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REVIEWS

Review

Changing Color for Photoprotection: The Orange Carotenoid Protein

Fernando Muzzopappa[1] and Diana Kirilovsky[1,*]

Under high irradiance, light becomes dangerous for photosynthetic organisms and they must protect themselves. Cyanobacteria have developed a simple mechanism, involving a photoactive soluble carotenoid protein, the orange carotenoid protein (OCP), which increases thermal dissipation of excess energy by interacting with the cyanobacterial antenna, the phycobilisome. Here, we summarize our knowledge of the OCP-related photoprotective mechanism, including the remarkable progress that has been achieved in recent years on OCP photoactivation and interaction with phycobilisomes, as well as with the fluorescence recovery protein, which is necessary to end photoprotection. A recently discovered unique mechanism of carotenoid transfer between soluble proteins related to OCP is also described.

Highlights

The orange carotenoid protein (OCP) is a blue-light-photoactive protein involved in cyanobacterial photoprotection. OCP binds one keto-carotenoid, which spans both C and N terminal OCP domains (CTD and NTD).

Upon photoactivation, the carotenoid is translocated to the NTD and the color of the protein changes to red. Then, the domains are separated and OCP becomes active. The red OCP binds to the phycobilisome, inducing thermal dissipation of excess energy.

Paralogs of the OCP domains, namely, CTDH (CTD-like) and HCP (NTD-like), are also carotenoid proteins. The principal role of CTDH is to transport the carotenoid from the membrane to HCP and, perhaps, to OCP. HCPs are excellent singlet oxygen quenchers. Only one subfamily is able to interact with the phycobilisome.

A unique mechanism of carotenoid transfer between soluble proteins has been discovered and characterized.

The Orange Carotenoid Protein (OCP) and Photoprotection

Living organisms need to constantly sense environmental changes and adapt to them for survival. For photosynthetic organisms, it is crucial to sense changes in the light intensity and rapidly respond to them. These organisms are able to modify their photosynthetic apparatus to efficiently harvest light under fluctuating light conditions, which rapidly change from subsaturating to oversaturating light intensities. At the same time, photosynthetic organisms must avoid photodamage, generated by an excess of energy reaching photochemical reactions centers. Indeed, light becomes dangerous when the entire photosynthetic electron transport chain becomes over-reduced and reactive oxygen species are formed, leading to severe cell damage. Thus, the survival and growth of photosynthetic organisms strongly depend on the balance between an efficient collection of light energy to sustain photosynthesis and protection against its photo-oxidizing effects. Plants, algae, and cyanobacteria have developed a photoprotective mechanism through which a normally highly efficient light-harvesting antenna is reversibly switched into a photoprotective, 'dark' state. In this state, potentially harmful absorbed energy is dissipated as heat, decreasing the energy reaching photochemical reaction centers. This increase in thermal energy dissipation is accompanied by a significant non-photochemical quenching (NPQ) of chlorophyll (phycobiliproteins) fluorescence. This mechanism differs in cyanobacteria from those existing in plants and algae, owing to the special cyanobacterial antenna, the phycobilisome (PBS; Figure 1). In cyanobacteria, a water-soluble photoactive carotenoid protein, the orange carotenoid protein (OCP), senses light intensity and induces thermal dissipation of excess excitation energy by interacting with PBS (see previous reviews [1–4]). The existence of the cyanobacterial OCP was reported in 1981 [5], but its function in cyanobacterial photoprotective thermal energy dissipation was only elucidated in 2006 [6]. Moreover, although it was first isolated in the late 1990s [7] and its tridimensional structure was described in 2003 [8], the photoactivity of OCP was only discovered in 2008 [9]. OCP has a second role in photoprotection as a very good quencher of singlet oxygen [8,10]. Large progress in the understanding of the OCP photocycle and interactions with its partners has been made in the past 5 years.

OCP-coding genes are present in a large number of PBS-containing cyanobacteria; however, one-third of sequenced strains have no *ocp* genes [11–14]. For example, *Synechococcus elongatus* and *Thermosynechococcus elongatus*, which are largely used as model organisms, lack *ocp*-like genes. OCP is completely absent in PBS-lacking cyanobacteria (alpha clade, including *Prochlorococcus marinus* strains) and in red algae, which are eukaryotic PBS-containing algae. There exist at least three paralog families of OCP, namely, OCP1, OCP2, and OCPX [11,12,15]. Most OCPs (approximately 65% of available sequences) belong to the OCP1 family, to which the well-characterized *Synechocystis* PCC 6803, *Arthrospira maxima*, and *Anabaena* PCC 7120 OCPs belong [11,12,15]. Only one OCP2 (*Tolypothrix* sp. PCC 7601) and one OCPX (*Scytonema hofmanni* PCC 7110) have been characterized

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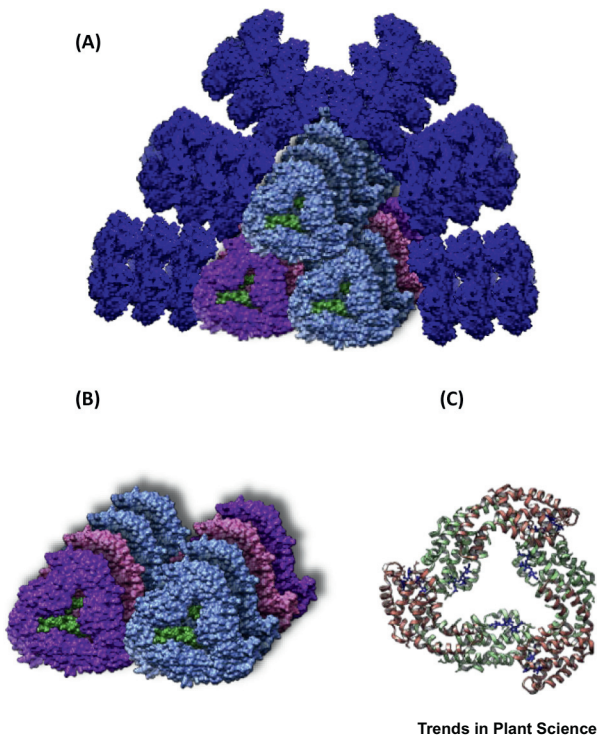

Figure 1. Phycobilisome (PBS) of *Synechocystis* PCC 6803.
(A) In *Synechocystis* PCC 6803, which is mostly used to study the cyanobacterial non-photochemical quenching mechanism, the PBS is hemidiscoidal, with six rods radiating from the core. Each rod is composed of three hexamers of blue phycocyanin (PC). Colorless linker proteins stabilize the PBS structure and optimize the directional energy transfer. Energy transfer occurs from the PC hexamer of the external rod to the PBS core. (B) The core is formed by two basal cylinders and one upper cylinder. Each cylinder contains four trimers of allophycocyanin (APC). All the trimers in the upper cylinder are formed by αAPC-βAPC heterodimers, emitting at 660 nm [a blue trimer is shown in (C)]. In the basal cylinders, one αAPC subunit is replaced in one of the external trimers by a special αAPC-like subunit, called ApcD (violet); in another trimer, one β subunit is replaced by ApcF, a βAPC-like subunit, and one α subunit is replaced by the N terminal domain of ApcE, an αAPC-like domain (rose). The bilins attached to ApcD and ApcE have a maximum fluorescence at 680 nm and are the terminal energy acceptors of PBS. In each cylinder, the two external trimers are stabilized by the ApcC linker protein (green).

[12,15]. It was first suggested that both OCP2 and OCPX are ancestors of OCP1 [12,16]. However, a recent phylogenetic analysis, using a rooted tree, clearly showed that a primitive OCP diverged into OCPX and an ancestor of the modern OCP2 and OCP1, which are the result of a second divergence [15]. OCP1 and OCP2 seemed to have diverged toward different specializations, maintaining or changing different properties that were present in their ancestor and are still present in the modern OCPX. The different properties will be described in the following sections. OCP1 and OCPX usually occur alone, while OCP2 generally coexists with OCP1 or OCPX [12,15].

Changing the Color: OCP Photoactivity

The photoactive OCP is composed of two globular domains, the α-helical N terminal domain (NTD; residues 18–165) and the α-helix/β-sheet C terminal domain (CTD; residues 190–317), a member of the nuclear transport factor 2 (NTF2) superfamily, which are connected by a long, flexible (largely unstructured) loop linker (approximately 25 residues; Figure 2) [8,17]. In the dark, OCP is orange and has a compact (closed) globular conformation. This conformation is stabilized by strong interdomain interactions in the middle of the protein, in the principal interface between the domains (including a salt

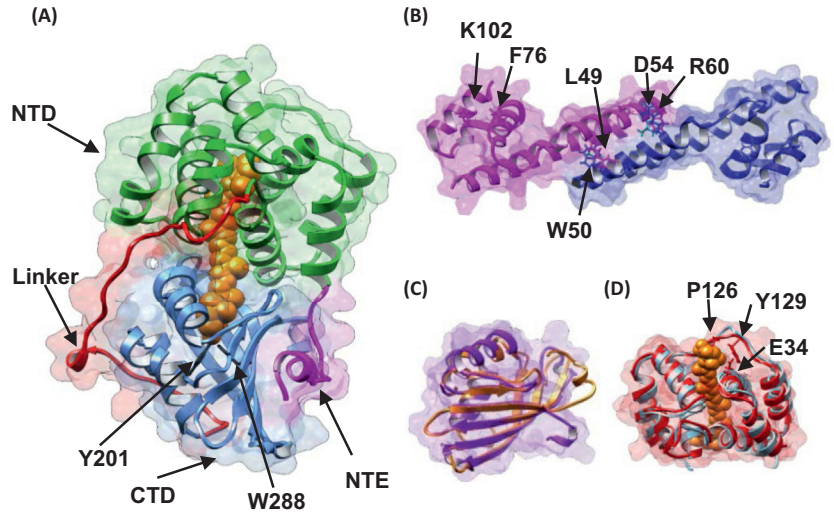

Figure 2. Tridimensional Structures of the Orange Form of Orange Carotenoid Protein (OCP^O), Fluorescence Recovery Protein (FRP), and Paralogs of OCP Domains [Helical Carotenoid Proteins (HCP) and CTD-like Proteins (CTDH)].

(A) *Synechocystis* OCP^O structure [Protein Data Bank (PDB): 3MG1]. The C terminal domain (CTD) is colored in cyan; the N terminal domain (NTD) is colored in green; the N terminal extension (NTE) is colored in pink; and the linker is colored in red. The Y201 and W288 amino acids, involved in H-bond formation with the carotenoid, are marked in black. (B) FRP dimer structure (PDB: 4JDX). The amino acids involved in the dimeric interaction and in FRP binding to OCP are marked. (C) Comparison of CTD of OCP (PDB: 3MG1; shown in violet) and CTDH (PDB: 6FEJ; shown in golden). (D) Comparison of NTD of OCP (PDB: 4XB4; shown in red) and HCP1 (PDB: 5FCX; shown in grey). Amino acids involved in carotenoid stabilization are marked. Figure was prepared using UCSF Chimera.

bridge between residues R155 and E244), and in the interface between the N terminal extension (NTE; residues 1–20), containing a small α-helix, and the CTD β-sheet (Figure 2) [8,17]. The closed structure is also stabilized by a ketocarotenoid, 3′-hydroxyechinenone (hECN), which spans both domains. The carotenoid carbonyl (keto) group is in a hydrophobic pocket of CTD, forming H-bonds with the OCP Y201 and W288 amino acids (numbering after *Synechocystis* OCP) [8,17]. The other carotenoid ring, containing a hydroxyl group, has π interactions with Y44 and W101 [8,17]. OCPX and OCP1 tend to forms dimers; they appear as monomers in solution at low concentrations, but at high concentrations, their dimerization is almost complete [15,18]. By contrast, OCP2 remains monomeric even at high concentrations [12,15].

The carotenoid is a sensor of light intensity. A strong blue-green light, absorbed by the carotenoid, induces conformational changes in the carotenoid and a breakage of the H-bonds between the carotenoid carbonyl group and Y201/W288 [9,19]. These changes lead to conformational changes in the two protein domains and the orange form (OCP^O) is converted into a metastable active red form (OCP^R) [9]. The nature of the carotenoid is essential for OCP activity; only OCPs carrying a ketocarotenoid, such as echinenone or canthaxanthin (CAN), in addition to hECN, are able to be photoactivated [20,21].

The structure of OCP^R was not resolved. Thus, the real structure of OCP^R is unknown. The following OCP^R characteristics are based on the results of different crosslinking and structural experiments and on the tridimensional structure of the isolated carotenoid containing NTD domain. OCP^R presents a more elongated and open configuration, in which the two domains are completely separated, being only connected by the linker loop (Figure 3) [22–27]. In addition, the relative orientation of the domains is different in OCP^O and OCP^R [22–27]. During photoactivation, the carotenoid moves by 12 Å. In OCP^R, the carotenoid is completely embedded in NTD, being solvent-exposed only in the vicinity of the rings [23,28]. New amino acids stabilize the carotenoid position in OCP^R, including

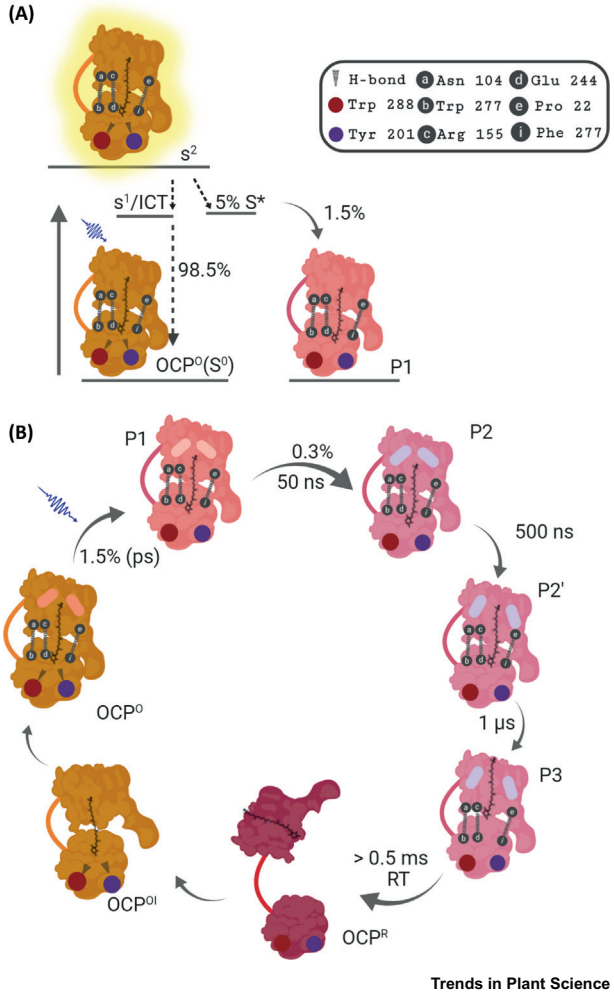

Figure 3. Orange Carotenoid Protein (OCP) Photoactivation.

(A) Blue-green light brings the carotenoid to the optically allowed S2 excited state, which relaxes within femtoseconds to the optically dark S1 excited state; the latter is coupled to an intramolecular charge-transfer (ICT) state and decays in approximately 4 ps to the ground state [9,49,76,77]. A small population (approximately 5%) of an S* excited state, decaying in 24 ps, was also detected [19]. In this excited state, the H-bonds with the protein weaken, resulting in their rupture (approximately 1.5%) and leading to the formation of the first, P1, red state [19]. In this state, the carotenoid is structurally relaxed and planar but seems to conserve the C6-C7 *trans*-configuration [19]. (B) Rapidly (within 50 ns), approximately 60% of the 'free' carotenoid molecules find a new quasi-equilibrium, P2, close to their original binding pocket. This small movement triggers slower changes in N terminal domain (NTD) helical elements (0.5–1 µs) (P2'), needed to allow the carotenoid translocation to NTD, occurring in 10 µs (P3) [19]. In this state, the protein is still closed [19]. The OCP opening, including N terminal extension and C terminal tail movements, occurs at least 10 times slower, leading to an open, active red form (OCP^R) [19,33]. During the recovery to the orange form (OCP^O), an intermediary orange state is formed [32]. This model is principally based on the results described in [19,32]. This figure was created using BioRender (https://biorender.com/).

E34, Y129, and P126 [23]. The carotenoid molecule, which has an all-trans configuration in both OCP^O and OCP^R, is more planar, with a larger effective conjugation, in OCP^R [9]. The longer conjugation length was proposed to be related to different positions of the rings around a single C6–C7 (C6'–C7') bond and to a less bent polyene chain [23,29]. Moreover, it was proposed that the first step in OCP photoactivation involves a rotation of the β-ionone ring [23]; however, recent structural

and spectroscopic data do not support this hypothesis [19,30]. It has recently been proposed that the breakage of hydrogen bonds between the carotenoid and OCP can be due to a structurally distorted, excited S* state of the carotenoid in the protein [19] (see below).

Recent studies have begun to elucidate the OCP photocycle [19,31–33]. First, asynchronous changes in carotenoid and protein components were observed and intermediary orange and red states were described in the OCP photocycle [32] (Figure 3). The existence of an intermediary red form was proposed during photoactivation, in which the carotenoid is still shared by the two domains [32]. An intermediary orange form was proposed during the recovery, in which the carotenoid is already attached to Y201 and W288 but the protein does not have its final conformation yet [32]. It was initially proposed that the latter state is less compact than the final OCP^O is; however, more recent data suggested a more 'compact' configuration of the intermediary orange form, in which water molecules are excluded principally in the carotenoid tunnel [31]. This study also suggested that domain association and restructuration of conserved water–protein interactions in the major and minor interface occur faster than the final rearrangements at carotenoid pocket [31].

More recently, it has been shown that during photoactivation, the carotenoid passes through several different red states before arriving to NTD [19] (Figure 3). It was speculated that the H-bonds between the carotenoid and the protein were weakened in a small carotenoid population (5%), which was in the S* excited state in which the carotenoid is distorted, partially resulting in H-bond rupture (approximately 1.5%) and leading to the formation of the first intermediary red state. The free carotenoid is then translocated to NTD through several steps and induces conformational changes in NTD α-helices (Figure 3) [19]. The opening of the protein, including the movement of NTE and of the C terminal tail (CTT), occurs much slower once the carotenoid is in NTD [19,33]. The studies of Gupta *et al*. [31], using X-ray radiolytic labeling, also suggested that first the carotenoid migrates to the NTD, then the domains separate, and finally the NTE dissociates and its α-helix becomes disordered [31,33]. Simultaneously, CTT also dissociates from the CTD β-sheet and moves to cap the CTD carotenoid tunnel once it is empty upon carotenoid translocation [34] (Figure 2).

In vitro, OCP can be chemically photoactivated. In the presence of high concentrations of sodium thiocyanate (higher than 1.5 M) [35] or Cu^+ [36], OCP photoactivation occurs even in the dark. Furthermore, OCP photoactivation is induced in the dark by low (<5) [8] and high (>10) [37] pH values.

Photoactivation is not needed for the singlet oxygen-quenching activity; both OCP^O and OCP^R show very good singlet oxygen-quenching activities, which do not depend on the type of the carotenoid bound, ketocarotenoid or zeaxanthin [10].

Photoprotection: Converting Solar Energy into Heat

Only OCP^R can bind to PBS, while OCP^O is unable to bind [9,38]. OCP^Rs from the three OCP families interact with PBS and induce NPQ [12]. The isolated NTD is constitutively active in fluorescence and energy quenching, even in the dark [28]. In OCP^O, CTD inhibits the NTD binding to PBS because a specific amino acid at the domain interface, Arg155, is essential for PBS binding [39]. The separation of the domains upon photoactivation uncovers this amino acid and the 'free' NTD is able to bind to PBS [23,28,38,39]. Thus, NTD is the effector domain and CTD is the regulator one. OCP^R interacts with one (or two) of the basal cylinders of PBS [38,40]. Binding of only one OCP^R molecule is sufficient to quench almost all PBS fluorescence [38,41,42]. However, single-molecule experiments with isolated PBS and PBS–OCP complexes suggested that the binding of a second OCP is possible (with a lower affinity) and necessary to quench even more PBS fluorescence [42]. The existence of an intermediary binding state, involving rearrangements of the PBS structure and leading to decreased energy transfer from the rods to the core, was also detected [41]. The most recent results suggested that NTD binds between two allophycocyanin (APC) trimers (Figure 4, Key Figure), the APC trimer containing ApcF-ApcE (disc 3) and the external trimer containing ApcD-ApcC (disc 4), in the basal cylinder [43]. OCP induces less fluorescence quenching in a mutated PBS, lacking ApcC [43] or missing the chromophore domain or the PB-loop of ApcE [44,45], suggesting that both proteins play a role in

Key Figure

Orange Carotenoid Protein (OCP)-Related Non-photochemical Quenching Mechanisms

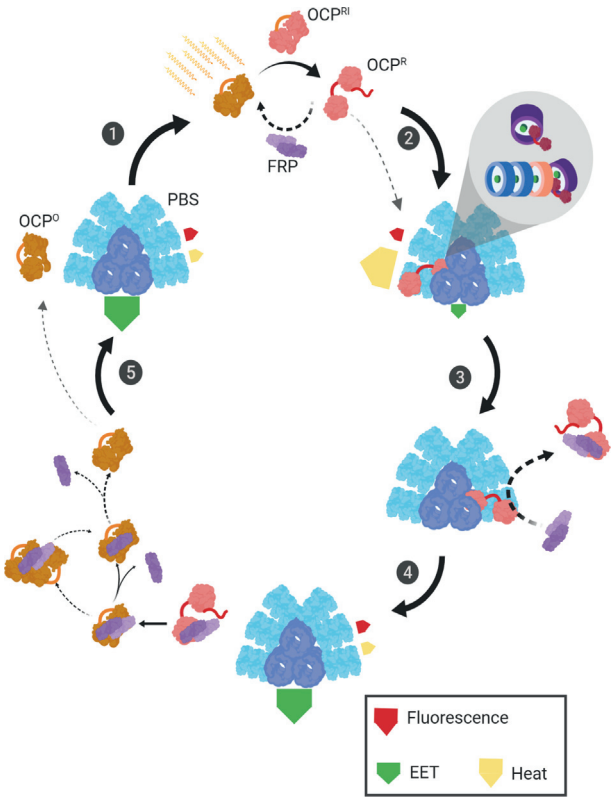

Figure 4. In the dark and under low irradiance, OCP is in its orange, inactive form and is not bound to the phycobilisome (PBS). PBS transfers most of the absorbed energy to the photosystems to support photosynthesis. (1) A strong light induces the photoactivation of the orange form (OCP^O), which is photoconverted to an active, open red form (OCP^R) via intermediary closed red forms. (2) OCP^R binds to the PBS core and induces the dissipation of excess absorbed light as heat, while PBS fluorescence and excitation energy transfer (EET) to the photosystems are minimal. (3) The dimeric fluorescence recovery protein (FRP) helps OCP detach from PBS under all light conditions. (4) Once detached from PBS, OCP^R is converted to the inactive OCP^O state and FRP accelerates this reaction. According to the current model, after OCP compaction, FRP can monomerize, or another OCP can bind to the OCP complex with the FRP dimer, which is split via a steric clash into two OCP complexes with FRP monomers [59]. Finally, the OCP–FRP complex dissociates, releasing free OCP^O to complete the cycle (5). This figure was created using BioRender (https://biorender.com/).

the stabilization of OCP binding. OCP binding alters the structure of the cylinder, separating the rings, and could decrease the affinity for a second OCP molecule. CTD, which may be relatively mobile in the attached OCP^R, stabilizes the OCP binding [23,43]. Further studies must be conducted to confirm the exact position of NTD of OCP^R in PBS.

The first site of fluorescence quenching seems to be an APC bilin emitting at 660 nm [46,47], although some results suggested that an APC bilin emitting at 680 nm could be also primarily quenched [48]. In

Synechocystis cells, the overall quenching rate is very fast, approximately $(16\ ps)^{-1}$ [47], which is competitive with the energy transfer to photosystems. Thus, OCP binding to PBS leads to a very efficient energy quenching, significantly decreasing the energy reaching reaction centers. However, the precise mechanism of energy dissipation remains to be elucidated. Most probably, the carotenoid plays a direct role in the quenching via energy or charge transfer [46,47,49–51]. As an alternative, OCP binding to PBS can affect the environment of one or more bilins, which could induce NPQ by hindering energy propagation and/or converting one α- or β-bilin into an energy-dissipating state. Further spectroscopic experiments are needed to elucidate the energy quenching mechanism.

Stopping Photoprotection: The Fluorescence Recovery Protein (FRP)

When low-light conditions return, the interaction of the OCP with the fluorescence recovery protein (FRP) is needed to increase the effective size of the antenna in cells containing OCP1 [52]. In the absence of FRP, the cells remain in a quenched state, even in the dark [52,53]. FRP has two different activities, being essential for the OCP^R-to-OCP^O conversion reaction and for detaching OCP^R from PBS [52,54]. *In vitro*, OCP^R spontaneously reverses to OCP^O in the dark, even in the absence of FRP [9]. For this recovery reaction, specific amino acids of the loop linker are essential in the three types of OCPs [15]. For example, the modification of E174 and/or R185 largely inhibits the OCP^R-to-OCP^O conversion [15]. However, the presence of FRP strongly accelerates the OCP^R-to-OCP^O reconversion [52]. FRP does not reduce the activation energy barrier of the OCP^R-to-OCP^O conversion [55] but brings together NTD and CTD, stabilizing the intermediary state that facilitates the OCP^R-to-OCP^O conversion [55,56]. This activity is light independent because FRP is active under light and dark conditions. *In vitro*, depending on experimental conditions, OCP^R can detach from PBS in the absence of FRP; however, the presence of FRP accelerates the detachment [38,54]. In cells, the concentration of FRP is much lower than that of OCP, which is important since high FRP concentrations completely inhibit the PBS fluorescence quenching [53,57]. Thus, the concentration of FRP not only controls the rate of PBS fluorescence recovery but also the amplitude of PBS fluorescence quenching. The *frp* gene is absent in strains containing only OCP2 or OCPX [12]. In these strains, OCPX or OCP2 is able to detach from PBS and allows fluorescence recovery in the absence of FRP [15]. Moreover, the rate of OCP^R-to-OCP^O conversion of these OCPs is fast and does not need to be accelerated by FRP [12,15].

FRP is an α-helix elongated protein that is stable as a dimer in solution [18,55,58] (Figure 2). The tridimensional structure of FRP revealed that there is a patch of highly conserved amino acids in the monomer–monomer interface, involved in intermolecular interactions [58]. For example, a cation–π interaction between R60 from one monomer and W50 from the other allows the formation of a salt bridge between D54 and R60 [58] (Figure 2). The FRP interface is also stabilized by hydrophobic interactions and, upon replacement of L49 by a glutamate, FRP becomes permanently monomeric [59]. The conserved R60, W50, and D54 are also important for the FRP activity as an accelerator of the OCP^R-to-OCP^O conversion, although these amino acids are not involved in the primary FRP binding site [54,58]. The FRP dimer interacts with high affinity with CTD of $OCP1^R$ [52,55,58,60]. The FRP binding interface involves F76 and K102, located in the C terminal head [56,59]. In an isolated OCP, the main (primary) FRP-binding site in CTD overlaps with the surface that is occupied by NTE on OCP^O [54,58,61]. This surface contains the F299 and D220 amino acids, which were demonstrated to be important for the FRP activity as an OCP deactivator [54]. Although these amino acids are present in OCP2 and OCPX, FRP is unable to bind to them [12,15]. In these OCPs, the double mutation R229E/K and D262G/N prevents the FRP binding [15]. Although the first and essential FRP-binding site is located in CTD, secondary NTD sites seem to be important for FRP activity [55,56,59,61]. This creates a scaffold for separated OCP domains, which facilitates their mutual approach and reconstruction of the carotenoid channel. FRP can also bind to OCP^O but with a very low affinity [55].

FRP binds to CTD of OCP^R as a dimer but then monomerizes [55,59,62]. It has been proposed that FRP binding to OCP weakens the interaction between the monomers, which can lead to spontaneous monomerization of FRP; alternatively, the binding of a second OCP to the FRP dimer may provoke a clash between the two OCP molecules, causing the splitting of the 2OCP:2FRP complex into two

OCP:FRP complexes [59]. The monomerization is not essential for FRP activity but can improve its efficiency, especially at elevated concentrations of OCP^R under high light intensities [59].

Figure 4 shows the working model of the OCP-related NPQ photoprotective mechanism, including recent discoveries.

Paralogs of OCP Domains

In addition to OCPs, many cyanobacterial strains contain homologs of the NTD and CTD domains of OCP [11,12,14,63–66]. The family of NTD-like proteins, named helical carotenoid proteins (HCPs), contains at least nine different subclades (HCP1 to HCP9) [65], while the family of CTD-like proteins (CTDHs) contains only two subclades (CTDH1 and CTDH2) [66]. Both HCPs and CTDHs are carotenoid proteins that preferentially bind CAN [64,66] (Figure 2). While several HCPs from different subclades can coexist in cells of some strains, only one CTDH is present [11,65,66]. Several HCPs and CTDHs were isolated and characterized from *Anabaena* PCC 7120 (HCP1–4 and CTDH2) [34,64–66], *T. elongatus* (HCP4/5 and CTDH1) [66], *Tolypothrix* (HCP2 and CTDH2) [67], and *Nostoc flagelliforme* (HCP1–3, HCP6, and CTDH) [68]. The structures of CAN-HCP1 from *Anabaena* PCC 7120 and CAN-HCP2 from *Tolypothrix* PCC 7601 [65,67], and that of the *Anabaena* PCC 7120 carotenoid-free CTDH2 (apo-CTDH2) [34] were resolved (Figure 2). HCPs are all α-helix proteins and their secondary and tertiary structures, as well as the position of the carotenoid, are similar to those in NTD of OCP [65,67]. While HCPs are monomers, CTDHs are dimers, sharing one CAN molecule [66]. In CTDH2, Cys103 stabilizes the dimer by the formation of an S–S bond between the monomers [66]. Two types of dimers were found in crystals of the *Anabaena* apo-CTDH2, one stabilized by the S–S bond and forming an empty carotenoid tunnel (F-type) and another one formed by an interaction between the β-sheets of the two monomers (A-type) [34]. Both types of dimers are present in solution [34]. The secondary and tertiary structures of apo-CTDH and CTD of OCP^O are similar. The principal difference between these structures is the position of CTT [34]. In CTD of OCP^O, CTT is in an external position, interacting with the β-sheet [8]; in apo-CTDH, CTT adopts an internal position, partially covering the empty carotenoid tunnel [34]. These results suggest that CTT is mobile and changes its position depending on the presence or absence of the carotenoid, not only in CTDH but also in OCP. Upon photoactivation, the carotenoid migrates to NTD and CTT moves to close the empty carotenoid tunnel.

HCPs from different subclades have different activities, at least *in vitro* [64,67,68], and only HCP4 proteins from *Anabaena* PCC 7120 and *T. elongatus* are able to bind to PBS and induce energy quenching, similar to OCP [64] (see below). HCP2 proteins from *Anabaena* and *Tolypothrix* and HCP3 from *Anabaena* are good singlet oxygen quenchers, and HCP1 can be a carotenoid carrier [64,67]. In drought-resistant *N. flagelliforme*, HCP1, HCP3, and HCP6 seem to play a photoprotective role in desiccated cells since their expression is upregulated upon dehydration [68]. CTDHs are also good singlet oxygen quenchers, but their principal role is as carotenoid carriers from the membrane to HCPs [34,66]. CTDHs are transient carotenoid proteins, which are able to transfer the carotenoid from the membrane to HCPs and OCP [66]. This activity is essential for HCPs, which are unable to take the carotenoid from the membrane [66], and thus *ctdh* genes are present in all strains containing HCPs. By contrast, OCPs are able to extract carotenoids from the membrane and CTDHs are absent in all strains containing only OCPs (without HCPs) [66]. Nevertheless, CTDH presence can increase the efficiency of holo-OCP formation in some strains [66]. In CTDHs, their CTT plays an important role in carotenoid uptake and translocation [34].

Carotenoid Transfer between Water-Soluble Proteins

A unique mechanism of carotenoid transfer between water-soluble proteins was discovered in 2017 [69,70] (Figure 5). Individual NTD and CTD domains of OCP were produced in *Escherichia coli* in the presence or absence of CAN. When CAN-CTD was incubated with apo-NTD, photoactive OCP-like complexes were formed [70]. In addition, the expression of the individual domains in the same CAN-producing *E. coli* cells resulted in the formation of OCP-like complexes [16]. NTD, similar to HCPs, is unable to take the carotenoid from the membrane [66]. By contrast, CTD, similar to CTDH, can extract the carotenoid from the membrane and then form an OCP-like complex by interacting with apo-NTD [66]. These results and the fact that an *ctdh* gene is frequently located next to the *hcp4* gene, which

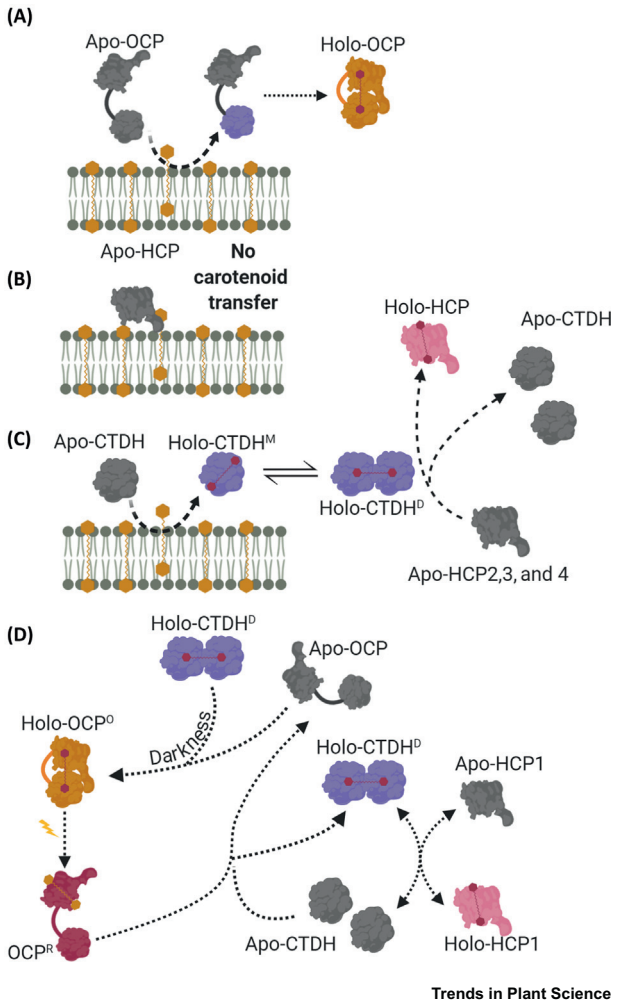

Figure 5. Membrane-to-Protein and Protein-to-Protein Carotenoid Translocation.

Apo-orange carotenoid protein (OCP) (A) and apo-CTD-like proteins (CTDH) (C) are capable of taking a carotenoid from the membrane, while apo-helical carotenoid protein (HCP) (B) is not able to form holo-proteins. After holo-CTDH is formed (C), it interacts with apo-HCP and transfers the carotenoid to apo-HCP to form holo-HCP. This model is based on the results described in [66]. (D) Multidirectional carotenoid transfer. Ana-HCP1 is the only HCP that is able to give the carotenoid to CTDH, in addition to being able to receive the carotenoid from CTDH. Under strong illumination, photoactivated OCP^R (red form) is able to interact with and transfer the carotenoid to apo-CTDH, forming holo-CTDH and apo-OCP. In the dark, holo-CTDH can transfer the carotenoid back to OCP. This model is based on the results described in [72]. This figure was created using BioRender (https://biorender.com/). Abbreviations: $CTDH^M$, CTDH monomer; $CTDH^D$, CTDH dimer; OCP^O, orange form of OCP.

encodes the only HCP that is able to interact with PBSs, suggested that a fusion of the *hcp4* ancestor and *ctdh* formed a gene coding for an ancestor of modern OCPs [12,16,65]. Holo-CTD1, -CTD2, and -CTDX are able to interact with the respective apo-NTDs and to form OCP-like complexes [15]. Upon illumination, all these complexes are photoactivated, leading to completely separated and free apo-CTDs and CAN-NTDs [15]. CAN-NTD2 is able to partially give the carotenoid to apo-CTD to form again an OCP-like complex [15,16]. By contrast, CAN-NTDX and CAN-NTD1 are unable to do this [15,16]. Since OCPX is the most ancient OCP, these results suggested that the HCP and CTDH ancestors that formed the first OCP were unable to realize a complete photocycle before the addition of an

appropriate linker upon gene fusion. Indeed, while the modern CAN-CTDH is able to give the carotenoid to the modern HCP4, holo-HCP4 cannot give the carotenoid to CTDH [66]. Moreover, the CTDH–HCP interaction never leads to the formation of an OCP-like complex; the carotenoid is completely transferred to HCPs [66]. This finding questions the hypothesis that the CTDH and HCP ancestors were able to form photoactive heterodimers.

Initial studies suggested that carotenoid transfer only occurred in one direction, namely, CTDH takes the carotenoid from the membrane and forms a dimer, thereby encapsulating the carotenoid [66] and then transferring it to apo-HCPs (or OCPs). Surprisingly, it was observed that *Anabaena* HCP1 (Ana-HCP1) was able to not only receive but also deliver the carotenoid to Ana-CTDH [71]. A further study of this mechanism showed that the carotenoid transfer could be multidirectional, involving specific partners, at least *in vitro* [72]. For example, NTD, HCP1, CTD, and photoactivated OCP are able to give the carotenoid to apo-CTDH [72]. The possibility and the direction of the carotenoid transfer are determined by the stability of the interaction between the partners and by their relative affinity for the carotenoid [72].

Outstanding Questions

What is the structure of the photoactivated OCP^R?

What are the different steps of the photocycle? What are the roles of NTE and CTT in the photocycle?

What is the mechanism of energy quenching? Is the carotenoid in OCP involved in energy dissipation?

What is the binding site for OCP in PBS? Which amino acids of APC and OCP are involved?

What are the roles of different HCPs *in vivo*? When are they expressed?

Is it possible to synthesize a family of OCPs and OCP-like proteins to be used in optogenetics and as regulators of artificial antennae?

Concluding Remarks and Future Perspectives

Cyanobacteria have evolved a very simple mechanism to decrease the energy that reaches photochemical centers under high irradiance to avoid oxidative damage. These organisms developed a two-partner system consisting of a photoactive carotenoid protein, OCP, which induces thermal dissipation of excess excitation energy, and another, nonchromophorylated protein, FRP, which blocks photoprotection under low light. The quantum yield of photoactivation is low to allow the accumulation of a photoactivated protein, which is the only protein capable of interacting with cyanobacterial antennae, PBSs, and quenching excess energy only under high-light conditions. In addition, OCP and the paralogs of its domains, HCPs and CTDHs, are excellent quenchers of singlet oxygen, decreasing the risks of oxidative damage. HCPs seem to help decrease the oxidative stress under specific stress conditions, such as desiccation. CTDH is an essential carotenoid carrier in cyanobacterial photoprotection. The study of these proteins allowed the discovery of a unique protein-to-protein carotenoid transfer mechanism, which could potentially be applicable to other processes involving water-soluble carotenoid proteins. These findings may also inspire the construction of stable carotenoid nanocarriers capable of targeted delivery of the antioxidant power to specific tissues, such as the macula lutea, in which the lutein-carrying NTF2-like StAR-related lipid transfer domain containing 3 (STARD3) protein plays a role in photoprotection.

The elucidation of the OCP photocycle and further characterization of the interactions between different HCPs, CTDHs, and isolated OCP domains of the three OCP families will enable the rational development of OCP and OCP-like variants able to serve as optogenetic tools. This will add a new element, having a different chromophore, the carotenoid, and a new type of triggering mechanism, in which initially coupled sensor and effector modules may reversibly or irreversibly dissociate.

OCP can also serve as a regulator of light uptake in artificial photosynthetic systems, by exploitation of its natural function. Artificial photosynthetic systems need light-harvesting modules and most initial efforts were dedicated to optimize the energy transfer efficiency and spectral coverage. Recently, however, the importance of regulating the process to avoid photodamage became evident and a synthetic system containing OCP as a light regulator was reported [73].

Finally, it has recently been demonstrated that OCP can quench different fluorescent energy donors via fluorescence resonance energy transfer [74,75], suggesting that OCP can be used as a regulator of fluorescence in light, temperature, and other types of sensors (see Outstanding Questions).

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