Structure and functions of Orange Carotenoid Protein homologs in cyanobacteria

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Abstract

Rapidly-induced photoprotection in cyanobacteria involves thermal dissipation of excess energy absorbed by the phycobilisome (PBS), the primary light-harvesting antenna. This process is called non-photochemical quenching (NPQ), and is mediated by a water-soluble photoactive protein, the Orange Carotenoid Protein (OCP). The OCP is structurally and functionally modular, consisting of a sensor domain, an effector domain, and a carotenoid. Blue-green light induces a structural transition of the OCP from the orange inactive form, OCP^O, to the red active form, OCP^R. Translocation of the carotenoid into the effector domain accompanies photoactivation. The OCP^R binds to the PBS core, where it triggers dissipation of excitation energy and quenches fluorescence. To recover the antenna capacity under low light conditions, the Fluorescence Recovery Protein (FRP) detaches the OCP from the PBS and accelerates back-conversion of OCP^R to OCP^O. Increased sequencing of cyanobacterial genomes has allowed the identification of new paralogous families of the OCP and its domain homologs, the Helical Carotenoid Proteins (HCPs), which have been found distributed widely among taxonomically and ecophysiologically diverse cyanobacteria. Distinct functions from the canonical OCP have been revealed for some of these paralogs by recent structural and functional studies.

Introduction

Photoprotective mechanisms have evolved in photosynthetic organisms to cope with fluctuating light and environmental stresses. Regulation of these mechanisms is critical for maintaining photosynthetic efficiency by minimizing photodamage. Typically, excess excitation energy that cannot be used for photochemistry in photosystem II (PSII) is dissipated as heat. This process is called non-photochemical quenching (NPQ) [1]. The mechanisms of NPQ differ among cyanobacteria, algae and plants. In contrast to the relatively well-characterized systems operational in eukaryotes [2], the Orange Carotenoid Protein (OCP)-mediated photoprotective response of cyanobacteria has only been extensively studied in the last decade [3,4].

The OCP is a water-soluble, 35 kDa protein that binds a single carotenoid molecule [3,4]. It is the only known photoactive protein that uses a carotenoid as its chromophore. The absorption of blue-green light or strong white light causes the OCP to convert from a dark stable orange form, OCP^O, to a light activated red form, OCP^R. The OCP^R form directly participates in photoprotection by binding to the light-harvesting antenna, the phycobilisome (PBS; **Figure 1**). Recovery from the energy-dissipative state is catalyzed by the Fluorescence Recovery Protein (FRP). The photoprotective OCP-FRP regulatory system (**Figure 1**) is found in the majority of cyanobacteria (with the exception of *Prochlorococcus* and marine *Synechococcus*) (**Figure 2**).

In addition to high light, expression of the OCP is known to be upregulated also under various stress conditions (e.g. nutrient starvation, oxidative stress, salt stress [4]). In addition, the OCP was proposed to govern the selective energy flow from the antenna to the different photosystems [5] in megacomplexes consisting of both photosystems plus the PBS, suggesting another means by which the OCP could play a role in acclimation to stress conditions at the level of the photosystems. The OCP is also known to quench singlet oxygen (¹O₂) [6,7] and has also been proposed to play a role in transport of carotenoids to the cytoplasmic or outer membrane to form a photoprotective shield [8]; this implies that the OCP also functions as part of systemic subcellular stress responses. Recent bioinformatic, structural, functional, and biophysical studies have

revealed numerous unanticipated findings, including new families of OCP (Bao et al., manuscript in revision) and OCP domain homologs [9,10] which confer an expanded range of functions. Collectively, an evolutionary history of the OCP is emerging which suggests that this group of soluble carotenoproteins has been of fundamental importance to the early evolution of cyanobacteria and perhaps even for the development of oxygenic photosynthesis.

Structural and Functional Modularity of the OCP System

Long before its precise function was understood, the crystal structure of OCP^O [6] revealed that it is comprised of two discrete structural domains: an all α -helical N-terminal domain (NTD) unique to cyanobacteria, and a mixed α/β C-terminal domain (CTD) that shares structural similarity to other proteins within the widespread nuclear transport factor 2 (NTF2)-like superfamily known to bind substrates with keto or hydroxyl functionalized 6-carbon rings [6,11]. The two domains are connected by a flexible linker and by a non-covalently bound ketocarotenoid molecule which is embedded within a tunnel-like binding pocket that spans both domains (**Figure 3A**). The OCP can bind different carotenoids, however the presence of a keto group is essential for photoactivity [12-14]. The carbonyl group of ketocarotenoids (e.g. 3'-hydroxyechinenone, echinenone, or canthaxanthin) forms two hydrogen bonds with residues in the CTD (Y201 and W288) that are absolutely conserved [12,15]. These hydrogen bonds are essential for photoactivity; OCP containing zeaxanthin, which lacks the carbonyl group, abolishes photoactivity [16]. The OCP^O consistently crystallizes as a dimer that, based on structural data (e.g. buried surface area, ΔG of association) is proposed to be a physiologically-relevant form, however whether OCP is a monomer or a dimer is controversial [6,11,17,18]. Interestingly, the two carotenoids within the OCP dimer are aligned nearly parallel to one another [6,8], which may have biological significance.

Using controlled proteolysis experiments, it was demonstrated that the OCP's structural modularity extends to function [19]: in the absence of the CTD, the NTD of the OCP retains the carotenoid, is spectrally similar to OCP^R, and constitutively quenches phycobilisome fluorescence in vitro – the hallmark feature of OCP activity. NTDs formed by proteolysis, Red Carotenoid Proteins (RCPs) [19,20], have been isolated from

cyanobacteria [21]. The CTD appears to regulate the fluorescence quenching/photoprotective role of the NTD by priming carotenoid photochemistry in OCP^O and by acting as a substrate for FRP in OCP^R. This structural and functional modularity is reminiscent of other bacterial photoreceptor that are composed of discrete sensor and effector domains [22].

The RCP consists of the NTD with a bound carotenoid [19,20]. Relative to its position in the OCP^O structure, the carotenoid is shifted 12 Å to be enveloped by the NTD. The position of the carotenoid in OCP^R is shown to correspond to that in RCP [18,20] (**Figure 3B**). Although the crystal structure of the OCP^R is not yet available, various biochemical and biophysical methods [15,17,18,23-28], such as small-angle X-ray scattering (SAXS) [18], X-ray hydroxyl radical footprinting [18,23,25], mass spectrometry (MS) [17,25,29], and circular dichroism [18] have suggested that the OCP undergoes a significant global conformational change upon photoactivation, with complete separation of the NTD and CTD [18,23] (**Figure 1A**). Thus, the OCP undergoes a light-driven domain dissociation, predicated upon an “unbolting” of the carotenoid which normally contributes to domain association in the OCP^O form [30].

With the advent of whole genome sequencing, Wu and Krogmann [21] first identified that the *slr1963* gene in *Synechocystis* corresponded to the OCP, and this gene was consistently accompanied by a second open reading frame [8] that is now known to be the FRP [31,32]. The *Synechocystis* FRP crystallized as two forms, a dimer and an elongated tetramer [33] (**Figure 3C**). The dimeric form is the active form which interacts with the CTD of the OCP [33-35]; the tetramer is likely an inactive, potentially storage form of the protein [33].

Mechanism of OCP Quenching

In plants, NPQ occurs at the membrane-embedded light-harvesting chlorophyll antenna complexes (LHCs) [1,2], which can switch from a light-harvesting state to a photoprotective state through protonation-induced conformational changes in LHC proteins with consequent dissipation of surplus excitation energy. An decrease in lumen pH induces a dimer-to-monomer transition of PsbS protein [36,37] or a conformational change associated with altered luminal intermolecular interactions of the PsbS dimer

[38]. This activates the xanthophyll cycle, whereby a chemical interconversion by enzymatic de-epoxidation of violaxanthin occurs under high light conditions and can lead to accumulation of zeaxanthin, which is an effective quencher of excited chlorophyll molecules and also facilitates the aggregation of LHCs, which can subsequently decouple from PSII in plants.

In contrast to pH-triggered NPQ in plants, the OCP-mediated quenching mechanism in cyanobacteria is simpler [39]. Illumination with blue-green light (or strong white light) triggers OCP-mediated quenching at the water-soluble PBS antenna complexes in the cytoplasm. Instead of changing the carotenoid by chemical reactions, as in the xanthophyll cycle of plants, cyanobacteria alter carotenoid properties by shifting its protein environment within the OCP as a result of photoactivation, stabilizing it in a more planar conformation that enhances its effective conjugation length [14]. Concomitant changes in the interaction between the CTD and the NTD include the breakage of a conserved salt bridge – Arg155–Glu244 – at the major interface between the two domains and detachment of the N-terminal extension (NTE) from the CTD at the minor interface, allowing separation of the two domains (**Figure 1A**); the unwinding of the NTE helix [18] is the only detectable change in secondary structure upon photoactivation.

The NTD in the open, elongated OCP^R form attaches to the PBS and brings the carotenoid to the PBS chromophores to induce energy quenching. The OCP^R form is stabilized upon binding to the PBS [39], which may be influenced by interaction between the positively charged Arg-155 accessible on the newly exposed NTD surface and negative surface charges in the PBS core [40].

The PBS binding site where the OCP/RCP docks is a topic of active investigation [17,41,42]. Recently, the basal cylinders of the allophycocyanin (APC) core were proposed to be the site of OCP^R binding [41,43,44]. These APC trimers include multiple APC₆₆₀ species and at least two APC₆₈₀ species, with an APC₆₆₀ bilin suggested to be the primary site of fluorescence quenching according to *in vivo* and *in vitro* picosecond fluorescence measurements [45,46]. Involvement of an APC₆₈₀ bilin has also been proposed as the location of OCP-mediated quenching [47], implicating the terminally-emitting large core-membrane linker, ApcE, also known as L_{CM} [41,42,48]. Blue-light-

inducible NPQ reminiscent of OCP behavior has been observed even in the primitive cyanobacterium *Gloeobacter violaceus* PCC 7421, which has atypical PBS structure and lacks thylakoid membranes [49]. Although the PBS rods and the rod–core linker proteins are different between *G. violaceus* and model OCP organisms like *Synechocystis*, the structure of their PBS cores are very similar, which supports that quenching occurs at or near the APC trimers.

In the mechanism of OCP-mediated NPQ, it is possible that the OCP^R binds in close proximity to an excited α - or β -bilin pigment located in an APC trimer of the PBS core and quenches its excited state directly. An energy transfer [50] or a charge transfer [45] from the bilin pigment to the carotenoid in OCP^R has been proposed [41,45,50-53] for de-exciting the antenna. The light-driven translocation of the carotenoid deeper into the NTD [20] could be essential for these mechanisms since this movement could bring the carotenoid closer to the bilins. More speculatively, the carotenoid may undergo an additional translocation – into the core of the PBS – upon binding [20]. Another possibility is that OCP^R activates quenching indirectly through a structural modification of a core APC cylinder upon binding; such a structural change could induce a conformational rearrangement of the bilin pigment and thereby interfere with its energy propagation [54].

Chemical cross-linking of the OCP^R bound to PBSs revealed several crosslinks to the interdomain linker; only one cross-link identified to the CTD (linking ApcB to CTD residue K249), suggesting that the CTD might not directly interact with the PBS [17,41]. Surprisingly, PBS cross-links were not detected with any NTD residues [41] despite strong evidence that NTD binds PBS on its own [19]; however, strong and specific interaction between the PBS and NTD [40] could have precluded access to the cross-linking agents. When the NTD of OCP^R binds to the PBS core, the CTD may be freely mobile (via the interdomain linker), providing accessibility for the FRP to bind and turn off quenching by accelerating the detachment of OCP^R from the PBS, thereby enabling its conversion back to OCP^O [31,33,39,40]. An ability to switch off the NPQ state would be very important for avoiding the wasteful quenching of excitation energy under low light conditions. As the activity of FRP favors the accumulation of excitation energy in

the antenna – and therefore the “fluorescence recovery” for which it is named – it is expected that the FRP would function transiently, as required by changing environmental conditions. However, it currently remains unknown if FRP is able to sense environmental conditions that may regulate its activity.

Multiplicity of OCPs, FRP and Domain Homologs

Since the first OCP structural description [6] and the earliest sequencing of cyanobacterial genomes, the individual OCP domains were recognized to also be encoded as independent polypeptides [8]. At least nine different clades of homologs to the NTD can now be phylogenomically identified across diverse cyanobacteria (**Figure 4A**), with members of different clades often co-occurring in the same genome [9] (**Figure 2**). These paralogs have been named “Helical Carotenoid Proteins” (HCPs), as they are all predicted to conserve both the all-helical structure as well as the carotenoid-binding pocket of the OCP-NTD [9,10]. C-terminal domain homologs (CTDHs) which are phylogenetically basal to the OCP-CTD sequences (**Figure 4B**) are also found in nearly every genome encoding an HCP, although they do not appear to have duplicate copies and their function remains enigmatic [9].

The expansion of the HCP family into different phylogenetic subtypes (**Figure 4A**) implies that the paralogs may have evolved divergent functions. Expression and characterization of four different subtypes from *Nostoc* PCC 7120 has shown each to be red-colored like the OCP-NTD and RCP, confirming their ability to bind carotenoids (**Figure 4D**) [9,10]. Functionally, HCP2 and HCP3 exhibited strong $^1\text{O}_2$ quenching activity similar to the OCP-NTD, while HCP4 (containing canthaxanthin) was demonstrated to bind PBSs and enable constitutive energy quenching [10]. A specific function has not yet been demonstrated for HCP1, although crystal structures with different carotenoids suggest its ability to bind a variety of different carotenoids, including myxoxanthophyll which contains a terminal bulky glycosyl group [9]. Therefore, it appears that a wider range of carotenoids bound to HCPs might be able to mediate $^1\text{O}_2$ quenching, compared to the more specific binding of ketocarotenoids that is required for inducible antenna quenching by the OCP [6,7]. Furthermore, the binding of HCP1 with myxoxanthophyll led to altered optical properties compared to HCP1 bound

with canthaxanthin (**Figure 4D**). This ability of the HCP/NTD family to bind different types of carotenoids implicates the chromophore as another module which may be varied in order to tune the functional properties of proteins which contain this domain.

In light of the interaction between the NTD and CTD of the OCP, it seems plausible that CTDHs may also interact with some HCPs, forming a heterodimer which would resemble the OCP without its linker (**Figure 4C**). The CTDH gene locus is commonly adjacent to an HCP4 or HCP5 locus [9], which are the clades which branch closest to the primitive OCP-NTD sequences (**Figure 4A**) and include the *Nostoc* 7120 paralog (All4941, an HCP4) which was the only HCP to demonstrate PBS quenching [10]. This evidence suggests that an ancient gene fusion event between an HCP and a CTDH may have occurred, giving rise to an ancestral OCP [9].

At least two additional families of full-length OCP paralogs were also recently identified (**Figure 4A, B**), occurring in diverse cyanobacterial genomes across most phylogenetic sub-clades (Bao et al., manuscript in revision). One of these new families, OCP2, forms a distinct clade that maps between the primitive “OCPX” family and the canonical, well-characterized OCP1 family, suggesting an intermediate evolutionary history. Using *Fremyella diplosiphon*, a model chromatic-adapting cyanobacterium which contains both OCP1 and OCP2, the properties of these paralogs were compared. The OCP2 was able to quench PBS, however with substantially different properties relative to OCP1; OCP2 showed faster photodynamics, inability to stably accumulate in the active OCP^R form at room temperature, absence of dimerization, and lack of FRP interaction. Relative to OCP2, OCP1 has multiple levels of fine regulatory control, enabling flexible access to a wider range of quenching activity, and is thus more evolutionarily refined than the newly identified OCP2 and OCPX. This is consistent with the domain fusion hypothesis and justifies the prevalence of OCP1 among the majority of cyanobacteria.

Conclusions and Prospects

Like many photoreceptors, the OCP is structurally and functionally modular. The two structural domains of the OCP and the noncovalently-bound carotenoid each contribute distinct functions which are regulated based on a reversible cycle of carotenoid photoconversion/translocation, light-driven domain dissociation, and inactivation by domain re-association, which may be further enhanced by interaction with the FRP. The widespread presence of domain homologs encoded as separate polypeptides supports their independent origins as HCPs and CTDHs which were likely involved in an ancient domain fusion event. The phylogenetic diversification of HCPs and of full-length OCPs further supports the functional modularity of OCP structural components; the emerging evidence of divergent functional properties among paralogs or while binding different carotenoids presents an opportunity to examine how structure-function relationships can evolve in pigment-binding proteins, with particular relevance to the early evolution of oxygenic photosynthesis and the concomitant need for photoprotective mechanisms.

Understanding of the structural features which influence properties such as the strength of domain association, tuning of optical properties, photoconversion dynamics, and interactions with FRP or PBS will enable OCP-based strategies to become part of the synthetic biology toolkit, which relies on modular components that can be swapped interchangeably. Modular photoreceptor components have already revolutionized the field of optogenetics [55,56].

Furthermore, advances in understanding how the protein environment can govern properties such as carotenoid selectivity and singlet oxygen quenching could enable the engineering of “smart” photoprotection systems – in plants and in cyanobacteria – in a manner comparable to how photosynthetic productivity was improved in plants by acceleration of recovery from photoprotection, thereby avoiding competition between photoprotection and photochemistry [57]. Engineering photoprotection in cyanobacteria could be a means of improving their performance as microbial cell factories. The regulation of OCP has been identified as one target to engineer increased performance and/or efficiency of production strains [58]. At the same time, a comprehensive

understanding of the natural distribution of OCPs/HCPs/CTDHs in relation to environmental metadata is expected to provide fundamental information on how cyanobacteria adapt to occupy a wide range of ecophysiological niches [4].

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Figure 1. Model of the OCP photocycle and the OCP/FRP-related photoprotective mechanism. **A)** Mechanism for OCP photoregulation: light absorption by OCP^O induces conformational changes in the carotenoid and domain interfaces, allowing the carotenoid translocation entirely into the NTE, accompanied by complete dissociation of the two domains. Photoactivated OCP^R reverts to inactive OCP^O in darkness and can be accelerated by the interaction between the FRP and CTD. **B)** OCP^R interacts with the PBS through the APC trimer of the core to mediate excess energy dissipation as heat. The FRP accelerates the detachment of OCP^R from the PBS.

Figure 2. Phylogenetic tree of sequenced cyanobacterial genomes with distribution of OCP-related genes. Presence of genes encoding the OCP, N- and C-terminal domain homologs (HCPs and CTDHs), and the FRP within genomes, denoted as colored shapes, was mapped on a species phylogeny of all Cyanobacteria based on the core housekeeping protein RpoC1 (Bao et al., manuscript submitted). Closely related taxa with the same inventory of OCP-related genes were collapsed, as indicated by the branch names. The five morphological subsections are indicated by the branch color: green (I, unicellular), brown (II, baeocystous), blue (III, filamentous), purple (IV,

heterocystous) and red (V, ramified).

Figure 3. Crystal structures of the OCP^O, RCP and FRP. **A)** OCP^O dimer: NTD colored in yellow and CTD colored in blue; the linker colored in pink; the carotenoid (Car) is shown as orange sticks. **B)** Comparison of the RCP and OCP^O structures: RCP (red) aligned with NTD of OCP^O (yellow); the carotenoids in the RCP and OCP^O are shown by red and orange sticks, respectively, to illustrate the translocation of the carotenoid upon conversion from OCP^O to OCP^R. **C)** Two conformations and oligomeric states of the FRP: the active dimeric form shown on the left, and tetrameric form shown on the right. Figure prepared with Pymol (www.pymol.org) using PDB code 4XB5 for OCP, 4XB4 for RCP and 4JDX for FRP.

Figure 4. Diversity of domain homologs of the OCP: HCPs and CTDHs. **A)** ML phylogeny of HCPs and the OCP NTD. **B)** NJ phylogeny of the OCP-CTD and NTF2 family. **C)** The HCPs and CTDH are homologous to each structural and functional domain of the OCP. **D)** Distinctive differences among the visible properties of carotenoproteins. From left to right: *Nostoc* HCP1 with myxothanthophyll, *Nostoc* HCP1 with canthaxanthin (CAN), the N-terminal domain of the *Synechocystis* OCP with CAN, *Nostoc* OCP with CAN, and free CAN.

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