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Review

The orange carotenoid protein in photoprotection of photosystem II in cyanobacteria☆

Diana Kirilovsky [a,b,*], Cheryl A. Kerfeld [c,d]

[a] Commissariat à l'Energie Atomique (CEA), Institut de Biologie et Technologies de Saclay (iBiTec-S), France
[b] Centre National de la Recherche Scientifique (CNRS), 91191 Gif sur Yvette, France
[c] United States Department of Energy—Joint Genome Institute, Walnut Creek CA 94598, USA
[d] Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA

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ABSTRACT

Photoprotective mechanisms have evolved in photosynthetic organisms to cope with fluctuating light conditions. Under high irradiance, the production of dangerous oxygen species is stimulated and causes photo-oxidative stress. One of these photoprotective mechanisms, non photochemical quenching (qE), decreases the excess absorbed energy arriving at the reaction centers by increasing thermal dissipation at the level of the antenna. In this review we describe results leading to the discovery of this process in cyanobacteria (qE_{cya}), which is mechanistically distinct from its counterpart in plants, and recent progress in the elucidation of this mechanism. The cyanobacterial photoactive soluble orange carotenoid protein is essential for the triggering of this photoprotective mechanism. Light induces structural changes in the carotenoid and the protein leading to the formation of a red active form. The activated red form interacts with the phycobilisome, the cyanobacterial light-harvesting antenna, and induces a decrease of the phycobilisome fluorescence emission and of the energy arriving to the reaction centers. The orange carotenoid protein is the first photoactive protein to be identified that contains a carotenoid as the chromophore. Moreover, its photocycle is completely different from those of other photoactive proteins. A second protein, called the Fluorescence Recovery Protein encoded by the *slr1964* gene in *Synechocystis* PCC 6803, plays a key role in dislodging the red orange carotenoid protein from the phycobilisome and in the conversion of the free red orange carotenoid protein to the orange, inactive, form. This protein is essential to recover the full antenna capacity under low light conditions after exposure to high irradiance. This article is part of a Special Issue entitled: Photosystem II.

1. Introduction

Oxygenic photosynthetic organisms—plants, algae and cyanobacteria—by converting solar energy into chemical energy—provide organic carbon molecules and oxygen that are essential for life on earth. Light quality and intensity varies depending on the time of the day, the weather, the season etc; light that is essential for life can become lethal when its energy exceeds the capacity of photosynthetic organisms to use it for carbon fixation. Under these conditions harmful reactive oxygen species are generated. Photosystem II (PSII) is the most light sensitive complex in the photosynthetic apparatus (photoinhibition is reviewed in Refs. [1–4] and in this issue Vass, Pospisil, Aro, Campbell). Temperature and water availability also vary daily and nutrients, likewise, can become growth limiting, for example, low concentration of iron in the oceans. Thus, photosynthetic organisms have developed physiological mechanisms that allow them to acclimate and to survive in a wide range of environmental conditions. The mechanisms responding to variations of the intensity and/or quality of the incident light involve both rapid and slow changes in the photosynthetic apparatus, ranging from seconds to hours. The rapid processes involve conformational changes and/or a reorganization of the photosynthetic complexes, while synthesis and/or degradation of proteins and protein complexes are slower responses. Two of the rapid processes involve changes in the effective size of the antenna of PSII: state transitions (reviewed in Refs. [5–7]) and non-photochemical-quenching (NPQ) [8,9]. In plants and green algae, the PSII antenna, the Light-Harvesting-Complex (LHCII), is composed of 6 hydrophobic membrane-intrinsic polypeptides of the Lhc family which non-covalently bind chlorophyll and carotenoids (reviewed in this issue, R Bassi). In cyanobacteria and red algae, this large membrane-bound chlorophyll antenna is absent. Instead, solar energy is absorbed by a large extramembrane (soluble) complex, the phycobilisome (PB), which is composed of several types of chromophorylated phycobiliproteins and of linker peptides; the latter are needed for the structural organization and functioning of the PBs (for reviews, see Refs. [10–14], this issue, Ikeuchi). Phycobilisomes, which are

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* Corresponding author at: iBiTec-S, Bât 532, CEA Saclay, 91191 Gif sur Yvette, France. Tel.: +33 1 69089571; fax: +33 1 69088717.
E-mail address: diana.kirilovsky@cea.fr (D. Kirilovsky).

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attached to the outer surface of the thylakoid membranes [15,16] are composed of a core from which rods radiate. In most freshwater cyanobacteria the rods contain only phycocyanin (PC), while in many marine cyanobacteria phycoerythrin (PE) or phycoerythrocyanin (PEC) are found in the distal end of the rods. The major core phycobiliprotein is allophycocyanin (APC). Three other chromophorylated proteins ApcD, ApcF and ApcE or Lcm (Linker core-membrane) serve as terminal energy acceptors which transfer the harvest energy to the chlorophylls of photosystems [17–19]. In addition the Lcm links the PB to the thylakoids.

In plants and algae, under saturating light conditions, decrease of the chloroplast lumen pH activates a mechanism which converts the very efficient collector of energy, LHCII, into a very efficient energy dissipator (see Refs [8,9,20,21] and this issue, Ruban, Niyogi and Jahns). This mechanism involves the activation of the xanthophyll cycle, the protonation of the PsbS protein (a protein of the Lhc family), and conformational changes of the LHC (and/or minor antennae) which modify the interaction between chlorophylls and carotenoids. Energy dissipation is accompanied by a diminution of PSII-related fluorescence emission, also known as non-photochemical-quenching fast (NPQf) or qE which usually serves as a measure of the dissipation process. This NPQ process is rapidly reversible (within seconds) in the dark in the absence of protein synthesis.

In cyanobacteria the equivalent mechanism is not induced by a drop in thylakoid lumen pH generated under high light conditions. Instead, strong blue-green (or white) light activates a soluble carotenoid protein, the orange carotenoid protein (OCP) which, by interacting with the phycobilisome, increases energy dissipation in the form of heat, thereby decreasing the amount of energy arriving at the reaction centers [22]. The fluorescence decrease associated with this energy dissipation in the antenna was called qEcya to differentiate this mechanism from that existing in plants.

The qE_{cya} mechanism is not the only one that protects cyanobacteria cells from high irradiance. Mutants lacking zeaxanthin are very sensitive to strong light, even more than mutants lacking the qE_{cya} mechanism, indicating an important role of this carotenoid in photoprotection [23]. In addition, under high light conditions and also during nutrient or temperature stress, single-helix chlorophyll binding proteins, HLIPs (high light inducible proteins), of the Lhc protein family, are synthesized. They play a photoprotective role in cyanobacteria [24,25]. Under iron stress conditions, IsiA (Iron-starvation-inducible protein), another chlorophyll/carotenoid binding protein of the Lhc family, is essential for cell survival. This protein acts as a PSI antenna but also can exist as empty (not attached to PSI) IsiA complexes that dissipate energy and quench fluorescence [26,27]. Thus, cyanobacteria have developed a large variety of mechanisms to protect themselves from different stresses and the relative importance of each depends on the environmental conditions. For example, OCP-related energy dissipation increases under conditions in which there exists an excess of functionally disconnected phycobilisomes (e.g. iron starvation, PSI less mutant) [28].

This review will focus on the characteristics of the phycobilisome-related light-induced photoprotective mechanism mediated by the OCP. Recent structural and functional results will be described and discussed in the context of similarities to other blue-light responsive proteins; important previous results will be mentioned. Additional information and more details about the blue-light induced photoprotective mechanism are described in Refs. [29–33].

2. The phycobilisome-related photoprotective mechanism: qE_{cya}

Evidence for the existence of a blue-green light induced phycobilisome related photoprotective mechanism were first described in 2000 [34]. El Bissati et al. [34] observed that the exposure of *Synechocystis* cells to strong intensities of blue-green light induced PSII fluorescence quenching under conditions in which the plastoquinone (PQ) pool was largely oxidized and the oxygen evolving activity was not saturated. Moreover, the presence of DCMU, an inhibitor of electron transport between the QA and QB quinones (thereby preventing PQ pool reduction), did not inhibit the blue-green light induced fluorescence quenching. These results indicated that the decrease of fluorescence was not related to state transitions, which are induced by the reduction of the plastoquinone pool (reviewed in Refs. [5–7]). Once the "quenched" cells were transferred to low light intensities, recovery of fluorescence occurred even in the presence of inhibitors of translation [34], demonstrating that the blue-green light-induced quenching was not related to photoinhibition (for reviews on photoinhibition see Refs. [1–4] and this volume, Vass et al.). In the case of photodamage, the increase of fluorescence is associated with the replacement of the damaged D1 polypeptide of PSII; this process requires D1 synthesis. Instead, El Bissati et al.[34] proposed that high light intensities induce phycobilisome fluorescence quenching concomitantly with a decrease of energy transfer from the phycobilisome to the photosystems. Subsequently, spectral and kinetics data confirmed the existence of this phycobilisome-related fluorescence quenching [35]. It was shown that in a *Synechocystis* mutant lacking reaction center II and the Chl antennae CP43 and CP47, blue light induced a reversible quenching of the phycobilisome emission. These results were confirmed by two other laboratories [22,36]. In contrast, blue-green light was unable to induce fluorescence quenching in a phycobilisome-deficient mutant or in a mutant containing only the phycocyanin rods of the phycobilisome while the presence of only the core of the phycobilisome is sufficient to induce the quenching [22]. In WT *Synechocystis* cells, a specific decrease of the phycobilisome-related-fluorescence was observed without change in the chlorophyll-related-fluorescence emission [22,36], implicating phycobilisomes as essential components of the blue-light-induced qE_{cya} mechanism. Picosecond time-resolved fluorescence decay data were consistent with a fluorescence quenching at the phycobilisome core [36]. The phycobilisome core contains APC emitting at 660 nm (at room temperature and 77 K) and terminal emitters (ApcD, ApcF and Lcm (ApcE)) emitting at around 680 nm (room temperature; at 77 K: 683 nm). Recently, Rakhimberdieva et al (2010) [37], suggested that fluorescence quenching originates from the short-wavelength APC (660 nm emission), but this remain to be demonstrated experimentally. Under conditions in which all PSII centers are closed, in the presence of DCMU + light, chlorophyll excitation could also be quenched via the phycobilisome quenching mechanism. In these conditions, excited chlorophyll molecules could be sufficiently long-lived to allow the exciton to be transferred from PSII chlorophyll to the core of the phycobilisome and there quenched [38].

The blue-green light induced quenching of fluorescence correlates with a decrease of the effective size of antenna, resulting in a decrease of the amount of energy arriving at the photochemical centers [22]. Quantitative measurements using *Synechocystis* mutants lacking PSI or PSII showed that in the quenched cells only 60%–70% of the energy absorbed by the phycobilisomes arrives to the PSII or to the PSI [37].

A specific characteristic of the qE_{cya} mechanism is that its induction depends on the quality of light; only blue-green light is able to trigger it. The action spectrum for phycobilisome emission quenching in the PS II-deficient mutants of *Synechocystis* suggested that a carotenoid molecule could be involved in this process [35]. Wilson et al [22] demonstrated that OCP, a soluble 35 kD protein containing a single, non-covalently bound keto-carotenoid [39–42], is specifically involved in the qE_{cya} mechanism. In the absence of the OCP (in a ΔOCP *Synechocystis* mutant or in cyanobacterial strains lacking the OCP gene), the fluorescence quenching induced by strong blue-green light is completely inhibited and the cells are more sensitive to high light intensities; this is manifested as a faster decrease in PSII activity under high irradiance [22,43]. Studies using immunogold labeling and analysis by electron microscopy showed that the OCP is present in the inter-thylakoid cytoplasmic region, on the phycobilisome side of the membrane [22]. The existence of an interaction between the OCP and the phycobilisomes

and thylakoids was supported by the co-isolation of the OCP with the phycobilisome-associated membrane fraction [22,28,43].

Under iron starvation conditions [28,38,44,45], blue-green light induces a very large fluorescence quenching, much larger than under complete-medium conditions [28,38,44,45]. This large light-induced phycobilisome-related fluorescence quenching correlates with a high OCP concentration (relative to chlorophyll and phycobiliproteins) [28,43]. Thus, under stress conditions the OCP-related photoprotection increases, underscoring its importance for the acclimation of cyanobacteria to environmental changes. The relationship between the concentration of the OCP and excitation energy dissipation was later confirmed using a *Synechocystis* mutant overexpressing the OCP, in which large quantities of OCP (8 to 10 times more than in WT cells) were present and a very large fluorescence quenching was observed [46] (see also Fig. 1B and C). The quenching of maximal fluorescence increases from 25% to 30% in WT cells to 60%–70% in overexpressing OCP cells (Fig. 1B) [46]. Quantification of OCP and` phycobilisomes suggests that in WT *Synechocystis* cells there is one OCP molecule per 2–3 phycobilisomes; in the overexpressing OCP strain there are 3–4 OCP molecules per phycobilisome (Wilson and Kirilovsky, unpublished data).

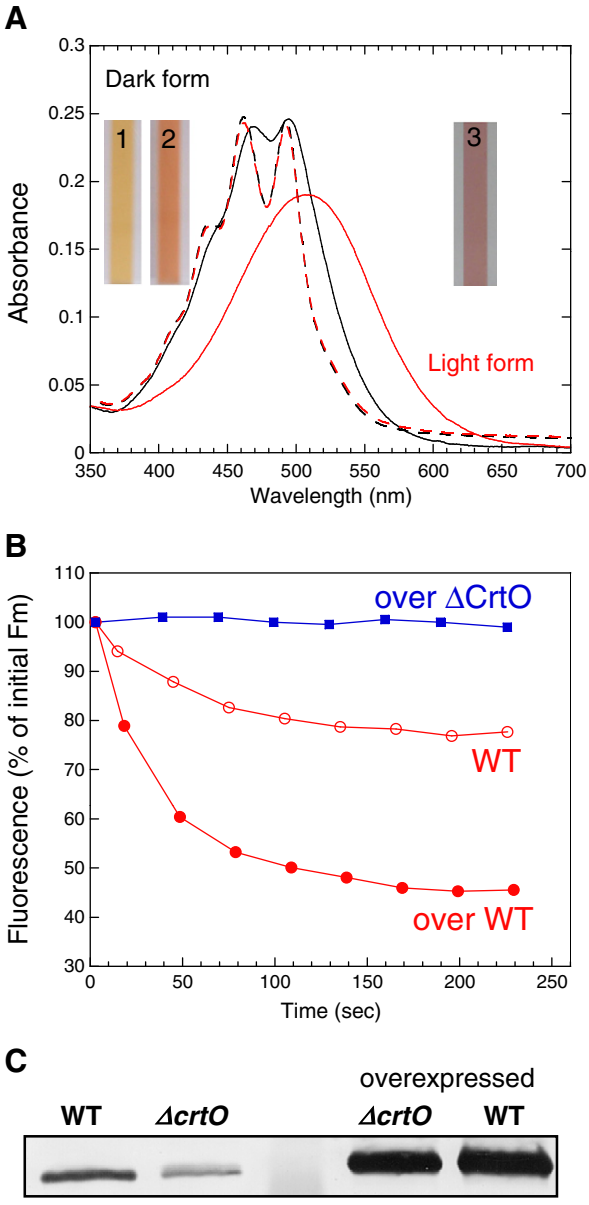


Fig. 1. Photoactivity of the isolated WT OCP and Photoprotection. Zeaxanthin cannot replace hECN for activity. (A) Absorbance spectra of the dark (black) and light (red) forms of the echinenone-containing OCP (isolated from the ΔCrtR-overexpressing OCP *Synechocystis* strain) (solid line) and of the zeaxanthin-containing OCP (isolated from the ΔCrtO-overexpressing OCP *Synechocysistis* mutant) (dashed line). To obtain the spectrum of the light form, the isolated OCP was illuminated with 1200 μmol photons $m^{-2} s^{-1}$, at 12 °C, for 5 min. The ech-containing OCP is orange in darkness (2) and red in strong light (3) while the zea-containing OCP is always yellow (1). (B) Decrease of maximal fluorescence (Fm) induced by strong blue-green light in the WT (open circles) strain, the overexpressing OCP strain (closed circles) and the ΔCrtO-overexpressing OCP strain (closed squares) in which the OCP contains only zea. (B) OCP immunodetection of OCP isolated from WT, ΔCrtO, overexpressing OCP and ΔCrtO-overexpressing OCP strains.

3. The OCP: primary to tertiary structure

In 1981, Holt and Krogman identified a 3-hydroxyechinenone (3-hECN) binding soluble protein in three different strains of cyanobacteria: *Arthrospira maxima, Microcystis aeruginosa* and *Aphanizomenon flos-aquae*[39]. In 2003, prior to an understanding of its function, Kerfeld and colleagues reported the crystal structure of the OCP isolated from *A. maxima* (PDB ID: 1M98) [47]. Recently the crystal structure of wild type OCP isolated from *Synechocystis* was determined at 1.6 Å resolution (PDB ID: 3MG1) [48] (Fig. 2). The OCP is composed of two domains: an all α-helical N-terminal domain (PFAM 09150) and an α/β C-terminal domain (PFAM 02136). While the fold of the C-terminal domain is a member of the nuclear transport factor 2 superfamily, the sequence and structure of the N-terminal domain of the OCP is unique to cyanobacteria. The carotenoid, 3-hECN, spans both domains of the protein, and is largely concealed.

In *Synechocystis*, the OCP is encoded by the *slr1963* gene [42]. It is constitutively expressed, even in *Synechocystis* mutants lacking phycobilisomes [28]. A survey of the currently available cyanobacterial genomic databases and 30 unpublished cyanobacterial genomes (Kerfeld and Gugger) showed that genes encoding for homologs to the *Synechocystis* OCP are present in most PB-containing cyanobacteria [33,43] (Fig. 3). The amino acid sequence of the OCP homologs is highly conserved: those from fresh-water cyanobacteria strains are 66%–82% identical to *Synechocystis* OCP. Those from marine cyanobacteria strains are less similar (62% to 64% identical to the *Synechocystis* OCP). The marine *Synechococcus* OCP sequences are very similar to each other; their sequence identity varies between 77%–95%. Constitutive expression of the full-length OCP has been confirmed in seven strains [43]. All of these strains were able to perform blue-green light-induced fluorescence quenching, substantiating the proposition that the OCP-related photoprotective mechanism is widespread among cyanobacteria [43]. Furthermore, in *A. maxima*, as in *Synechocystis*, iron starvation induces an increase in OCP concentration and a larger fluorescence quenching [43]. Among PB-containing cyanobacteria, a few strains lack an OCP ortholog, including the freshwater *Synechococcus elongatus* PCC 7942, the thermophile *Thermosynechococcus elongatus* (although the latter contains two consecutive genes, one encoding the N-terminal the other the C-terminal domain of the OCP). In these strains, the qE_{cya} mechanism was absent and they are more sensitive to episodes of high light irradiance similar to the ΔOCP *Synechocystis* mutant [43]. Interestingly, under iron starvation these strains protect themselves by quickly decreasing the phycobiliprotein content to avoid the accumulation of potentially harmful functionally disconnected phycobilisomes [43].

Some cyanobacteria have, in addition to the whole OCP-like gene, multiple gene copies encoding the N-terminal domain located in disparate parts of the genome and a single copy of the gene coding for the C-terminal domain [33,40,41] (Fig. 3). The gene encoding the C-terminal domain is typically present near one of the N-terminal domain encoding genes. In *Nostoc punctiforme* several of the N-terminal paralogs are known to be expressed [49]. In *Cyanothece* ATCC51142, the C-terminal gene and one of the N-terminal genes are both expressed in light conditions but not in darkness [50]. The function of these fragments is unknown and their origin seems to be

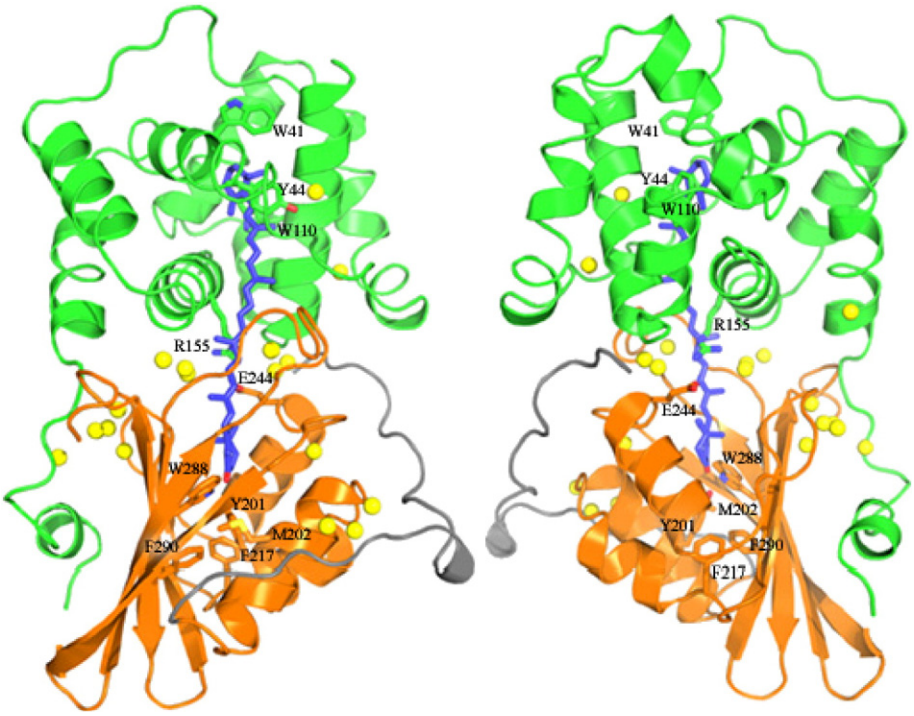


Fig. 2. Structure of *Synechocystis* OCP. Two views of the structure of *Synechocystis* OCP. The N-terminal domain is colored green, the C-terminal domain is orange; side chains discussed in the text are shown in sticks and labeled. The linker region between the two domains is shown in gray. The hECN molecule is shown in sticks (blue) with oxygen atoms colored red. Water molecules conserved among the OCP structures are shown as yellow spheres. The view on the right is rotated 180° to that on the left. Figure made with Pymol (http://www.pymol.org/).

an ancient gene duplication. The observation of multiple separate genes encoding the two domains of the OCP is of interest because other blue light photoactive proteins are composed of receiver and output domains that are either fused or encoded separately [51,52]. The potential to combine different variants of the N-terminal domain with the C-terminal domain could provide the potential for diverse output functions of the OCP in response to blue light.

4. The structural basis of photoactivity and fluorescence quenching in the OCP

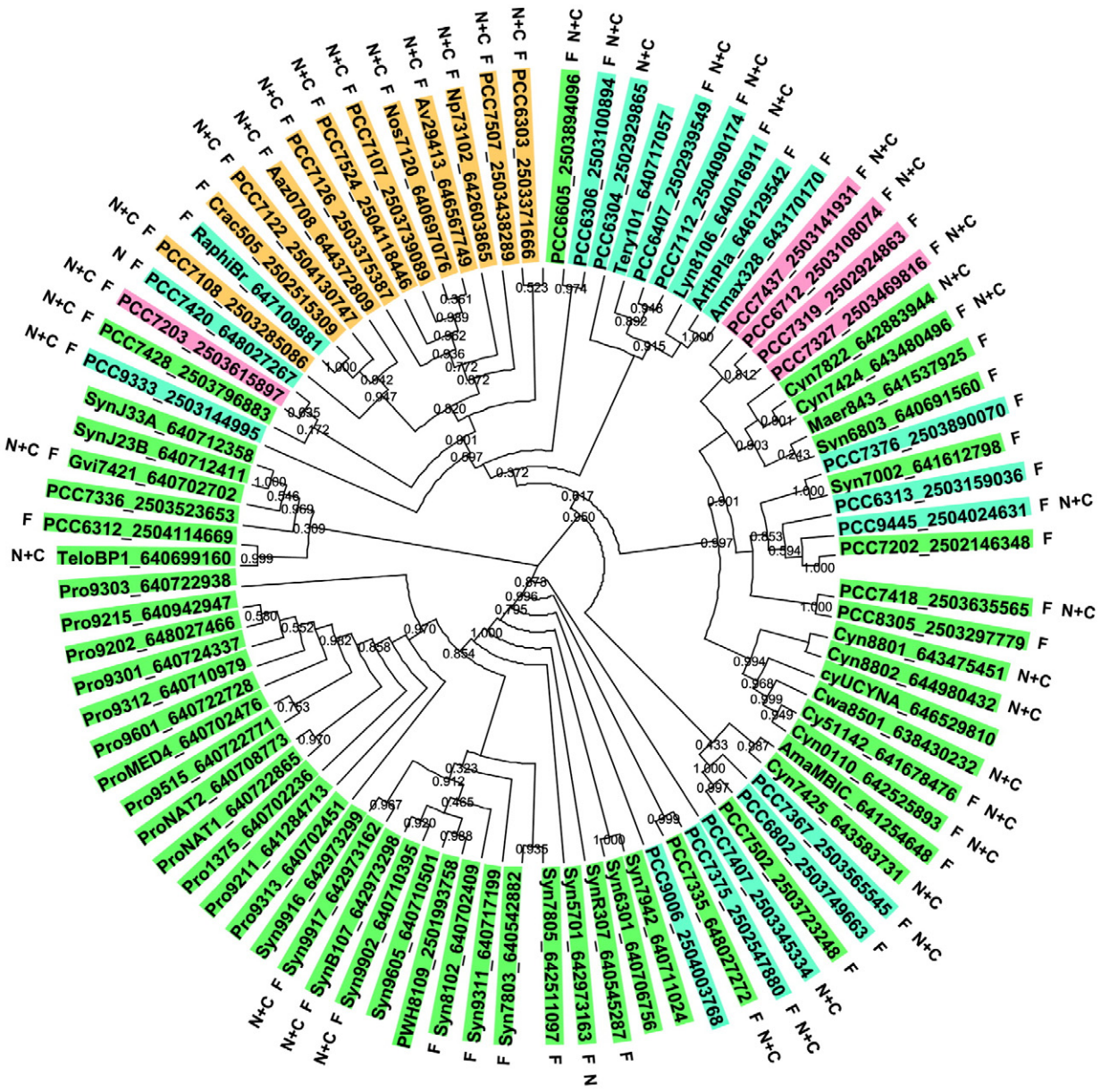
Both *A. maxima* and *Synechocystis* WT OCPs bind the keto carotenoid hECN. The non-covalent binding of the hECN to the OCP changes its spectroscopic properties [53]. The binding is responsible for shortening of the S1 lifetime of the hECN (from 6.5 ps in solution to 3.3 ps in the OCP) due to an elongation of the effective conjugation length of the carotenoid. In addition, the hECN binding results in stabilization of an intramolecular charge-transfer (ICT) state making the protein bound hECN a more effective energy dissipator. The OCP is also able to bind other types of carotenoids. The overexpressing OCP mutant strain contains approximately 8–10 times more OCP than the WT strain. Since hECN is a minor carotenoid in *Synechocystis*, it is not sufficiently abundant to bind all of the OCP produced in cells overexpressing OCP; in this strain, the OCP binds echinenone (ech) and zeaxanthin (zea) in addition to hECN [54]. In the ΔCrtO *Synechocystis* mutant, in which the *crtO* gene, encoding the β-carotene ketolase, was interrupted (leading to the lack of ech and hECN), the OCP binds mostly zeaxanthin [54]. In the ΔCrtR *Synechocystis* mutant lacking zea and hECN, due to the absence of the β-carotene hydroxylase, CrtR, OCP binds mostly ech[55]. While zea has two hydroxyl groups, one in each ring (sites 3 and 3′) ech and hECN have a carbonyl group in one of the rings (site 4). hECN has an additional hydroxyl group in the other ring.

Dark-adapted OCP binding ech (isolated from the overexpressing OCP strain) or hECN (isolated from WT cells) is orange while the OCP binding zea (isolated from the ΔCrtO mutant) appears yellow [54] (Fig. 1A). Illumination of the hECN (or ech) OCP with strong blue-green light induces conformational changes in the carotenoid and in the protein that converts OCP from an orange to a red form [46] (Fig. 1A). The carotenoid is in an all-trans-configuration in the dark (orange) form [47,53]. In the red form it is also in an all trans-configuration (no trans-cis isomerisation occurs) but the apparent conjugation length of hECN increased by about one conjugated bond and results in a less distorted, more planar structure [46]. At the level of the protein, the changes correspond to a less rigid helical structure and a compaction of the β-sheet domain upon illumination [46]. Since hECN and ech can generate the red form with similar kinetics and the ech-OCP can efficiently induce fluorescence quenching in *Synechocystis* cells, the hydroxyl group of hECN is not required for OCP activity [54].

In contrast, the OCP containing zea is not photoactive [54] (Fig. 1A) and it is unable to induce fluorescence quenching under strong illumination (Fig. 1B) even when present in high quantities in the cells (Fig. 1C) [54]. These results strongly suggested that the presence of the carbonyl group, that distinguishes ech and hECN from zea, is essential for OCP activity. The carbonyl group also seems to influence the stability of carotenoid binding [54].

The red form is accumulated in whole cells under conditions which induce energy dissipation and fluorescence quenching, suggesting that the red form is the active form of the protein [46]. In addition, as noted above, OCP containing zea, which does not undergo photoconversion to a red form, is unable to induce photoprotection (Fig. 1B). Thus, we concluded that the red form is essential for photoprotection. The energies, lifetime and stability of the S1 and the ITC states in the red OCP form remain to be studied.

The excited-state properties of hECN bound to a 16 kDa red carotenoid protein (RCP) isolated from *A. maxima* were recently described [56]. This protein, which was co-purified with the OCP, lacks the C-terminal domain of the OCP but still binds hECN. The S1 lifetime of the hECN bound to the RCP was 5.5 ps only slightly faster than that of the hECN in solution and the ICT state disappears. The red color of



Gvi7421, Gloeobacter violaceus PCC 7421; Cwa8501, Crocosphaera watsonii WH 8501; PCC7202, Cyanobacterium stanieri PCC 7202; PCC7502, Synechococcus sp. PCC 7502; PCC7001, Cyanobium sp. PCC 7001; PCC6307, Cyanobium gracile PCC 6307; Cy51142, Cyanothece sp. ATCC 51142; Cyn0110, Cyanothece sp. CCY 0110; Cyn7424, Cyanothece sp. PCC 7424; Cyn7425, Cyanothece sp. PCC 7425; Cyn7822, Cyanothece sp. PCC 7822; Cyn8801, Cyanothece sp. PCC 8801; Cyn8802, Cyanothece sp. PCC 8802; PCC8305, Dactylococcopsis salina PCC 8305; PCC7418, Halothece sp. PCC 7418; Maer843, Microcystis aeruginosa NIES-843; Syn6301, Synechococcus elongatus PCC 6301; Syn7942, Synechococcus elongatus PCC 7942; SynB107, Synechococcus sp. BL107; Syn9311, Synechococcus sp. CC9311; Syn9605, Synechococcus sp. CC9605; Syn9902, Synechococcus sp. CC9902; SynJ23B, Synechococcus sp. JA-2-3Ba(2-13); SynJ33A, Synechococcus sp. JA-3-3Ab; Syn7002, Synechococcus sp. PCC 7002; PCC7335, Synechococcus sp. PCC 7335; SynR307, Synechococcus sp. RCC307; Syn9916, Synechococcus sp. RS9916; Syn9917, Synechococcus sp. RS9917; Syn5701, Synechococcus sp. WH 5701; Syn7803, Synechococcus sp. WH 7803; Syn7805, Synechococcus sp. WH 7805; Syn8102, Synechococcus sp. WH 8102; PWH8109, Synechococcus sp. WH 8109; PCC7336, Synechococcus sp. PCC 7336; Syn6803, Synechocystis sp. PCC 6803; TeloBP1, Thermosynechococcus elongatus BP-1; cyUCYNA, cyanobacterium UCYN-A; PC10605, Cyanobacterium sp. PCC 10605; PCC7428, Gloeocapsa sp. PCC 7428; PCC7126, Microchaete sp. PCC 7126; Aaz0708, Anabaena azollae 0708; Av29413, Anabaena variabilis ATCC 29413; PCC7122, Anabaena cylindrica PCC 7122; PCC7108, Anabaena sp. PCC 7108; Crac505, Cylindrospermopsis raciborskii CS-505; Nsp9414, Nodularia spumigena CCY9414; Np73102, Nostoc punctiforme PCC 73102; Nos7120, Nostoc sp. PCC 7120; PCC7107, Nostoc sp. PCC 7107; RaphiBr, Raphidiopsis brookii D9; PCC6303, Calothrix sp. PCC 6303; PCC7507, Calothrix sp. PCC 7507; Amax328, Arthrospira maxima CS-328; ArthPla, Arthrospira platensis str. Paraca; PCC9333, Crinalium epipsammum PCC 9333; PCC7407, Geitlerinema sp. PCC 7407; PCC6306, Leptolyngbya sp. PCC 6306; PCC7375, Leptolyngbya sp. PCC 7375; PCC7376, Leptolyngbya sp. PCC 7376; Lyn8106, Lyngbya sp. PCC 8106; PCC7420, Microcoleus chthonoplastes PCC 7420; PCC6304, Oscillatoria acuminata PCC 6304; PCC6407, Oscillatoria sp. PCC 6407; PCC7112, Oscillatoria sp. PCC 7112; PCC6802, Pseudanabaena sp. PCC 6802; PCC7367, Pseudanabaena sp. PCC 7367; PCC6313, Spirulina major PCC 6313; Tery101, Trichodesmium erythraeum IMS101; PCC6712, Chroococcidiopsis sp. PCC 6712; PCC7203, Chroococcidiopsis thermalis PCC 7203; PCC7319, Pleurocapsa sp. PCC 7319; PCC7327, Pleurocapsa sp. PCC 7327; PCC7437, Stanieria cyanosphaera PCC 7437; Pro9601, Prochlorococcus marinus AS9601; Pro9202, Prochlorococcus marinus str. MIT 9202; Pro9211, Prochlorococcus marinus MIT 9211; Pro9215, Prochlorococcus marinus MIT 9215; Pro9301, Prochlorococcus marinus MIT 9301; Pro9303, Prochlorococcus marinus MIT 9303; Pro9312, Prochlorococcus marinus MIT 9312; Pro9313, Prochlorococcus marinus MIT 9313; Pro9515, Prochlorococcus marinus MIT 9515; ProNAT1, Prochlorococcus marinus NATL1A; ProNAT2, Prochlorococcus marinus NATL2A; Pro1375, Prochlorococcus marinus marinus CCMP1375; ProMED4, Prochlorococcus marinus pastoris CCMP1986; AmaMBIC, Acaryochloris marina MBIC11017; uncyPop, uncultivated cyanobacterium (Poplar biomass community MLE1-like); PCC6605, Chamaesiphon sp. PCC 6605; PCC9006, Prochlorothrix hollandica PCC 9006; PCC9445, Spirulina sp. PCC 9445; PCC6312, Synechococcus sp. PCC 6312; PCC7524, Nostoc sp. PCC 7524

Fig. 3. Distribution of the OCP among cyanobacteria. A 16S species tree of cyanobacteria with sequenced genomes (public and Kerfeld and Gugger, unpublished). Organism IDs are colored by section (green, Section I; red, Section II; blue, Section III; yellow, Section IV). F = full-length OCP gene, N, C correspond to genes encoding the N- and C-terminal domain, respectively. 16S rDNA sequences (identified after organism name) were retrieved manually from the Integrated Microbial Genomes browser. Sequences were aligned using the maxiterate function in MAFFT [60]. A maximum-likelihood tree was generated using FastTree [61]. *Prochlorococcus* (Pro) strains do not have phycobilisomes and do not contain OCP homologs..

the protein seems to be caused by aggregation. Thus, the properties of the excited-states of the red active OCP must be completely different to those of the RCP.

As noted above, the keto group of the carotenoid is essential to OCP function. In the structures of the OCP from both *A. maxima* and *Synechocystis*, the hECN is hydrogen bonded via its keto group to the absolutely conserved aromatic amino acids Tyr201 and Trp288 (*Synechocystis* OCP numbering; Fig. 2) of the central strand of the β-sheet [47,48] (Fig. 2). The hydrogen bond distances are 2.6 and 2.9 Å respectively, the former is a relatively short hydrogen bond. The orientation of Tyr201 seems to be stabilized via aromatic interactions with two absolutely conserved Phe side chains (residues 217 and 290), whereas the orientation of Trp288 may be stabilized by the interaction with the sulfur of Met202 [47,48] (Fig. 2). Measurements of kinetics of the orange to red photo-conversion at different pH, suggested that deprotonation of Tyr201 destabilizes the orange form, accelerating the accumulation of the red form. The orange to red conversion of the WT-OCP is about eight times faster at pH 12 than at pH 8 or 7 [55]. At pH 12 the orange form is so unstable that the red form is accumulated even in the absence of light. We hypothesize that in the red form the hydrogen bond between Tyr201 (and Trp288) and the carotenoid carbonyl is weaker than in the orange form or it is broken [55].

Replacing Tyr201 or Trp288 by Ser or His largely destabilizes carotenoid binding [55]. The concentration of OCP in these mutants was low and only a fraction was able to be isolated; of the isolated protein, 30% to 50% lacked the carotenoid. Also the carotenoid binding was relatively nonselective; in addition to ketocarotenoids (hECN, ech and canthaxanthin, the latter contains two carbonyl groups), the mutated proteins bound mainly zeaxanthin (or β-carotene in strains lacking zeaxanthin). The echinenone- (canthaxanthin-) binding Trp288 and Tyr201-OCPs were more red than the WT-OCP suggesting that both hydrogen bonds are essential to maintain the carotenoid in its orange state. However, this red protein was unable to induce fluorescence quenching, supporting the idea that simply a red-shifted carotenoid protein is insufficient to induce photoprotection. These proteins were not photoactive, suggesting that no conformational changes were induced by illumination [55].

The importance of the hydrogen bonding of the C-terminal domain of the OCP to the carotenoid, as well as the overall structure of the C-terminal domain, is reminiscent of other blue light photoreceptors. Both BLUF and LOV domains contain a central beta sheet core flanked by alpha helices (reviewed in Ref. [57]), as in the C-terminal domain of the OCP. In LOV and BLUF proteins, changes in the chromophore are propagated to the beta scaffold resulting in modifications in the intra-protein hydrogen bonding, leading to changes in inter-domain interactions and/or exposure of additional regions of the protein for interprotein interactions (e.g. [51,57]). In the OCP, the hydrogen bonding network emanating from the hECN to the surface of the OCP as well as the presence of conserved water molecules and the potential for alterations in the interaction between the N- and C-terminal domains of the OCP suggest that there could be mechanistic similarities between the OCP and other blue light photoreceptors [48]. For example, conformational changes that vary hydrogen bonding patterns and/ or the position of Tyr201 and Trp288 could induce a cascade of conformational changes in the protein and/or in a network of hydrogen bonds leading to a higher affinity of the OCP for the phycobilisome and/or a relative "opening" of the protein to allow interaction between the carotenoid and the phycocyanobilins. In the Trp288 and Tyr201 mutants (mutants in which one of the hydrogen bonds between the protein and carotenoid is missing), these conformational changes, critical to the interaction between the carotenoid and the allophycocyanin chromophores, are apparently not induced leading to the lack of energy dissipation.

In the N-terminal domain of the OCP, the hydroxyl ring of hECN is surrounded by a structurally conserved water molecule and absolutely conserved aromatic residues: Trp41, Trp110 and Tyr44 (Fig. 2) [48]. Although the presence of the hydroxyl group is not necessary for photoactivity, the interaction of the terminal ring of the carotenoid with these amino acids is essential. The aromatic nature of Trp110 and Tyr44 is critical. Changing these amino acids to Phe did not affect OCP photoactivity and photoprotection; however if they were replaced by Ser, photoactivity and fluorescence quenching were lost [48,55]. However, the mutations affected the photoactivity of the OCP differently. The W110S-OCP, isolated from the *Synechocystis* W110S-OCP overexpression mutant, is photo-inactive at temperatures lower than 25 °C but at temperatures higher than 30 °C, strong light is able to convert the mutated orange protein into its red form [55]. Moreover, at this temperature the red form was relatively stable. Thus, substitution of Trp110 by a Ser seemed to affect the amount of energy needed for conversion to the red form. Although *Synechocystis* grows at 32 °C, no induction of the photoprotective mechanism by the W110S OCP was observed at this temperature, possibly indicating that this red form is not able to correctly interact with the phycobilisome. In contrast, although *in vitro* the Y44S-OCP, isolated from the ΔCrtR-overexpressing Y44S-OCP mutant was photo-inactive from 8 to 35 °C, *in vivo* it was able to induce some fluorescence quenching with slow kinetics [55]. Thus, substitution of the Tyr44 by a Ser seemed to destabilize the isolated red form; however in the cell it was partially stabilized, perhaps by interaction with the phycobilisomes or other proteins.

By analogy to other blue-light responsive proteins, the interaction between the N- and C-terminal domains of the OCP may be important to its function. In the structure of the OCP there is a salt bridge between Arg155 and Glu244 which seems to contribute to the stabilization of the central interface of the protein between the N- and C-terminal domains (Fig. 2; [48]). Moreover, Arg155 and Glu244 both hydrogen bond to a conserved water molecule and flank a small region of the structure in which the carotenoid is solvent-exposed. Given that the two domains of the OCP are joined by a long flexible linker, alteration of the interaction between the two domains with concomitant loss of this salt bridge is possible; the resulting increase in accessibility of the carotenoid may be important for interaction with other proteins. In a mutant strain containing OCP in which the Arg155 was substituted by a Leu or a Glu, strong blue-green light is unable to induce fluorescence quenching although there is a relatively high concentration of the protein in the cell [48]. The mutated OCP was photoactive and its absorption spectrum is similar to that of the red WT OCP, indicating that similar changes were induced in the carotenoid of the WT and mutated OCP. In the 1.7 Å resolution crystal structure of the R155L OCP (PDB ID: 3MG3), a glycerol molecule occupies the space vacated by the Arg side chain and forms a hydrogen bond with Glu244, thus the disposition of the two domains with respect to one another is similar to WT [48].

In solution, the lack of the salt bridge may reduce the protein rigidity by weakening the interaction between the two domains thus rendering the carotenoid more mobile within the protein; this could allow it to adopt the red conformation without the requisite conformational changes induced in WT OCP. *In vitro* reconstitution of the OCP-induced fluorescence quenching mechanism will be useful to elucidate if these conformational changes are needed for binding or for efficient carotenoid-phycocyanobilin contact for energy transfer. On the other hand, FTIR measurements will help to elucidate the conformational changes needed to induce fluorescence quenching by comparing the spectra of the R155L OCP mutant to that of WT-OCP.

5. Recovery of full antenna capacity: the Fluorescence Recovery Protein (FRP)

In darkness, the isolated red OCP spontaneously reverts to the orange form. This step, which is not accelerated by illumination, is highly temperature dependent *in vitro*, occurring in seconds at 30 °C and in hours at 4 °C [46]. *In vivo*, the recovery of the lost fluorescence

occurs in 10–15 min at 30 °C [28,58,59]. The slower fluorescence recovery *in vivo* compared to the red to orange conversion *in vitro* indicates that the red form is stabilized in cells. The stabilizing factor could be the phycobilisome but this remains to be demonstrated. We have recently shown that *in vivo* a protein is needed to destabilize the red form and to allow the recovery of the fluorescence quenching and of the full antenna capacity [58]. When light intensity decreases, the "quenched" *Synechocystis* cells are unable to recover the lost fluorescence in the absence of a protein that we called Fluorescence Recovery Protein, FRP [58]. In *Synechocystis*, this protein is encoded by the *slr1964* gene adjacent to the OCP-encoding gene, *slr1963*.

Slr1964-like genes are present in almost all of the OCP-containing cyanobacteria strains. In the freshwater strains, the gene is just downstream of the OCP-encoding gene; in the marine strains a gene coding a putative β-carotene ketolase is found between them. In *Synechocystis* cells, the *frp* gene could be expressed independently of the *ocp* gene indicating the existence of its own-specific promoter. However the presence of long mRNA containing both genes in the overexpressing OCP-FRP strain suggested that the two genes could be co-transcribed under some conditions [58].

FRP orthologs contain 106 to 120 amino acids with a molecular weight of about 13–14 kDa; they are predicted to be soluble proteins and alpha helical (Fig. 4). *Synechocystis*'s FRP seems to be a special case. It contains an N-terminal extension of 25 amino acids with four methionine residues. The fourth methionine coincides with the first methionine of other predicted *frp* gene products. Experiments are underway to elucidate if the "short" (beginning at Met26) or the "long" FRP (beginning at Met1) is present in WT *Synechocystis* cells. Nevertheless, both proteins, which are present as trimers in solution and do not bind a chromophore, are active [58].

All of the FRP present in the cell is associated with phycobilisome-bounded-thylakoid complexes [58]. It strongly interacts with the thylakoids, even more so than the OCP. Co-immunoprecipitation experiments showed that much more OCP was co-precipitated with FRP in high light conditions than in darkness. Thus it appears that FRP interacts with the red OCP form and not (or almost not) with the orange form. The presence of FRP greatly accelerates the red to orange form conversion in the dark while it does not affect the initial rate of the light induced orange to red form conversion [58]. As already noted, the rates of the dark red to orange form conversion of the isolated OCP are temperature dependent with the $t_{1/2}$ at 18 °C of about 120 s and at 8 °C more than 1 hour in the absence of FRP. In a ratio of one FRP per two OCP the $t_{1/2}$ were much shorter: 5 s at 18 °C and 110 s at 8 °C [58]. Thus, when FRP was present during illumination of the isolated OCP almost no accumulation of the red form was observed. These results suggested that the amount of FRP in the cells must be substantially lower than that of OCP.

6. Working model and perspectives

Based on published results, it is clear that the OCP, a soluble carotenoid protein, is specifically involved in a photoprotective PB-associated mechanism and that the OCP is a photoactive protein sensing light intensity. Strong light induces the conversion between a dark stable orange form, OCP^o, and a metastable active red form, OCP^r. The accumulation of the OCP^r is essential for the induction of the

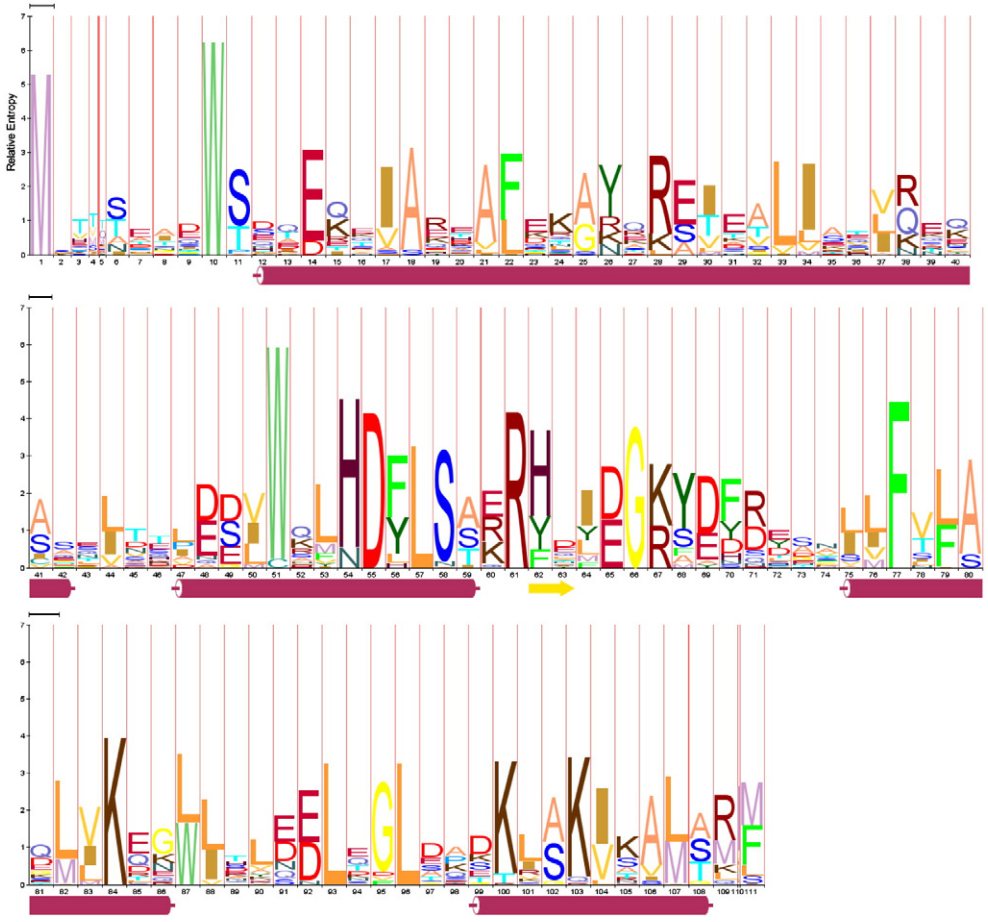


Fig. 4. HMM sequence logo for orthologs of the FRP with secondary structure prediction. The HMM logo was derived from the multiple sequence alignment of the primary structures of FRP (from organisms shown in Fig. 3). Predicted secondary structure elements are shown below the logo; alpha-helices are denoted by red cylinders, a single predicted beta-strand is shown as a yellow arrow. The HMM was built using HMMER [62], and the logo was visualized using LogoMat-M [63]. Secondary structure prediction was performed using Jpred [64].

photoprotective mechanism. The presence of the FRP (fluorescence recovery protein), encoded by the *slr1964* open reading frame in *Synechocystis*, is essential for the recovery of a fully functional antenna (and high fluorescence levels) when the cells are no longer under the high light exposure. The FRP, which functions as an oligomer (probably a trimer) destabilizes the OCP^r. The FRP–OCP interaction accelerates the OCP^r to OCP^o conversion.

Our working model is shown in Fig. 5. We propose that in darkness the OCP^o is not attached (or weakly attached) to the PB; however, once it is converted into the red form it becomes strongly attached to the PB and the red form is stabilized. While the OCP^r is attached to the PB, energy dissipation as heat occurs; either the energy is dissipated through the OCP or, alternatively, the binding of the OCP to the PB could alter the environment of the core chromophores leading to energy dissipation at the level of the phycobilisome. To restore energy transfer to the RC, OCP^r must be detached from the PB and/or interact with a destabilizing factor to be re-converted into the orange form releasing it from binding to the PB. By binding to the OCP^r, the FRP could help the OCP detach from the PB and then accelerate the conversion to OCP^o. Our working hypothesis proposes that OCP interacts with the trimers of allophycocyanin (forming the core of the PB) via its C-terminal domain [46]. This proposition was suggested by the structural similarity between the C-terminal domain of the OCP and the core linker protein, $L_c^{8.9}$. The OCP can interact with the internal or external chromophores of the APC trimers. We also propose that FRP interacts with the "free" N-terminal part of the OCP. Because OCP^r has a higher affinity for the FRP than OCP^o, light induced conformational changes of the OCP apo-protein are essential not only for OCP binding to the PB and fluorescence quenching but also for interaction with the FRP. Since the FRP has no chromophore, it cannot be activated or deactivated by light, therefore its function is likely concentration dependent. In order to maintain maximal photoprotection under high light conditions a low FRP:OCP ratio must be present. A high FRP concentration will prevent OCP^r accumulation.

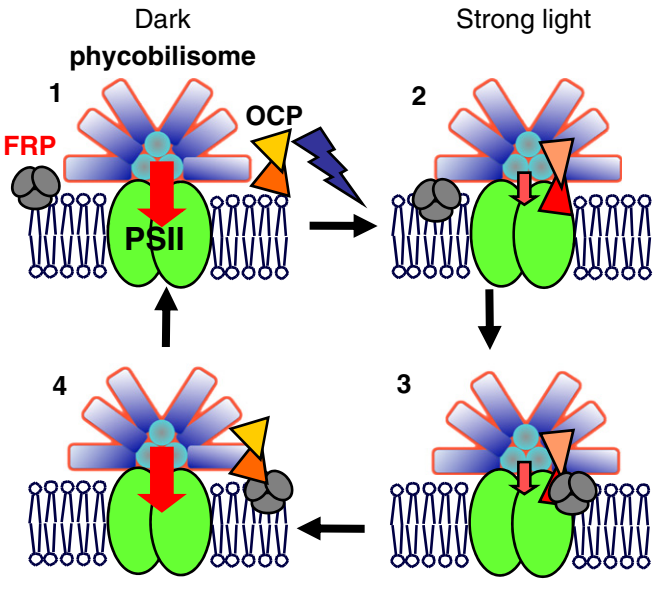


Fig. 5. Working model. 1) In darkness and under low irradiance the OCP is predominantly in the orange form (OCP^o) and it is not (or weakly) attached to the PB. 2) Absorption of blue-green light induces changes in the carotenoid and the protein, converting OCP^o to the red form (OCP^r). The OCP^r binds to the APC trimers of the core of the PBs via its C-terminal region, inducing fluorescence quenching. The phycobilisome protein and chromophore that interact with the OCP^r reminds to be identified. 3) The FRP, as a trimer, binds to the N-terminal OCP^r. 4) The FRP helps to detach the OCP^r from the PB and accelerates the OCP^r to OCP^o conversion. In darkness (or low light), conditions in which the detached OCP is not converted to the red form, the action of the FRP induces the recovery of fluorescence.

Several elements of this working model remain to be demonstrated. The most important being the following. 1) Elucidation of the site of energy quenching: OCP or PB chromophore? 2) Identification of the site of interaction with the PB: does the OCP interact with internal or external chromophores of the APC rings or of APC-last emitters mixed rings? 3) Which component of the core is involved in the fluorescence quenching? In addition, there are numerous open questions about the photocycle of the OCP, its relationship if any, to that of other blue-light active proteins and the interactions of the OCP with the PB and the FRP. Further studies on the OCP-related photoprotective mechanism will be necessary to answer these questions. I*n vitro* reconstitution of the mechanism using isolated phycobilisomes, OCP and FRP and the crystallization of the activated red OCP form will be essential steps toward the elucidation of the qE_{cya} mechanism.

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