Summary

The orange carotenoid protein (OCP) is a water-soluble, photoactive protein involved in thermal dissipation of excess energy absorbed by the light-harvesting phycobilisomes (PBS) in cyanobacteria. The OCP is structurally and functionally modular, consisting of a sensor domain, an effector domain and a keto-carotenoid. On photoactivation, the OCP converts from a stable orange form, OCPO, to a red form, OCP R. Activation is accompanied by a translocation of the carotenoid deeper into the effector domain. The increasing availability of cyanobacterial genomes has enabled the identification of new OCP families (OCP1, OCP2, OCPX). The fluorescence recovery protein (FRP) detaches OCP1 from the PBS core, accelerating its back-conversion to OCPO; by contrast, other OCP families are not regulated by FRP. N-terminal domain homologs, the helical carotenoid proteins (HCPs), have been found among diverse cyanobacteria, occurring as multiple paralogous groups, with two representatives exhibiting strong singlet oxygen (\(^1\text{O}_2\)) quenching (HCP2, HCP3) and another capable of dissipating PBS excitation (HCP4). Crystal structures are presently available for OCP1 and HCP1, and models of other HCP subtypes can be readily produced as a result of strong sequence conservation, providing new insights into the determinants of carotenoid binding and \(^1\text{O}_2\) quenching.

I. Introduction

Amongst the simplest of pigments, carotenoids are unbranched polyisoprenoid chains with conjugated double bond systems that may be linear or extend into terminal ring structures. Given their length (typically C40) and hydrophobicity, they are thought to have arisen in the Archaebacteria as a structural reinforcement for the first cell membranes (Vershinin, 1999), and are now one of the most widespread and versatile lipid-soluble pigments in nature (Esteban et al., 2015). The functional repertoire of carotenoids is greatly expanded through their ability to be bound in specific
conformations within proteins (Britton & Helliwell, 2008). Carotenoproteins can provide structural support, anchor membrane proteins or produce ecologically relevant colorations. The binding of carotenoids within proteins also allows them to be held in defined orientations proximal to other chromophores in order to function as accessory pigments in photosynthetic complexes, extending the spectral range of light-harvesting antennae by absorbing photons not captured by chlorophylls (Züllig, 1989; Hickman & Schweger, 1991, 1996). Conversely, carotenoproteins also confer photoprotective functions by absorbing and/or dissipating excess absorbed light energy or by ‘quenching’ reactive oxygen species (ROS) (Steiger & Pating, 1991; Zhu et al., 2010). Carotenoproteins also play critical roles in protecting the photosynthetic machinery from high light stress (D’Haene et al., 2008; Daddy et al., 2015; Dilbeck et al., 2016; Niedzwiedzki et al., 2016). The structure and function of many of these carotenoproteins still requires elucidation, and many others are likely still to be identified, as there is no predictive method for recognizing carotenoid-binding pockets in proteins at the sequence level.

On the other hand, much has been learned over the past decade about a group of water-soluble cyanobacterial carotenoproteins exemplified by the orange carotenoid protein (OCP; Kerfeld, 2004a,b; Kirilovsky & Kerfeld, 2016). The OCP governs photoprotective responses in cyanobacteria, including thermal dissipation of excess light energy captured by the light-harvesting antenna, as well as disarming ROS, which are inherently produced during oxygenic photosynthesis (Hirayama et al., 1994; Edge et al., 1997; Vershinin, 1999). In the last 3 years, the increased availability of sequenced cyanobacterial genomes has enabled the identification of new families of OCP, as well as multiple groups of proteins homologous to its two constituent structural domains (Melnicki et al., 2016; Bao et al., 2017). Accumulating evidence has suggested that the OCP has evolved from the fusion of two primitive carotenoid-binding proteins (Lechno-Yossef et al., 2017; Moldenhauer et al., 2017). Several families of domain homologs have been shown recently to bind carotenoids on their own, with some demonstrating flexibility for carotenoid specificity (Lopez-Igual et al., 2016; Melnicki et al., 2016). Investigations of the functional properties of these evolutionarily diverse OCP homologs have recently begun. This review focuses on new structural, functional and regulatory findings, as well as structural bioinformatics for some of the most interesting subtypes, suggesting a much wider role than previously thought for these carotenoproteins.

II. The structure of the OCP

The OCP was discovered by David Krogmann and colleagues in soluble cyanobacterial crude extracts; it was purified from three different cyanobacterial species – *Arthrobacteria, Aphanizomenon* and *Microcystis* (Holt & Krogmann, 1981), providing the first description of a water-soluble carotenoprotein in cyanobacteria. Using the first available cyanobacterial genome sequences, the Krogmann group was able to identify the OCP as the 34-kDa gene product of the slr1963 locus in *Synechocystis* sp. PCC 6803 (hereafter Syn6803) (Wu & Krogmann, 1997). The first crystal structure of the OCP, purified from *Arthrobacteria maxima* (hereafter *A. maxima*), showed that the protein comprised two discrete structural domains: an all-helical N-terminal domain (NTD), composed of two discontinuous four-helix bundles, and a C-terminal domain (CTD) with a mixed α/β-fold (Kerfeld et al., 2003) (Fig. 1a). The NTD (Pfam09150) is found only in cyanobacteria, whereas the CTD is a member of the ubiquitous Nuclear Transport Factor-2 (NTF2) superfamily (Pfam02136) (Kerfeld et al., 2003; Wilson et al., 2010). The carotenoid spans the two domains and is only sparingly solvent accessible, with typically c. 96% of its surface area shielded by the protein. The OCP has been shown to bind various keto-carotenoids when purified from cyanobacteria (3’-hydroxyl-echinone, 3hECN; echinone, ECN; and canthaxanthin, CAN) (Punginelli et al., 2009). The keto group is essential for function; it is hydrogen bonded to the CTD via the conserved residues Y201 and W298 (Kerfeld et al., 2003; Wilson et al., 2010) (Fig. 1a; Y203 and W290, numbering corresponds to that of All3149).

A linker of 25 amino acids connects the NTD and CTD; it has been shown recently that this linker region is not essential for association between the two domains of the OCP (Lechno-Yossef et al., 2017; Moldenhauer et al., 2017; Sluchanko et al., 2017). The N-terminal x-helix of the polypeptide chain hydrogen bonds to the CTD, providing the minor interface between the two domains. The second major interface between the two domains is stabilized by a salt-bridge between the highly conserved residues R155 (NTD) and E244 (CTD) (Fig. 1a; R155 and E246, numbering corresponds to that of All3149) (Kerfeld et al., 2003; Boulay et al., 2010). It is now known that the carotenoid is essential for domain association (Lechno-Yossef et al., 2017; Moldenhauer et al., 2017), and serves as a ‘bolt’ which slides into the NTD to permit domain dissociation (see later) (Cogdell & Gardiner, 2015).

The two structural domains of the OCP were identified as discrete gene products in some of the first cyanobacterial genomes sequenced (Kerfeld et al., 2003; Kerfeld, 2004a,b) (see Section IV, later). These shorter variants were initially thought to be related to another observation by Krogmann and colleagues; they had purified a smaller (16 kDa) red carotenoid protein (RCP) from various cyanobacteria (Holt & Krogmann, 1981; Wu & Krogmann, 1997). However, N-terminal amino acid sequencing indicated that the RCP was actually a proteolytic fragment of the OCP, retaining the majority of the NTD as well as the carotenoid, but lacking the entire CTD (Wu & Krogmann, 1997). Early efforts to understand the function of these water-soluble carotenoproteins showed that both the OCP and the RCP quench singlet oxygen (1O₂), with the 16-kDa RCP exhibiting a significantly faster rate than the OCP, consistent with greater carotenoid accessibility (Kerfeld et al., 2003). It was also shown that the full-length OCP could mimic the RCP spectrally on acidification *in vitro* (Kerfeld et al., 2003; Kerfeld, 2004a,b) or repeated freeze–thaw cycles (Holt & Krogmann, 1981). These observations prompted the suggestion that the OCP was involved in photoprotection (Kerfeld et al., 2003), which was ultimately demonstrated by Kirilovsky and colleagues in 2008 (Wilson et al., 2008).
III. OCP function

1. OCP photoactivation

Excess light absorbed by pigments in the photosynthetic apparatus of plants, algae and cyanobacteria can drive the formation of chlorophyll triplet states, leading to the accumulation of $^{1}O_2$ radicals and other ROS. As ROS can ultimately lead to oxidative damage, all oxygenic photosynthetic organisms must have photoprotective mechanisms in order to withstand the hazards associated with autotrophy driven by photocatalytic water oxidation. In plants, a nonphotochemical quenching (NPQ) mechanism depends on carotenoid interconversion, triggered by thylakoid lumen acidification under high irradiance or other stresses (Horton et al., 1996). Cyanobacteria use a distinctively different NPQ mechanism to dissipate excess light energy from the water-soluble phycobilisome (PBS) antenna (Manodori & Melis, 1986). It too involves carotenoids (Rakhimberdieva et al., 2004), but is activated instead by strong light (Campbell et al., 1998). The OCP, found in the majority of cyanobacteria, is now known to be responsible for this cyanobacterial NPQ process (Wilson et al., 2006).

On absorption of strong blue–green light, the OCP converts from a dark, stable orange form, OCP<sup>1°</sup>, to a light-activated red form, OCP<sup>R</sup> (Wilson et al., 2008). Photoactivated OCP<sup>R</sup> directly participates in photoprotection by binding to the PBS and facilitating the dissipation of excess captured energy (Gwizdala et al., 2011) (Fig. 2a). This light-inducible process manifests as quenching of fluorescence (Wilson et al., 2006; Rakhimberdieva et al., 2007, 2010; Gorbunov et al., 2011). Only the OCP<sup>R</sup> form is able to induce this fluorescence and excitation energy quenching (Wilson et al., 2008; Puginelli et al., 2009; Gwizdala et al., 2011).

On photoactivation, the two domains of the OCP fully dissociate (Gupta et al., 2015; Liu et al., 2016), resulting in a form that is more elongated and less hydrophobic than the OCP<sup>1°</sup> form.
(Maksimov et al., 2016). As a prerequisite, the carotenoid moves from its position spanning the two domains of the OCP\(^{\alpha}\) (Fig. 1a), burrowing 12 Å deeper into an extension of the carotenoid-binding tunnel which resides completely within the NTD (Leverenz et al., 2015). The overall structures of each individual domain do not fundamentally change on photoactivation, with the exception of unfolding of the \(\alpha\)-helix (the N-terminal extension, NTE) (Gupta et al., 2015), which clasps the CTD in the minor interface of the OCP\(^{\alpha}\) (Fig. 1a).

The conserved K268 in the minor interface has been observed to be post-translationally modified by acetylation (Mo et al., 2015); this modification is increasingly being regarded to be as important as phosphorylation in the regulation of protein function (Hu et al., 2010). Constitutive disruption of the minor interface would presumably poise the OCP\(^{\alpha}\) form towards photoactivation. Several other sites for post-translational modifications of the OCP could also play a role in regulating the balance between the OCP\(^{\alpha}\) and OCP\(^{\beta}\) states. For example, there are two phosphorylation sites in the linker that joins the NTD and CTD (T182 and S184) (Angeleri et al., 2016) (Fig. 2f). Likewise, C245 in the CTD (Fig. 2f) (Anabaena PCC 7120 numbering C247; Chardonnet et al., 2015) can be glutathionylated. This modification presumably occurs only...
in the OCP\textsuperscript{R} form because separation of the NTD and CTD would be required to expose this residue (Fig. 1a). Interestingly, the modification presumably locks the OCP in the activated red state by producing the two domains from reassocation.

Photoactivation kinetics for the OCP\textsuperscript{O} to OCP\textsuperscript{R} conversion are much slower than typical conformational changes in proteins, evoking a comparison with proline isomerization (Gorbunov et al., 2011). The kinetics have been modeled with at least three phases: with an initial femtosecond-scale photoconversion of the carotenoid’s excited S\textsubscript{2} state to an optically forbidden S\textsubscript{1} state, progressing through an intramolecular charge transfer (ICT) state at the picosecond scale (Wilson et al., 2008), and reaching the OCP\textsuperscript{R} quenching state via a slower light-independent step (Gorbunov et al., 2011). Non-Arrhenius behavior was observed for the quenching rate, which suggested that carotenoid-absorbed photons trigger a process that resembles soluble protein folding (Rakhimberdieva et al., 2007). Furthermore, the activation energy calculated for the OCP\textsuperscript{R} to OCP\textsuperscript{O} conversion rate was noted to be comparable with the effective enthalpy of transition during protein unfolding, suggesting that, during photoactivation, the OCP undergoes significant rearrangements comparable with protein unfolding (Maksimov et al., 2016). Recently, labeling of a cysteine on the CTD with a fluorescent probe, tetramethylrhodamine-maleimide (TMR), has demonstrated that photoactivation causes an 18\text{Å} separation of the CTD from the carotenoid, occurring in concert with the 12\text{Å} translocation of the carotenoid, confirming an increase in the hydrodynamic radius of the OCP\textsuperscript{R} form (Gupta et al., 2015; Maksimov et al., 2017).

The CTD of the OCP has been proposed to confer and tune a light sensor function to the carotenoid of the OCP (Kirillovsky & Kerfeld, 2013), largely as a result of its participation in forming the inactive OCP\textsuperscript{O} ground state, as well as its importance in governing photoactivation. The hydrogen bonding to the keto-carotenoid is essential for photoactivation (Punginelli et al., 2009; Wilson et al., 2011). Recently, it has been demonstrated that the isolated CTD is capable of ‘turning off’ an otherwise constitutively active NTD, driving the carotenoid back into the OCP\textsuperscript{O}-like configuration in an NTD–CTD heterodimer (Lechno-Yossef et al., 2017; Moldenhauer et al., 2017).

2. Regulation by the fluorescence recovery protein

The CTD also provides the regulation of OCP-mediated photoprotection through its interaction with the fluorescence recovery protein (FRP). Spontaneously, the OCP\textsuperscript{R} form is metastable and slowly back-converts to the OCP\textsuperscript{O} ground state in the dark at a temperature-dependent manner (Wilson et al., 2008). The 14-kDa FRP, which was first identified as an open reading frame consistently proximal to the OCP gene (Kerfeld, 2004a), essentially is capable of ‘shutting off’ or circumventing OCP’s quenching function. There was almost no observable PBS fluorescence quenching when FRP was overexpressed under high irradiance (Gwizdala et al., 2013; Sluchanko et al., 2017). The FRP is suggested to have two distinct activities: acceleration of the detachment of the OCP from a PBS complex and subsequent deactivation of the OCP (Thurotte et al., 2017). Recently, evidence for FRP binding to the OCP\textsuperscript{O} form was observed, causing the OCP\textsuperscript{R}-like absorption spectrum of a constitutively active W288A OCP mutant to become more ‘orange-like’, potentially implicating secondary interactions with the NTD (Sluchanko et al., 2017).

The crystal structure of the FRP (Sutter et al., 2013) revealed two different conformations, as well as oligomeric states, within the crystal asymmetric unit: a tetrameric form with an extended \(\alpha\)-helix conformation and small helical cap at each end, and a dimeric form with a more compact fold, with each individual monomer hinged back on itself through the breaking of the longest helix (Fig. 1d). The dimer (Sutter et al., 2013; Bao et al., 2017) forms the active site, a region spanned by residues 50 and 61 with strong sequence conservation. The tetramer was proposed to represent an inactive form, as the putative active site is abolished in this conformation; such conformational inactivation could provide the regulation of FRP activity through an as-yet unknown mechanism.

Based on structural properties (e.g. surface area buried; Sutter et al., 2013), the tetrameric FRP has been proposed to be physiologically relevant, although this is disputed by biochemical characterizations (Sluchanko et al., 2017). However, native mass spectrometry studies observed a higher order oligomerization state that is concentration dependent (Lu et al., 2017). It has been observed recently that FRP interaction with OCP occurs with an apparent stoichiometry of 1 : 1, suggesting that the active form may be a monomer (Sluchanko et al., 2017), and there is also a report of FRP acting as a trimer (Boulay et al., 2010). Computational docking simulations positioned the bulged end of FRP dimers (and theoretical monomers) inside the ‘central cavity’ that is hypothesized to form in OCP\textsuperscript{R} monomers as a result of the dissociation of the NTD–CTD major interface (Fig. 1a) on photoactivation, although no such structures have been determined to date (Zlenko et al., 2015). Thus, many details of the molecular mechanism of FRP-mediated recovery remain to be elucidated. Nevertheless, this theoretical FRP–OCP interaction model is appealing because it proposes that this ‘central cavity’ of the OCP is the same docking surface for PBS attachment, suggesting that FRP competition for this binding site may play a role in the fluorescence recovery process (Stadnickuk et al., 2015; Zlenko et al., 2015; Thurotte et al., 2017).

3. OCP interaction with PBS

Once the OCP\textsuperscript{R} is formed, it can interact with PBS even in darkness (Wilson et al., 2006; Gwizdala et al., 2011; Tian et al., 2011, 2012, 2013; Maksimov et al., 2014). One OCP\textsuperscript{R} monomer is sufficient to quench the fluorescence of a PBS (Gwizdala et al., 2011). The OCP\textsuperscript{R}–PBS complex is stable both in vivo and in vitro, in darkness as well as under low light. OCP-mediated quenching of PBS excitation has been demonstrated in vitro using PBS from Syn\textsubscript{6803} (Gwizdala et al., 2011), Anabaena PCC 7120 (Lopez-Igual et al., 2016) and Fremyella diplosiphon (Bao et al., 2017). As the PBS quaternary structure also varies across different cyanobacterial species (Watanabe & Ikeuchi, 2013), it is unclear how quenching by the strongly conserved OCP accommodates for regulatory and evolutionary changes which alter PBS architecture, suggesting either some versatility in how the OCP binds PBS or that the OCP...
must bind specifically to PBS components which are universally conserved across all PBS architectures. Nevertheless, it is generally accepted that the OCP$^1$ form binds to some region of the allophycocyanin (APC) core of the PBS as the primary site of energy and fluorescence quenching (Wilson et al., 2006; Gwizdala et al., 2011; Tian et al., 2011, 2012, 2013; Jallet et al., 2014; Maksimov et al., 2014; Zhang et al., 2014; Harris et al., 2016). The possibility that binding to the antenna elicits further translocation of the carotenoid of the OCP$^1$ into the PBS (Leverenz et al., 2015) should also be considered in modeling the site and mechanism of fluorescence and energy transfer.

The APC core comprises three or more cylinders, each built from stacks of four trimeric APC rings, as well as several linker proteins, and contains c. 70 bilin chromophores per PBS core. As full quenching of a PBS unit can be accomplished by a single OCP (Gwizdala et al., 2011), this suggests that there should be a single binding site close to a bilin, at which all excitation energy converges, to which all chromophores can transfer their excitation energy. The ApcE linker protein is an obvious candidate for such an OCP quenching site as it contains the most red-shifted terminal emitter bilin, which mediates excitation transfer to the reaction centers in the thylakoid membrane, and is present in only two copies per PBS. The ApcE linker, also known as the large chromophorylated core-membrane linker protein (L$_{CM}$), anchors the PBS core to the thylakoid membrane, and is also an appealing OCP-binding target because its bilin-binding pocket is uniquely shifted from the otherwise strictly conserved C81 bilin site of all other ApcA and ApcB homologous subunits inside the APC core, thus providing a novel and specific surface to accommodate OCP binding (Zlenko et al., 2015). However, mutation of the shifted cysteine site to serine in ApcE – as PBS assembly is abolished in ΔApcE deletion mutants – had no effect on PBS quenching (Jallet et al., 2012); by contrast, deletion of just the chromophore-containing domain of ApcE permitted PBS assembly, but abolished OCP-related quenching as well as PBS excitation transfer to photosystem II (PSII) (Elanskaya et al., 2016).

Although two other chromophorylated core linkers, ApcD and ApcF, have been alternatively proposed as possible OCP-binding sites, deletion of these core linker genes also failed to have an effect on OCP-related quenching (Jallet et al., 2012). Other evidence supports the conclusion that ApcD and ApcF proteins are not important for the induction of OCP-related NPQ (Dong et al., 2009; Jallet et al., 2012; Stadnichuk et al., 2012; Maksimov et al., 2015). Both wild-type (WT) and ApcD mutants showed similarly slower fluorescence recovery rates than ApcF or ApcDF mutants, further arguing against these other linker proteins playing any specific role in OCP-related quenching (Acuña et al., 2016). Nevertheless, spectrally resolved picosecond fluorescence measurements using WT and mutant cells of Synechocystis WH8102 & Synecochoccus BL107 (Varkey et al., 2015). These latter observations in two marine Synechococcus species are notable, because they are globally widespread members of the alpha-cyanobacteria (subclade C1) that are parental to Prochlorococcus strains which all lack the OCP (perhaps because they employ a chlorophyll $a$ type of light-harvesting antenna and lack PBS), providing some insight into OCP evolution.

As a secondary protective function, the OCP is able to quench $^1$O$_2$ (Kerfeld et al., 2003; Sedoud et al., 2014; Lopez-Igual et al., 2016). This quenching activity is retained even in OCP mutants which prevent photoactivation and/or bind non-keto-carotenoids of the terminally emitting bilins) could also be primarily quenched in addition to APC660 (Kuzminov et al., 2012).

Using in vitro and in vivo evidence, Stadnichuk et al. (2012, 2013) have suggested that ApcE could be the primary site of fluorescence quenching. Furthermore, ApcE contributed one of the three crosslinked pairs pulled down by OCP in a study using bifunctional lysine-specific crosslinking reagents (the other two PBS adducts were derived from different regions of ApcB) (Zhang et al., 2014). Similarly, ApcE was one of the seven PBS components which contributed OCP adducts in a similar study using glutaraldehyde as the crosslinking agent (the others being ApcA, ApcB, ApcC, ApcD, CpcG and CpcB) (Harris et al., 2016). In both studies, PBS adducts were mapped to various regions of the CTD, the flexible interdomain linker as well as the NTE helix, but there was a noticeable lack of crosslinks to any parts of the NTD (Fig. 2b), which has been convincingly demonstrated to be necessary and sufficient for binding and quenching PBS (Wilson et al., 2012; Leverenz et al., 2014). An explanation for the lack of NTD crosslinking could be supplied by sufficiently strong NTD–PBS binding, which blocks access to the crosslinking agent, reminiscent of protection in X-ray footprinting (Gupta et al., 2016).

4. The OCP and subcellular stress: singlet O$_2$ quenching

The OCP appears to be constitutively expressed in cells at relatively low concentrations (approximately one OCP per three to four PBS) (Gwizdala et al., 2011). However, under high light, OCP expression can be strongly upregulated (Hihara et al., 2001; Muramatsu & Hihara, 2012), causing a higher OCP to PBS ratio and leading to the larger amplitude of fluorescence and energy quenching observed under different stress conditions. In addition to high light, expression of the OCP can also be upregulated under various stress conditions which exacerbate photodamage and/or prolong the excited state of antenna chromophores. For example, cold acclimation (Rowland et al., 2011), salt stress (Fulda et al., 2006), low culture density (Zhang & Bryant, 2015), heat shock (Zhang & Bryant, 2015), nitrogen starvation (Flaherty et al., 2011; Sadler et al., 2016), CO$_2$ limitation (Sadler et al., 2014), oxidative stress (Shrivastava et al., 2016) and cadmium exposure (Chen et al., 2014) were also associated with elevated OCP expression levels.

Expression of OCP orthologs has also been observed in global expression datasets of other cyanobacterial species, such as NodulariaCCY9414 (Voss et al., 2013), Anabaena variabilis (Park et al., 2013), Anabaena 7120 (Shrivastava et al., 2016), Synechococcus WH8102 and Synechococcus BL107 (Varkey et al., 2015). These latter observations in two marine Synechococcus species are notable, because they are globally widespread members of the alpha-cyanobacteria (subclade C1) that are parental to Prochlorococcus strains which all lack the OCP (perhaps because they employ a chlorophyll $a$ type of light-harvesting antenna and lack PBS), providing some insight into OCP evolution.
(such as zeaxanthin) (Sedoud et al., 2014). Furthermore, the OCP can suppress the cellular accumulation of ¹O₂ even under conditions in which the protein cannot be photoactivated, such as strong monochromatic orange–red light (Sedoud et al., 2014). Quenching of ¹O₂ is substantial when catalyzed by either the OCPóst or OCPóst form, as both showed activity largely exceeding that of smaller ¹O₂ quenchers, such as ascorbate, histidine or trolox (a soluble carotenoid derivative) (Sedoud et al., 2014), and in the same range as other antioxidant proteins, such as superoxide dismutase (Kerfeld et al., 2003). The ¹O₂ quenching ability of OCP is avid enough to render cyanobacterial cells more resistant to photoinhibition and oxidative stress (Sedoud et al., 2014). The mechanism of ¹O₂ quenching remains unclear, although there is preliminary evidence for either a physical mechanism of energy transfer or indirect mechanism through oxidation of the carotenoid (Edge et al., 1997; Sedoud et al., 2014).

As noted earlier (see Section II), a shorter version of the OCP containing only the NTD can be purified as a 16-kDa proteolytic fragment, known as the RCP (Holt & Krogmann, 1981; Wu & Krogmann, 1997; Chábera et al., 2011), which exhibits a faster rate of ¹O₂ quenching than the full-length OCP (Kerfeld et al., 2003). This NTD-only form was artificially reproduced through intentional proteolysis of A. maxima OCP (Leverenz et al., 2014) or by synthetic truncation of the OCP sequence from Syn6803, expressing only the NTD (Leverenz et al., 2015). Both of these RCP-like preparations resemble the OCPóst form spectroscopically, but are permanently red and thus exhibit no photoactivity. Furthermore, they were both able to bind PBS and demonstrated constitutive quenching of fluorescence in both light and darkness, indicating that the NTD is not involved in light sensing. We suggest that the 16-kDa RCP fragments isolated from various cyanobacteria (Holt & Krogmann, 1981) may not be artifacts, but rather may be physiologically relevant forms. It is possible that, under environmental conditions which present traumatic stresses that may seriously threaten a cell’s ability to survive, the OCP may be proteolytically remodeled into constitutively active RCP variants that would permit survival long enough for cell division. Such a mechanism would be especially relevant for cold temperature stress, where the light may not be strong enough to cause photoactivation. Relevant to this hypothesis, the constitutively active W288A mutant of Syn6803 OCP required a much lower ratio of OCP to PBS in order to saturate the rate of PBS quenching, indicating that there could indeed be a physiological advantage of having faster PBS quenching rates as a result of circumventing the regulatory photoactivation function (Sluchanko et al., 2017).

IV. Distribution of OCPs, FRP and domains in cyanobacteria

The OCP is now known to be structurally and functionally modular, consisting of an effector domain (the NTD or RCP), a sensor domain (CTD) and a carotenoid. The more than doubling of the number of available cyanobacterial genomes in the last 3 years has enabled the identification of new families of OCP and homologs to its constituent domains (Melnicki et al., 2016; Bao et al., 2017). Phylogenomic analysis of 333 sequenced cyanobacterial genomes has revealed that there are at least three paralogous families of OCP (Bao et al., 2017). Amongst these phylogenetically distinct groups, the well-characterized SLr1963 protein from Syn6803 (Kerfeld et al., 2003; Kerfeld, 2004a; Gupta et al., 2015; Leverenz et al., 2015; Kirilovsky & Kerfeld, 2016) is the most widespread and is referred to as OCP1. Members of this family are found in almost every phylogenetic subclade of the cyanobacteria, and always co-occurs with the FRP (113 genomes), which is nearly universally encoded one or two loci away from an OCP1 gene (Fig. 3).

The OCP2 is a newly identified clade paralogous to OCP1. It has been identified in 18 different taxonomically well-distributed cyanobacterial genomes (Bao et al., 2017) (Fig. 3). These OCP2-containing genomes never contain a gene encoding FRP unless an OCP1 gene is also present (10 genomes). A potential third clade of OCPs is phylogenetically discernible, branching furthest from the canonical OCP1 family, although its boundaries cannot yet be clearly defined (Bao et al., 2017). This third family, provisionally named ‘OCPX’, rarely co-occurs with OCP1 and OCP2, and appears to be ancestrally derived. OCPX is more frequently observed than OCP2 (identified to date in 35 genomes). The OCPX-containing organisms are likewise widely distributed across the cyanobacterial tree, but are highly represented amongst the filamentous heterocystous cyanobacteria of phylogenetic subclade B1 (Shih et al., 2013) (morphological subsections IV/V; Rippka et al., 1979).

The first functional characterization of an OCP2, from F. diplosiphon PCC 7601 (UTEX 481), revealed that, relative to Fremyella OCP1, OCP2 exhibits faster photoconversion, as well as the inability to stably accumulate as the active OCPóst form at room temperature. OCP2 was able to quench PBS, but the degree of fluorescence quenching was lower than that of Fremyella OCP1 under identical assay conditions, possibly as a result of the faster back-conversion kinetics. In contrast with OCP1, OCP2 activity does not appear to be regulated by the Fremyella FRP. The oligomeric state of the Fremyella OCPs by native polyacrylamide gel electrophoresis and dynamic light scattering analysis indicates that OCP2 exists as a monomer, unlike OCP1, which was confirmed to exist as a dimer.

The oligomeric state of OCP1 has been the subject of dispute, with OCP1 of A. maxima characterized as a dimer in solution. All of the OCPóst crystal structures (A. maxima, Syn6803 and Anabaena PCC 7120) (Kerfeld et al., 2003; Wilson et al., 2010; Leverenz et al., 2015; Lopez-Igual et al., 2016) contain dimers in the asymmetric unit, which, based on structural properties, are predicted to be physiologically relevant. By contrast, the solution state of the OCP1 of Syn6803 has been described as a monomer (Wilson et al., 2010; Gupta et al., 2015). The oligomeric state is likely to be functionally relevant; in order to photoactivate and bind to the PBS, OCP is presumably a monomer. If the OCPóst is a dimer, this suggests that the first step in activation of the OCP would necessarily be dimer dissociation; the dimer to monomer transition could conceivably serve as a regulatory step in the activation of the OCP. A native mass spectrometric analysis of the oligomerization states of Syn6803 OCP1 demonstrated that the monomeric OCP dominates at low protein concentrations, with an appreciable population of dimeric OCP (Lu et al., 2017).
suggest that the OCP1 can be either a dimer or monomer, and that a change in oligomeric state is a factor in the regulation of OCP1 activity.

The distinctive properties of OCP1 and OCP2 suggest that they may have different physiological roles. For example, although OCP2 transcript levels were undetectable in transcriptome analyses of WT *Fremyella* grown under low light, OCP2 expression was detectable (and higher than that of OCP1) by real-time polymerase chain reaction under increased irradiance (Bao et al., 2017). Furthermore, OCP2 transcripts are highly expressed in an *rcaE*

![Fig. 3 Distribution of genes encoding the orange carotenoid proteins (OCPs), helical carotenoid proteins (HCPs), C-terminal domain homologs (CTDHs) and the fluorescence recovery protein (FRP) in available sequenced cyanobacterial genomes. Data compiled from cyanobacterial genomes from all morphological subsections: subsection I, Chroococcales; subsection II, Pleurocapsales; subsection III, Oscillatoriales; subsection IV, Nostocales; and subsection V, Stigonomatales (Shih et al., 2013). The x-axis represents the number of genes; the y-axis shows the names of the cyanobacteria in phylogenetic order.](image-url)
regulatory mutant of *Fremyella* (lacking the photoreceptor required to perform complimentary chromatic acclimation and so unable to respond to light quality), suggesting a specific physiological role for the OCP2. Under most of these conditions, OCP1 expression levels did not change appreciably, implying that low levels of constitutive expression might be required to prepare cells for photoprotection by OCP1 under fluctuating conditions. This is consistent with its activity being regulated post-transcriptionally and/or post-translationally, through FRP interaction and/or a change in oligomeric state. By contrast, OCP2 function appears to have fewer potential attenuations, and so perhaps is more likely to be important under conditions such as prolonged exposure to environmental stress, regulated solely at the transcriptional level. Collectively, these data suggest that the properties of the OCP2 may be closer to those of a primitive OCP, retaining functional attributes related to its early evolution.

The first OCP crystal structure determination coincided with the earliest sequencing of cyanobacterial genomes. It was immediately noted that the two structural domains of the OCP, the NTD and CTD, each had homologs to individual genes in the majority of OCP-encoding genomes (Kerfeld *et al.*, 2003; Kerfeld, 2004a,b). Since then, at least nine different clades of NTD homologs have been identified across diverse cyanobacteria. These paralogs have been named helical carotenoid proteins (HCPs), as they are all predicted to conserve both the all-helical fold of the NTD as well as the residues specific for binding carotenoid (Leverenz *et al.*, 2015; Melnicki *et al.*, 2016). Homologs to the CTD (CTDHs) are also found in nearly every genome encoding an HCP, although there is typically only one per genome; their function(s) remain unknown (Fig. 3). The nine HCP clades are each found across cyanobacterial species and do not appear to be confined to a particular taxonomic group, except for HCP9, which has been identified only in the basal cyanobacterial genus *Gloeobacter* (which also contains OCPX) (Fig. 3). HCP4 and HCP5 are frequently encoded near genes encoding a CTDH, and it has been proposed that the OCP is the product of an ancient fusion event which combined genes for an HCP with a CTDH into a single protein coding sequence (Lechno-Yossef *et al.*, 2016). The primary structure of the HCP4 is most similar to the OCP-NTD (Fig. 4), consistent with the proposal that it is closest to the ancestor of the NTD (Melnicki *et al.*, 2016).

HCP2 and HCP3 (*all3221* and *all4783*, respectively) have been shown to be effective $O_2$ quenchers, similar to the OCP-NTD (Lopez-Igual *et al.*, 2016) and the RCP (Kerfeld *et al.*, 2003), but, unlike the OCP-NTD and the RCP, do not quench PBS. A specific function has not yet been demonstrated for HCP1. It is known to be, like the OCP, able to bind a range of carotenoids (45–63% CAN, 20–27% ECN, 9–16% $\beta$-carotene and 7–10% hECN), with a different mixture (including the carotenoid glycoside deoxymyoxanthophyll) observed when overexpressed in a ΔCrtr Syn6803 background (Melnicki *et al.*, 2016). We suggest that carotenoproteins, such as the OCP and the HCPs, tune their function by varying the carotenoid they bind.

The diversity of carotenoids produced by cyanobacteria includes the same carotenes as plants, but also keto-carotenoids, hydroxy-carotenoids and carotenoid glycosides (Hirschberg & Chamovitz, 1994). Keto-carotenoids are necessary for OCP photoactivation (Punginelli *et al.*, 2009). The synthesis of keto-carotenoids increases under stress conditions, such as intense UV-B irradiation, nitrogen deficiency or the extreme desiccation of desert mats (Stransky & Hager, 1969; de Loura *et al.*, 1987). The levels of carotenoid glycosides, particularly myoxanthophyll, likewise increase under these conditions (Ehling-Schulze *et al.*, 1997; Steiger *et al.*, 1999). The function of these pigments is unknown, although they are abundant in the cell wall and plasma membrane, and have been proposed to stabilize membranes, function as sunscreens or regulate the permeability of membranes to oxygen (Jurgens & Mantele, 1991; Subczynski *et al.*, 1991). Interestingly, HCP1 has a predilection for binding myoxanthophyll, suggesting a possible role in carotenoid transport to the plasma membrane or cell wall. As carotenoid synthesis is associated with the thylakoid membrane (Hirschberg & Chamovitz, 1994), a carotenoid delivery system to the outer membrane is required. At the same time, it may be that some of the HCPs, such as HCP1, could be components of the cell wall, as there are numerous reports of carotenoproteins associated forms NTD–CTD heterodimers (Lechno-Yossef *et al.*, 2017; Moldenhauer *et al.*, 2017), leads us to propose that, as predicted by Kerfeld and colleagues in 2003, these separately encoded NTD and CTD homologs can mix and match (perhaps with differing carotenoid selectivity, see Section V) to form different OCP-like heterodimers with a range of photoprotective activities.

In addition, at least some HCPs probably function without an associated CTDH. Global proteomic and transcriptomic studies have confirmed that the different HCPs are not pseudogenes and are indeed expressed under various conditions (summarized in Melnicki *et al.*, 2016). The first systematic attempt to determine the function of paralogous HCPs focused on the four HCPs (HCP1–4) in *Anabaena* PCC 7120. All appeared red–violet in color on expression in CAN-producing *Escherichia coli*, and were confirmed to each bind 100% CAN (Lopez-Igual *et al.*, 2016). Functionally, HCP4 (*all4941*) was the only paralog able to interact with the PBS, where it induced constitutive fluorescence quenching, reminiscent of the RCP (Leverenz *et al.*, 2014), and with quenching superior to the OCP-NTD at low phosphate. The regulatory mutant of *Fremyella* (lacking the photoreceptor required to perform complimentary chromatic acclimation and so unable to respond to light quality), suggesting a specific physiological role for the OCP2. Under most of these conditions, OCP1 expression levels did not change appreciably, implying that low levels of constitutive expression might be required to prepare cells for photoprotection by OCP1 under fluctuating conditions. This is consistent with its activity being regulated post-transcriptionally and/or post-translationally, through FRP interaction and/or a change in oligomeric state. By contrast, OCP2 function appears to have fewer potential attenuations, and so perhaps is more likely to be important under conditions such as prolonged exposure to environmental stress, regulated solely at the transcriptional level. Collectively, these data suggest that the properties of the OCP2 may be closer to those of a primitive OCP, retaining functional attributes related to its early evolution.

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There are a small number of HCP-containing genomes that lack any OCP, the majority of which contain either an HCP4 or HCP5 encoded adjacent to a CTDH (Melnicki *et al.*, 2016). As these two HCP subclades share the most sequence similarity with the OCP-NTDs (particularly those of OCPX), this suggests that a heterodimeric interaction may occur between a CTDH and one of these HCPs, perhaps recapitulating the evolution and the functional properties of the OCP. For example, *Thermoysteococcus elongatus* BP-1 lacks an OCP, but contains an HCP4 and CTDH encoded in tandem; however, NPQ induction could not be demonstrated in this organism under the conditions tested (Boulay *et al.*, 2008). Nevertheless, the frequent proximal co-occurrence of genes for a CTDH and one or more HCPs, and the recent demonstration that synthetically split OCPs...
**Fig. 4** Conservation of carotenoid-interacting residues among orange carotenoid protein (OCP) and helical carotenoid proteins (HCPs). The five OCP/N-terminal domain (NTD) homologs in *Anabaena PCC 7120* known to bind carotenoids are shown aligned at the top, with experimentally determined carotenoid-binding residues marked for either cpcO or cpcR (carotenoid protein configurations) by orange or red squares. Sequence logos are shown below for the five regions in which cpc residues cluster, indicating the subtype-specific conservation patterns across all sequences in each clade. Residues indicated by triangles correspond to the *Anabaena PCC 7120* OCP1 (All3149). Red boxes indicate subtype-specific deletions, and blue arrows and blue vertical bars indicate subtype-specific insertions which were hidden. Logo positions were manually aligned in Adobe illustrator.

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V. Carotenoid–protein interactions in the OCP and the HCPs

Before the realization of the important physiological role of the OCP, it was recognized to be an ideal model system to study carotenoid–protein interactions. The understanding of carotenoid–protein interactions in photoprotective processes, such as the xanthophyll cycle, and in light harvesting is hampered by the lack of structural information and, in general, the inability to predict carotenoid-binding sites from sequence information. Currently, crystal structures are available for OCP1O, the RCP (corresponding to the NTD in the activated OCPR) and HCP1. The structures of OCP2 and HCP2, HCP3 and HCP4 can be homology modeled based on known structures. However, the native carotenoids bound to these proteins, which will probably affect their function and spectroscopic properties, have yet to be identified. Nevertheless, the homology models of HCP2, HCP3 and HCP4 and the structure of HCP1 (Melnicki et al., 2016), in conjunction with the results of the first efforts to elucidate their functions, provide some insight into the structural basis of function and the general determinants of carotenoid binding in the OCP and its homologs.

Table 1 Carotenoid solvent accessibility and tabulation of selected amino acids in red carotenoid protein (RCP) and helical carotenoid proteins HCP1, HCP2, HCP3 and HCP4 from Anabaena PCC 7120 within 4 Å distance to the carotenoid

<table>
<thead>
<tr>
<th></th>
<th>RCP</th>
<th>HCP1</th>
<th>HCP2</th>
<th>HCP3</th>
<th>HCP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent accessibility (Å²)</td>
<td>183</td>
<td>193</td>
<td>185</td>
<td>180</td>
<td>192</td>
</tr>
<tr>
<td>Methionine</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Histidine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>11</td>
<td>6</td>
<td>12</td>
<td>7</td>
</tr>
</tbody>
</table>

Solvent accessibility of the carotenoid in RCP and HCP1 structures (PDB 4XB4 and 5FCX, respectively) and homology models of HCP2–4 calculated using the software PDBePISA (http://www.ebi.ac.uk/pdbe/pisa/).

The carotenoid of the OCP occupies two distinct positions in the protein depending on the state of photoactivation: resting (OCP↑) and activated (OCP↓). The residues defining these two distinct positions are referred to as carotenoid–protein configurations cpO and cpR, respectively (Figs 2, 4, 5). These residues are largely conserved across the OCP and HCP families. In the RCP (Syn6803) and HCP1 structures and the homology models of Anabaena PCC 7120 HCP2, HCP3 and HCP4, the carotenoid is similarly solvent accessible (all 19–20%); the solvent-accessible regions are near the two β-ionone rings (Fig. 1b,c). By contrast, in the OCP↓, only 4% of

![Fig. 5 Residues involved in O₂ singlet quenching within 4 Å of the carotenoid.](image)
the carotenoid is solvent exposed, localized to a portion of the polyene (C25, C29, C31, C32, C34, C39) at the major interface, and partially at β-ionone ring 2 in the NTD (C10 and C13).

In OCP\(^\text{P}\), the β2 ring is flanked by two aromatic–sulfur interactions that are mediated by conserved residues (W41:M117, W110:M161 and W110:M83) (Fig. 5; Table 1). In the OCP\(^\beta\) /RCP, these intraprotein aromatic–sulfur interactions are preserved, but, in the cpcR, they flank the central portion of the polyene (Fig. 5). Notably, these aromatic–sulfur interactions are present in the structures of HCP1, HCP2, HCP3 and HCP4 (with the exception of the W110:M83 interaction for HCP1 and HCP2, and W110:M161 in HCP2). No similar aromatic–sulfur interactions are found among the carotenoid-binding sites in the other watersoluble carotenoid proteins, peridinin-chlorophyll protein (PDB-ID 1PPR) or crustacyanin (PDB ID 1GKA). Aromatic–sulfur interactions are known to stabilize protein structure and potentially confer regulation of structural integrity based on the oxidation state of the methionine (Valley et al., 2012; Lewis et al., 2016). In the context of the photoprotective function of the OCP, this is potentially significant, as the reversible oxidation of methionine residues has been proposed to be a regulatory mechanism (Anbanandam et al., 2005) in diverse photosynthetic organisms (Rinalducci et al., 2008; Tarrago et al., 2009), and could be involved in the regulation of the function of the OCP and HCPs.

At the time of the initial structure determination, the number of conserved methionine residues in the OCP and their disposition relative to the carotenoid were noted as probably functionally relevant (Kerfeld et al., 2003). In addition to a structural role in aromatic–sulfur interactions, they could potentially serve as endogenous antioxidants (Levine et al., 1996; Kim et al., 2014). In OCP and the HCPs, accordingly, they could protect the carotenoid from oxidation. This would be comparable with the function of methionine residues that flank the active site in glutamine synthetase (GS) (Levine et al., 1996). However, in the GS structure, the methionines that were oxidized were primarily surface exposed and not part of aromatic–sulfur interactions; in OCP and the HCPs, M83, M117 and M161 are mostly buried (all have solvent accessibility below 10%). By analogy, therefore, the aromatic–sulfur interactions in the OCP and its homologs are more likely to be involved in a structural role than a protective function.

Alternatively, or in addition, the methionine residues could contribute to the function of the protein in \(^1\text{O}_2\) quenching as part of the protective role for the cell; the \(^1\text{O}_2\) quenching capacity of a given HCP or OCP could not only involve methionine, but also the bound carotenoid, as well as the aromatic amino acids. Tryptophan, tyrosine and phenylalanine (in order of avidity) quench \(^1\text{O}_2\) (Michaeli & Feitelson, 1994). However, again, it is not possible to distinguish whether the function of these residues is part of the overall role of the protein to protect the cell, or whether their function is to protect the carotenoid. For example, ‘chains’ of chains of tyrosine and tryptophan have been proposed to protect proteins from oxidative damage (Gray & Winkler, 2015), and the OCP and, in particular, the HCPs are enriched in clusters of these amino acids.

The preponderance of aromatic amino acids surrounding carotenoids in proteins has long been appreciated, where they participate in stabilizing π–π interactions with the polyene of the carotenoid (Ciani et al., 2002; Mao et al., 2003; Rozak et al., 2004; Garcia-Martín et al., 2008). The amino acids within 4 Å of the carotenoid in HCP–4 and the OCP\(^\beta\) (cpcR) are enriched in aromatics (Fig. 5), although differences in the number and disposition of the residues in the carotenoid-binding pocket would be assumed to be related to the different functional properties reported in the initial characterization of HCP–4. For example, HCP4 quenches PBS comparable with the RCP/OCP\(^\beta\); the carotenoid environments of the NTD in cpcR and that of HCP4 are similar in being relatively less crowded with aromatic and methionine residues; however, HCP2, which does not quench PBS, is similarly sparsely populated. HCP2 and HCP3 were alike in quenching \(^1\text{O}_2\); however, their carotenoid environments are distinctly different (Fig. 5). HCP1 was distinctive for being unable to quench PBS and not effective for \(^1\text{O}_2\) quenching (Lopez-Igual et al., 2016). Amongst HCP–4, HCP1 is particularly enriched in aromatics, in particular phenylalanine, which is a relatively poor quencher of \(^1\text{O}_2\). A second distinguishing feature of HCP1 is the disposition of the aromatic rings relative to the carotenoid (Fig. 5); they are primarily orthogonal to the polyene, rather than co-planar, which is characteristic of the interaction of carotenoids with aromatic amino acids generally. This particular configuration, in which the carotenoid is presumably more loosely held, is consistent with a very distinctive role, such as carotenoid transport.

VI. Conclusions and prospects

The recent identification of new families of the OCP, as well as the large expansion in the number and distinct types of homologs to its structural and functional domains, raises the possibility that there may be a heretofore unappreciated range of OCP-related photoprotective responses in cyanobacteria. An understanding of the structural basis of the function of these proteins should enable the fine-tuning of photoprotection in cyanobacteria, which could enhance their productivity as, for example, microbial cell factories. As model systems for the study of carotenoid–protein interactions, studies of the OCP and its homologs may contribute to the understanding and eventual manipulation of carotenoid–protein interactions in photoprotective processes, such as the xanthophyll cycle, and in light harvesting in natural and artificial photosynthetic systems. Furthermore, the continued expansion of the OCP as a model system for the examination of carotenoid–protein interactions may be coupled with ongoing efforts to synthesize novel carotenoids for the design of new water-soluble colorants with potential agronomic value, new antioxidants or photoprotective proteins that can enhance the efficiency of the light reactions. More generally, the modular structure of the OCP can potentially be used to gain control over carotenoid and protein properties and functions in applications as diverse as optogenetics and photodynamic therapies.

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