In vitro and in vivo characterisation of the OCP-related photoprotective mechanism in the cyanobacterium Synechocystis PCC6803

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In vitro and *in vivo* characterisation of the OCP-related photoprotective mechanism in the cyanobacterium *Synechocystis* PCC6803

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Abbreviations

APC	Allophycocyanin
Arg	Arginine
ATP	Adenosine-5'-triphosphate
bp	base pairs
DNA	Deoxyribonucleic acid
E. Coli	Escherichia coli
ECN	Echinenone
FMN	Flavin mononucletide
FNR	Ferredoxin-$NADP^+$ reductase
FRP	Fluorescence Recovery Protein
GFP	Green Fluorescent Protein
Glu	Glutamate
hECN	3'-hydroxyechinenone
ICT	Intramolecular charge-transfer
IR	Infra-red
LH	Light harvesting
LHCII	Major antenna complex in plants
Met	Methionine
mRNA	Messenger RNA
$NADP^+$	Oxidized Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NIES	National Institute for Environmental Studies
OCP	Orange Carotenoid Protein
OCP^o	Orange form of OCP
OCP^r	Red form of OCP
PAM	Pulse-amplitude modulation
PB	Phycobilisome
PC	Phycocyanin
PCC	Pasteur Culture Collection
Pheo	Pheophytin
PQ	Plastoquinone
PQH_2	Plastoquinol
PSI	Photosystem I
PSII	Photosystem II
Q	Quinone
RC	Reaction centre
RCP	Red Carotenoid Protein
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
sp.	(lat.) Species
Synechocystis	*Synechocystis* PCC6803
TE	Terminal emitters of phycobilisome
Trp	Tryptophan
Tyr	Tyrosine
UN	United Nations
UV	Ultra violet
WT	Wild type
Zea	Zeaxanthin

Preface

All things are poison, and nothing is without poison; only the dose permits something not to be poisonous. This concept, originally a paradigm of toxicology, can be applied to the field of photosynthesis, and hence why there is a need for photoprotection. Light drives photosynthesis, but light can be poisonous to any photosynthetic organism because excess absorbed energy that cannot be used to drive photosynthesis can enhance the production of toxic reactive oxygen species, such as singlet oxygen. Moreover, photosynthetic organisms have to react to constantly modulating doses of light to perform photosynthesis yet avoid poisoning.

Diana Kirilovsky's discovery of the key role of the Orange Carotenoid Protein (OCP) in the cyanobacterial non-photochemical-quenching-like, heat-dissipating mechanism triggered vast and profound studies in the topic (Wilson et al., 2006). Three years after that, I joined the group in CEA Saclay and started research projects of my Ph.D.

I worked in two major projects during my Ph.D. One of the projects involved the development of *in vitro* reconstitution system of the OCP-related photoprotective mechanism of cyanobacteria using isolated complexes. This system gave us an access to mechanistic details of photoprotection. Also, it confirmed the direct involvement of previously described players: OCP and Fluorescence Recovery Protein (FRP) and proved that they are sufficient to switch the photoprotection on and off, respectively (Gwizdala et al., 2011). The *in vitro* system was an essential element to elucidate the OCP amino acids involved in the binding to the phycobilisomes (Wilson et al., 2012) and the phycobilisomes (PB) components involved in this interaction (Jallet et al., 2012). In collaboration with other laboratories we have applied the *in vitro* system to elucidate the physical properties of excited states of the OCP (Berera et al., 2012; Berera et al., *submitted*) and the mechanism of energy and fluorescence quenching (Tian et al., 2012). My goal in the second project was to further characterize the FRP, an off-switch of the OCP-related photoprotection, and in particular to establish the real length of the FRP of *Synechocystis* PCC6803, the model organism for our research (Gwizdala et al., 2012).

During the three years of my theses, big progresses were achieved on the detailed description of the players involved in OCP-related photoprotection that largely contributed to our understanding of this mechanism, which is crucial for a quick response to fluctuating light conditions.

Introduction

Cyanobacteria

Cyanobacteria are distantly related to gram-positive bacteria, but they share some characteristics with gram-negative bacteria (*e.g.* structure of the cell wall). The name "Cyanobacteria" comes from the classification and morphological characteristic of the phylum; "*cyano*-" (Greek), referring to the bluish colour of the strains described as typical for the phylum (at the time of discovery) and "-*bacteria*", as they are prokaryotes. Historically, they have been incorrectly called "blue-green algae", which suggested that cyanobacteria are eukaryotes. This name, even though taxonomically misleading, was in a way informative. By classifying cyanobacteria as algae, the most remarkable and unique attribute of the phylum was expressed: cyanobacteria run oxygenic photosynthesis!

Not all cyanobacteria are actually blue-green. The wide-ranging colours of cyanobacteria come from pigments called phycobilins which are bound to soluble proteins, the phycobiliproteins. These coloured proteins form the cyanobacterial photosynthetic antenna, the phycobilisome (reviewed in (Glazer, 1984)). The phycobilisomes vary in colour from turquoise and blue, to pink or brown. The phycobilisome-containing strains carry also chlorophyll *a* and carotenoids. There is a group of yellow-green coloured cyanobacteria, the prochlorophytes, which does not contain phycobilisomes but a membrane antenna, containing chlorophyll *a* and *b* and carotenoids, called PCB. Atypical forms of chlorophyll were found in specific cyanobacteria strains: chlorophyll *d* (absorption maximum shifted to far-red/near IR, *e.g. Acaryochloris*) (Miyashita et al., 1996), or lately described chlorophyll *f* (absorption maximum even further into IR *e.g. Hongdechloris*) (Chen et al., 2012).

The ancestors of present day cyanobacteria started producing oxygen around 2.7 billions years ago in the ocean. They evolved a light-driven process called oxygenic photosynthesis. The evolution of photosynthesis by cyanobacteria had a profound influence on the development of planet Earth. For the first 300 million years, oxygen slowly oxidised oceanic minerals. After these became fully oxidised, an oxygenic atmosphere slowly developed, causing widespread death of anaerobic organisms (Great Oxygenation Event). However, with the help of the newly formed ozone layer, UV radiation was filtered, allowing organisms exploiting oxygenic

photosynthesis to flourish on the ocean surface, and finally on land (reviewed in (Des Marais, 2000)). Therefore, life today has been dramatically shaped by the activity of cyanobacteria!

Primitive cyanobacteria diverged into the present day phycobilisome containing species. Species of cyanobacteria are divided into 5 morphological groups (Rippka et al., 1979): (1) Unicellular-dividing by binary fission (*e.g. Synechocystis*, *Synechococcus* and *Gleobacter*); (2) Unicellular, colonial, dividing by multiple fission (*e.g. Xenococcus*); (3) Filamentous-not forming heterocysts (*e.g. Arthrospira*); (4) Filamentous-forming heterocysts (*e.g. Anabaena*, *Nostoc* and *Calothrix*); (5) Branching-filamentous (*e.g. Fisherella*) (**Figure 1**). Some filamentous strains are able to form specialized cells: heterocysts (to fix nitrogen from the atmosphere) (**Figure 1**.4), akinetes (thick walled survival cells) or hormogonia (during reproduction). These specialised cells serve whole colonies. Some unicellular strains divide by multiple fissions to form specialized cells, the baeocytes (*e.g. Dermocarpa*) (Waterbury and Stanier, 1978). Baeocytes (in number 4-1000) stay within one host cell wall during reproduction (Rippka et al., 1979) (central cell in **Figure 1**.2a).

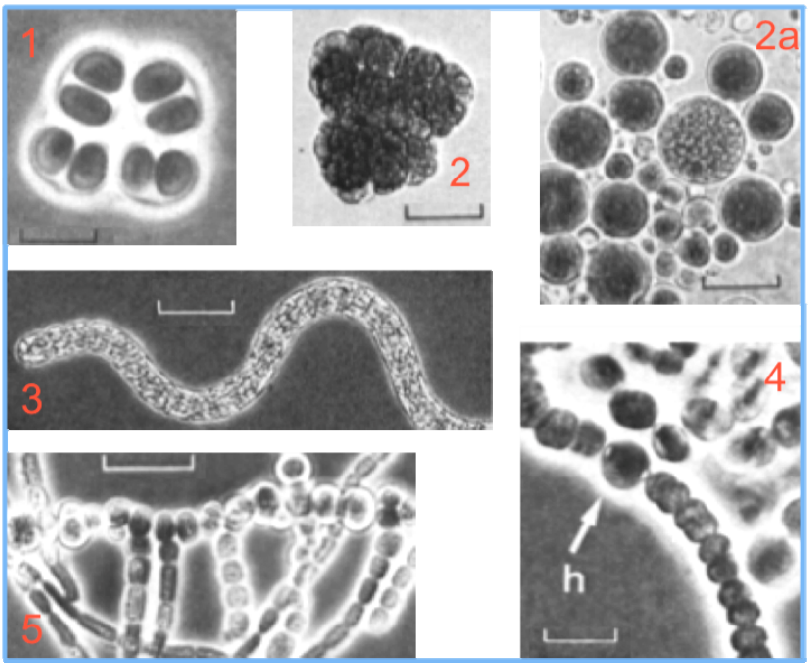

Figure 1 Five morphological groups of cyanobacteria.
In 1979 Rippka, et al., proposed a new classification and names for cyanobacterial species. Strains were divided into 5 groups presented on the microphotographs: (1) unicellular-dividing by binary fission *Gloeocapsa* PCC7428; (2) and (2a) unicellular, colonial, dividing by multiple fission – (2) *Chroococcidiopsis* PCC7431, (2a) baeocytes of *Dermocarpa* PCC7437; (3) filamentous-not forming heterocysts *Spirulina* PCC7345; (4) filamentous-forming heterocysts *Nostoc* PCC6705 – "h" points a heterocyst; (5) branching-filamentous *Fischerella* PCC7414. Bars represent 5 µm in (1) and (4), or 20 µm in (2), (2a), (3) and (5). Pictures were taken from Rippka, et al., 1979.

The broad distribution of cyanobacteria in many kinds of habitats is due to the heterogeneity of the phylum. Cyanobacteria strains are present in fresh and salt water. Some have evolved to withstand extreme conditions, including high and low temperatures, acidification, harsh chemical compositions and low water accessibility. Unsurprisingly, they are often the first organisms to colonize virgin habitat. Certain strains of cyanobacteria occur in symbiosis with other organisms or simply inside other organism tissues (*e.g.* mosses, lichens and fish). According to the theory of endosymbiotic origin of chloroplasts (proposed by Mereschkowsky in 1905; reviewed in (Raven and Allen, 2003)), a cyanobacterium became engulfed by another larger cell and continued to photosynthesise. It is in a way, an extreme example of colonization. Cyanobacterial tolerance to high heat was used for a long time to isolate cultures from natural sources (Stanier et al., 1971).

Figure 2 Cyanobacteria in art.
An unusual application of cyanobacteria. Culture of *Nostoc sp.* in "Cyanobacterial bonsai project" by Hidedo Iwasaki. Pictures of treated colonies were presented on exhibitions around Europe.

Cyanobacteria are exploited by man today (**Figure 2**) and perhaps will provide us with future energy supplies. As photoautotrophs, cyanobacteria are perfect candidates to become producers of biofuels. The multiple metabolic pathways in cyanobacteria provide us with the building blocks for 'ready-to-use bioreactors' (Hall

et al., 1995). Other examples of cyanobacteria uses is their reintroduction in the Arizona (USA) deserts to stabilise a crust, decrease the formation of dust storms and prevent the loss of valuable agricultural land. Furthermore, cyanobacterial extracts or whole cells are also used in cosmetics and medications, being a rich source of substances, which are hard or expensive to synthetically produce. The Aztecs has already appreciated cyanobacteria as a reach source of nutrition. Today, 15% of humanity is facing nutritional shortfalls (UN Food and Agriculture Organisation 2011 report) and cyanobacteria is already used as a cheap source of protein. The presence of unique metabolic pathways also makes the cyanobacteria a good tool for decontaminating water from heavy metals or chemicals. Perhaps the resistance of cyanobacteria could permit their tolerance to space and other planets, which may help in creating conditions for spreading terrestrial life! However, we cannot forget their important role in fundamental science research. They have been used as a model organism for studies of oxygenic photosynthesis *e.g.* cyanobacterial complexes were used to resolve crystal structures of photosystems (Zouni et al., 2001; Krauss et al., 1996; Jordan et al., 2001; or lately Umena et al., 2011). One strain in particular, *Synechocystis* PCC6803, which is also our model organism, became what is called "the *E. coli* of photosynthesis".

Synechocystis PCC6803

Synechocystis PCC6803 is unicellular, divides by binary fission and is tolerant to a wide range of salt concentrations (double the seawater concentration, (Marin et al., 2006; Reed et al., 1985)). This is the reason for its presence in freshwater and saline media. Mostly it can be found in the relatively freshwater lakes of warm temperate zones. Originally called *Aphanocapsa* N-1, it was isolated from a freshwater lake in 1968 in California, USA (by Kunisawa in (Stanier et al., 1971)). In 1979 due to its morphological properties it was called for the first time *Synechocystis* (Rippka et al., 1979). *Synechocystis* cells are spherical with a diameter of 2-3 μm. The cell is surrounded by an envelope, which is composed of the plasma membrane, a layer of peptidoglycan and the outer membrane (similarly to gram-negative bacteria). Cell composition is typical for prokaryotes *i.e.* without nucleus or any membrane-enclosed organelles (**Figure 3**). There are however, some elements unique for cyanobacterial cells and not present in other bacteria and connected to their

photosynthetic activity. In *Synechocystis* cells there is a system of parallel membranes, called the thylakoid (in **Figure 3**.A marked as "T"), which follow the curvature of the envelope. Soluble photosynthetic antenna complexes, called phycobilisomes, are attached on the stromal side of the thylakoid membrane. It has been demonstrated that the curvature of thylakoid membranes is correlated with the size of phycobilisomes (Collins et al., 2012). The complexes associated to photosynthetic and respiratory electron transfer chains are both within the thylakoid.

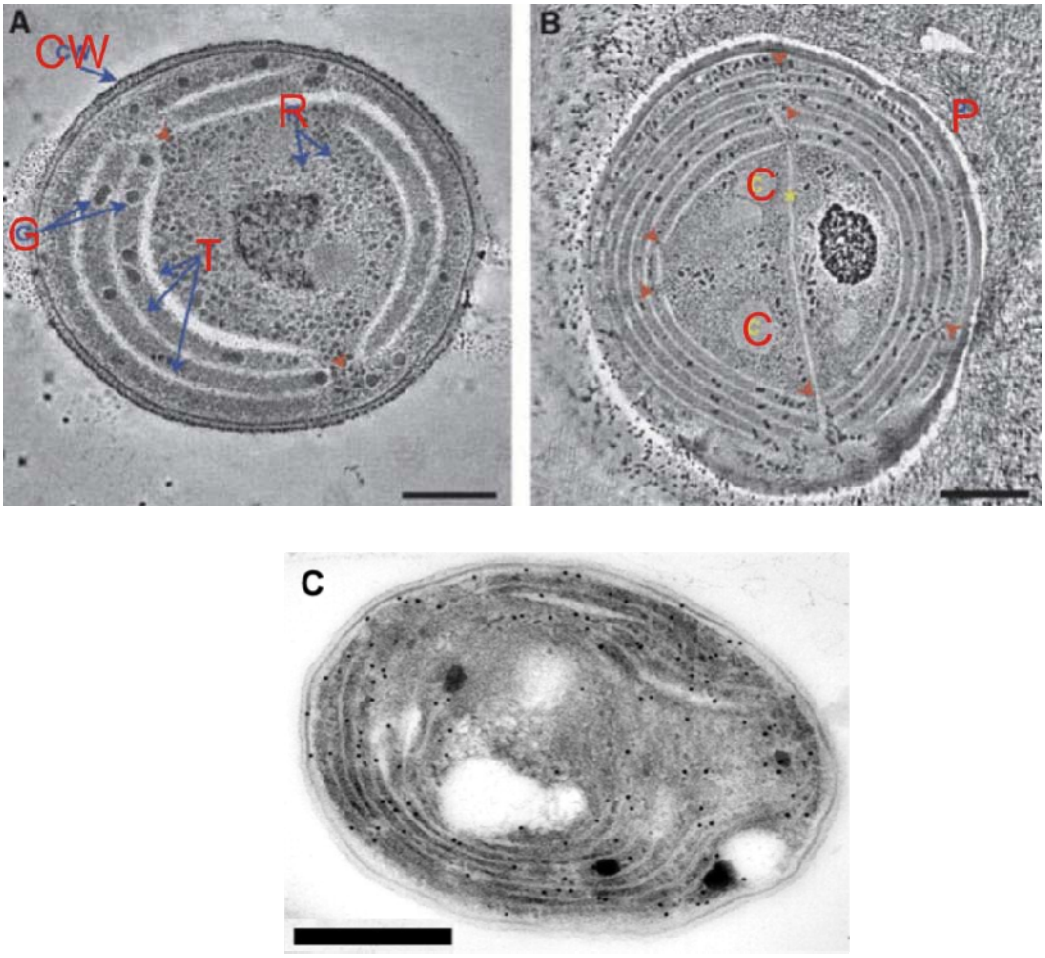

Figure 3 Electron micrographs of cyanobacteria.
In picture (A) *Synechococcus* PCC7942 and in (B) *Microcoleus sp*. In both pictures: "T" is thylakoid: "CW" is cell wall "R" are ribosomes, "G" is glycogen, "C" are carboxysomes and "P" is extracellular polysaccharide envelope. Red arrows point perforations of thylakoid. Bars represent (A) 0.2 μm and (B) 0.5 μm. Pictures (A) and (B) were taken from Nevo et al., 2007.
(C) Architecture of a *Synechocystis* PCC6803 cell. Small black dots represent OCP-GFP complex detected by immunogold labelling. OCP is located in the stromal space. Bar represents 0.5 μm. Picture was taken from Wilson et al., 2006.

In *Synechocystis* cells there are carboxysomes (in **Figure 3**.B marked as "C"). These microcompartments, which also contain Rubisco enzyme, serve as a carbon

dioxide concentrating system for efficient photosynthesis. *Synechocystis* store energy captured in photosynthesis in form of a polysaccharide, glycogen (in **Figure 3**.A marked as "G").

Synechocystis has been used as a model organism for studies of oxygenic photosynthesis due to its relatively simple genetic machinery as well as to its spontaneous transformability. The 3.5 Mbp genome of *Synechocystis* was sequenced in 1996 and it was the first known genome of a photosynthetic organism (Kaneko et al., 1996). Out of the 3200 genes assigned on the genome, over 1000 did not reveal any similarity to previously characterised genes. The transcription starting sites were mapped in 2010 (Mitschke et al., 2011). *Synechocystis* uptakes external DNA and integrates it into the genome by homologous recombination (Grigorieva and Shestakov, 1982). *Synechocystis* is a perfect model to study mutations in photosynthetic apparatus because of its possible heterotrophic growth in presence of sugars (Rippka et al., 1979). Finally, its relatively fast growth, as well as easy genetic manipulation (in comparison with the other oxygenic autotrophs), is why *Synechocystis* is one of the most used model organism to study photosynthesis.

Photosynthesis and photoinhibition

Photosynthesis

The term *Photosynthesis* from Greek means "assemblage in light", referring to the light driven anabolic process where carbon dioxide is reduced to sugars. Sunlight is a universal source of energy. This energy however, is accessible for the organisms, which have developed a photosynthetic apparatus (photosynthetic bacteria including cyanobacteria) and for other organism that have incorporated it from endosymbiosis. The photosynthetic apparatus of plants, algae and cyanobacteria is an electron transfer chain composed of three protein-cofactors complexes working in series and spanning photosynthetic membranes (thylakoid): Photosystem II, Photosystem I and Cytochrome b_6f. The fixation of carbon to produce sugars requires ATP and reducing power in the form of NADPH (Calvin and Benson, 1949; Bassham et al., 1950). The oxidation of H_2O in the Photosystem II releases protons in the thylakoid lumen. Further protons are released at cytochrome b_6f. This creates a proton-motive force for ATP synthesis (Mitchell, 1961). The electrons from water via the photosynthetic electron transport chain reduce $NADP^+$. Additionally, oxygen is released as a by-product of oxygenic photosynthesis.

Photosystems

Photosystems are composed of reaction centres where charge separation occurs, and light-harvesting antennae that collect light energy and provide the reactions centres with excitation energy. The process of charge separation occurs when an electron emitted from the excited special chlorophyll reduces the primary electron acceptor. The subsequent electron transfer stabilizes charge separation. The protein scaffold fine-tunes the redox properties of cofactors (lately reviewed in (Cardona et al., 2012)). The reaction centres are membrane complexes binding all the cofactors necessary for charge separation and stabilisation of the separated charges.

Reaction centre of Photosystem II

Photosystem II (PSII) performs reduction of plastoquinone and water oxidation. Energy of light is used to run this uphill reaction. Out of the 20 protein-subunits of the Photosystem II, only three are absolutely necessary for the assembly

of the minimal reaction centre capable of charge separation, D1, D2 and the cytochrome b_{559} (Guskov et al., 2009) (**Figure 4**). The heterodimer formed by D1 and D2 binds the cofactors necessary for the photochemical reaction. A photon is able to induce a charge separation of the chlorophyll P680 and the electron is donated to Pheophytin (Pheo) (in D1), the primary electron acceptor. The electron is transferred downhill in energy from $Pheo^-$ to the quinone Q_A (a one-electron carrier) in D2 and then to Q_B in D1. The Q_B forms a stable semiquinone, which after a second charge separation event becomes doubly reduced and also protonated to plastoquinol (QH_2). The Q_BH_2 leaves the Q_B site and is replaced by an oxidised quinone from the PQ-pool. The plastoquinol is then re-oxidised by the Cytochrome b_6f. The $P680^+$ cation after each charge separation takes the missing electron from the Tyrosine Z. The tyrosyl radical is reduced by oxidising the Mn_4Ca cluster of the oxygen evolving complex (structure in (Umena et al., 2011)), which undergoes the so-called 'Joliot-Kok's cycle' of S-states from S0 to S4 (Kok et al., 1970). A molecule of oxygen is released after four charge separations when the S4 state rapidly decomposes to S0, requiring the oxidation of two water molecules (Joliot et al., 1969). Even though Photosystem II is a quasi-symmetrical heterodimer the cofactors of D2 protein, with the exception of Q_A, do not participate in the principal linear electron transport chain. Some of them, including the haem of the cytochrome b_{559}, are involved in photoprotective secondary electron transport chains (reviewed lately in (Cardona et al., 2012)).

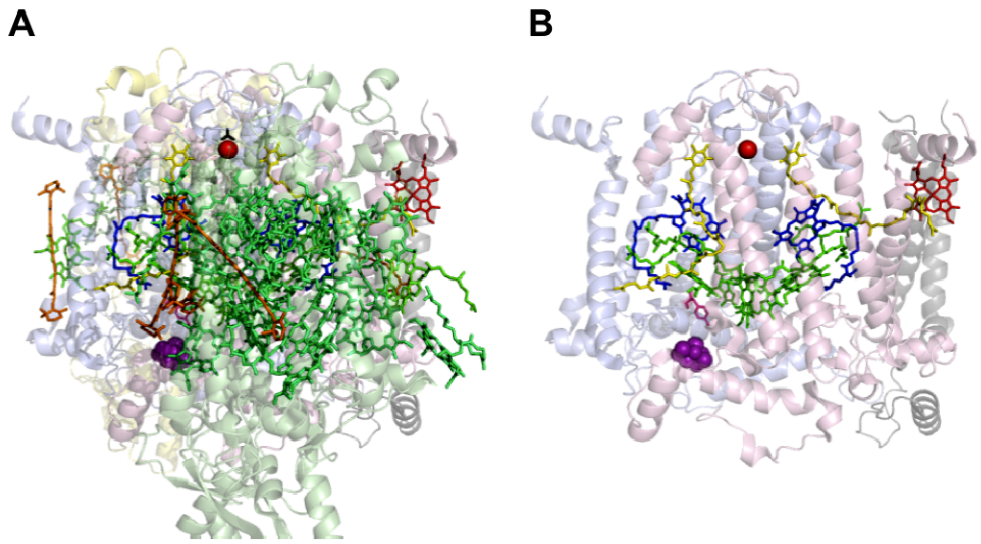

Figure 4 Photosystem II:
(A) A picture of D1 (light blue), D2 (light pink), cytochrome b_{559} (gray), CP43 (light yellow) and CP47 (light green) proteins with their cofactors (excluding lipids): chlorophylls (green), carotenoids (orange),

pheophytins (blue), quinones (yellow), Mn_4Ca cluster (purple), non-haem iron (red sphere), bicarbonate (black) and haem (red sticks) from *Thermosynecococcus vulcanus*. (B) Reaction centre of Photosystem II of *Thermosynecococcus vulcanus* composed of D1 (light blue), D2 (light pink) and cytochrome b_{559} (gray). Cofactors coloured like in (A) with additional Tyrosine Z (pink). Charge separation occurs between the chlorophyll P680 (green) and the D1 pheophytin (blue) the charge separation is stabilized by electron transfer to the Q_A quinone in D2 (yellow on the left) and then to the Q_B quinone in D1 (yellow on the right). In the donor side of PSII, chlorophyll $P680^+$ receives an electron from Tyr Z (pink) that takes an electron from the Mn_4Ca cluster (purple). The haem in cytochrome b_{559} (red) is involved in secondary electron transfer (see text). (A) and (B) were redrawn with PyMol from Umena et al., 2011.

Reaction centre of Photosystem I

Photosystem I (PSI) performs an electron transfer from the reduced soluble plastocyanin to a soluble ferredoxin on the other side of the membrane. Light provides energy to run this uphill electron transfer. Depending on the availability of elements (Iron or Copper), a cytochrome c_6 can replace the plastocyanin in cyanobacteria. Similarly, flavodoxin can substitute for ferredoxin. Photosystem I is a heterodimer composed of 11 protein-subunits (Shen et al., 1993). In the Photosystem I, the cofactors of the electron transfer chain are bound to PsaA and PsaB, which form a heterodimer and to PsaC proteins (**Figure 5**). These 3 subunits are conserved in Photosystems I among all photosynthetic organisms but other subunit composition can vary. Energy of light induces charge separation: the electron is transferred from the excited special pair of chlorophylls, P700 to the primary electron acceptor, chlorophyll A_0 (bound to PsaB). The electron is then transferred to a phylloquinone (A_1) and to the F_X iron-sulphur centre. The F_X is shared by the PsaB and PsaA proteins. Finally, electrons from F_X reduce two iron-sulphur centres F_A and F_B in the PsaC protein, which are subsequently oxidised by ferredoxin. Electron from reduced ferredoxin is transferred onto $NADP^+$ in a reaction catalysed by an enzyme, ferredoxin-$NADP^+$ reductase (FNR). Formed upon charge separation, $P700^+$ is reduced with an electron from reduced plastocyanin (reviewed in (Brettel, 1997)).

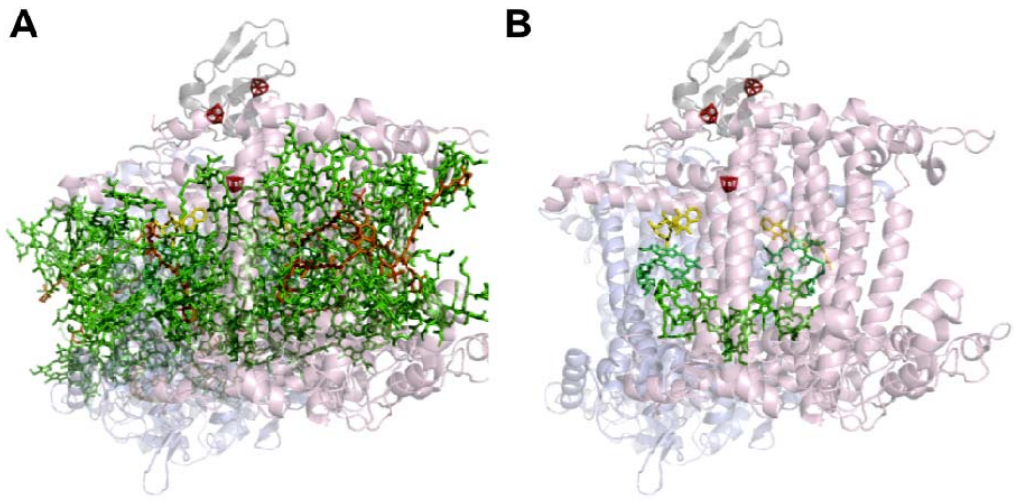

Figure 5 Photosystem I:
Reaction centre of Photosystem I from *Thermosynecococcus elongatus* composed of PsaA (light blue), PsaB (light pink), and PsaC (gray): (A) with all cofactors (excluding lipids), (B) only with the cofactors involved in the electron transfer chain. (B) In PsaB (light pink on the right) electrons from central special pair of chlorophylls P700 (green) through chlorophyll A_0 (lime green) and phylloquinone A_1 (yellow) reduce F_X iron-sulphur centre (red bottom). Electrons from F_X reduce two iron-sulphur centres F_A and F_B (red upper) in the PsaC protein (gray) (see text). In (A) additionally carotenoids (orange) are visible. (A) and (B) were redrawn with PyMol from Chapman et al., 2011.

Cytochrome b_6f

Cytochrome b_6f complex is an enzyme translating the two electron chemistry of PSII into the one electron chemistry of PSI. Out of eight protein subunits per monomer of the complex, the cytochrome *b*, the Rieske protein and the c-type cytochrome *f* bind all the cofactors of the electron transfer chain (**Figure 6**). Cytochrome b_6f is likely to be a functional homodimer (in analogy to cytochrome bc_1 as described in (Swierczek et al., 2010)). In the Q_o site of the cytochrome *b* electrons from plastoquinol bifurcate into two discrete paths. One of these electrons is transferred through the mobile iron-sulphur cluster bound to Rieske protein to the haem of cytochrome *f*, and finally reduces the soluble electron carrier: plastocyanin or cytochrome c_6. The second electron from Q_o site is transferred *via* the b_l and b_h haems of cytochrome *b* to an oxidised quinone in a Q_i site on the other side of the membrane. In the Q_i site a stable semiquinone is formed and after two turnovers of the enzyme, plastoquinol is released from a Q_i site. This re-reduction of quinone in Q_i site is a part of the Q-cycle (Mitchell, 1975; Osyczka et al., 2005) in which protons are "pumped"

to build up the protonmotive force across the membrane. Lately it was demonstrated that cytochrome b_6f that had a disrupted Q-cycle still retained active (but impaired) linear electron transfer from PSII to PSI (Malnoe et al., 2011).

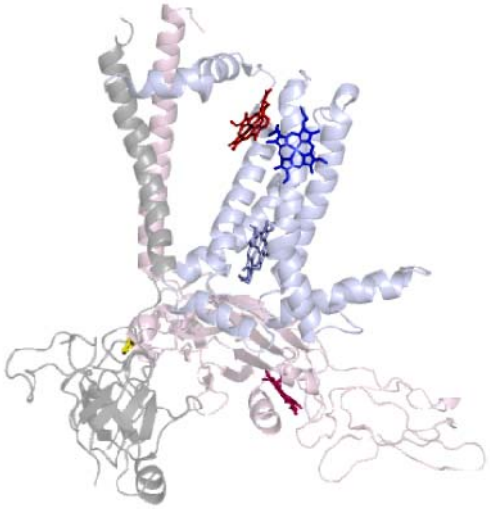

Figure 6 Cytochrome b_6f:
Picture of the cytochrome *b* (light blue), cytochrome *f* (light pink) and the Rieske protein (gray) from a monomer of cytochrome b_6f from *Nostoc* PCC7120. Electrons bifurcate into two pathways: the high potential chain with iron-sulphur cluster (yellow) and a *c*-type haem (pink) in cytochrome *f*; the low potential chain with haems the b_l (silver-blue) and b_h (blue) (see text). Next to the b_h haem there is additional haem (red). Picture was redrawn with PyMol from Baniulis et al., to be submitted – last version (2011) from RCSB PDB.

Photosynthetic antennae

Photosynthetic reaction centres are fully functional on their own. However even in high light, an incident photon (of relevant energy) stimulates each reaction centre only once per second. To fulfil energetic requirements a rate of at least 100 times higher is needed (Cogdell in (Nicholls and Ferguson, 2002)). Light-harvesting antennae complexes provide reaction centres with excitation-energy even under low light conditions. The high number of diverse pigments in the antennae with finely-tuned optical properties enables them to efficiently harvest light energy and transfer it to the reaction centres. Moreover, photosynthetic antennae are functionally flexible in their energy conversions enabling them to avoid photodamage of reaction centres.

Depending on light conditions energy thermal-dissipation can be triggered at antenna level to decrease the energy arriving at the reactions centres.

In contrast to conserved photosynthetic reaction centres, the composition and arrangement of antenna complexes vary between organisms. Therefore, the mechanisms of energy transfer and dissipation occurring at antenna level are extremely varied. The diversification of antennae types has allowed organisms to exploit the variable energetic niches.

Antennae in plants and green algae

Photosynthetic antennae in higher plants and green algae are membrane embedded proteins, named Light Harvesting Complexes (LHC), which bind carotenoids and, chlorophyll *a* and *b*. Excitation-energy in illuminated antenna propagates from excited carotenoids and chlorophyll *b* to chlorophyll *a*. Finally, excitation-energy from a network of connected chlorophyll *a* molecules reaches the reaction centres.

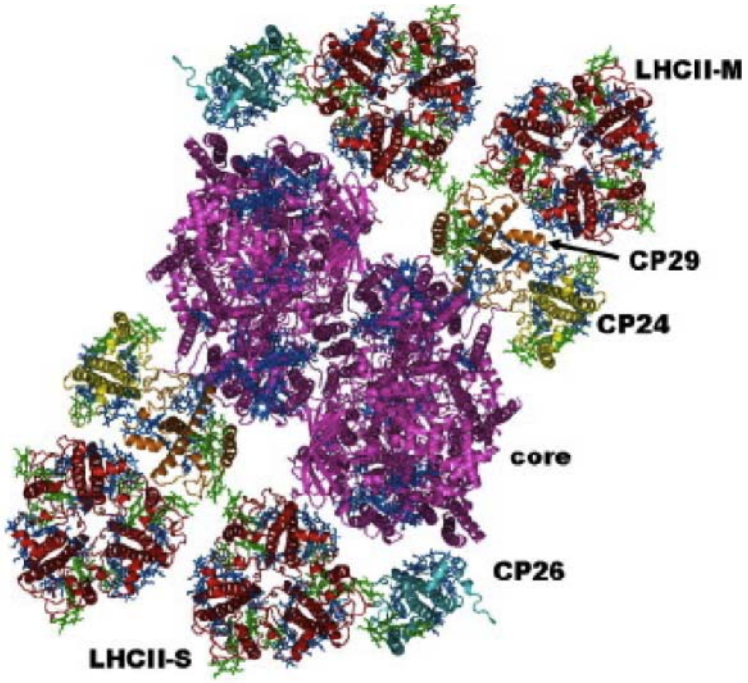

Figure 7 In higher plants photosynthetic antennae are membrane embedded.
Model of the organization of the antennae complexes of Photosystem II in the grana thylakoid of higher plants. Reaction centre of Photosystem II (magenta); LHCII major trimeric antenna complex (red); and minor antennae: CP24 (yellow), CP27 (orange), CP26 (cyan). Picture was taken from Croce and van Amerongen, 2011.

Photosystem II complex has proximal and outer photosynthetic antennae. While the proximal antennae, CP43 and CP47 are present in higher plants, green algae and cyanobacteria, the outer chlorophyll antennae are found only in higher plants and green algae. In higher plants six genes encodes the outer antennae of PSII (*Lhcb*). Proteins Lhcb1-3 form a trimeric, major antenna complex, called LHCII (**Figure 7**). The proteins Lhcb4-6 are minor antenna complexes, termed CP29, CP26 and CP24, respectively. The minor complexes do not form a trimer (reviewed in (Croce and van Amerongen, 2011)). Antennae of PSI are built from two proteins (*Lhca*), which bind chlorophylls and carotenoids. In different algae species the photosynthetic antenna can have variable pigment and protein composition.

Phycobilisomes

Due to their intense colouration and strong fluorescence emission, phycobilisomes (PB) became an object of studies as early as the 1830's. Later, in the 1850's Stokes performed spectroscopic studies on this pigmented matter and his observations gave basis to the idea of the Stokes shift. Already at the end 19th century it was suggested that these pigments provide energy to chlorophylls to run photosynthesis (reviewed in (de Marsac, 2003)).

Indeed, in many strains of cyanobacteria phycobilisomes serve as the main light-harvesting antenna. Phycobilisomes are also present in red algae as the major antenna of PSII. The colour of these soluble "small granules (about 320 Å) regularly arranged along the parallel chloroplast lamella" (from (Gantt and Conti, 1965); the name "phycobilisome" was proposed by these authors) comes from chromophores, called phycobilins. Phycobilins are linear tetrapyrrols (**Figure 8**.B) synthesised in biochemical reactions, in which carbon monoxide is a by-product (Troxler and Dokos, 1973). In cyanobacteria there are four types of phycobilins possessing different optical properties: phycoerythrobilin, phycocyanobilin, phycourobilin and phycobiliviolin. Phycobilins, which number 1-4 per polypeptide, bind to different phycobiliproteins through thioether bonds with cysteine (reviewed in (Glazer, 1988b, 1988a; MacColl and Guard-Friar, 1987). In cyanobacteria there are 3 major types of phycobiliproteins: phycoerythrins, phycocyanins (PC) and allophycocyanin (APC) (reviewed in (de Marsac, 2003). Phycobilins interact with phycobiliproteins, which tune their spectral properties (MacColl and Guard-Friar, 1987) (**Figure 8**.A).

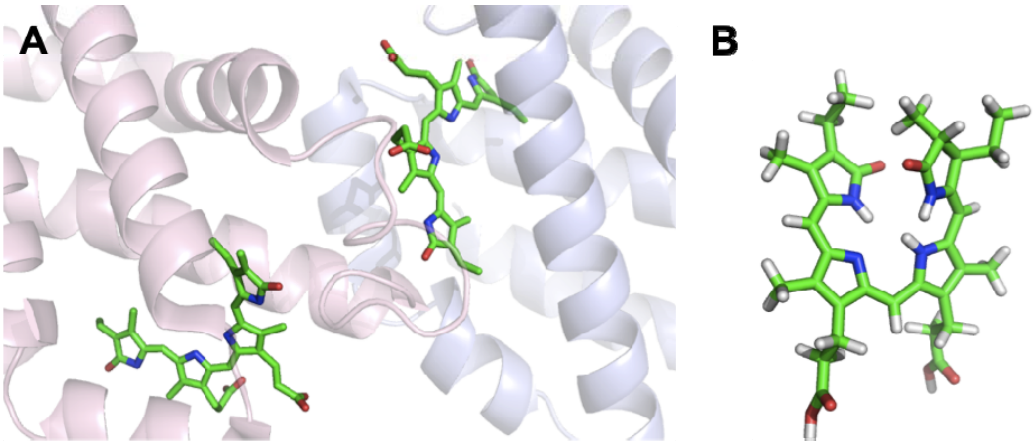

Figure 8 *Synechocystis* PCC6803 phycobilisomes bind only phycocyanobilin.
Pigments in phycobilisomes are linear tetrapyrrols. Allophycocyanin and phycocyanin in *Synechocystis* bind only phycocyanobilin. (A) Phycocyanobilins bound to an α-allophycocyanin subunit (light blue) and to a one β-allophycocyanin subunit (light pink) of two neighbouring monomers in a trimer. Pigments are bound *via* Cysteins to the apoproteins. Distance between these two pigments is ~20 Å. Picture was redrawn with PyMol from Reuters et al., 1999. (B) Conformation of phycocyanobilin for the theoretical minimal energy is very different than for this pigment attached to a protein. Phycocyanobilin is composed of carbon (green), nitrogen (blue), oxygen (red) and hydrogen (white).

Electron micrographs of phycobilisomes isolated from various organisms indicated four general types of phycobilisome architecture: hemi-ellipsoidal, hemi-discoidal, block shaped and bundle shaped (Sidler in (Bryant, 1995)). Phycobilisomes are arranged in order to provide efficient excitation-energy transfer to the photosystems. The shorter-wavelength absorbing components are at distal positions and in the central position there are the most red-shifted species. It means that a phycobilisome forms an energetic funnel (reviewed in (Glazer, 1984)). In the central position of the hemi-discoidal phycobilisome there is a core build of cylinders (2-5 per core) of allophycocyanin trimers (4 or 2 per cylinder) (**Figure 9**). From the core there are various numbers of rods radiating. These rods are composed of hexamers of phycocyanin (always the core proximal hexamer) and optionally phycoerythrin or phycocyanoerythrin (reviewed in (Glazer, 1984)).

Complete phycobilisomes are huge complexes of approximately 60x60x12 nm (for *Synechocystis*, (MacColl, 1998)). When isolated, phycobilisomes are highly unstable and require high phosphate concentration to remain intact (Gantt and Lipschultz, 1972). High concentration of phosphate is believed to create "micro-hydrophobic" environment mimicking the tight packing in the cell (Rigbi et al., 1980; Zilinskas and Glick, 1981; Stagg et al., 2007). Addition of high concentrations of another non-aggregating protein (*e.g.* Bovine Serum Albumin - BSA) allows the

elimination of the phosphate while keeping the phycobilisomes unimpaired (Prof. Noam Adir – personal communication).

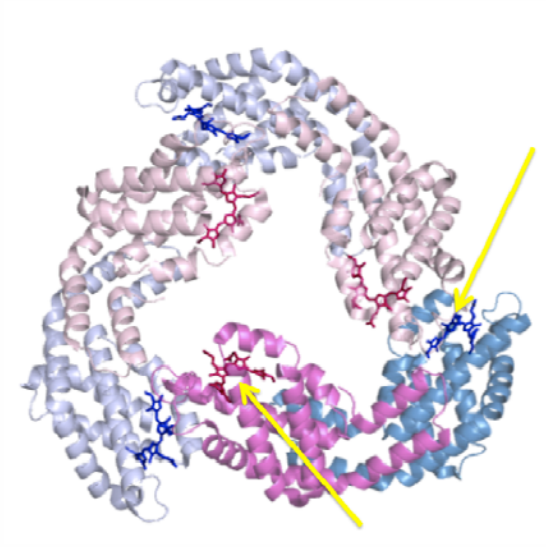

Figure 9 Tree-dimensional structure of a trimer of allophycocyanin.
In the core of a *Synechocystis* PCC6803 phycobilisome there are 3 cylinders, each composed of 4 trimers. Each trimer is composed of 3 monomers (one of monomers is highlighted) and each monomer is build of α- (light blue) and β- (light pink) allophycocyanin subunits. Phycobilins in one monomer (pointed with yellow arrows) are ~50 Å from each other while phycobilins of two neighbouring monomers are closer (~20 Å). The α-allophycocyanin subunits (blue) are more external than β-allophycocyanin subunits. Picture was redrawn with PyMol from Reuters et al., 1999 (for clarity structure of a capping linker L_C is not shown).

The instability, complexity and size of a phycobilisome make the resolution of its tree-dimensional structure very toilsome (Adir, 2005). In fact, no tri-dimensional structure of the whole phycobilisome exists. Recently, the structure of a rod was published (David et al., 2011). Proposed models of a phycobilisome structure are based on data from electron microscopy (Bryant et al., 1979; Yamanaka et al., 1980; Arteni et al., 2009) (**Figure 10**.A), crystal structures of monomers (Brejc et al., 1995; Adir, 2003) and trimers (the most complex is an APC trimer with a linker, (Reuter et al., 1999; McGregor et al., 2008)) (**Figure 9**) and enzymatic degradation assays (Lundell and Glazer, 1983).

Additional to the photosynthetic function, cells utilize phycobilisomes as an amino acid stock, which can be accessed by enzymatic degradation of phycobilisomes under starvation (Collier and Grossman, 1992; Dines et al., 2008).

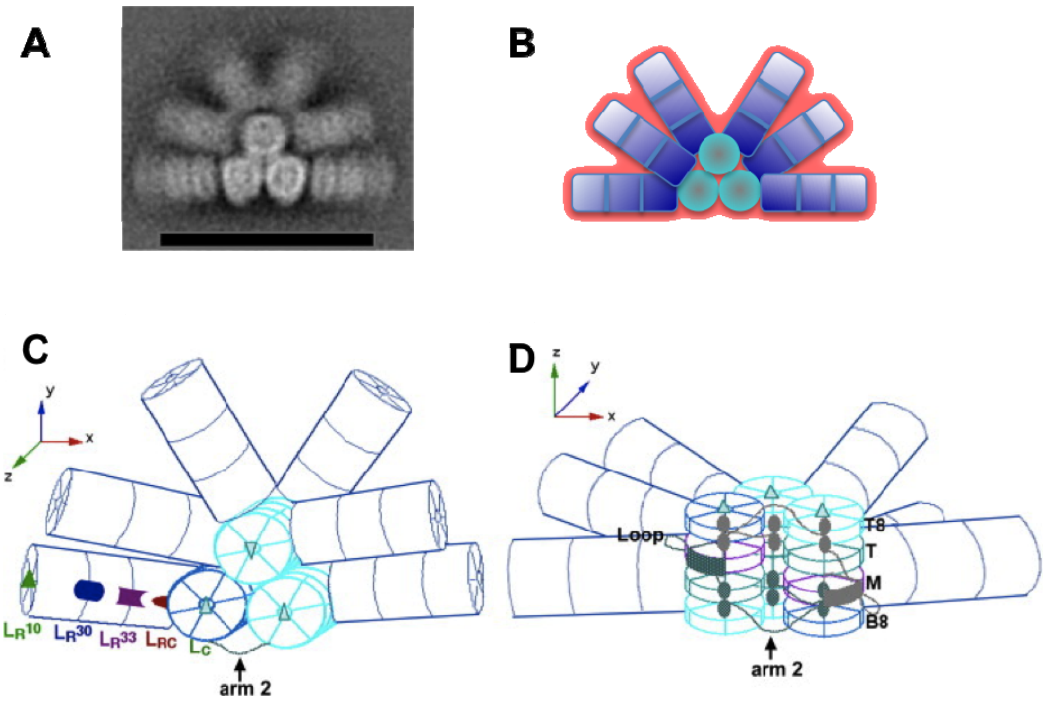

Figure 10 Phycobilisomes are major photosynthetic antenna in many cyanobacteria. The *Synechocystis* PCC 6803 phycobilisome:
The architecture of phycobilisomes allows efficient energy transfer to the reaction centre. (A) Electron micrograph of a phycobilisome (front view) (Arteni et al., 2009). Bar represents 50 nm. (B) Phycobilisome redrawn from (A) – 6 rods (assembled hexamers) of phycocyanin (dark blue) radiate from a tri-cylindric core of allophycocyanin (cyan). (C) and (D) detailed model of a phycobilisome (C) front view, (D) bottom view. In (C) linkers: capping L_R^{10} (dark green), L_R^{30} (dark blue), L_R^{33} (purple), rod-core L_{RC} (red) and in the core capping L_C (light green) are placed in the central cavity of a hexamer or a trimer. Arm 2 is a loop of L_{CM}. (D) model of the organization of the core. Upper cylinder composed of subunits α- and β-allophycocyanin and two L_C. Lower cylinders (closer to the reaction centres or the membrane) are anti-symmetrical. Trimers "T" and "T8" are composed of α- and β-allophycocyanin subunits. Trimer "T8" is capped by L_C. Trimer "M" have one of α-allophycocyanin subunits replaced by a domain of L_{CM} and one β-allophycocyanin subunits replaced by ApcF protein (both likely in the same monomer). Trimer "B8" have one of α-allophycocyanin subunits replaced by ApcD, and is capped by L_C. Trimers "M" and "B8" are so-called terminal emitters. (A), (C) and (D) were taken from Arteni et al., 2009.

In *Synechocystis* there are two major phycobiliproteins only: Phycocyanine (PC) and Allophycocyanine (APC). Both proteins bind only one type of phycobilin, the phycocyanobilin (**Figure 8**). Protein subunits of APC, called α or β, bind one phycocyanobilin each, while PC α subunit binds one phycocyanobilin and PC β subunit binds two phycocyanobilin molecules (MacColl, 1998). Two subunits together (α and β of APC or of PC) form a stable, so-called monomer (McGregor et al., 2008). Three monomers of APC form a stable trimer (**Figure 9**). In case of PC, trimers aggregate in face-to-face orientation, forming a stable hexamer (reviewed in (Glazer, 1984; MacColl and Guard-Friar, 1987)). To fulfil the functional requirements, a phycobilisome of *Synechocystis* follows the hemi-discoidal design of

a central tri-cylindrical core and six rods radiating from it (EM picture in (Arteni et al., 2009)) (**Figure 10**). On average, the rods are composed of 3 hexamers of PC. The rod absorption maximum is at 620 nm and the emission at 650 nm. The spectral properties of each hexamer of the rods are tuned by non-chromophorylated linker proteins, which also stabilise the binding between the hexamers (Adir, 2005). Rods are attached to the core *via* a linker protein. The core of a *Synechocystis* phycobilisome is composed of 3 cylinders (EM picture in (Arteni et al., 2009)) (**Figure 10**). Each cylinder contains four APC trimers (**Figure 9**), which absorb at 650 nm and emit at 660 nm. In some trimers, the α or β subunit is replaced by another APC-like protein that emits at around 680 nm, so-called terminal emitters, ApcD, ApcF and ApcE (known also as L_{cm}) (**Figure 10**.D). The terminal emitters are responsible for the energy transfer to the photosystems (Lundell and Glazer, 1983; Maxson et al., 1989; Gindt et al., 1994; Ashby and Mullineaux, 1999) and L_{cm} also acts a membrane anchoring linker protein, which is absolutely necessary for core formation (Redlinger and Gantt, 1982; Capuano et al., 1993; Ajlani and Vernotte, 1998).

The difference in absorption (or fluorescence) peak position between a PC hexamer and an APC-terminal emitter trimer is approximately 30 nm, even though both complexes bind the same phycobilin. The origins of this spectral shift are discussed in the literature and are likely caused by dissimilar pigments environments in the proteins and different interactions between monomers in hexamers and trimers. In the APC trimers, pigments of adjacent monomers are within the distance for exciton coupling to occur (~20 Å) (discusses in (MacColl, 1998)) (**Figure 8**).

Photoinhibition

The linear transfer of electrons of the three complexes is able to convert light energy into a proton-motive force and reduce $NADP^+$. It is usually presented as so-called Z-scheme, reflecting the free-energy change of the various electron carriers (reviewed in (Govindjee, 2006)) (**Figure 11**). Other reactions within the chain, but not included in the Z-scheme, are possible and can result in energy dissipation or in the formation of potentially dangerous, so-called "reactive oxygen species" (ROS), which irreversibly damage the components of the photosynthetic apparatus. (reviewed in (Rutherford et al., 2012)). While the Photosystem I is the main site of production of the ROS superoxide, especially at low temperatures (reviewed in (Scheller and

Haldrup, 2005)), the Photosystem II is the main site of oxygen singlet formation (Vass, 2011). The photodamage processes are collectively called photoinhibition. Here, I will briefly summarize Photosystem II related photoinhibition processes.

Although photoinhibition mechanisms induced by the visible part of solar radiation (light) are still unclear, the majority of research groups propose that an irreversible damage of D1 protein (followed by the D2 damage) is a result of singlet oxygen generated from triplet chlorophylls formed during charge recombination. The amount of chlorophyll triplets increases under conditions in which the PQ pool is "over-reduced" e.g. low carbon dioxide concentration, decreased temperature or high light. However, charge recombination can also occur at very low light intensities before Q_B is doubly reduced. It has also been suggested that the destruction of the Mn_4Ca cluster *via* direct absorption of light lies at the basis of inactivation of Photosystem II (discussed in (Vass, 2012)).

The absorption of the UV range of solar radiation can damage the Mn_4Ca cluster (reviewed in (Vass, 2012)) so that the Tyr Z^+ and $P680^+$ cannot be reduced. As secondary effects, quinone acceptors and tyrosine donors are damaged and highly reactive hydroxyl radicals are generated from partly oxidised water initiating further damage (Hideg and Vass, 1996).

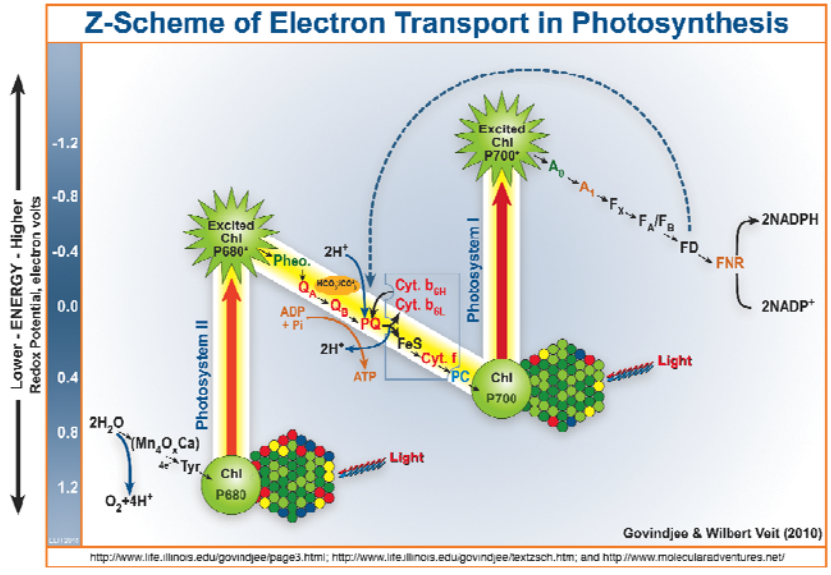

Figure 11 The Z-scheme of photosynthetic electron transfer chain.
Scheme represents photosynthetic electron transfer chain emphasising energetic levels of formed intermediates. From www.life.illinois.edu/govindjee/ZSchemeG.html. Version from 2010 corrected.

Photoprotection

Too much light reaching the photosynthetic apparatus can cause photodamage and ultimately can lead to the death of a cell. To cope with deleterious effects of high light, photosynthetic organisms have a variety of photoprotective mechanisms. These mechanisms can be classified as follows:

1. Modulation of the capacity of light absorption
2. Thermal dissipation of an excess absorbed energy from photosynthetic antennae
3. Secondary-Electron-transfer involved in photoprotection
4. Scavenging ROS
5. Repair of damage in PSII

The subject of my thesis is about a photoprotective mechanism involving thermal dissipation of excess absorbed energy, which will be presented after a brief description of the other photoprotective mechanisms existing in cyanobacteria. Although I focus upon cyanobacteria, thermal dissipation mechanism in higher plants will be also briefly summarised.

Modulation of the capacity of light absorption

Lowering light absorption, allow cyanobacteria to decrease the energy arriving to the reaction centres under high light conditions. Cyanobacteria can avoid excess illumination by negative phototaxis, which is the movement away from high light (reviewed in (Bhaya, 2004)).

In addition, cyanobacteria regulate the size of antennae by lowering the synthesis of pigments and antenna proteins. Under high light conditions, the synthesis of chlorophyll and phycobilins is inhibited at the level of the synthesis of the 5-aminolevunilic acid, common precursor of these pigments. In one generation, the pigment content of the cell decreases by 50% (Muramatsu et al., 2009; Hihara et al., 1998). The expression of genes encoding enzymes involved in pigment synthesis and encoding photosynthetic proteins, especially phycobiliproteins and Photosystem I proteins is also decreased (Muramatsu et al., 2009).

Degradation of the already existing PB can also occur under high light. One of mechanisms of PB degradation involves an enzyme SspA1, which is upregulated under high light (Pojidaeva et al., 2004). It was also reported that very high light illumination can induce exciton-decoupling of PB, followed by detachment from thylakoid and degradation (Tamary et al., 2012). A smaller effective antenna decreases the energy arriving to the reaction centres.

There are two proteins from the LH family which are expressed under stress conditions, such as high light and iron starvation, that also seems to have a role in photoprotection by interacting with chlorophyll under situations in which the photosystems and their antennae are downregulated. The first family is High Light-Induced Proteins (Hlips), also called Light-harvesting-like (Lil) or Small CAB-like proteins (Scp) are single α-helix membrane proteins, which accumulate in cyanobacteria in response to high light (Funk and Vermaas, 1999). The function of Hlips in cyanobacteria is under debate. Mutants missing all Hlips have altered pigmentation (Xu et al., 2004). It was proposed that Hlips are involved in coordination of chlorophyll biosynthesis during high light stress (Hernandez-Prieto et al., 2011). IsiA, also called CP43' due to the similarity to CP43, is a chlorophyll-binding membrane complex. Iron starvation induces the expression the IsiA encoding gene (Laudenbach and Straus, 1988) but other stresses also increase its concentration in the cells. It was shown that IsiA encircles trimeric Photosystem I (Bibby et al., 2001; Boekema et al., 2001) and increase its absorption cross section. When high concentrations of IsiA accumulate in the cell in response to iron starvation, they form empty aggregates (without PSI), which thermally dissipate absorbed energy (Ihalainen et al., 2005).

Secondary-electron-transfer pathways

In photosynthesis, electrons produced from charge separations in PSII are primarily used to reduce $NADP^+$. However, under excess excitation the photosynthetic electron transport chain can become over-reduced, inducing secondary-electron-transfer pathways. These pathways are putatively involved in photoprotection because they decrease excitation pressure. Carbon assimilation and photorespiration at low CO_2 concentration will not be discussed.

Secondary electron transports at Photosystem II

Cyclic electron transfer within the reaction centre II provides electrons to reduce D2 β-carotene$^+$ that has been oxidised by P680$^+$ and P680$^+$ (Telfer et al., 1991). These reactive cations are stabilised under conditions in which electrons are not provided by Tyr Z and the Mn_4Ca cluster. In these conditions the increased lifetime of P680$^+$ can oxidise water or the β-carotene of the D2 protein because it has a sufficiently high redox potential (1260 mV, (Rappaport et al., 2002)). The secondary electron pathway involving β-carotene$^+$ and P680$^+$ reduction has been largely discussed in the literature (for a review see "Scheme 1" in (Faller et al., 2001; Frank and Brudvig, 2004)). It also involves Chl_Z, the haem of the cytochrome b_{559} and chl_{D2}. The oxidized haem of the cytochrome takes the electron from PQH_2 (reviewed in (Cardona et al., 2012)).

Another route of secondary electron transfer in PSII allows electrons to leave the photosynthetic electron transport chain *via* flavodiiron proteins (Flv), which carry two non-haem irons and one FMN cofactor. Out of four Flv proteins in *Synechocystis* Flv2 and Flv4 are involved in decreasing excitation pressure at PSII. Flv2 and Flv4, which form a membrane-attached heterodimer in *Synechocystis*, are present in the cells under low carbon dioxide concentration and high light conditions (Zhang et al., 2012). Flv2/Flv4 heterodimer is suggested to provide a direct "escape" for electrons from the Q_B site of Photosystem II. The heterodimer could also decouple the phycobilisomes from the Photosystems (Zhang et al., 2012).

Pseudo-cyclic electron transfer, Mehler like reaction at Photosystem I

Under optimum growth conditions, electrons from the photosynthetic electron transfer chain reduce NADP$^+$, which then is used as reducing power in the Benson-Calvin cycle. When photosynthesis rate is higher than the rate of NADPH$^+$ consumption (*e.g.* high light, low CO_2), electrons from the electron transfer chain can reduce oxygen *via* the cyanobacterial Mehler-like reaction. In *Synechocystis* this reaction is mediated by a heterodimer of flavodiiron proteins Flv1 and Flv3 (Helman et al., 2003). NADPH likely serves as a source of electrons for the photoreduction of oxygen leading to formation of water and avoiding the production of ROS, typical of the Mehler reaction of other photosynthetic organisms (Helman et al., 2003). This

mechanism decreases excitation pressure by withdrawing the electrons from the photosynthetic electron transfer chain.

Cyclic electron flow around PSI also forms another alternative electron pathway, which operates *via* two different routes. One involves a plastoquinone reductase, the so-called NDH complex (Deng et al., 2003) and the other *via* a putative ferredoxin quinone reductase, feeding electrons directly into the cytochrome b_6f complex. It has been reported in plants and green algae in the literature that cyclic electron flow protects against photoinhibition of PSI (Roach and Krieger-Liszkay, 2012; Sonoike, 2011), by keeping the acceptor side of PSI oxidised.

Scavenging ROS

Under high light conditions, the photosynthetic electron transfer chain can be locally blocked or globally over-reduced, which can lead to the generation of reactive oxygen species (ROS). Within PSII, charge recombination can occur between the donor and acceptor sides, especially when charge separations exceed the capacity of downstream electron flow (Telfer et al., 1994; Hideg et al., 1998). During charge recombination triplet chlorophyll can be formed and by reacting with molecular oxygen, singlet oxygen ($^1O_2$), a major component of photoinhibition is formed (reviewed in (Rutherford and Krieger-Liszkay, 2001; Vass, 2011)). The energy of triplet chlorophyll, which is also formed at the chlorophyll antenna level (CP43, CP47) can be dissipated by carotenoids avoiding the formation of $^1O_2$. Interestingly, the distribution of carotenoids varies between different cyanobacteria strains (Hertzber and Liaaenje, 1971). Among the various carotenoids in cyanobacteria, zeaxanthin is the most prominent in protecting against the photooxidative stress (Schafer et al., 2005; Zhu et al., 2010). Singlet oxygen is a highly reactive molecule that can oxidise many biological compounds, such as membrane lipids. In *Synechocystis* tocopherol is crucial in protecting against oxidative damage by terminating the chain reaction of lipid peroxidation (Maeda et al., 2005). Other ROS that can be produced by photosynthesis include superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). $O_2^{\cdot-}$ can be converted to H_2O_2 by superoxide dismutase and H_2O_2 is broken down to water by various enzymes, including catalase, glutathione peroxidases, peroxiredoxins, rubrerythrins and DNA-binding proteins (reviewed in (Latifi et al., 2009)).

Repair of damage in PS II

Even under low light conditions, the reaction centre of Photosystem II (RCII) is susceptible to photodamage. The D1 protein is the primary site of damage in RCII, requiring selective replacement in the RCII repair cycle (reviewed in (Nixon et al., 2005; Huesgen et al., 2009; Mulo et al., 2012)). Damaged subunits of the RCII (mostly D1 protein) are degraded by the FtsH2/FtsH3 heterohexamer (Boehm et al., 2012). In plants, the Deg/HtrA proteases seem also to be involved. However, in cyanobacteria their role is less clear (reviewed in (Huesgen et al., 2009)). The role of these enzyme families and potential involvement of others have been discussed in the literature (see Fig 2 in (Huesgen et al., 2009)). The expression of the genes encoding FtsH and DegP/HtrA is up regulated in high light (reviewed in (Muramatsu and Hihara, 2012)).

In the RCII repair cycle, the degraded D1 protein is replaced by a newly synthesised one. Under high light the D1 protein (encoded by *psbA1*) is replaced by a more light-tolerant isoform (encoded by *psbA3*) in most of the examined cyanobacteria strains (*e.g. Synechococcus* 7942, *Thermosynechococcus elongatus*, *Gleobacter violaceus, Anabaena* 7120, *Synechococcus* WH7830). In *Synechocystis* there are two identical D1 isoforms, *psbA2* (equivalent to *psbA1* in *Synechococcus)* and *psbA3*. Under strong illumination both genes are upregulated, but *psbA3* more so than *psbA2* (reviewed in (Mulo et al., 2012)).

Thermal dissipation of an excess absorbed energy from photosynthetic antennae

Overview

An exited pigment in photosynthetic antenna can be deactivated *via* three different pathways: photochemistry, thermal dissipation and fluorescence emission. The excitation energy can be transferred to neighbouring pigments so the energy can reach the reaction centre where charge separation occurs. This pathway leads to the photochemical quenching of excitation (photochemistry). Under sub-saturating light conditions most of the excitation-energy absorbed by the pigments of the antennae is quenched by the photochemical reaction in the reaction centres; only around 3% of the energy comes back as fluorescence, and very little is dissipated as heat. Under

higher light intensities, photoprotective mechanisms are induced that increase thermal dissipation at the level of antennae. The increase in thermal dissipation of the excitation-energy leads to a simultaneous decrease of fluorescence emission and of energy transfer to reaction centres. Hence, the effective size of the photosynthetic antenna is smaller.

Thermal dissipation (qE) in plants

The increase of ΔpH across the photosynthetic membrane (*i.e.* acidification of the thylakoid lumen) activates thermal dissipation in plants. Upon protonation of the PsbS protein (Funk et al., 1995; Li et al., 2000), enzymatic de-epoxidation of violaxanthin to zeaxanthin, in the so-called xanthophyll cycle (Demmig-Adams, 1990) and a rearrangement of antenna complexes in the membrane, an increasing amount of energy is dissipated as heat at the level of the light harvesting complex (LHCII). The proteins involved, the site(s) and the mechanisms of thermal dissipation in higher plant have been lively discussed in the literature, but a common complete description has so far not been achieved. Open questions concern many aspects of this process. What are the complexes involved in energy dissipation? Minor (monomers), major (trimers) or both antennae? Is the "quencher" of energy the chlorophylls or the carotenoids? How does the mechanism of energy quenching occur? Through excitation transfer or charge transfer (reviewed in (Ruban et al., 2012; Jahns and Holzwarth, 2012))?

Thermal dissipation mechanism in cyanobacteria

It has been believed that cyanobacteria, which do not have LHCII complexes but phycobilisomes as the major antennae, do not have a photoprotective mechanism involving an increase of thermal dissipation at the antenna level (Campbell et al., 1998). However, in 2000, El Bissati and co-workers reported the first results suggesting the existence of such mechanism also in cyanobacteria. They observed that strong blue-green light induced fluorescence quenching which was reversible in darkness or low light conditions. They demonstrated that this fluorescence quenching was not related to photoinhibition or state transitions. Simultaneous quenching of F_m and F_o and its occurrence at temperatures in which state transitions were inhibited, allowed the authors to suggest that this blue-green light induced fluorescence quenching most probably occurred at the level of phycobilisomes. This process was

proposed to be photoprotective and to decrease the energy arriving at the reaction centres (El Bissati et al., 2000). Later, in 2004 Rakhimberdieva and co-workers, performed spectral and kinetic studies of the blue-green light induced fluorescence quenching in a mutant missing Photosystem II. They confirmed that fluorescence occurred at the level of phycobilisomes. Unlike in plants or green algae, induction of fluorescence quenching was not driven by ΔpH across the membrane (El Bissati et al., 2000; Wilson et al., 2006). Action spectra of when this fluorescence quenching was induced, indicated that the process is triggered by a carotenoid (Rakhimberdieva et al., 2004). In 2006, Wilson and co-workers identified the trigger of the thermal dissipation mechanism in cyanobacteria, the Orange Carotenoid Protein (OCP). A *Synechocystis* mutant missing OCP was unable to induce the fluorescence quenching. This mutant was more sensitive to high light than the WT. Under high light conditions, PSII photoinactivation (measured as the oxygen evolution) occurred faster in the mutant than in the WT. Studies of phycobilisome mutants allowed to suggest that the core of a phycobilisome is the site of fluorescence quenching (Wilson et al., 2006). Later, it was confirmed by other research groups, that the phycobilisome core is the site of fluorescence quenching (Scott et al., 2006; Stadnichuk et al., 2009). Scott and co-workers also demonstrated that addition of a crosslinker can block phycobilisomes in quenched or unquenched states. This suggested inter- or intra-protein reorganization during induction of fluorescence quenching (Scott et al., 2006). In 2007, it was suggested that allophycocyanin (APC), emitting at 660 nm, could be the site of quenching (Rakhimberdieva, Vavilin, et al., 2007). The same year, it was observed that the recovery of lost fluorescence under low light is temperature dependent (Rakhimberdieva, Bolychevtseva, et al., 2007). In 2008, Wilson and co-workers demonstrated that under strong light conditions, the orange OCP undergoes conformational changes and it is converted to a red active form. The light inducing photoconverison of OCP had the same colour (blue-green) as the photoprotection triggering light (Wilson et al., 2008). In the same laboratory, it was showed that the strains, which do not carry OCP are more sensitive to high light intensities (Boulay et al., 2008). Punginelli and co-workers demonstrated in 2009 that the carbonyl group of the carotenoid in OCP is required in photoactivity and stability of the protein (Punginelli et al., 2009). In 2009 and 2010 two groups showed that OCP-related mechanism diminishes energy transfer to both Photosystem II and Photosystem I (Stadnichuk et al., 2009; Rakhimberdieva et al., 2010). In 2010, Boulay and co-

workers identified and initially characterised the Fluorescence Recovery Protein (FRP), which is responsible for regaining full antenna capacity after quenching. A *Synechocystis* mutant missing FRP was unable to regain full antenna capacity (Boulay et al., 2010). Also in 2010, the groups of Cheryl Kerfeld and Diana Kirilovsky reported together new tri-dimensional structures of OCPs from *Synechocystis* WT and mutants. It gave basis for further functional studies of the OCP (Wilson et al., 2010).

The Orange Carotenoid Protein

In 1981, Holt and Krogmann first described the presence of the soluble Orange Carotenoid Protein in several strains of cyanobacteria (Holt and Krogmann, 1981) (detection of OCP in a cell in **Figure 3**.C). In 1997, Wu and Krogmann identified the *Synechocystis* gene encoding for OCP and isolated and characterised the protein. They identified the carotenoid carried by the OCP as the 3'-hydroxyechinenone (hECN). They also characterized a product of OCP cleavage, the Red Carotenoid Protein (RCP), which is present in the cells at 10 times lower concentration than OCP. RCP misses the first 15 residues at the N-terminal and lacks all the C-terminal domain of OCP (Wu and Krogmann, 1997). The mass of RCP (16 kDa) was approximately half the OCP mass (35kDa).

The function of OCP was unknown when the first tri-dimensional crystal structure of the *Arthrospira maxima* OCP (**Figure 12**) was resolved at 2.1 Å (Kerfeld et al., 2003; Kerfeld, 2004b, 2004a). Its function in photoprotection in *Synechocystis* was revealed in 2006 (Wilson et al., 2006).

In *Synechocystis* OCP is encoded by the *slr1963* gene (Wu and Krogmann, 1997). OCP is present in most of the phycobilisomes-containing strains (Boulay et al., 2008; Kirilovsky and Kerfeld, 2012). Many strains have sequences encoding for one of two domains of OCP separately (usually N-terminal). Products of the expressions of these genes have unknown functions. Strains that do not carry OCP are more photosensitive than those in which the protein is present (Boulay et al., 2008).

The OCP encoding gene is constantly expressed, but becomes upregulated under stress conditions (Fulda et al., 2006; Wilson et al., 2007). In particular, high light illumination dramatically increases the level of *ocp* transcription (Hihara et al., 2001).

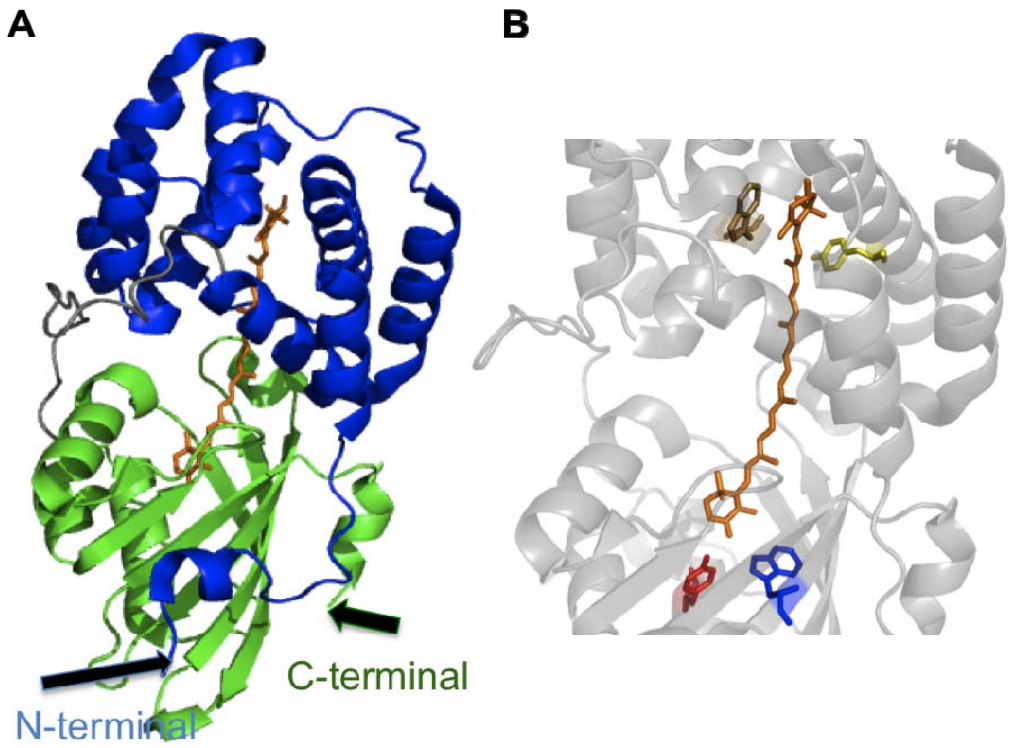

Figure 12 The OCP structure.
(A) OCP is composed of two domains: N-terminal (blue) and C-terminal (green); a flexible linker (gray) and a carotenoid hECN (orange). Carotenoid spans the two domains and is almost completely buried in the protein matrix. (B) Zoom-in at the carotenoid. In the C-terminal domain the carotenoid with its keto-group is bound to the apoprotein *via* Tyr201 (red) and Trp288 (blue). In the N-terminal domain the interacting residues are Tyr44 (yellow) and Trp110 (brown). (A) and (B) were redrawn with PyMol from Kerfeld et al., 2003.

The OCP is composed of 13 α-helices and 6 β-strands (Kerfeld et al., 2003; Wilson et al., 2010). The all-α-helical N-terminal domain has 8 α helices, which are divided into two bundles of 4 α-helices each. The N-terminal domain structure does not resemble any previously described protein structure. The C-terminal domain is more common and resembles the NTF2 domain. The two domains are joined by a flexible loop (Kerfeld et al., 2003; Wilson et al., 2010). The hECN having 11 carbon-carbon double bonds in an all-trans conformation spans the two domains of the OCP. Between the bundles of the N-terminal domain, there is the binding site of the hydroxyl-ring of the hECN, which interacts with conserved aromatic residues (**Figure 12**.B). The keto-end of hECN is bound to the C-terminal domain through hydrogen bonds with Tyrosine 201 and Tryptophan 288. The carotenoid is buried within the protein matrix and only 3.4% of its surface is solvent accessible (Kerfeld et al., 2003).

OCP can also bind other carotenoids: echinenone or zeaxanthin (Punginelli et al., 2009).

Spectroscopic properties of the carotenoid in solvent are very different than in OCP (Polivka et al., 2005). The isolated hECN appears to be yellow in organic solvent. The interaction of hECN with the OCP apoprotein red-shifts the absorption maximum of the hECN by 50 nm. Absorption spectrum of OCP shows two maxima at 476 and 496 nm with a shoulder at 440 nm (Kerfeld et al., 2003; Polivka et al., 2005; Wilson et al., 2008).

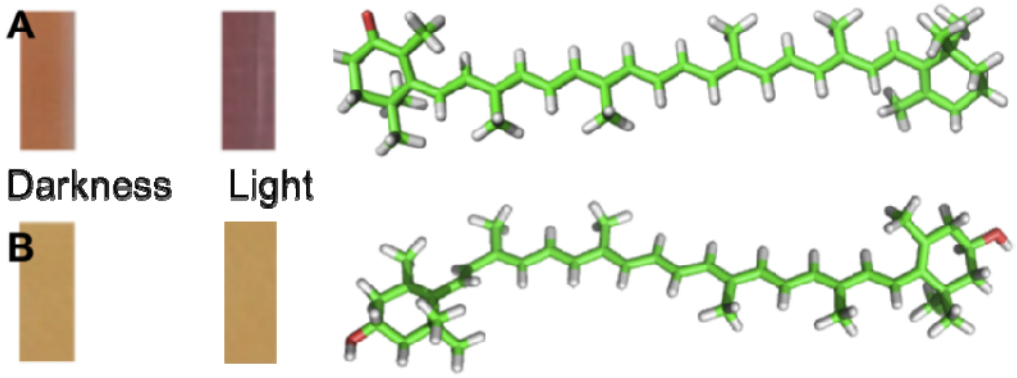

Figure 13 OCP can binds different carotenoids.
(A) The OCP, which binds 3'-hydroxyechinenone or echinenone is photoactive. In darkness, the OCP appears orange but when illuminated turns into red. (B) when zeaxanthin is bound to the OCP, the protein is yellow in darkness and under high light intensities. It is not photoactive. The keto-group of the carotenoid (carbon (green), oxygen (red) and hydrogen (white)) is required for photoactivity. Photographs were taken from Wilson et al., 2008 and Punginelli et al., 2009.

As mentioned already, the absorption spectrum of the OCP changes after illumination with strong light (Wilson et al., 2008). The absorption maximum shifts to 510 nm and the resolution of the vibrational bands (476 and 496 nm) is lost (Wilson et al., 2008). The OCP is orange in darkness (OCP^o), but upon illumination photoconverts to the red form (**Figure 13**.A) with a quantum yield of 0.03 (OCP^r). Absorption of light induces changes in the carotenoid: increasing the apparent conjugation length by one bond and reaching a more planar and less disordered structure. Raman spectroscopy suggested that trans-cis isomerisation is very unlikely to occur during photoconversion. The light-induced changes in the carotenoid are followed by rearrangement of the secondary structure of the protein. FTIR experiments showed loosening of α-helices, strengthening of the β-sheets and changes in the loop. Reconversion from the red to the orange form occurs spontaneously in dark and has strong temperature dependence (Wilson et al., 2008). When hECN is

replaced by echinenone (ECN) in the OCP, the protein remains photoactive and can induce the photoprotective mechanism. When zeaxanthin, which does not have a carbonyl group, is bound to OCP, the protein is not photoactive and does not induce photoprotection. OCP carrying zeaxanthin appears yellow (Punginelli et al., 2009) (**Figure 13**.B).

Fluorescence Recovery Protein

The Fluorescence Recovery Protein is a 13 kDa (theoretical mass) non-chromophorylated protein encoded by *slr1964* gene in *Synechocystis* (Boulay et al., 2010). In fresh water strains, the FRP-like protein encoding gene is downstream from the OCP-containing gene. In marine strains, there is a putative β-carotene ketolase gene between the OCP- and FRP-like encoding genes. Only a few OCP-containing strains do not to have an FRP-like encoding gene (Boulay et al., 2010). *ocp* and *frp* genes are very likely to be transcribed independently from their own promoters (Boulay et al., 2010).

Except for *Synechocystis* and closely related *Mycrocystis* NIES843, FRPs have conserved number of residues: 106-111. According to Cyanobase, FRP in these two strains has a N-terminal prolongation of 22-25 residues. The sequence of *Synechocystis* FRP suggested that it is a soluble protein but seemed to have very strong interactions with the thylakoids (Boulay et al., 2010).

FRP does not influence fluorescence quenching but mutants missing FRP can only regain 20-30% of the lost fluorescence. FRP binds only to the red form of OCP and accelerates its reconversion to the orange form. This led to the conclusion that FRP is responsible for switching off the OCP-related photoprotective mechanism (Boulay et al., 2010).

*The working model of the OCP related photoprotective mechanism at the beginning of my thesis (*Figure 14*) and open questions*

The discoveries listed above allowed a proposal of a working model of the OCP-related photoprotective mechanism as follows: Under low light conditions, the orange OCP is inactive and the phycobilisomes transfer most of the absorbed energy to the photosystems. When *Synechocystis* cells are under high blue-green (or white) light intensities, OCP is photoconverted – forms the red form – and induces the photoprotection by interacting with the core of the phycobilisome. This mechanism

decreases the energy arriving at the reaction centres. After illumination, in darkness or low light intensities FRP induces recovery of full antenna size through interaction with the red form of OCP.

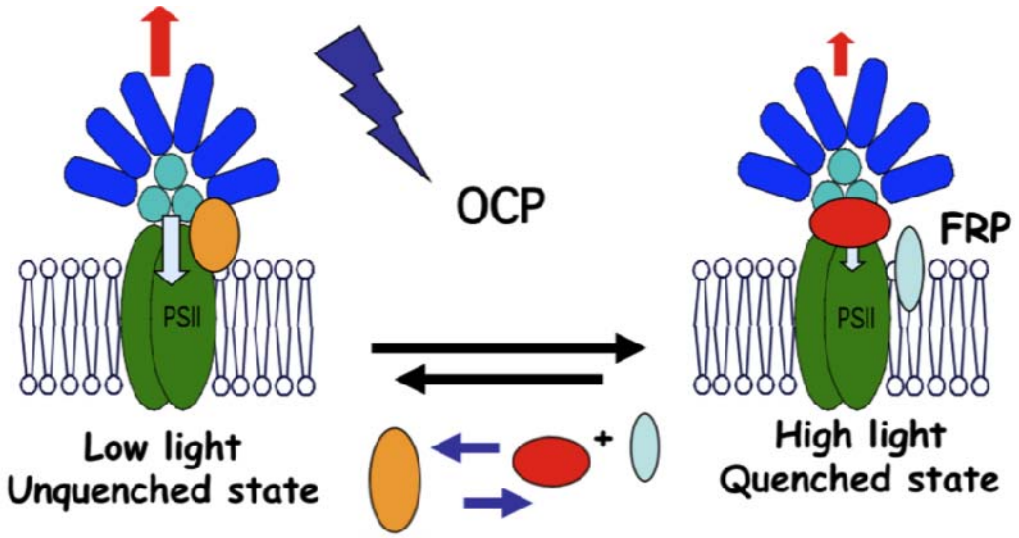

Figure 14 Model of the mechanism presented by Diana Kirilovsky in February 2010.
OCP^o (orange oval), OCP^r (red oval), FRP (blue oval), energy transfer to PS (blue arrow pointing down), fluorescence (red arrow pointing up). Sizes of arrows indicate the relative capacity of the process (non-quantitatively).

In 2010, still many aspects of OCP-related photoprotection were remaining to be elucidated: demonstration that OCP, PB and FRP are sufficient to induce the mechanism; confirmation of direct involvement of OCP as an effector of quenching; identification of the residues (FRP, OCP, PB), domains (OCP) and subunits (PB) involved in interactions of OCP with PB and OCP with FRP; elucidation of the site of quenching, the stoichiometry, the mechanisms and rates of energy dissipation. In order to answer to these multiple questions, the development of an *in vitro* reconstituted system was required.

Summary of research

In my work in the field of the Orange Carotenoid Protein (OCP)-related photoprotective mechanism of cyanobacteria I followed two directions, which provided an improvement in the description of the mechanism. The first project characterized the Fluorescence Recovery Protein (FRP), one of the 3 essential elements for the mechanism that switches off the photoprotection. The second one was to reconstitute *in vitro* the mechanism in full from induction to recovery using isolated proteins and complexes. In the "*in vitro* reconstitution project" I applied mostly biochemical approaches while in the "FRP project", I used molecular biology as the main tool. In both projects fluorescence spectroscopy (steady state, low temperature or Pulse-amplitude modulation, PAM-fluorometry) was used. The use of a large scope of techniques allowed me to define the direction for my future career. I intend to study biological (photosynthetic) systems by means of biophysical methods. In particular, single molecule spectroscopy attracted my attention as a preferable tool to get the insights into molecular mechanisms beyond averaged, ensemble spectroscopy.

The Fluorescence Recovery Protein (Gwizdala et al., 2012)

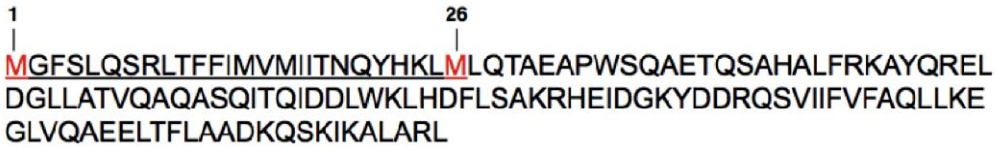

Figure 15 Amino acid sequence of the *Synechocystis* FRP according to Cyanobase.
Synechocystis FRP was predicted to have an N-terminal prolongation of 25 residues in comparison to FRP-like proteins of other cyanobacteria. It is the Met26 that is the first Methionine of FRP in *Synechocystis*. Met1 and Met26 are marked in red. Picture was taken from Gwizdala et al., submitted.

I joined the group during the preparation of an article describing the discovery of the function of an unknown protein encoded by the *slr1964* gene in *Synechocystis*, situated downstream the *ocp* gene, *slr1963* (Boulay et al., 2010). I have participated in the purification of the shortened version of FRP, as a soluble protein, from *E. coli*. Previous attempts to purify a full version of FRP were unsuccessful because the protein formed inclusion bodies (from *E. coli*) or was strongly attached to the membranes (*Synechocystis*) and the use of detergents inactivated the protein.

In all species of cyanobacteria, the length of FRP-like proteins is conserved and varies between 106 and 111 amino acids. However, according to the Cyanobase, *Synechocystis* is a strain for which FRP had an N-terminal prolongation. In this prolongation of 25 amino acids, four Methionines are present. The comparison of sequences pointed the Met26 as the possible first Methionine of the *Synechocystis* FRP. The difference in lengths of the protein indicated that the *Synechocystis* FRP could really begin at Met 26. This idea was supported by the previously experienced unsuccessful attempts to isolate the FRP starting at Met1 (Boulay et al., 2010). To elucidate which is the first Methionine of the *Synechocystis* FRP, I constructed and characterised a series of mutants. Their phenotypes, in terms of fluorescence quenching and recovery, were compared. A mutant with the Met26 replaced by an Isoleucine had a phenotype similar to the mutant deficient in FRP strongly suggesting that Met26 is necessary for the presence of FRP. This was confirmed by the fact that the long FRP (starting at Met1) was present only when the corresponding version of the gene was under the control of the strong *psbA2* promoter (but not under its own promoter). In addition, the activity of this long FRP was lower than the activity of the FRP present in WT cells. On the other hand, a mutant overexpressing the short version of the *frp* gene was unable to induce the photoprotective mechanism due to the increased content of very active FRP in the cells. All these results drove us to the conclusion that the FRP of *Synechocystis* starts at Met26 and its length is conserved among species. Finally, I isolated the FRP (short version) form *Synechocystis* and have demonstrated that *in vitro* it is as active as the FRP previously isolated from *E. coli*. By modelling the structures of *ocp* and *frp* mRNAs (Zuker, 2003) I have identified a possible *ocp*'s terminator of transcription. Moreover, the results suggest that *frp* could be translated from a leaderless mRNA, which was described for only 4 *Synechocystis* genes (Mitschke et al., 2011).

The in vitro reconstitution of the photoprotective mechanism (Gwizdala et al., 2011)

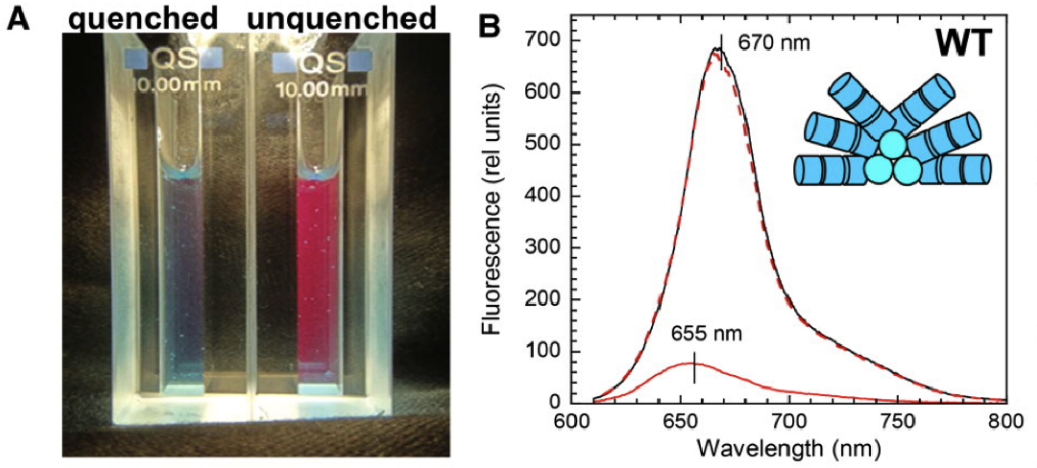

Figure 16 Fluorescence quenching induced by illumination of PB in the presence of OCP.
(A) Photograph of the quenched (left) and unquenched (right) PBs illuminated by white light showing the fluorescence emitted by the samples. The colour of the unquenched PBs is a result of the red fluorescence of PC and APC.
(B) The room temperature fluorescence spectra of dark unquenched (solid black line), light unquenched (dashed red line), and light quenched PBs (solid red line) isolated from wild-type *Synechocystis*. The PBs (0.013 µM) were illuminated 5 min with white light (5000 µmol photons $m^{-2}s^{-1}$) in the absence (light unquenched) or in the presence of OCP (0.53 µM) (light quenched). rel units, relative units. Picture was taken from Gwizdala et al., 2011.

When I started my project, the OCP-related photoprotective mechanism was well described by means of *in vivo* studies. It was known that the mechanism occurs at the level of the photosynthetic antenna, the phycobilisomes (PB) (El Bissati et al., 2000; Wilson et al., 2006) and that the mechanism protects the reaction centres from excess energy under high light conditions (Wilson et al., 2006). It was also known that the OCP was absolutely required for this mechanism (Wilson et al., 2006). The isolated OCP was identified to be photoactive *i.e.* upon illumination undergoing conformational changes in the carotenoid and in the protein (Wilson et al., 2008). Mutants having non-photoactive OCP were not capable to induce photoprotection (Punginelli et al., 2009). Thus, photoactivation was expected to be crucial for the mechanism. Also, the role of the FRP, protein responsible for recovering full capacity of the antenna had been already described (Boulay et al., 2010). These 3 components (PB, OCP and FRP) were believed (but not demonstrated) to be sufficient to run the mechanism. However, attempts to induce fluorescence quenching by interaction of the isolated OCP with phycobilisomes had been not successful. This was mainly due to the fact that the high phosphate concentrations needed to maintain the

phycobilisome intact largely inhibits OCP photoactivation. To overcome these limitations I have added an excess of OCP to the PB at 23°C. The fluorescence from PB in presence of an excess of OCP was not quenched in the darkness but after illumination with a strong light, fluorescence intensity was decreased by 90% on average. Illumination of isolated phycobilisomes in the absence of OCP did not induce fluorescence quenching. Thus, the fluorescence quenching indicated activation of the OCP-related mechanism *in vitro*. This initial experiment was followed by the development of the *in vitro* set-up for this study and systematic characterisation of PB's fluorescence quenching and recovery induced by the activated OCP or the FRP, respectively. This gave a set of data that were previously inaccessible from *in vivo* experiments. In this project it was proved that OCP, FRP and PB are the elements required and sufficient for the mechanism. The experiments confirmed that only the photoactivated red OCP interacts with a PB; light is needed for OCP photoactivation but the binding of the photoactivated OCP to the PBs is light independent. The site of fluorescence quenching is within the core of PB but the presence of the rods stabilise the OCP-PB interaction. Also, it was shown that one OCP per PB is sufficient to induce complete fluorescence quenching. Presence of FRP was demonstrated to prevent fluorescence quenching when present during illumination or to induce the recovery when added in darkness to the quenched OCP-PB system. Finally, I have managed to re-isolate stable OCP-PB complexes, free from the excess of OCP used to induce the fluorescence quenching. The *in vitro* reconstituted system improved the understanding of the mechanism of photoprotection and became a tool itself to study further the characteristics of the elements involved and their interactions.

With the *in vitro* reconstituted system I have participated in projects performed in collaboration with two other members of the Kirilovsky's group, Adjélé Wilson and Denis Jallet, and with two Dutch laboratories directed by Prof. van Amerongen and Prof. van Grondelle.

OCP's residues involved in photoactivity and binding to PB (in collaboration with Adjélé Wilson from our group; Wilson et al., 2012)

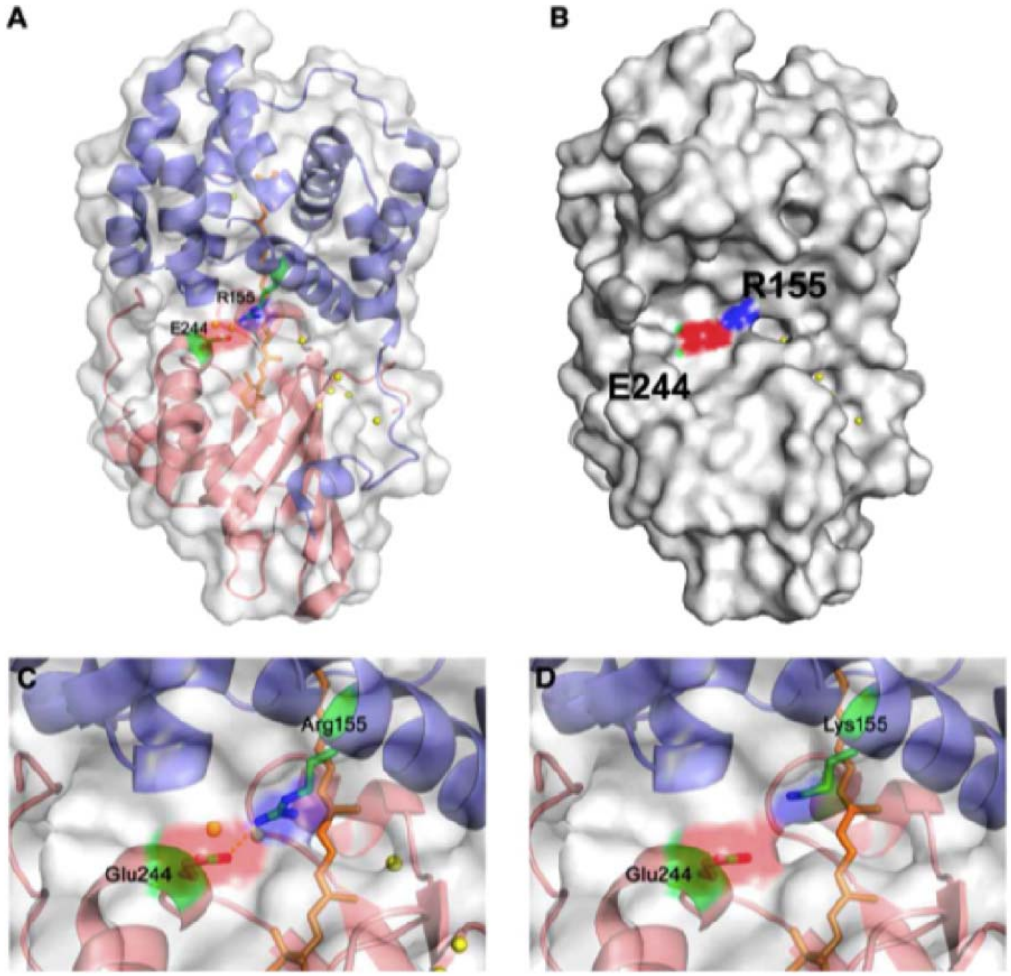

Figure 17 **The OCP structure and position of the mutated amino acids Arg155 and Glu244.**
(A) N-terminal domain is in blue, and the C-terminal domain is in red. The carotenoid is shown as orange sticks, and Arg155 (N-terminal domain) and Glu244 (C-terminal domain) are shown as sticks coloured green for side chains, red for oxygen atoms, and blue for nitrogen atoms. (B) Surface view with similar orientation and colouring as in (A). (C) Close-up of the central interdomain interface and the salt bridge between Arg155 and Glu244. Also shown are structurally conserved water molecules (yellow spheres).
(D) Close-up of the central interdomain interface with Lys substituted for Arg155. The Lys rotamer with the closest approach to Glu 244 was used in the model. Picture was taken from Wilson et al., 2012.

OCP is a photoactive protein (Wilson et al., 2008) and only its photoactivated form binds to the phycobilisome (Gwizdala et al., 2011) to induce photoprotection. There is however a mutant with a point mutation in *ocp*, which is unable to induce the photoprotective mechanism even though the associated OCP is photoactive (Wilson et

al., 2010). This OCP has Arg155 replaced by Leucine. Arg155 is a residue of the N-terminal domain, exposed in the interdomain cavity of OCP. It forms a salt bridge with Glu244 from the C-terminal domain. This salt bridge was thought to stabilise the OCP. Adjélé Wilson has constructed additional mutants disrupting this salt bridge and I further studied the interaction of the photactivated OCP with phycobilisomes. Differential FTIR measurements (collaboration avec Dr Maxime Alexandre and Dr Alberto Mezetti) showed that the photoactivation of OCPs isolated from the mutants and WT reflects similar changes in protein conformation. *In vitro* experiments showed that changes in Arg155 and Glu244 affected the kinetics of photoactivation and the stabilisation of the red OCP form. Mutations that destabilized the salt bridge accelerated the photoactivation kinetics and increased the stability of the red form. For example, when Arg155 was replaced by a glutamate the red OCP form was very stable due to the repulsive effect of the charges. Therefore, it is highly possible that in the orange form, OCP remains in a closed conformation with the interdomain cavity being hardly accessible. Upon photoactivation, the salt bridge is broken and the cavity becomes exposed, and OCP is in an open conformation. In this cavity the carotenoid is likely to be solvent exposed. Thus, additionally, in the OCPs with destabilized salt bridge the carotenoid was easily lost and the proteins were less stable.

My contribution to this work was based on the reconstitution system I had developed with WT and mutant OCPs. This demonstrated that the positive charge and the orientation of the Arg155 are crucial for the OCP's attachment to the PBs while the mutation in position 244 did not influence the binding to the PB.

Studies of the function of these residues enabled us to propose a molecular model for photoactivation of OCP and provided with initial data concerning the chemical character of the OCP-PB bond. We also obtained a range of OCPs with different affinities to PB that could be used for further studies.

Fluorescence quenching in phycobilisomes lacking terminal emitters (in collaboration with Denis Jallet from our group; Jallet et al., 2012)

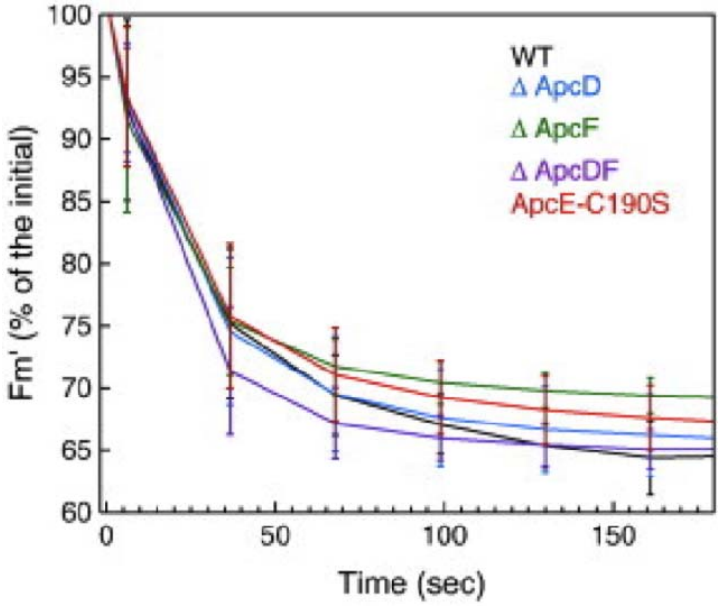

Figure 18 OCP related fluorescence quenching in the WT and mutant cells.
Decrease of maximal fluorescence induced by illumination with strong blue-green light (400-550 nm, 1400 µmol photons m^{-2} s^{-1}) in WT (black), ΔApcD (blue), ΔApcF (green), ΔApcDF (violet) and ApcE-C190S (red) cells. Data shows the mean (± SD) of at least three independent experiments. Picture was taken from Jallet et al., 2012.

From *in vivo* experiments it was proposed that the OCP interacts with the core of PBs (Wilson et al., 2006; Scott et al., 2006). This was confirmed *in vitro* by my reconstitution experiments (Gwizdala et al., 2011). The core of PBs is formed by APC trimers containing only αAPC-βAPC monomers emitting at 660 nm (APC_{660}) and by trimers that also contain the terminal emitters (TE), ApcD, ApcF or ApcE and emit at 680 nm (APC_{680}). From our previous works was not clear with which type of trimer OCP interacts. The aim of the project was to elucidate the site of fluorescence quenching in the core of the phycobilisomes. To this purpose, blue-green light induced fluorescence quenching was studied in phycobilisome mutant cells, not only *in vivo*, but also *in vitro* when the TE are not present in the phycobilisomes. In this project I have participated in the construction of the mutants. Our results from both *in vivo* and *in vitro* experiments, demonstrated that none of the terminal emitters (at least their bilins) are required to induce fluorescence quenching. Therefore, an APC_{660} trimer was proposed to be the site of the OCP-induced fluorescence quenching. This

result was confirmed by the fluorescence lifetimes measurements realized by Lijin Tian (Tian et al., 2012, 2011).

Fluorescence lifetimes of quenched and unquenched phycobilisomes (in collaboration with Lijin Tian from Prof. Herbert van Amerongen's group; Tian et al., 2012)

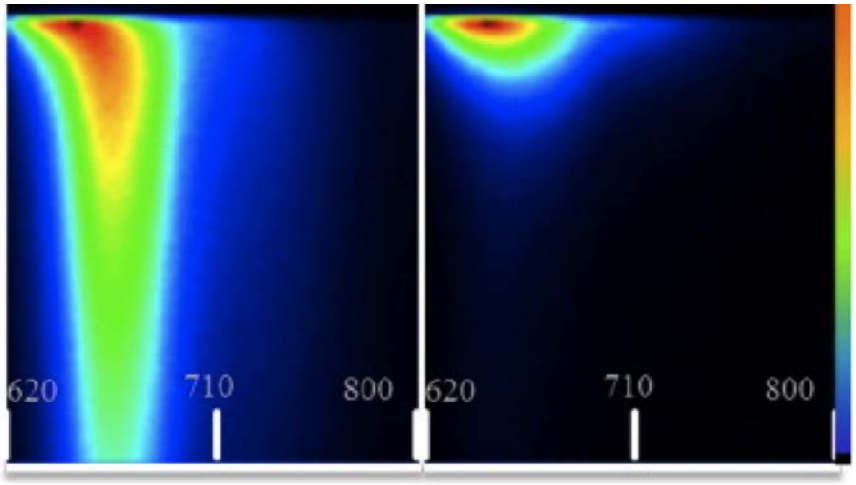

Figure 19 Fluorescence lifetimes of isolated PB and PB-OCP complexes.
Time-resolved fluorescence data obtained with the streak-camera setup using a time window of 2 ns with 590-nm excitation of PB. Left panel corresponds to the unquenched PBs and right panel corresponds to quenched PBs (OCP-PB complex). All images represent the fluorescence intensity (using a linear colour gradient) as a function of time (vertical axis, 2 ns) and wavelength (horizontal axis, nm). Picture was taken from Tian et al., 2012.

Time-resolved fluorescence measurements were applied in the OCP-related photoprotection studies in order to elucidate the kinetics and the site of fluorescence quenching. These measurements combined with data analysis enabled to conclude about the rates of excitation-energy transfers and of quenching. Moreover, comparison of these rates for the isolated OCP-PB complexes, used in this project, with the previously obtained rates for fluorescence quenching *in vivo* (Tian et al., 2011) provided additional validation of the *in vitro* reconstitution system. In this project I have provided quenched and unquenched isolated phycobilisomes and participated in data interpretation.

Results obtained for the isolated complexes were similar to these for whole cells. They confirmed that the mechanism and the site of fluorescence quenching *in vitro* is the same as in the photoprotective mechanism *in vivo*. In both cases data analysis and the tests of alternative hypothesis showed that the APC_{660} is the site of

fluorescence quenching (also as in Jallet et al., 2012). However, the fluorescence quenching rates were slower *in vitro* than in intact cells. Still, the fluorescence-quenching rate *in vitro* is extremely fast and *in vivo* would lead to 66% of the excitations quenched in PB while the previously demonstrated percentage *in vivo* is 80% (Tian et al., 2011). For efficient photoprotection these fast quenching rates are needed to compete with energy transfer to reaction centres.

Application of the *in vitro* reconstituted system in this project made the measurements less demanding than *in vivo*, regarding sample manipulation, data analysis and interpretation. The validation of the mechanism in the *in vitro* reconstituted system allows the study of the physical basis of photoprotection using ultrafast transient absorption spectroscopy, which is not easily feasible *in vivo.*

Ultrafast transient absorption spectroscopy on red and orange OCP at room temperature or at 77 K (in collaboration with Dr Rudi Berera from Prof. Rienk van Grondelle's group; Berera et al., 2012; Berera et al., submitted)

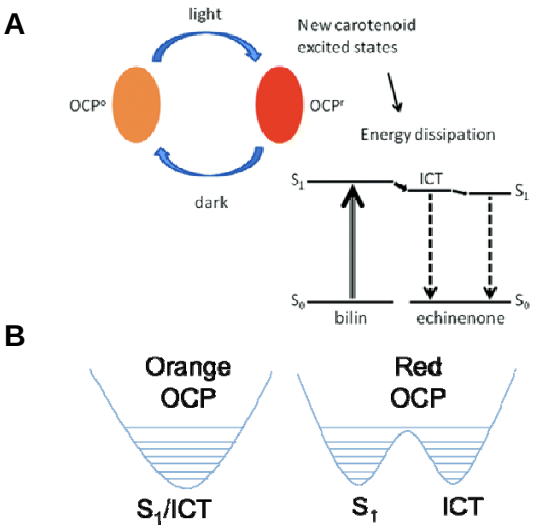

Figure 20 Excited states of carotenoid in the red form of OCP.
(A) Proposed quenching model. The carotenoid in the activated OCP receives energy from the excited bilin in the terminal emitter in a process mediated by the carotenoid Intramolecular charge-transfer (ICT) state. Picture was taken from Berera, *et al*., 2012.
(B) Schematic representation of the effect of photoactivation on the potential energy surface of the carotenoid S_1 and ICT states. Picture was taken from Berera et al., 2012 and Berera et al., submitted.

The aim of these studies was to provide a description of photoactivated OCP's excited states that will help to elucidate the molecular mechanism of energy and fluorescence quenching. The "pump-probe" ultrafast absorption technique enables

observation ("probe") of the spectroscopic properties of excited states upon excitation ("pump") from ground state to S_2 state (direct absorption to S_1 is forbidden in carotenoids). My role in these projects was to prepare samples and to monitor sample condition during the measurements. I have also participated in data interpretation.

We showed that at room temperature and at 77 K the spectral properties of OCP change upon photoactivation. While in the dark form of OCP, S_2 excitation decays to the undistinguishable S_1/ICT state, in the photoactivated OCP, S_1 and ICT act as two separate states. Moreover, in the activated OCP the ICT state is very pronounced. It was demonstrated previously that this state mediates the energy flow between carotenoid and chlorophyll (or chlorophyll mimicking pigment) in energy harvesting and dissipation processes. Therefore, it could potentially have a role in the OCP-related photoprotective mechanism. Experiments at 77 K were conducted to eliminate sample heterogeneity lowering the resolution of data analysis.

Presently, with this technique we study the possible energy transfer between a PB's pigment and OCP's carotenoid using isolated OCP-PB complexes. These data will complement our understanding of physics behind the photoprotective mechanism. In order to avoid sample bleaching and aggregation observed in preliminary experiments I modified the cuvette-flow cell used in this set-up. Data from this experiment has been already collected and are currently being analysed.

Conclusions and perspectives

Conclusions

The work of this thesis has contributed to the understanding of the OCP-related photoprotective mechanism, primarily by the development of an *in vitro* reconstitution system (Gwizdala et al., 2011). **Figure 21** summarises the present model of the OCP-related mechanism we have developed based on Gwizdala, *et al*., 2011 and other projects I was involved during my thesis.

In Gwizdala, *et al*., 2011, the *in vitro* reconstitution of the OCP-related photoprotective mechanism was presented. This system showed that OCP, PB and FRP are the only compounds required for triggering and switching off this photoprotective mechanism. Only the red photoactivated form of OCP binds to the core of PB (which is the site of interaction) and the binding itself is light independent. Once, one OCP is bound to the core of the PB, it induces quenching of almost all PB fluorescence. On average there is 1.2 OCP per PB in an OCP-PB complex. FRP triggers the recovery of lost fluorescence and detachment of OCP from PB (Gwizdala et al., 2011).

In Wilson, *et al*., 2012, we studied the influence of Arg155 and Glu244 of the OCP on photoactivity and OCP-PB interaction. We demonstrated that disruption of a salt bridge between Arg155 from the N-terminal domain and Glu244 from the C-terminal domain in OCP increases the rate of photoactivity and stability of the red form of OCP. OCPs missing the positive charge at residue 155 were not capable of binding to PB and of inducing fluorescence quenching (Wilson et al., 2012).

In Gwizdala, *et al*., submitted, we showed that *Synechocystis* FRP protein begins at Methionine 26 and that in WT cells there is no FRP starting at Methionine 1. It was demonstrated that the balance between the OCP and the FRP content in the cell has to be fine-controlled in order to induce OCP-related photoprotection. We isolated, for the first time the FRP from cyanobacteria and showed that it is active in reconverting red form of OCP to the orange one *in vitro* (Gwizdala et al., submitted).

In Jallet, *et al*., 2012, we showed that there is not one specific terminal emitter (ApcD, ApcF and phycobilin of ApcE) required for OCP-related photoprotection. Strong blue-green light induced similar fluorescence quenching in phycobilisomes

from *Synechocystis* mutant cells in which one or two of the terminal emitters were absent. The isolated phycobilisomes of these mutants were also quenched with similar kinetics when illuminated in the presence of the OCP (Jallet et al., 2012).

In Tian, *et al*., 2012, we showed that fluorescence quenching occurs at APC_{660} and not at terminal emitters. *In vitro* rates of quenching were slower than in measurements *in vivo* but still fast to reflect photoprotective mechanism. Rates of fluorescence quenching are higher for phycobilisomes having rods (Tian et al., 2012).

In Berera, *et al*., 2012 and Berera, *et al*., submitted, we demonstrated changes of spectroscopic properties of the OCP carotenoid upon photoactivation. In photoactivated OCP the S1 and ICT states of the carotenoid act as separate states and the ICT state is very pronounced.

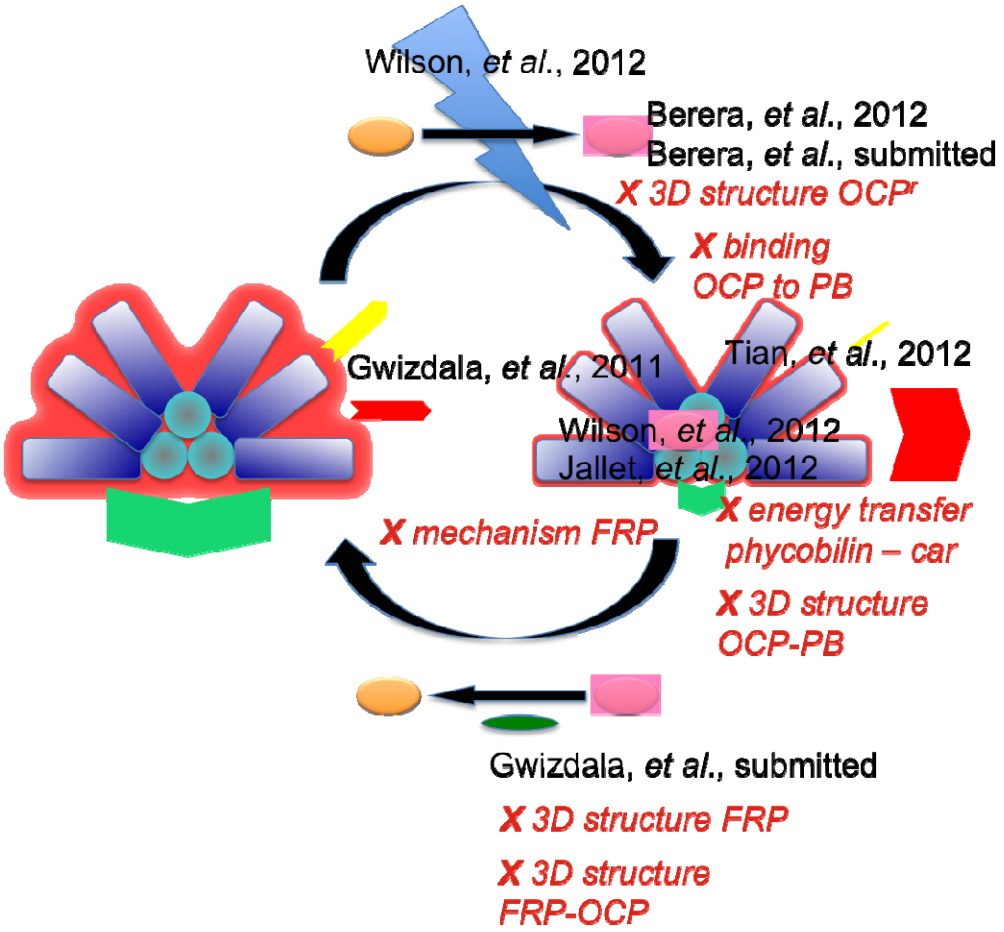

Figure 21 Model of OCP-related photoprotective mechanism-2012.
OCP^o (orange oval), OCP^r (pink square), FRP (dark green oval) and PB are involved in the mechanism. When illuminated with blue-green light (blue lightening) OCP^o converts to OCP^r. Binding of OCP^r to the core of PB, which is light independent, increases thermal dissipation (red arrow) and diminishes energy transfer to reaction centres (green arrow) and fluorescence emission (yellow arrow). Articles, in which I was involved, are listed next to the areas of impact of particular projects (black texts). Perspectives are listed in red.

Perspectives

Within last three years our understanding of the molecular processes underlying photoprotection in cyanobacteria increased remarkably, due to the new data acquired from the *in vitro* experiments. However, several points about this mechanism remain to be elucidated.

In **Figure 21** some of future goals are listed (in red with an ***X***). The tri-dimensional structure of OCP^r has not been resolved yet due to the instability of this form of OCP. Utilisation of isolated mutant OCPs, which form stable OCP^r (like OCP R155E) could be potentially useful. A project in which we are trying to resolve the tri-dimensional structure of OCP-PB complexes has already been started. I supplied the laboratory of Prof. Noam Adir with the OCP-PB complexes. The potential outcome of this project is a tri-dimensional structure of OCP bound in its binding site in PB. This result would provide exact binding site and orientation of OCP. Very preliminary FTIR results suggested that the structure of the OCP bound to PB is somewhat different to that of the isolated OCP^r.

Before providing the tri-dimensional structure of FRP it was important to know which the first Methionine was. The structure of the FRP and, if possible, the FRP-OCP complex (it is a short living complex) will give an insight into the FRP-driven mechanism of regaining full capacity of antenna. The exact mechanism of FRP remains to be solved. Very preliminary results from measurements of fluorescence kinetics, using the *in vitro* reconstitution system under various light conditions suggested that FRP triggers the reconversion of OCP while OCP is still bound to the PB. Then, the OCP alone detaches from a PB. Further studies are required to elucidate the mechanism, particularly single molecule fluorescence spectroscopy will be very useful in this study. A single molecule spectroscopy project has already started in collaboration with Dr Tjaart Kruger from Prof. Rienk van Grondelle's group and so far we established the conditions of the experiment and started studies on intrinsic dynamics of PB fluorescence. Single molecule fluorescence spectroscopy experiments will give an access to the heterogeneity of fluorescence dynamics induced by OCP or FRP. These dynamics are determined by mechanistic details of the processes, which are averaged out in ensemble spectroscopy *i.e.* possible intermediate states or conformational changes. The results obtained in this project can be interpreted in terms of a molecular model at the basis of photoregulation in cyanobacteria. These

results in combination with ATR FTIR and Raman spectroscopy can image the changes occurring in the protein matrix and at the pigment level when OCP, FRP and PB interact together.

Another on-going project concerns the energy transfer between phycobilins in PB and the carotenoid in OCP. Upon detection of this energy transfer it will be characterised by a series of "pump-probe" experiments in collaboration with Prof. Rienk van Grondelle's laboratory.

Closing

Studies of OCP-related photoprotective mechanism allowed us to understand one of ways cyanobacteria cope with stress conditions. Even though only three compounds are involved in the mechanism it is very complex and requires fine-tuning to operate. OCP is multifunctional: it senses high light, directly triggers the mechanism and is also a very likely candidate for the dissipater. As we showed, a single change in the OCP protein sequence can induce dramatic modifications of its photoactivity or PB affinity. This also begs the questions whether the OCP is not also a high light responsive photoreceptor regulating expression of the genes induced in these conditions.

OCP as a soluble, single carotenoid carrier is a perfect candidate to become one of models for biophysical studies. I believe that together with our collaborators we have made the first steps in this direction.

Last but not least, the *in vitro* set up made it a superb pleasure to observe the *orange* turning *red* to make the *blue* stop glowing *pink*.

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Articles

De fortes illuminations peuvent être dommageables voire même létales pour les organismes photosynthétiques. Une des stratégies utilisées pour se protéger de tels effets délétères consiste à augmenter la dissipation thermique de l'énergie absorbée en excès au niveau des antennes. Chez les cyanobactéries une protéine photo-active, l'Orange Carotenoid Protein (OCP), contrôle ce processus. Une fois photo-activée l'OCP interagit avec le cœur des phycobilisomes (PBs, les antennes collectrices majoritaires chez les cyanobactéries) et déclenche le mécanisme, entrainant à la fois une baisse de l'énergie parvenant aux photosystèmes et une diminution de la fluorescence des PBs. L'énergie absorbée en excès est dissipée sous forme de chaleur. Pour que les PBs regagnent leur pleine capacité de transfert, une autre protéine nommée Fluorescence Recovery Protein (FRP) est requise. La FRP accélère la désactivation de l'OCP. Dans ce manuscrit, je vais présenter ma contribution à la compréhension du mécanisme de photo-protection lié à l'OCP.

J'ai continué la caractérisation de la FRP chez *Synechocystis* PCC 6803, organisme modèle utilisé dans nos études. J'ai montré que la FRP de Synechocystis est plus courte que ce qui est indiqué dans Cyanobase, commençant en fait à la méthionine 26. Mes résultats ont aussi révélé que la photo-protection n'a lieu que lorsque le ratio OCP/FRP est élevé.

Le plus grand aboutissement de ma thèse a été la reconstitution *in vitro* du mécanisme de photo-protection lié à l'OCP en utilisant de l'OCP, de la FRP et des PBs isolés. J'ai montré que la lumière est requise uniquement pour la photo-activation de l'OCP et que l'attachement de l'OCP au PB ne demande aucune illumination. Ce n'est qu'une fois photo-activée que l'OCP peut interagir avec le PB et entrainer la diminution de fluorescence (quenching). En se basant sur les résultats obtenus *in vitro* nous avons proposé un modèle moléculaire pour le mécanisme de photo-protection lié à l'OCP. Le système de reconstitution *in vitro* a été utilisé pour évaluer l'importance d'un pont salin conservé (Arg155-Glu244) entre les deux domaines de l'OCP et a révélé que celui-ci stabilise la forme inactive de l'OCP. La photo-activation entraine rupture du pont salin, l'Arg155 étant ensuite impliquée dans l'interaction entre OCP et PB. Le site d'attachement de l'OCP au cœur du PB a aussi été étudié en utilisant le système *in vitro*. Nos résultats ont montré que les émetteurs terminaux du PB ne sont pas requis et que le site primaire de quenching est un trimère d'allophycocyanine émettant à 660nm. Enfin nous avons étudié les propriétés des états excités du caroténoïde dans l'OCP photo-activée, montrant qu'un de ces états a un caractère de transfert de charge très prononcé et peut avoir un rôle principal dans la dissipation de l'énergie. Nos résultats suggèrent fortement que non seulement l'OCP induit dissipation de l'énergie absorbée sous forme de chaleur mais aussi que l'OCP agit directement comme dissipateur d'énergie.

***In vitro* and *in vivo* characterisation of the OCP-related photoprotective mechanism in the cyanobacterium *Synechocystis* PCC6803**

Strong light can cause damage and be lethal for photosynthetic organisms. An increase of thermal dissipation of excess absorbed energy at the level of photosynthetic antenna is one of the processes protecting against deleterious effects of light. In cyanobacteria, a soluble photoactive carotenoid binding protein, Orange Carotenoid Protein (OCP) mediates this process. The photoactivated OCP by interacting with the core of phycobilisome (PB; the major photosynthetic antenna of cyanobacteria) triggers the photoprotective mechanism, which decreases the energy arriving at the reaction centres and PSII fluorescence. The excess energy is dissipated as harmless heat. To regain full PB capacity in low light intensities, the Fluorescence Recovery Protein (FRP) is required. FRP accelerates the deactivation of OCP. In this work, I present my input in the understanding of the mechanism underlying the OCP-related photoprotection.

I further characterized the FRP of *Synechocystis* PCC6803, the model organism in our studies. I established that the *Synechocystis* FRP is shorter than what it was proposed in Cyanobase and it begins at Met26. Our results also revealed the great importance of a high OCP to FRP ratio for existence of photoprotection.

The most remarkable achievement of this thesis is the *in vitro* reconstitution of the OCP-related mechanism using isolated OCP, PB and FRP. I demonstrated that light is only needed for OCP photoactivation but OCP binding to PB is light independent. Only the photoactivated OCP is able to bind the PB and quench all its fluorescence. Based on our *in vitro* experiments we proposed a molecular model of OCP-related photoprotection. The *in vitro* reconstituted system was applied to examine the importance of a conserved salt bridge (Arg155-Glu244) between the two domains of OCP and showed that this salt bridge stabilises the inactive form of OCP. During photoactivation this salt bridge is broken and Arg155 is involved in the interaction between the OCP and the PB. The site of OCP binding in the core of a PB was also investigated with the *in vitro* reconstituted system. Our results demonstrated that the terminal energy emitters of the PB are not needed and that the first site of fluorescence quenching is an APC trimer emitting at 660 nm. Finally, we characterised the properties of excited states of the carotenoid in the photoactivated OCP showing that one of these states presents a very pronounced charge transfer character that likely has a principal role in energy dissipation. Our results strongly suggested that the OCP not only induces thermal energy dissipation but also acts as the energy dissipator.