adapted iron-starved cells are in State 1 (Ivanov *et al.*, 2006).

Extended iron starvation resulted in a marked increase of the emission at 685 nm relative to that at 695 nm. The 685-nm fluorescence emission reflects the presence of PSI-less IsiA aggregates (Yeremenko *et al.*, 2004; Wilson *et al.*, 2007) (Fig.4.6.A).

The typical shift of the PSI-related peak from 730 nm to 725 nm was observed in both strains and is related to IsiA complexes surrounding PSI (Burnap *et al.*, 1993; Falk *et al.*, 1995; Park *et al.*, 1999) (Fig.4.6.A).

In the 77K fluorescence emmision spectrum of *T. elongatus* obtained using 600-nm excitation, which is absorbed mainly by phycobilisomes, emission bands related to the phycocyanin (650 nm), allophycocyanin (660 nm), the phycobilisome terminal emitter Lcm (685 nm), PSII (685 and 695 nm), and PSI (730 nm) were observed (Fig.4.6.B). The emission at 685 nm is principally related to the PB terminal emitter and, to a lesser extent to the CP43 antenna of PSII (Redlinger and Gantt, 1982; Vandorssen *et al.*, 1987). Empty IsiA rings (without PSI) in prolonged iron-starved cells also contribute to the peak at 685 nm.

In the fluorescence spectra of *T. elongatus*, generated at 77K by 600-nm excitation, the first manifestation of iron starvation in cells was a decrease in the allophycocyanin (660 nm) fluorescence (Fig.4.6.B). And this is in accordance with absorption spectra which showed faster decrease of phycobiliproteins than chlorophyll.

Iron starvation also resulted in a decrease of fluorescence emission emitted by PSI indicating that in cells containing less phycobilisomes less energy is transferred to PSI (Fig.4.6.B).

The peak at 685 is a little more complicated. A small increase of this peak during the early phases of iron starvation is related to the induction of IsiA, which contributes to this peak. Extended iron starvation resulted in a decrease of the 685-nm peak indicating a decrease in phycobilisome terminal emitter emission, which has a consequence that less energy is transferred to PSII (CP43) which also contributes to this peak (Fig.4.6.B).
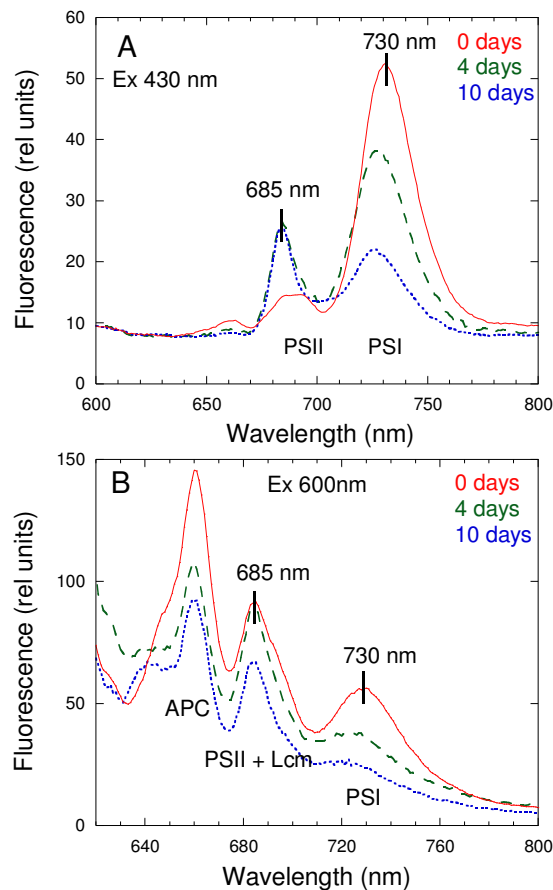
Fig.4.6. Changes in 77K fluorescence emission spectra induced by iron starvation of T. elongatus cells. 77 K fluorescence spectra of T. elongatus cells grown in iron-containing medium (red line) or in iron-lacking medium for 4 (green) and 7 (blue) days. The excitation wavelength was at 430 nm (A) or 600 nm (B). The figure shows a representative iron-starvation experiment. The cells were at 3 µg Chl/ml

In *Synechocystis* cells the 77K fluorescence emission spectrum induced by excitation at 430nm (Fig.4.7.A) is similar to the spectrum of *T. elongatus* using the same excitation wavelength.

In contrast, the fluorescence spectrum of *Synechocystis* induced by 600-nm excitation light (Fig.4.7.B) is very different from the *T. elongatus* spectrum.

In the fluorescence spectrum induced by 600-nm excitation, a large increase at 685 nm was found in iron-starved *Synechocystis* cells (Fig.4.7.B). This increase is related to the phycobilisome terminal emitter and attributed to the accumulation of functionally disconnected phycobilisomes, which show a high fluorescence emission (Wilson *et al.,* 2007). Although iron starvation resulted in a higher ratio of 685 nm emission to 725 nm, in *T. elongatus* cells the 685

nm emission (and 660 nm emission) decreased with the time of iron starvation instead of an increase as observed in *Synechocystis* cells.

Thus, in *T. elongatus* cells phycobiliprotein content decreased faster than chlorophyll content. This is probably essential for the long term survival of the cells in the absence of the photoprotective blue light-induced NPQ mechanism.
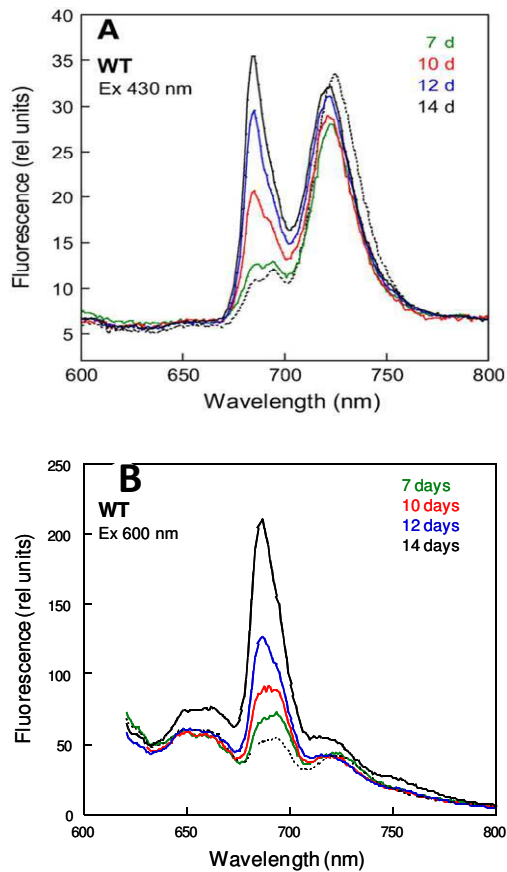
Fig.4.7. Changes in 77K fluorescence emission spectra induced by iron starvation in Synechocystis cells. 77 K fluorescence emission spectra of Synechocystis cells grown in iron-containing medium (dotted line) or in iron-lacking medium for 7 (green), 10 (red), 12 (blue) and 14 (black) days. The excitation wavelength was at 430 nm (A) or 600 nm (B). The figure shows a representative iron-starvation experiment. The cells were at 3 μg Chl/mL (Wilson et al., 2007).

As mentioned above, these observations were extended to other cyanobacterial species with different OCP configurations: *A. maxima* (with OCP) and *S. elongatus* (without OCP).

Absorbance spectra and 77 K fluorescence emission spectra of these cells under iron starvation were measured as well. Absorbance and 77K fluorescence emmission spectra of iron-starved *A. maxima* containing the OCP gene resembled that of *Synechocystis* cells. In *S. elongatus* cells, which have no OCP gene, iron starvation induced similar changes as observed in *T. elongatus* (Boulay *et al.,* 2008).

From these results we can conclude that some changes in the photosynthetic apparatus induced by iron starvation occurred independent of the presence or absence of OCP. Indeed, in the iron-starved cells of all the studied strains containing or lacking OCP, we observed the accumulation of the ring-forming IsiA protein as indicated by the blue shift of the 683 nm Chl absorption peak, shift of the PSI emission peak at 77 K, and a specific increase of the 685 nm 77 K fluorescence emission peak. In addition, the content of PC and Chl as well as the content of PSI and PSII was less in iron-deplete than in iron-replete cells.

Iron-starved *Synechocystis* and *A. maxima* cells show an increased blue light-induced NPQ and have an increased OCP to Chl ratio. *Synechocystis* and *A. maxima* cells after extended iron-starvation have a higher F_0 level and 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. The phycobiliprotein to Chl absorbance ratio decreased during iron starvation of *T. elongatus* and *S. elongatus* cells, whereas it remained constant in *Synechocystis* and *A. maxima* cells. Functionally disconnected phycobilisomes are 'dangerous' for the cells because the energy absorbed by disconnected phycobilisomes cannot be thermally dissipated at the level of the phycobilisomes, or the IsiA complexes (Havaux *et al.,* 2005; Wilson et *al.,* 2007) and therefore oxidative damage (e.g. peroxidation of lipids) may occur. Thus, when cyanobacteria are unable to reduce the amount of energy transferred to the reaction centers by the PBs 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.

4.2. The role of the PsbU protein in the Photosystem II of *Synechococcus* 7942

(Abasova *et al.*, 2011)

4.2.1. Relaxation kinetics of flash-induced fluorescence of the *Synechococcus* WT and ΔPsbU mutant cells

To investigate the role of the PsbU subunit of PSII we monitored the electron transport within PSII in WT and ΔPsbU mutant cells of *Synechococcus.* 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.

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 (Fig.4.8A). In the presence of DCMU the initial amplitude of the fluorescence signal showed the same difference as observed in the absence of DCMU (Fig.4.8.B).

It has been previously shown that the deletion of the PsbU protein in Synechocystis cells results in the accumulation of excitonically decoupled phycobilisomes, which leads to disruption of energy transfer from phycobilisomes to PSII (Veerman *et al.*, 2005). If similar effect (disconnected phycobilisomes) exists also in *Synechococcus* then the decreased PSII fluorescence in the PsbU mutant could be explained by the lack of saturation of PSII, which happens when we use the red actinic and measuring flashes. In order to test if such an effect exists in *Synechococcus*, or not, we performed the same experiment, but in this case using saturating blue actinic flashes (from an external Xenon flash lamp through a blue filter) and blue measuring flashes provided by the fluorescence equipment. These measurements were made both in the absence and presence of DCMU.
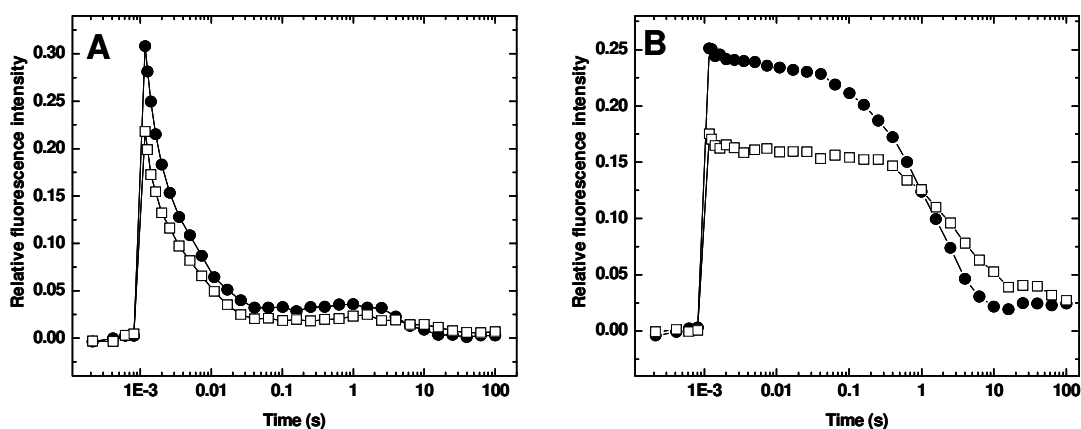
Figure 4.8. Relaxation of flash-induced Chl fluorescence. WT (full circles) and PsbU mutant cells (empty squares) were excited with a saturating single turnover flash at the 1 ms time point. The measurements were performed in the absence of electron transport inhibitors (A), or in the presence of 10 μM DCMU (B). The fluorescence traces were measured using the same amount of Chl (5μg/ml) and shown after shifting the F_0 values to 0.

The new results showed the same difference in the fluorescence amplitude between the ΔPsbU mutant and the WT as obtained with the red actinic and measuring flashes (not shown).

In addition, we have also performed 77 K fluorescence measurements induced by 600 nm excitation light, which is absorbed mainly by the phycobilisomes. These measurements were performed in order to check if there is an increase in the number of uncoupled phycobilisomes in the mutant cells, which can be seen as an increase of the emission peak at 685 nm.

77K fluorescence emission spectra at the same chlorophyll concentration showed no difference between ΔPsbU mutant and WT cells indicating that there are no functionally disconnected phycobilisomes in the Synechococcus ΔPsbU mutant (Fig.4.9.). These data confirm that in our case the decreased flash fluorescence amplitude in the ΔPsbU mutant is not caused by a disturbed energy transfer between the phycobilisomes and PSII, and therefore it reflects a decreased amount of active PSII centers.
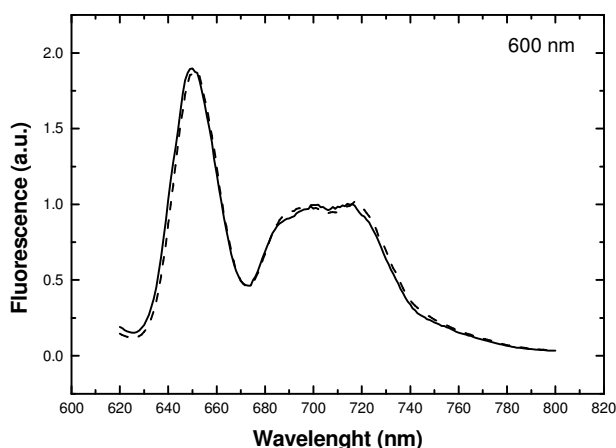
Figure 4.9. 77K fluorescence emission spectra (excitation at 600nm) in WT (solid) and mutant cells (dashed). The fluorescence was measured using saples containing the same amount of Chl.

The relaxation of the fluorescence kinetics, in the absence of DCMU, exhibits three main decay phases, which reflect the reoxidation of Q_A^- in the dark via different routes (Fig.4.10.A, Table 1.) (Vass *et al.*, 1999). After a few minutes of dark adaptation the S_0 and S_1 states are present in proportions of 25:75. An illumination by one flash creates a high number of PSII centers in the S_2 state. The fast and middle phases reflect forward electron transport from Q_A^- to PQ which occupies the Q_B site at the time of the flash, or binds after the flash, respectively. Whereas, the slow phase of the decay originates from back reaction of Q_B^- with the oxidized S_2 state (Fig.4.10.A, Table 1).

In the presence of DCMU, which inhibits the Q_A^- to Q_B electron transfer step fluorescence relaxation is dominated by a slow phase, which reflects the recombination between Q_A^- and the S_2 state of the water oxidizing complex (Fig.4.10.B, Table 1).

In a study by Balint et al. it was reported that the rate of the forward electron transport from Q_A^- to Q_B was not affected by the absence of the PsbU subunit, while the $S_2Q_A^-$ charge recombination process is slowed down in *Synechococcus* 7942 (Balint *et al.*, 2006). However, flash fluorescence data presented in that study were rather noisy, and the different phases of the fluorescence decay could not be clearly resolved.

According to our high resolution data, in the absence of DCMU, the time constant and relative amplitude of the fast phase are similar in the WT and the PsbU mutant, whereas the middle phase is slightly slower in the PsbU-less mutant. The slow phase is significantly slower in the mutant than in the WT (Fig.4.10.A, Table 1).

In the presence of DCMU, the main fluorescence decay component, which reflects the recombination of Q_A^- with the S_2 state is slower in the mutant (T_3 ~3.4 s) than in the wild type (T_3 ~1.6 s) , which is in agreement with previous data (Balint *et al.*, 2006) (Fig.4.10.B, Table 1).

There is a 3-8 % fast component in the presence of DCMU. We have confirmed that this fast component is not due to a flash artifact, and could not be abolished by further increase of the DCMU concentration to 50 µM. Although at this stage we can not exclude that DCMU binding may not be complete in a small fraction of PSII reaction centers, it is more likely that the fast phase arises from the recombination of Q_A^- with $Y_Z^{+\bullet}$ (Cser *et al.*, 2005).

Fluorescence relaxation in the presence of DCMU contains also a non-decaying part. The non-decaying fraction is significantly larger in the ΔPsbU mutant (A_0 ~ 25%) than in the WT (A_0 ~ 6%) (Fig.4.10.B, Table 1).
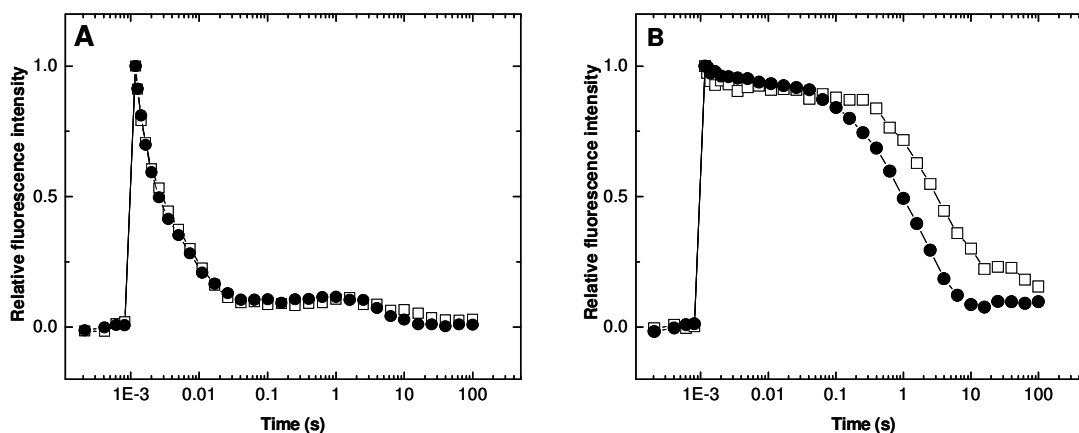
Figure 4.10. Relaxation of flash-induced Chl fluorescence. WT (full circles) and ΔPsbU mutant cells (empty squares). The measurements were performed in the absence of electron transport inhibitors (A), or in the presence of 10 µM DCMU (B). The fluorescence traces were shown after normalization to identical initial amplitudes.

Our high resolution flash fluorescence data confirm the earlier finding that the absence of the PsbU subunit does not affect forward electron transport from Q_A^- to Q_B, but induces a significant slowdown of the Q_A^- reoxidation via charge recombination with the S_2 state in the presence of DCMU (Balint *et al.*, 2006).

The time constant of the $S_2Q_A^-$ decay was 2.1 times larger in the ΔPsbU mutant than in the WT (Table 1). Moreover, in the absence of DCMU, the slow phase of the fluorescence relaxation, which reflects the $S_2Q_AQ_B^-$ recombination via charge equilibrium with the $S_2Q_A^-Q_B$ state is also slower in the mutant. The time constant of the $S_2Q_B^-$ decay was 1.5 times larger in the ΔPsbU mutant than in the WT (Table 1).

	Fast phase	Middle phase	Slow phase	Non-decaying phase
Strain				
	T_1 (ms)/A_1 (%)	T_2 (ms)/A_2 (%)	T_3 (ms)/A_3 (%)	A_0 (%)
		No addition		
WT	0.73±0.01/30.9±1.7	8.6±0.3/51.2±2	6.9±0.6/17.9±0.7	0±0
PsbU mutant	0.71±0.1/36.1±4.7	10.8±1.3/47.3±4	10.1±3.8/16.1±1.7	0.5±0.5
		+DCMU		
WT	0.6±0.2/3.5±0.23	-/-	1.6±0.1/90±3.3	6.5±3.3
PsbU mutant	0.4±0.1/8±1.1	-/-	3.4±0.31/68±1.6	24±1.3

Table 1. Kinetic parameters of flash-induced fluorescence traces. Fluorescence was measured and analyzed as described in the Materials and Methods. The standard errors were obtained from averaging three different measurements obtained on biologically different cultures. The T_1,..,T_3 values are the time constants of the decay phases, whereas the A_1,..,A_3 values are the relative amplitudes. The A_0 values shows the fraction of fluorescence, which does not decay after the flash.

These values reflect an 18 and 10 meV increase in the free energy of the stabilization of the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs, respectively, in the ΔPsbU mutant relative to the WT. The energetic stabilization of the charge pairs was calculated by assuming that the decay of the charge separated states is dominated by a thermally activated process, which obeys the Boltzmann equilibrium between the free energy levels of the charge separated state and of the primary radical pair. The relative change of the free energy of the stabilization was obtained by using the $\Delta G_{PSBU<->WT} = kT \ln(T_{3,PSBU}/T_{3,WT})$ formula, where k corresponds to the Boltzmann constant and T corresponds to the temperature. The T_3 values denote the time constants of the respective decay processes.

$S_2Q_A^-$ and $S_2Q_B^-$ stabilization is the consequence of the changes in the free energy span between the $P_{680}^+Phe^-$ radical pair state and $S_2Q_A^-$ and $S_2Q_B^-$ charge separated states, respectively. A decrease in the free energy of $P_{680}^+Phe^-$ can lead to the energetic stability of the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs. Stabilization of $S_2Q_B^-$ and $S_2Q_A^-$ can also arise from an increased free energy level of the S_2, or the Q_A, Q_B states.

Both, $S_2Q_A^-$ and $S_2Q_B^-$ recombination pathways involve $P_{680}^+Phe^-$ and the S_2 states. But the two acceptor states, Q_A and Q_B, are different. A difference in the stabilization of $S_2Q_A^-$ and $S_2Q_B^-$ can give us information about the free energy (redox potential) change at the level of the quinone electron acceptors. 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 ($\Delta G(Q_A <-> Q_B) = kT \ln(T_{3,no\ add}/T_{3,DCMU})$). This ratio is different in the WT and mutant strain indicating that the $Q_A <-> Q_B$ 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 ΔPsbU mutant cells, respectively.

The decrease of the $Q_A <-> Q_B$ free energy (redox) gap in the mutant as compared to the WT is in agreement with previous work (Veerman *et al.*, 2005), which showed that in a significant number of PSII reaction centers Q_A remained reduced in the mutant in the dark. The decrease of the $Q_A <-> Q_B$ free energy (redox) gap results in reduction of Q_A by an electron from Q_B^- or the PQ pool with closed PSII centers as a result.

It has been demonstrated in previous studies that inactivation of the *psbU* gene resulted mostly in structural destabilization of the donor side of PSII (Shen *et al.*, 1997; Shen *et al.*, 1998; Nishiyama *et al.*, 1999). However, our data show, in agreement with previous findings (Veerman

et al., 2005), that the absence of the PsbU subunit modifies not only the OEC, but also the acceptor side components of PSII. There are similar studies, showing that structural changes at the PSII donor side can affect the redox potential of acceptor side components like in the case of the removal of the PsbO subunit (Vass *et al.*, 1992a), or of the Mn cluster (Krieger-Liszkay and Rutherford, 1998).

Another interesting feature of the ΔPsbU mutant is the non-decaying part of fluorescence in the presence of DCMU. This indicates that in these PSII reaction centers Q_A remained reduced because the S_2 state of the OEC was reduced by another donor. This donor can be Y_D (or Tyr$_D$), which acts as alternative electron donor to P_{680}^+. Y_D is usually in the oxidized radical state ($Y_D^{+\bullet}$), but can be in reduced form in the PsbU mutant. In normally functioning PSII Y_D is found in the oxidized state in dark, and, therefore cannot donate an electron to the S_2 state. The stability of $Y_D^{+\bullet}$ is due to the highly hydrophobic nature of the protein environment around the Y_D pocket (Vass and Styring, 1991). In the ΔPsbU mutant the modification of the donor side can alter the protein environment of the Mn cluster and Y_D, which in turn can make $Y_D^{+\bullet}$ more accessible to exogenous reductants. Reduction of $Y_D^{+\bullet}$ during dark adaptation before the fluorescence measurement can produce Y_D, which acts as a stable electron donor to P_{680}^+ by competing with donation by the Mn cluster. Another explanation for the increased non-decaying part of fluorescence in the mutant could be that a high number of the PSII centers are in S_0 state in the dark. In this case one-flash illumination will create non-recombining $S_1Q_A^-$ state in the presence of DCMU.

4.2.2. Thermoluminescence characteristics of the *Synechococcus* WT and ΔPsbU mutant cells

In our study, in addition to flash induced chlorophyll fluorescence measurements, thermoluminescence measurements were used.

The various types of charge pairs, created and stored by an illumination at low temperature, can be revealed as successive thermoluminescence bands on progressively warming the sample (Vass and Govindjee, 1996; Ducruet, 2003; Vass, 2003; Rappaport *et al.*, 2005). Thermoluminescence results from excited P_{680}^* which is created by recombination between a previously light-separated charge pair. Charge pairs are formed between positively charged (+)

carriers on the donor side of photosystem II and electrons (−) on the acceptor side of photosystem II. Charge pairs can be stored as (i) electron on Q_A (as Q_A^-) when PSII centers are closed, or electron on Q_B (as Q_B^-) when PSII centers are open, (ii) four + holes are stored as S_{1+}, S_{2+}, S_{3+} and S_{4+} states of the donor side, of which only the S_2 and S_3 states can recombine with Q_A^- or Q_B^-.

For TL measurements a dark-adapted sample is cooled to a temperature of +5°C and then illuminated. A single turnover flash produces a charge pair, which is stabilized as the luminescence-emitting state $S_2Q_B^-$ (Demeter and Vass, 1984). Subsequent heating of the illuminated sample from −40 °C to 80 °C induces a temperature dependant luminescence emission, which results in the so called B band of thermoluminescence in the 30 °C temperature range. Blocking the Q_B pocket by DCMU and giving a saturating flash at -10 °C results in charge stabilization of the $S_2Q_A^-$ state, whose recombination leads to the Q band (Droppa *et al.*, 1981).

However, *Synechococcus* cells were sensitive to freezing, which led to the loss of TL signal intensity (not shown), and made it even undetectable in the ΔPsbU mutant in the absence of DCMU (Balint *et al.*, 2006). In order to avoid this freezing effect, the standard protocol was changed and TL curves were recorded from 0 to 80 °C (with a saturating flash at +5 °C) in the absence of DCMU, and from -4 to 80 °C (with saturating flash at -2 °C) in the presence of DCMU.

The B band, which arises from the $S_2Q_B^-$ recombination (Demeter and Vass, 1984) appeared at 29 °C in the WT cells. In the ΔPsbU mutant the position of this band was up-shifted to a higher temperature (33 °C) (Fig.4.11A).

In the presence of DCMU, the Q band was observed at around 10 °C in the WT. In the mutant, the peak position was shifted to a higher temperature and appeared at ≈29 °C (Fig.4.11B).

In a previous study, which was performed with the same ΔPsbU mutant as we used here, an upshift of the Q band was detected (Balint *et al.*, 2006), indicating an increased stability of $S_2Q_A^-$ state in the mutant compared to the WT. Our TL measurements confirmed these results. In contrast, the B band, which arises from the $S_2Q_B^-$ recombination, could not be detected in the earlier study by (Balint *et al.*, 2006) because of the sample freezing effect.

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 larger peak temperature shift for the Q band than for the B band reflects the increased stabilization of the $S_2Q_A^-$ recombination as compared to that of $S_2Q_B^-$, which is in agreement with the fluorescence data discussed above (Fig.4.10, Table 1). The upshift of both TL bands (Q and B) has also been found in *Synechocystis* 6803 mutants, which lack the PsbU subunit (Shen *et al.*, 1997).
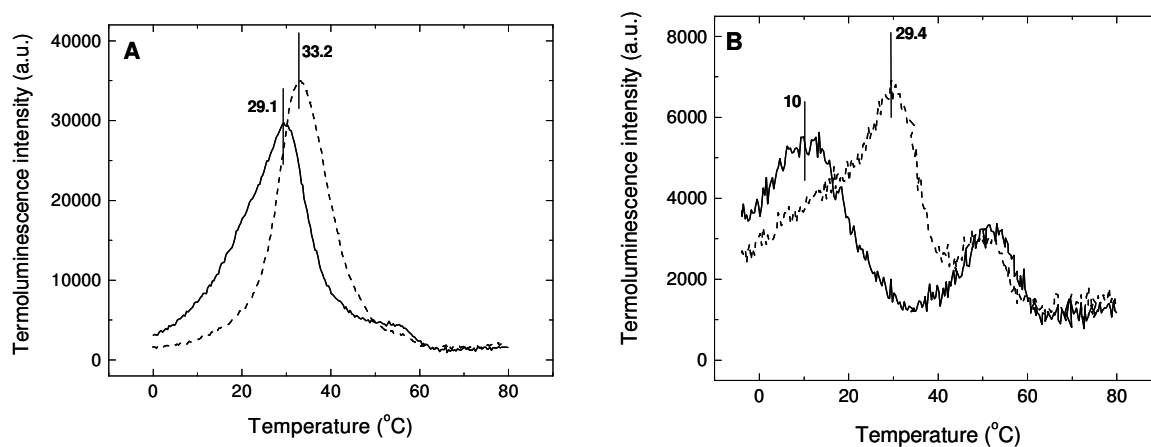
Figure 4.11. Flash-induced thermoluminescence. WT (full line) and mutant cells (dashed line) were excited with a saturating single turnover flash either at T = 5 ºC in the absence of DCMU followed by a TL measurement from 0 to +80ºC (A), or at T = -2 ºC in the presence of 10µM DCMU followed by a TL measurement from -4 to +80 ºC (B). The TL curves were measured using samples containing the same amount of Chl.

A characteristic feature of the ΔPsbU mutant is the increased TL intensity both in the presence and absence of DCMU. The Q band position in the WT (+10 °C) is close to the excitation temperature (- 2 °C) and also to the starting temperature of the measurement (- 4 °C). Due to this the Q band amplitude could be partially lost during initial phase of the TL measurement in the WT (Fig.4.11B). However, loss of the signal cannot happen in the absence of DCMU, where the TL bands are at around or above +30 °C, which allows stabilization of the separated charges at the excitation temperature (Fig.4.11A). As a consequence, the increased

intensity of the B band in the ΔPsbU mutant reflects a real effect due to modified charge recombination characteristics.

The TL intensity was measured at the same chlorophyll concentration in WT and mutant cells. But we have to take into account that at the same chlorophyll concentration the amount of functional PSII centers is about 70 % in the PsbU mutant in comparison with the WT, as shown by the initial amplitude of the flash fluorescence signal (Fig.4.8.). When the TL intensity is normalized to the flash fluorescence amplitude of the particular strain, the B band intensity appears to be 1.7 -fold higher in the ΔPsbU 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$^-$ (Cser and Vass, 2007a). In these studies, mutants were used in which the free energy level of Phe and P_{680} was modified by site directed mutagenesis of amino acid residues near Phe and/or P_{680} (Klug et $al.$, 1998). When either E_m(Phe/Phe$^-$), or E_m(P_{680}^+/P_{680}) is shifted to more negative values the free energy gap decreases, and consequently the recombination rate increases. In contrast, a shift of E_m(Phe/Phe$^-$) or E_m(P_{680}^+/P_{680}) to more positive values increases the free energy gap. Thus, the recombination rate of P_{680}^+Phe to P_{680}^* will be decreased. In the mutants, in which the free energy of the primary radical pair was shifted to more negative values, the TL intensity increased and a strong stabilization of charge recombination of both the $S_2Q_A^-$ and $S_2Q_B^-$ states was observed as shown by the slow down decay of the flash induced chlorophyll fluorescence signal and the upshifted thermoluminescence peak temperatures (Cser and Vass, 2007a). In contrast, in mutants in which the free energy of the primary radical pair was shifted to more positive values charge recombination of the $S_2Q_A^-$ and $S_2Q_B^-$ states was accelerated (demonstrated by the faster decay of chlorophyll fluorescence yield, and the downshifted peak temperatures of the thermoluminescence Q and B bands) and TL intensity decreased relative to the WT (Cser and Vass, 2007a).

Based on the data discussed above the increased TL amplitude of the ΔPsbU mutant (1.7 fold higher than in the WT), which is accompanied by a slower $S_2Q_A^-$ and $S_2Q_B^-$ recombination rate (Fig.4.10., Table 1), is indicative of a decreased free energy level of the P_{680}^+Phe$^-$ primary charge separated state. Although the amplitude of the Q band was also significantly higher in the ΔPsbU mutant than in the WT, its intensity could not be reliable due to the possible loss of the

signal intensity during the initial phase of the TL measurement as discussed above. Therefore, we based our calculation on the increase of the TL intensity in the absence of DCMU by using the $\Delta G = kT \ln(TL_{PSBU}/TL_{WT})$ formula (Cser and Vass, 2007a) (where k, T, and TL are the Boltzmann constant, the temperature and TL intensities, respectively), and it resulted in a 14 meV decrease of the free energy level of $P_{680}^+Phe^-$ relative to P_{680}^*.

4.2.3. High light sensitivity of the ΔPsbU mutant cells

The sensitivity of the WT and the ΔPsbU strains to photoinhibition was checked by illuminating cells with strong light (500 µmol photons m^{-2} s^{-1}) for 1.5 hour (Fig.4.12.). The main target of photoinhibition is the D1 protein of PSII. Our experiments were performed in the presence and absence of the protein synthesis inhibitor lincomycin, which blocks the synthesis of new D1 protein and thus prevents the replacement of the damaged one.

Our photoinhibition experiments with lincomycin showed that the *Synechococcus* ΔPsbU mutant has an increased susceptibility to light (Fig.4.12.A).

In the absence of lincomycin the rate of inactivation of PSII is usually smaller than in the presence of lincomycin. This is related to the ability of cells to synthesize new D1 protein during the light treatment. Moreover, if cells are brought back to normal growth light conditions, full recovery of oxygen-evolving activity occurs. In the absence of lincomycin, the ΔPsbU mutant was found to be more sensitive to high light, showing a larger decrease of oxygen-evolving activity than the WT. We also compared the ability of PSII in WT and mutant cells to repair the damage. Despite of the fact, that the ΔPsbU 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 (Fig.4.12.B).

The larger extent of high light induced loss of PSII activity in the ΔPsbU mutant as compared to the WT was also confirmed by the variable fluorescence data, calculated from the initial amplitude of flash-induced fluorescence decay (not shown).
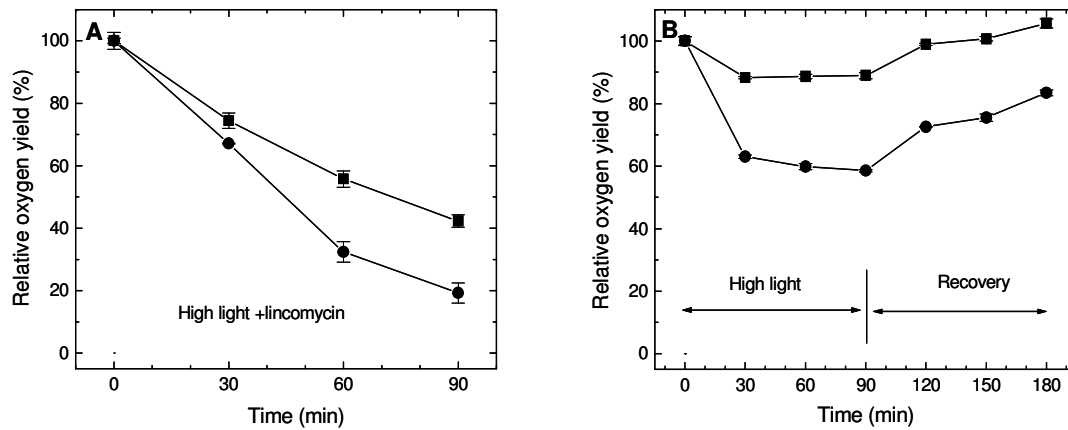
Figure 4.12. Light induced inhibition of PSII activity. WT (full squares) and mutant cells (full circles) of Synechococcus 7942 were exposed to 500 µmol photons m^{-2} s^{-1} visible light in the presence of lincomycin (A), or its absence (B). After 90 min high light treatment the cells were transferred back to the growth light intensity 40 µmol photons m^{-2} s^{-1} in order to let the PSII activity recover.

Our photoinhibition experiments with lincomycin show that in the *Synechococcus* ΔPsbU mutant PSII is more susceptible to light (Fig.4.12.A). Similar results have been obtained earlier in the PsbU-less mutant of *Synechocystis* (Inoue-Kashino *et al.*, 2005*)*. However, in the absence of lincomycin, the *Synechocystis* mutant was inhibited to a similar extent by photoinhibition as the WT. This result showed that the repair of the D1 protein can fully compensate the increased sensitivity to light, which is induced by the lack of PsbU in *Synechocystis* (Inoue-Kashino *et al.*, 2005). In our experiments without lincomycin, the light susceptibility of the *Synechococcus* ΔPsbU mutant was still larger than that of the WT (Fig.4.12.B). This sensitivity of PSII in the ΔPsbU mutant cells in the absence of lincomycin shows that the capacity of the cells to repair PSII cannot keep up with the rate of damage during high light exposure in the absence of the PsbU subunit in *Synechococcus*. However, the ΔPsbU mutant can completely recover its PSII activity when the cells are transferred to the growth light intensity following the high light treatment (Fig.4.12.B). In agreement with this data it has been shown that the D1 protein level is similar in the WT and the ΔPsbU mutant under standard growth conditions in the absence of protein synthesis inhibitor (Balint *et al.*, 2006).

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 (Vass, 2011) as well as inactivation of the Mn cluster (Hakala *et al.*, 2005; Ohnishi *et al.*, 2005; Murata *et al.*, 2007).

PsbU is located at the donor side of PSII and it is obvious that the part of the light induced inhibition is due to an increased light sensitivity of the Mn cluster. But, we have to consider that the ΔPsbU mutant has a functional donor side, showing only a small modification of the charge stabilization properties. Therefore, increased light sensitivity of the mutant could be related not only to enhanced light susceptibility of the Mn cluster but also to other modifications of PSII.

In a previous study it has been shown that this *Synechococcus* ΔPsbU mutant possesses a largely enhanced antioxidant capacity, which suggests that there is an increased formation of reactive oxygen species in PSII in the absence of the PsbU protein (Balint *et al.*, 2006).

Several studies have shown that H_2O_2 can be produced instead of O_2 in PSII which lack extrinsic proteins (Schroder and Akerlund, 1986). H_2O_2 can further produce hydroxyl radicals by transition metal-catalyzed reduction. Balint et al. showed that deletion of *psbU* in *Synechococcus* cells resulted in increased activity of KatG, an enzyme belonging to the group of prokaryotic catalase-peroxidases, as well as to a higher activity of cellular peroxidase(s) (Balint *et al.*, 2006). This could mean that there is an increased H_2O_2 production in the ΔPsbU mutant, which might be the reason for its light sensitivity. But, the enhanced antioxidant capacity of the ΔPsbU mutant may not necessarily help the cells to cope with all the consequences of H_2O_2 production due to donor side modification. The electron transport characteristics of the ΔPsbU mutant indicated an increased stability of the $S_2Q_B^-$ and $S_2Q_A^-$ states with a significantly increased intensity of the B and Q TL-bands (Fig.4.10., 4.11.). Interestingly, the *Synechocystis* mutant (D1-Gln130Leu) with similar electron transport characteristics and an increased TL intensity just as the ΔPsbU mutant studied here also shows an increased light sensitivity (Cser and Vass, 2007b). In the case of this mutant the free energy gap of the primary radical pair was shifted to more negative values. Based on the similar charge recombination characteristics of the D1-Gln130Leu mutant of *Synechocystis* and the *Synechococcus* ΔPsbU mutant studied here we can suggest that the increased light sensitivity of the ΔPsbU mutant has at least a partly similar background as that of the D1-Gln130Leu mutant.

The modulation of photoprotection by the free energy level of the primary radical pair has been explained by the modification of the rate of a nonradiative charge recombination pathway from the singlet state of the primary radical pair, $^1[P_{680}{}^+Phe^-]$, which is slowed down when the Phe redox potential is increased (D1-Gln130Leu) and accelerated when the Phe redox potential is decreased (D1-Gln130Glu) (Cser and Vass, 2007a; Vass and Cser, 2009). The non-radiative recombination of $^1[P_{680}{}^+Phe^-]$ competes with the recombination pathway that proceeds from $^3[P_{680}{}^+Phe^-]$ and produces $^3P_{680}$ whose interaction with ground state molecular oxygen leads to the production of highly reactive singlet oxygen (Fig.4.13.).
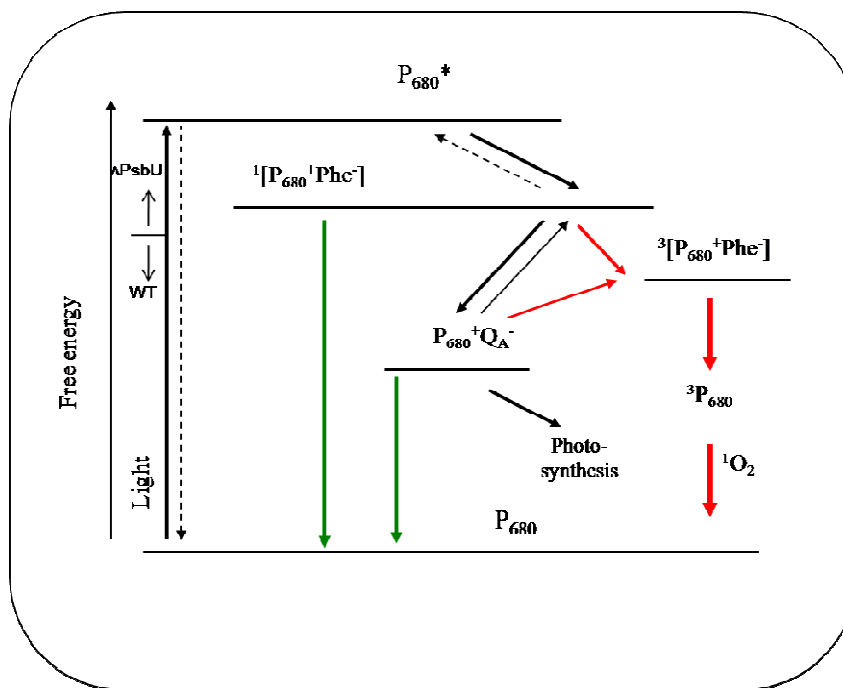
Figure 4.13.Charge recombination-induced photoprotection (green arrows) and photodamge (red arrows) of PSII. Deletion of the PsbU protein may modify the free energy level of the $P_{680}{}^+Phe^-$ primary charge separated state toward the negative values, which change the free energy gap between $P_{680}*$ and $P_{680}{}^+Phe^-$. This in turn may lead to the increased recombination rate of $P_{680}{}^+Phe$ to $P_{680}{}^*$ with increased $^3P_{680}$ production and as a consequence high singlet oxygen.

Our data (Abasova *et al.*, 2011) suggests that the absence of the PsbU protein induces modifications not only at the donor side but also at the acceptor side of PSII. 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 (Fig.4.13.). This in turn leads to charge recombination that forms $^3P_{680}$ (Vass and Cser, 2009; Vass, 2011), which induces the production of harmful singlet oxygen that causes oxidative damage of PSII.

As discussed above the lack of the PsbU protein induces to some extent different effects in *Synechocystis* and *Synechococcus*. These effects may be due to differences in the fine structure of the PSII complex, which may lead to differences in the consequences of the lack of the PsbU subunit, and point to the necessity of caution when generalizing the conclusions about the role of PsbU obtained with a particular species.
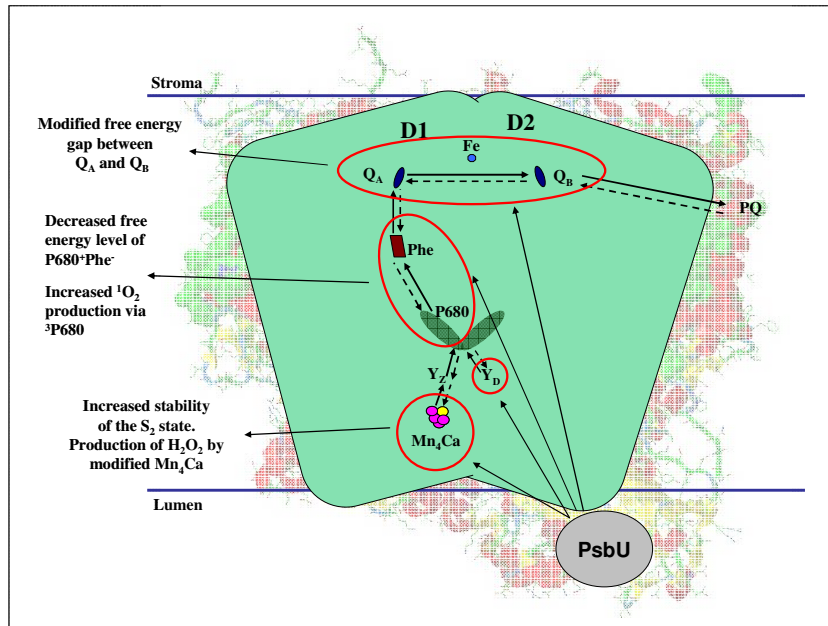
Figure 4.14. Consequences of the lack of PsbU in the PSII complex of Synechococcus 7942.
The scheme shows the main sites where the lack of PsbU affects PSII electron transport characteristics, as well as light sensitivity. The main sites of interest are: (i) The vicinity of the Mn cluster, whose disturbance may lead to H_2O_2 production and decreased stability of the alternative Tyrosine-D electron donor (Y_D). (ii) The vicinity of P_{680} and/or Phe, whose disturbance may lead to decreased free energy level of the $P_{680}{}^+Phe^-$ primary radical pair, which can initiate increased singlet oxygen production. (iii) The vicinity of the Q_A and/or Q_B quinone electron acceptors, which results in decreased free energy gap between Q_A and Q_B.

5. 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 strains which contain the complete OCP gene show a blue light-induced quenching of fluorescence under normal and iron-stress conditions.

    In contrast, 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 species, 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 charachterization 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 (Balint *et al.*, 2006).

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 ($\approx$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 lumenal part of the complex. The photosensitivity of the ΔPsbU *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.

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KIVONAT

**A NARANCSSÁRGA KAROTENOID KÖTŐ FEHÉRJE
(*O*RANGE *C*AROTENOID *P*ROTEIN) ÉS A PsbU FEHÉRJE SZEREPE
A CIANOBAKTÉRIUMOK FÉNY ELLENI VÉDELMÉBEN**

PhD disszertáció összefoglaló

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BEVEZETŐ

A napenergia kémiai energiává való átalakításával a növények, algák és cianobaktériumok létfontosságú szerves szénvegyületekkel és oxigénnel látják el a Földet. Az átalakítás folyamatát oxigéntermelő fotoszintézisnek nevezzük. A fotoszintézis elsődleges reakciói a tilakoid membránba ágyazódott pigment-fehérje komplexekben, más néven a fotokémiai rendszerekben zajlanak (*P*hoto*S*ystem II és *P*hoto*S*ystem I, PSII és PSI). A cianobaktériumokban a fényt a fikobiliszómák (*P*hyco*B*ilisomes, PBS) nyelik el, amelyek a

tilakoid membránok külső felszínén helyezkednek el (Glazer, 1984; Grossman *et al.*, 1993; Tandeau de Marsac, 2003; Adir, 2005).

Ha a fotoszintézishez szükségesnél több fény éri a fotoszintetikus szervezetet, akkor a reaktív oxigénformák képződése miatt ez káros lehet az illető szervezetre (Aro *et al.*, 1993; Melis, 1999). A cianobaktériumok, a növényekhez és algákhoz hasonlóan, többféle fényvédelmi mechanizmussal rendelkeznek. Három fő élettani folyamat ismert, amelyek részt vesznek a fotoszintetikus apparátus változó fényviszonyokhoz való alkalmazkodásában: energia disszipációs hatékonysági állapot átmenet (state transition) (Mullineaux *et al.*, 1997; Schluchter *et al.*, 1996), a károsodorr D1 fehérje kijavítása (Aro *et al.*, 1993), és a nem fotokémiai fluoreszcencia-kioltás (*N*on-*P*hotochemical *Q*uenching, NPQ) (Kirilovsky, 2007).

A narancssárga karotenoid kötő fehérje

A fölösleges fényenergia hőként való kibocsátását nem fotokémiai kioltásnak nevezik (NPQ). A *Synechocystis sp.* PCC6803 (a továbbiakban röviden *Synechocystis*) nevű cianobaktériumban a fotoszintetikus reakciókat telítő kék-zöld fény indukálja a fikobiliszómák által elnyelt fölösleges fényenergia hőként való kibocsátását, aminek következményeként a fikobiliszómák és a reakciócentrumok közti energiaátadás csökken. Ebben a folyamatban játszik szerepet a narancssárga karotenoid kötő fehérje (*O*range *C*arotenoid *P*rotein, OCP). OCP hiányában az erős fehér vagy kék fény által indukált fluoreszcencia-kioltás nem történik meg a fotoszintetizáló sejtekben (Wilson *et al.*, 2006). Olyan stressz körülmények közt, mint például a vashiány, a kék-zöld fény nagymértékű fluoreszcencia-kioltást okoz, ami sokkal jelentősebb mértékű, mint amit stresszmentes környezetben tapasztalunk. Kimutatták, hogy a nagymértékű fluoreszcencia-kioltás összefüggésbe hozható a magas OCP koncentrációval (Wilson *et al.*, 2007; Boulay *et al.*, 2008). Az OCP szerepét a fikobiliszómák hő kibocsátás által történő fényvédelmi mechanizmusában egyelőre csak *Synechocystis*-ben vizsgálták, és nem ismert, hogy ez a fontos folyamat mennyire gyakori jelenség más cianobaktériumokban. A rendelkezésre álló cianobakteriális genom adatbázisokat átnézve a fikobiliszómát tartalmazó cianobaktériumok legtöbbjében találtak olyan géneket, amelyek a *Synechocystis* OCP-vel homológ fehérjéket kódolnak. Ezek közül néhány baktérumtörzs nem hordozza a teljes génszekvenciát. Ilyen például

a *Termosynechococcus elongatus*, amelynek genomja csak az OCP-t kódoló N- és C-terminális részeket tartalmazza.

Munkánk során azt vizsgáltuk, hogy milyen szerepe van az OCP-nek a kék fény által indukált nem fotokémiai fluoreszcencia-kioltásban vashiányos és vassal ellátott optimális környezetben. Ehhez a következő fotoszintetikus modellszervezeteket használtuk: *Arthrospira maxima (A. maxima)* és *Synechocystis*, amelyek tartalmazzák a teljes, OCP-t kódoló gént; *Synechococcus elongatus* PCC 7942 *(S. elongatus)*, amelyből hiányzik az OCP-t kódoló gén; és *Termosynechococcus elongatus (T. elongatus)*, amely az OCP-t kódoló gén N- és C-terminális szakaszait tartalmazza.

A PsbU fehérje szerepe

Az oxigénfejlesztő komplex (**O**xygen **E**volving **C**omplex, OEC) a kettes fotokémiai rendszer (**P**hoto**S**ystem II, PSII) lumenális oldalán helyezkedik el és főként különböző oxidációs állapotban lévő Mn, valamint Ca^{2+} és Cl^- ionok alkotják. A közvetlen közelében elhelyezkedő perifériális membránfehérjék szerepe a Ca^{2+} és Cl^- ionok, mint létfontosságú kofaktorok optimális szinten való tartása a hatékony vízbontás érdekében. A cianobakteriális PSII komplexek 5 perifériális fehérjét tartalmaznak: PsbO, PsbP, PsbQ, PsbU, és PsbV. A PsbU fehérje a PSII 12 kDa nagyságú perifériális alegysége, amely kristályszerkezeti vizsgálatok szerint a PsbO és a PsbV között helyezkedik el, és a legtöbb érintkezési pontja ezekkel a fehérjékkel van. A PsbU szerepe a Mn-komplex (Shen *et al.*, 1997), és a PSII szerkezeti stabilitásának fenntartása. Ezen alegység hiányában csökken a fotoszintetikus sejtek oxigéntermelése és jelentős fényérzékenység alakul ki. Magas hőmérséklethez való alkalmazkodás során a PsbU jelenléte hozzájárul az oxigéntermelő komplex hőstabilitásához (Nishiyama *et al.*, 1997; Nishiyama *et al.*, 1999). A fehérje eltávolítása nemcsak a lumenális oldalra, hanem a PSII sztróma felöli oldalára is hatással van. Kimutatták, hogy a PsbU hiánya változásokat okoz az elsődleges fotokémiai folyamatokban, miközben szabadfikobiliszóma felhalmozódás tapasztalható és csökken a fikobiliszómák és a PSII közti energiaátadás (Veerman *et al.*, 2005). A közelmúltban kimutatták azt is, hogy a PsbU-val nem rendelkező *Synechococcus* PCC7942 sejtek magasabb érzékenységet mutatnak oxidatív stresszel szemben, mint a vad típusú sejtek (Balint *et al.*, 2006).

A PsbU fehérje fényvédelemben betöltött szerepének tisztázásához a *Synechococcus* PCC7942 (*Synechococcus*) baktériumtörzs ΔpsbU deléciós mutánsát vizsgáltuk.

CÉLKITŰZÉSEK

A munkánk fő céljai a kék fény által indukált nem fotokémiai fluoreszcencia-kioltás mechanizmusának jobb megismerése, valamint a cianobakteriális PSII PsbU alegysége szerepének feltérképezése voltak. A részletes célkitűzéseink a következők:

1. Az OCP jelenléte és a kék fény által indukált fluoreszcencia-kioltás közti korreláció vizsgálata.

2. A vaselvonás hatásainak feltérképezése a nem fotokémiai fluoreszcencia-kioltásra, valamint az állapot átmenetre (state transition), OCP-t kódoló génnel rendelkező, illetve azt részben vagy teljes egészében nélkülöző cianobaktériumokban.

3. A cianobakteriális PSII komplex PsbU perifériális fehérje szerepének tanulmányozása a fénykárosítás elleni védelemben *Synechococcus* PCC7942 ΔpsbU deléciós mutánsa segítségével.

ANYAGOK ÉS MÓDSZEREK

Az irodalomban használt standard, illetve a disszertáció alapjául szolgáló publikációkban leírt módszerek:
- § Cianobaktériumok nevelése
- § Fe^{2+} elvonás
- § Abszorbancia mérések
- § Klorofill tartalom meghatározása
- § PAM fluoreszcencia mérések
- § Fluoreszcencia spektrum 77 K-en
- § Flash-indukált klorofill-fluoreszcencia lecsengés kinetikájának elemzése

§ Termolumineszcencia mérések

§ Oxigéntermelés mérése

§ Fénykezelések

EREDMÉNYEK ÉS MEGVITATÁSUK

OCP és fényvédelem különböző cianobaktériumokban

Annak érdekében, hogy megvizsgáljuk az OCP jelenléte és a kék fény által indukált fluoreszcencia-kioltás közti összefüggést, olyan cianobaktérium törzseket használtunk, amelyek vagy rendelkeztek OCP-t kódoló génnel, illetve annak egyes szakaszaival, vagy teljes egészében nélkülözték azt. A fluoreszcencia változások kinetikáját PAM fluoriméterrel követtük nyomon. Ha a sötétadaptált sejteket alacsony intenzitású, folyamatos kék-zöld fénnyel világítottuk meg, akkor mind a négy baktériumtörzs esetében (*A. maxima, Synechocystis, S. elongatus, T. elongatus*) növekvő maximális fluoreszcencia (F_m') értékeket figyelhettünk meg a State2-State1 állapot változás miatt. Az OCP-t kódoló gént részben tartalmazó (*T. elongatus*), vagy teljes egészében nélkülöző (*S. elongatus*) sejtek esetében a sejtek fluoreszecenciája magas szinten maradt akkor is, ha nagy intenzitású folyamatos kék-zöld fénnyel világítottuk meg őket. Ezekben az esetekben nem volt tapasztalható fluoreszcencia-kioltás, ami arra utal, hogy az OCP jelenléte nélkül ez a fajta kioltás nem indukálódik. Ezzel szemben, ha az OCP-t kódoló gént tartalmazó sejteket (*Synechocystis* and *A. maxima*) tettük ki nagy intenzitású kék-zöld fénynek, akkor fluoreszcencia-kioltást mérhettünk az NPQ folyamat indukciója miatt. A kék fény által indukált nem fotokémiai fluoreszcencia-kioltással ellentétben, az OCP hiánya nem befolyásolta az állapot átmenet (state transition) megjelenését egyik általunk vizsgált cianobaktériumban sem.

Az OCP-vel rendelkező sejtekben, vashiányos körülmények között is ugyanúgy indukálható volt a fluoreszcencia-kioltás intenzív kék-zöld fénnyel, mint optimális körülmények között, miközben az F_0 értékeik megemelkedtek. A huzamosan vashiányos környezetben nevelt, OCP-t szintetizálni képes sejtekben (*Synechocystis, A. maxima*) megnövekedett az NPQ kapacitás mértéke, ellentétben a vassal ellátott környezetben nevelt sejtekkel. Az OCP-t szintetizálni képtelen sejtekben (*T. elongatus* and *S. elongatus*) nem mutatkozott NPQ indukció a tartósan vashiányos környezet hatására sem. Az OCP-vel rendelkező vizsgált sejtek azon

képessége, hogy kék fény hatására fluoreszcencia-kioltás indukálódhat alátámasztja azt az elképzelést, miszerint ez a védekezési mechanizmus széles körben jelen van a cianobaktériumokban. Ezzel szemben, az OCP-t kódoló gén hiányában nem jelentkezik fluoreszcencia kioltás. Emellett vizsgálataink azt is kimutatták, hogy azok a törzsek, amelyek nem képesek kék fény által indukált fluoreszcencia-kioltásra, hajlamosabbak fotoinhibíciót, azaz fénykárosodást elszenvedni.

Vassal ellátott és vashiányos sejtek abszorpciós spektrumait hasonlítottuk össze az OCP-t kódoló génnel rendelkező, vagy azt nem tartalmazó törzsek esetén. Tartós vashiány mellett nevelt *Synechocystis* és *A. maxima* sejt 77 K fluoreszcencia emissziós spektruma határozott csúcsot mutat 685 nm-nél (600 nm-nél történő gerjesztés esetén), ami erős fluoreszcenciát kibocsátó, szabad fikobiliszómák megjelenésére utal (Wilson *et al.,* 2007). Ezzel szemben az OCP-t kódoló génnel nem rendelkező sejtek esetében ez a jelenség nem volt megfigyelhető a fikobiliprotein tartalom gyors csökkenése miatt. Vashiányos környezetben, ha a cianobaktériumok nem képesek a kék fény által indukált NPQ segítségével csökkenteni a reakciócentrumokhoz eljutó energiát, akkor ahhoz, hogy ne halmozódjanak fel a potenciálisan veszélyes szabad fikobiliszómák, csökkentik a fikobiliprotein mennyiségüket.

A PsbU szerepe a PSII fénykárosításának megelőzésében

A PsbU szerepének vizsgálatához a *Synechococcus* cianobaktérium törzs vad típusú (WT) és psbU deléciós mutáns (ΔPsbU) sejteinek fotoszintetikus elektrontranszportját követtük nyomon. A sejtek fényimpulzus által indukált klorofill-fluoreszcencia lecsengési kinetikáját tanulmányoztuk az elektrontranszport gátló 3-(3,4-diklorofenil)-1,1-dimetilurea (DCMU) jelenlétében és hiányában. Ezáltal rálátást nyertünk az akceptor oldali elektrontranszport kinetikájára illetve a redukált akceptor és az oxidált donor elemek közti töltésrekombinációs folyamatokra. A sötétadaptált sejtekben egy telítő fényfelvillanás redukálja a Q_A elektron akceptort az összes PSII komplexben, ami a változó fluoreszcencia hirtelen növekedéséhez vezet. A maximális fluoreszcencia növekedés (F_m-F_o) amplitúdója arányos az aktív PSII reakciócentrumok összmennyiségével. Ez közel 30%-kal volt alacsonyabb a ΔPsbU sejtekben a WT-hoz képest. A DCMU hiányában mért fluoreszcencia lecsengésből meghatározható az $S_2Q_B^-$ töltésrekombináció időállandója, mely 1.5x volt nagyobb a ΔPsbU sejtekben, mint a WT-ban.

DCMU jelenlétében a $S_2Q_A^-$ lecsengésének időállandója a mutáns sejtekben 2.1x volt nagyobb, mint a WT ugyanezen értéke. A $S_2Q_B^-$ és $S_2Q_A^-$ lecsengési időállandók arányából ki lehet számítani a Q_A és a Q_B elektron akceptorok közti szabadenergia különbséget. Mivel a WT paramétereivel számolt arány különbözött a ΔPsbU-tól, arra következtettünk, hogy a Q_A <-> Q_B szabadenergia különbség megváltozott a mutánsban. Számításaink szerint ez 37 meV a WT-ban és 28 meV a mutáns sejtekben.

A vizsgálataink során a flash által indukált klorofill-fluoreszcencia mérések mellett termolumineszcencia méreseket is végeztünk, mindezt DCMU jelenlétében és hiányában egyaránt. A ΔPsbU sejtekben a B sáv, ami DCMU hiányában az $S_2Q_B^-$ töltésrekombinációnak tulajdonítható, és a Q sáv, ami DCMU jelenlétében az $S_2Q_A^-$ töltésrekombinációhoz kapcsolható, magasabb hőmérsékletek felé tolódtak. Ezek az adatok megegyeznek a flash indukált klorofill-fluoreszcencia mérési eredményekkel és megerősítik az $S_2Q_B^-$ és az $S_2Q_A^-$ energetikai stabilizációját a mutáns sejtekben. Azonos számú funkcionális PSII komplex mellett a B sáv intenzitása 1.7-szer nagyobb a ΔPsbU sejtekben a WT-hoz képest. Korábbi vizsgálatok kimutatták, hogy a $P_{680}^+Phe^-$ elsődleges töltésszétválási állapot szabadenergia szintje befolyással van a termolumineszcencia intenzitására, azáltal, hogy megváltoztatja a P_{680}^* és a $P_{680}^+Phe^-$ közti szabadenergia különbséget (Cser and Vass, 2007a). A ΔPsbU sejtek megnövekedett termolumineszcencia amplitudója, a lassabb $S_2Q_A^-$ és $S_2Q_B^-$ töltésrekombinációval együtt, a $P_{680}^+Phe^-$ elsődleges töltésszétválási állapot csökkent szabadenergiájára utal.

A WT és a ΔPsbU sejtek fotoinhibícióra való érzékenységét 1.5 órás intenzív (500 µmol m^{-2} s^{-1} foton) megvilágítás mellett teszteltük, a D1 fehérje szintézist gátló linkomicin jelenlétében, illetve annak hiányában. Kísérleteink azt mutatták, hogy linkomicin jelenlétében a ΔPsbU sejtek fokozottan érzékenyek az erős fényre. Annak ellenére, hogy a mutáns sejtek hajlamosabbak voltak a fotoinhibícióra linkomicin hiányában, a fényintenzitás növekedési szintre való visszaállítása után jelentős mértékben regenerálódott a PSII aktivitásuk. Több folyamat is résztvesz a fotoinhibícióban: az akceptor oldali elektrontranszport folyamatok következtében reaktív oxigénformák képződhetnek (Vass, 2011), ugyanakkor az oxigéntermelő komplex is inaktiválódhat (Hakala *et al.*, 2005; Ohnishi *et al.*, 2005; Murata *et al.*, 2007).

Az eredményeink arra utalnak, hogy a PsbU fehérje hiánya változásokat idéz elő nemcsak a PSII donor oldalán, hanem az akceptor oldalon is. A fehérje hiánya az akceptor oldalon valószínűleg nem csak a Q_A és a Q_B környezetét befolyásolja, hanem módosítja a Phe, vagy a

P$_{680}$ reakciócentrum P$_{D1}$Chl-je hidrogénkötéses kölcsönhatásait. Ez utóbbi olyan töltésszétváláshoz vezet, ami triplett klorofillt (^{3}P$_{680}$) eredményez (Vass and Cser, 2009; Vass, 2011), ami indukálhatja a szinglett oxigén termelését, így a PSII oxidatív károsodást szenvedhet.

KÖVETKEZTETÉSEK

1. Korábban nem volt ismert, hogy az igen fontos, kék fény által indukált fluoreszcencia-kioltási fényvédő mechanizmus általánosan elterjedt-e a cianobaktériumok körében vagy sem. Munkánk során kimutattuk, hogy csak azok a cianobaktériumok képesek kék fény által indukált klorofill-fluoreszcencia kioltásra, amelyek genomja tartalmazza a teljes OCP-t kódoló gént. Ez egyaránt igaz optimális és vashiányos körülmények között nevelt cianobaktériumokra. Ezzel ellentétben azok a cianobaktériumok, amelyek csak részben tartalmazzák vagy teljesen nélkülözik az OCP-t kódoló gént, nem képesek a kék fény által indukált fluoreszcencia kioltásra sem optimális, sem vashiányos viszonyok mellett. Az utóbbi csoportba tartozó cianobaktérium törzsek más módszerrel birkóznak meg az olyan stresszkörülményekkel, mint amilyen a vashiány: az OCP-t kódoló gént nem tartalmazó törzsek sejtjeiben vashiányos környezetben csökken a fikobiliprotein mennyisége és ezáltal elkerülhetővé válik a veszélyes, szabad fikobiliszómák felhalmozódása. Hasonló stresszkörülmények közt, amelyek során a sejtekben megjelenhetnek a szabad fikobiliszómák, az OCP-t előállítani képes törzsekben az erős fény elleni védekezés az OCP-fikobiliszóma szintjén zajló energia elvezetés útján történik.

2. A PsbU protein alegység szerepét a PSII komplex elektrontranszportjával és fényérzékenységével kapcsolatban tanulmányoztuk. A kísérleteket egy korábban leírt PsbU-hiányos *Synechococcus* PCC 7942 mutánson végeztük, amelyről kimutatták, hogy emelkedett antioxidáns kapacitással rendelkezik (Balint *et al.*, 2006). Fényimpulzus által indukált klorofill-fluoreszcencia méréseink szerint a PsbU hiánya lelassítja az S$_2$Q$_A^-$ és az S$_2$Q$_B^-$ töltésrekombinációs folyamatokat. Termolumineszcencia adataink, azaz a magasabb hőmérsékletek felé tolódott Q- és B-sávok arra utalnak, hogy ezekben a

mutáns sejtekben megnövekszik az $S_2Q_A^-$ és az $S_2Q_B^-$ töltéspárok stabilitása. Továbbá, a termolumineszcencia sávok intenzitása általában magasabb a ΔPsbU sejtekben (≈1.7X a B-csúcs esetében), a WT-ban mérthez képest. A mutáns sejtek erős megvilágítás mellett érzékenyebbek a fotoinhibícióra, linkomicin jelenlétében és hiányában egyaránt. Eredményeink arra engednek következtetni, hogy a PsbU alegység hiánya a *Synechococcus* PCC 7942 cianobaktérium törzsben befolyással van az $S_2Q_A^-$ és $S_2Q_B^-$ töltéspárok stabilitására, azáltal, hogy módosítja a PSII donor és akceptor oldali elemeit. A hatást valószínűleg a donor oldali oxigénfejlesztő komplex közvetlen környezetében, valamint PSII-n belül bekövetkező szerkezeti változások okozzák. Eredményeink szerint ezeket a változásokat a PsbU fehérje alegység hiánya a lumenális oldalról indukálhatja. A ΔPsbU sejtek fényérzékenysége valószínűleg a reaktív oxigénformák jelenlétéhez kapcsolható, amelyek létrejöttét vagy a megváltozott donor oldal, vagy/és az elsődleges töltéspár módosított energetikai állapota indukálja.

ABSTRACT

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 (Glazer, 1984; Grossman *et al.*, 1993; Tandeau de Marsac, 2003; Adir, 2005).

Excess light can be lethal for photosynthetic organisms because of production of harmful reactive oxygen species (Aro *et al.*, 1993; Melis, 1999). 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 (Mullineaux *et al.*, 1997; Schluchter *et al.*, 1996), D1 protein repair (Aro *et al.*, 1993), and non-photochemical PSII fluorescence quenching (NPQ) (Kirilovsky, 2007).

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 (Wilson *et al.*, 2006). 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 (Wilson *et al.*, 2007; Boulay *et al.*, 2008).

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 *Termosynechococcus 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 lumenal side of PSII. It consists of a Mn_4Ca cluster and several lumenal 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 (Shen *et al.*, 1997). The presence of PsbU contributes to the heat stability of the OEC during the acclimation to high temperature (Nishiyama *et al.*, 1997; Nishiyama *et al.*, 1999). The removal of PsbU affects not only the lumenal 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 (Veerman *et al.*, 2005). Recently, it was proposed that PsbU provides protection from reactive oxygen species in *Synechococcus* 7942 cells (Balint *et al.*, 2006). In this study, I 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 (Wilson *et al.,* 2007). 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 ΔPsbU 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 (≈ 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 ΔPsbU 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 ΔPsbU 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 <-> 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 ΔPsbU 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 ΔPsbU 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 ΔPsbU 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 $P_{680}^+Phe^-$ (Cser and Vass, 2007a). The increased TL amplitude of the ΔPsbU 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 ΔPsbU strains to photoinhibition was checked by illuminating cells with strong light (500 μmol photons m^{-2} 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* ΔPsbU mutant has an increased susceptibility to light. Despite of the fact, that the ΔPsbU 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 (Vass, 2011) as well as inactivation of the Mn cluster (Hakala *et al.*, 2005; Ohnishi *et al.*, 2005; Murata *et al.*, 2007). 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 $^3P_{680}$ (Vass and Cser, 2009; Vass, 2011), 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

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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 charachterization 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 (Balint *et al.*, 2006).

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 ($\approx$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 lumenal part of the complex. The photosensitivity of the ΔPsbU *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.

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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**

Phosphatidylglycerol (PG) is an essential lipid component of photosynthetic membranes. In this work I studied the role of the PG in the electron transport of Photosystem II using the Synechocystis sp. PCC 6803 and Synechococcus sp. PCC 7942 mutants which are unable to synthesize PG. My results demonstrated that in Synechocystis cells the absence of PG led to the inhibition of $Q_A{}^-$ to Q_B electron transfer. In Synechococcus cells a clear modification of the Q_B binding site was observed in PG-depleted cells.

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**

My aim in this study was to observe the effect of the UV-B light on the oxygen-evolving activity and the decay of Chl fluorescence in Arthrospira (Spirulina) platensis cells. It was shown that PSII repair, which occurs via synthesis of the D1 protein,

represents an efficient defense mechanism against UV-B induced activity loss of PSII in Arthrospira platensis.

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**

The small CAB-like proteins (SCPs) of Synechocystis belong to the family of stress-induced light-harvesting-like proteins. They are constitutively expressed in a mutant deficient of PSI. I studied the involvement of SCPs in the photoprotection of PSII by comparing the PSI-less control strain with a PSI-less mutant, in which all SCPs were deleted. To investigate the role of SCPs in the repair cycle of PSII, we exposed the cells to gabaculine and examined the recovery of the photosynthetic activity after photobleaching. Gabaculine inhibits the synthesis of chlorophyll. Hence, cells grown in the presence of gabaculine can only repair or assemble new PSII with chlorophyll molecules recycled from damaged PSII. As expected, the presence of gabaculine enhanced photoinhibition in the PSI-less control. However, after photoinhibitory treatment a low recovery rate was observed, indicating chlorophyll recycling into newly synthesized PSII. In the absence of SCPs, however, severe photoinhibition was already observed in untreated cells (similarly to the PSI-less strain in the presence of gabaculine), addition of gabaculine had no additional effect on photoinhibition and recovery. SCPs, therefore, seem to be involved in the repair cycle of PSII by regulating the synthesis of new chlorophyll.