Structural and Functional Modularity of the Orange Carotenoid Protein: Distinct Roles for the N- and C-Terminal Domains in Cyanobacterial Photoprotection

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The orange carotenoid protein (OCP) serves as a sensor of light intensity and an effector of phycobilisome (PB)-associated photoprotection in cyanobacteria. Structurally, the OCP is composed of two distinct domains spanned by a single carotenoid chromophore. Functionally, in response to high light, the OCP converts from a dark-stable orange form, OCPO, to an active red form, OPCR. The C-terminal domain of the OCP has been implicated in the dynamic response to light intensity and plays a role in switching off the OCP’s photoprotective response through its interaction with the fluorescence recovery protein. The function of the N-terminal domain, which is uniquely found in cyanobacteria, is unclear. To investigate its function, we isolated the N-terminal domain in vitro using limited proteolysis of native OCP. The N-terminal domain retains the carotenoid chromophore; this red carotenoid protein (RCP) has constitutive PB fluorescence quenching activity comparable in magnitude to that of active, full-length OCPR. A comparison of the spectroscopic properties of the RCP with OCPR indicates that critical protein–chromophore interactions within the C-terminal domain are weakened in the OCPR form. These results suggest that the C-terminal domain dynamically regulates the photoprotective activity of an otherwise constitutively active carotenoid binding N-terminal domain.

INTRODUCTION

The water-soluble orange carotenoid protein (OCP) is a photoactive carotenoid binding protein found in nearly all cyanobacteria (Kirilovsky and Kerfeld, 2013). The OCP plays an important role in regulating energy transfer from the light-harvesting phycobilisome (PB) antennae of these organisms by participating in a light-activated, photoprotective nonphotochemical quenching mechanism (Scott et al., 2006; Wilson et al., 2006). The photoactivity of the OCP is directly linked to its role as the inducer of photoprotection. Absorption of blue-green light by the single 3'-hydroxyechinenone (3'-hECN) chromophore of the OCP causes a conversion from OCP’s dark-stable form, OCPO, to its light-activated form, OCPR (Wilson et al., 2008). Conformational changes in both the carotenoid and the protein (Wilson et al., 2008, 2012) accompany the formation of the OCPR photoproduct, which binds to PBs and acts as a direct quencher of excess energy and PB fluorescence (Wilson et al., 2008; Gwizdala et al., 2011). The OCP’s state is thus active photochemically (i.e., it is a photoswitch), while the OCPR photoproduct is active in photoprotection. The structural bases for these diverse functions of the OCP remain largely uncharacterized.

The crystal structure of OCPR has been solved to 2.1 Å for Arthrospira maxima OCP (Kerfeld et al., 2003) and, more recently, to 1.65 Å for Synechocystis sp PCC6803 OCP (Wilson et al., 2010). The OCP structure (Figure 1A) includes a mixed α/β C-terminal domain (belonging to the Nuclear transport factor 2 [NTF-2] fold; Pfam [http://pfam.sanger.ac.uk] ID: PF02136) and an additional all-helical N-terminal domain unique to cyanobacteria (Pfam ID: PF09150). The two domains are connected via a long, flexible linker (residues 160 to 196 in A. maxima and Arthrospira platensis). The 3'-hECN chromophore of the OCP is buried in the interior of the protein and spans both domains. Figure 1B shows a detailed view of the protein–chromophore interactions in the C-terminal domain, where absolutely conserved residues Tyr-203 and Trp-290 (A. maxima and A. platensis numbering; Tyr-201 and Trp-288 in Synechocystis) simultaneously hydrogen bond to the carbonyl group of 3'-hECN in a geometry evocative of the active site of the enzyme ketosteroid isomerase (also a NTF-2 fold; Cho et al., 1999) or the hydrogen-bonding environment of the p-coumaric acid chromophore in photoreactive yellow protein (Borgstahl et al., 1995).

The structural modularity of the OCP, combined with the observation that a large number of cyanobacterial genomes...
Figure 1. Structure of the OCP from *A. maxima* (Protein Data Bank ID: 1M98).

(A) The OCP monomer in the OCP\textsuperscript{Q} state. The C-terminal domain (residues 197 to 315; cyan) is connected to the N-terminal domain (residues 1 to 160; red) via a long flexible linker (residues 161 to 196; purple). Preferential trypsin cleavage sites of native OCP (Arg-9, Lys-167, Arg-187, and Lys-312) are marked with asterisks. The carotenoid, 3'-hydroxyechineone, is shown in balls and sticks.

(B) Close-up view of protein–chromophore interactions in the C-terminal domain and the interface between the N- and C-terminal domains. Tyr-203 and Trp-290 residues (sticks) H-bond to the 4-keto group of 3'-hECN. Residues Leu-207, Leu-250, and Ile-305 in the C-terminal domain are proposed to sterically constrain the carotenoid in the twisted conformation characteristic of OCP\textsuperscript{Q} (see Discussion). The interdomain salt bridge between Glu-246 and Arg-155, known to be disrupted upon photoactivation, is also shown. Figures prepared with the University of California-San Francisco Chimera package (Pettersen et al., 2004).
encode multiple copies of genes for the N- and C-terminal domains (often in addition to one or more full-length OCP; Kirilovsky and Kerfeld, 2013), hints that the N-terminal and C-terminal domains may function independently or perhaps mix and match with carotenoids to assemble functionally diverse full-length OCPs (Kerfeld et al., 2003). Modular assembly is a recurring theme in many plant and cyanobacterial photoreceptor proteins ( Möglich et al., 2010). But do OCP’s structurally modular domains perform distinct functions in the context of photoactivity and/or photoprotection?

The first clue to the importance of the OCP’s N-terminal structural domain in PB binding and quenching was recently established by Wilson et al. (2012). Specifically, a positively charged residue, Arg-155, in the N-terminal domain was shown to be critical to PB binding and quenching activity in full-length OCP. Isolation of pigmented N-terminal domain fragments during lengthy native source purifications of OCP (Holt and Krogmann, 1981; Wu and Krogmann, 1997; Wilson et al., 2006) suggested a preferential and stable binding of 3’-hECN to the N-terminal domain. These fragments, referred to as red carotenoid proteins (RCPs), were presumed to be proteolytic degradation products of the full-length OCP. The absence of genes encoding only the N-terminal domain in A. maxima genomes verifies that these RCPs are the products of proteolysis. Wu and Krogmann determined that one particular 16.7-kD RCP from A. maxima possessed a mass and primary structure consistent with OCP’s N-terminal domain minus the first 15 residues (Wu and Krogmann, 1997). While RCP is not photoactive, it still displays the molecular characteristics currently known to be required for quenching activity: a 3’-hECN chromophore and a positively charged residue, Arg-155.

Here, we produced and characterized a homogeneous carotenoid binding N-terminal domain fragment (RCP) obtained via limited proteolysis of native OCP. Using an in vitro PB fluorescence quenching assay (Gwizdala et al., 2011), we show that this RCP is comparable to OCPR as an active quencher of PB fluorescence assay (Gwizdala et al., 2011), we show that this to be required for quenching activity: a 3’-hECN chromophore and a positively charged residue, Arg-155.

Results of SDS-PAGE analysis of limited digestions of OCPD and OCPR at different time points during a 24-h digest by trypsin are shown in Figure 2A. Hydrolysis of OCPD and OCPR occurred at significantly different rates, with OCPR exhibiting relatively accelerated degradation to 17- and 14-kD digestion products. In fact, while OCPR was completely digested in <60 min, more than 50% of full-length OCP was still present after 10 h of digestion of the OCPD form. Additional proteolytic digests performed with chymotrypsin and glu-C protease also exhibited a substantial enhancement in the rate of proteolysis of the OCPR form (see Supplemental Figure 2 online). Extended (72 h) digestions performed with each type of serine protease also produced two dominant protease-resistant fragments (Figure 2B). The observation that two large polypeptide fragments appear regardless of protease substrate

RESULTS

Limited Proteolysis of Native OCP

A. platensis RCP isolated by the method of Holt and Krogmann (1981) was found to be inhomogeneous even after extensive attempts to purify the protein using high-resolution ion-exchange chromatography (IEC) and size-exclusion chromatography (SEC). Coomassie-stained SDS-PAGE of the most highly purified fractions exhibited one or two diffuse bands at ~15 to 17 kD and in some cases an additional weak band at 12 to 14 kD (see Supplemental Figure 1 online). These RCP samples exhibited broad visible absorption spectra with a maximum at 505 to 507 nm and Amax/A280 values ranging from 1.88 to 2.28 (see Supplemental Figure 1 online). By contrast, no 15- to 17-kD RCP fragments were observed at any point during the rapid OCP purification from A. platensis described in this work. Instead, this OCP purification resulted in a largely homogeneous 35-kD OCP as visualized by SDS-PAGE (Figure 2A, Ctrl samples). The Amax/A280 ratio of OCP purified by this method was >1.8.

Figure 2. Coomassie Blue-Stained SDS-PAGE of Protease Products Formed by Protease Digestion of OCPD and OCPR.

(A) Time course of native proteolytic digestion of OCPD and OCPR by trypsin. Digestions of OCPD (O) and OCPR (R) were stopped at times indicated on the horizontal axis. No trypsin was added to control (Ctrl) samples. MW, molecular mass.

(B) OCPD or OCPR digestion by trypsin (lanes 1 [O] and 2 [R]), chymotrypsin (lanes 3 [O] and 4 [R]), and glu-C protease (lanes 5 [O] and 6 [R]) after 72 h.

(C) Products of an OCP (lane 1) digest by immobilized trypsin (lane 2) and the purified 17.5-kD (lane 3) and 14.1-kD (lane 4) fragments isolated by IEC.
specificity strongly suggests that these two large segments of the OCP are inherently resistant to proteolysis (i.e., they form stable, well-folded domains in both OCPO and OCPR).

Isolation and Identification of the N- and C-Terminal Domains of the OCP

Preparative-scale digests of native A. platensis OCP with immobilized trypsin yielded similar degradation fragments to those observed with trypsin in solution (Figure 2C). The primary structure of each fragment was determined by a combination of N-terminal sequencing and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). From the N-terminal sequence and the MALDI molecular mass we obtained for each fragment, we could identify the cleavage site for trypsin (C terminus to Lys in both cases). The mass of the larger protein produced during digestion by immobilized trypsin was determined to be 17.46 kD by MALDI-TOF MS and the N-terminal sequence determined to be Ser-Ile-Phe-Pro-Glu-Thr-Leu-Ala by Edman degradation. This molecular mass and sequence correspond to residues S10-K167 of the N-terminal domain of A. platensis OCP. The molecular mass of the smaller fragment was determined to be 14.11 kD by MALDI-TOF MS with an N-terminal sequence of Thr-Lys-Val-Gin-Ile-Glu-Gly-Val, which corresponds to residues T188-K312 of the C-terminal domain. These preferential sites of proteolysis by trypsin are indicated in Figure 1A, and the results indicate that trypsinization of native OCP preferentially digests the flexible linker but leaves the structural domains largely intact.

The 17.5- and 14.1-kD protein fragments were separated by subsequent IEC and SEC purification. Both eluted as single peaks during final SEC (Figure 3A). The 17.5-kD N-terminal domain from preparative trypsin digests was found to elute at an apparent molecular mass of 22 kD by analytical SEC, while the C-terminal domain fragment eluted at an apparent molecular mass of 15 kD. On the same analytical SEC column, full-length OCPO (in darkness) eluted with an apparent molecular mass of 41 kD, while illuminated OCP (enriched in the OCPR form) was observed to elute at a higher apparent molecular mass (43 kD) with a significantly broadened elution profile compared with OCPO (Figure 3A). No clearly resolved peaks suggestive of multiple stable oligomeric states were observed during the OCPO or OCPR size-exclusion runs. The isolated 17.5-kD fragment was visibly red in appearance (Figure 3B), whereas the purified 14.1-kD fragment was colorless. Collectively, these data show that the OCP can be reproducibly cleaved into its two structural domains, with the N-terminal domain retaining the carotenoid, forming a 17.5-kD RCP.

Spectroscopic Characterization of OCPO, OCPR, and RCP

The presence of well-ordered secondary structure in each domain isolated by proteolytic digestion of the OCP was confirmed by measuring the far-UV circular dichroism (CD) spectra of the domains (Figure 3C). The N-terminal domain (RCP) CD spectrum is characterized by a large positive band at 193 nm and negative bands at 209 and 222 nm, consistent with helical secondary structure content (Kelly et al., 2005; Greenfield, 2006). The C-terminal domain CD spectrum is characterized by a positive band at 197 nm and a negative band at 215 nm; this spectrum is consistent with a mixture of antiparallel β-sheet and helix (Greenfield, 2006). The sum of the N-terminal and C-terminal domain CD spectra approximate the full-length OCP spectra, and the few observed differences can be attributed to the presence of the flexible linker that contributes additional disordered secondary structure (expected to have a negative band at 195 nm and a small positive band at 210 to 230 nm; Greenfield 2006) to full-length OCPO or OCPR. These results are entirely consistent with removal of the OCP’s disordered flexible linker by trypsin, resulting in separated, but structurally intact N- and C-terminal domains.

The 17.5-kD N-terminal domain RCP produced by trypsin digestion of highly purified A. platensis OCP represents homogeneous RCP, a valuable tool for understanding the structural basis of the activity of the OCP. In order to further characterize the protein and chromophore structure of this RCP, steady state spectroscopic characterization was performed in parallel with a similar characterization of full-length A. platensis OCPO and OCPR. The UV-visible absorption spectra of A. platensis OCPO, OCPR, and of the RCP are shown in Figure 4A. The absorption spectrum of RCP described here differs in some ways from those previously reported (Holt and Krogmann, 1981; Chábera et al., 2011). Significantly less vibronic structure is observed in the S6→S5 transition of the carotenoid in the visible region of the spectrum. Pronounced vibronic structure in the OCPO spectrum results from the constrained C$_{2}$–C$_{7}$ s-trans conformation of 3'-hECN observed in the OCPO crystal structure (see Figure 4C for C atom numbering in the 3'-hECN structure). In previous studies, vibronic structure in the RCP spectrum is readily apparent in the form of distinct shoulders at 410 and 470 nm, with the shoulder at 410 nm being attributed to RCP aggregates (Chábera et al., 2011). The visible absorption spectra of RCP isolated in this work is nearly identical to that of the full-length OCPR photoprotein (Figure 4A). That is, while the OCPO spectrum possesses clear vibronic structure, the RCP and OCPR spectra are almost identically broadened and red-shifted (with maximum absorbance at 510 nm for OCPR and 507 nm for RCP) with a striking absence of any vibronic features. The broadening and red-shifting of the 3'-hECN chromophore in the OCP and RCP, attributed to conformational heterogeneity of 3'-hECN in these proteins, as well as orientation and local environment of the carbonyl oxygen, has been discussed in detail elsewhere (Polivka et al., 2005; Chábera et al., 2011; Bérea et al., 2012). Similar effects are observed in the absorption spectra for other carotenoids with conjugated carbonyl groups when dissolved in polar solvents (Bautista et al., 1999; Frank et al., 2000). The reduced A$_{280}$ in the RCP spectrum is consistent with the loss of aromatic amino acids associated with the C-terminal domain. Furthermore, compared with the OCPO spectrum, both the RCP and OCPR absorbance spectra exhibited significantly increased intensity in a near-UV band at 318 nm. Illumination of the RCP with strong blue-green or white light had no observable effect on its absorption spectrum (see Supplemental Figure 3 online).

The molecular structure of the RCP’s 3'-hECN chromophore was further characterized by resonance Raman spectroscopy. Resonance Raman spectra of the RCP, as well as A. maxima OCPR and OCPO, are shown in Figure 4B. The OCPR and RCP spectra exhibit nearly identical frequencies and relative intensities for all vibrational modes observed. However, a comparison of these spectra to that of OCPO reveals a number of differences.

Modularity of Orange Carotenoid Protein
First, a blue shift of the intense C=C stretching mode of OCP$^O$, from 1520 to 1524 cm$^{-1}$, is observed. This suggests a larger effective conjugation length in the 3'-hECN chromophore of RCP/OCPR, an observation previously proposed to result from a more planar s-trans conformation of the 4-keto-β-ionylidene ring in OCP$^O$ (Wilson et al., 2008). Additionally, while no large differences were observed for the intense methyl rocking and C=C stretching modes observed at ~1008 and ~1157 cm$^{-1}$, respectively, there are still some notable differences in the 1100 to 1400 cm$^{-1}$ fingerprint region of the spectrum. Two distinct peaks at 1186 and 1213 cm$^{-1}$ in the OCP$^O$ spectrum are replaced by a single, dominant 1193-cm$^{-1}$ peak in the OCPR/RCP spectrum. A moderately intense peak at 1286 cm$^{-1}$ is also unique to the OCPO spectrum. While the OCPR and RCP fingerprint spectra are in excellent qualitative agreement with that of all-trans β-carotene in solution (Koyama et al., 1988), the fingerprint region of the OCP$^O$ spectrum is rather unusual for an all-trans C40 carotenoid. However, this is perhaps unsurprising given the unique protein environment of OCP$^O$ and the twisted and bowed conformation of 3'-hECN observed in the crystal structure.

However, the most striking feature distinguishing the Raman spectra of RCP/OCPR from that of OCP$^O$ is the extremely large intensity difference observed for an ~980-cm$^{-1}$ hydrogen-out-of-plane (HOOP) wagging mode. The relatively high frequency of this particular HOOP vibration is most consistent with a combination A$_2$-type HOOP mode resulting from the in-phase wagging of two hydrogens across an unsubstituted double bond in the all-trans polyene chain (Figure 4B, inset). This assignment is supported by the similar 955- to 965-cm$^{-1}$ frequency calculated for these modes by normal-coordinate analysis of all-trans β-carotene (Saito and Tasumi, 1983). HOOP modes can serve as direct reporters of out-of-plane distortions, since the intensity of these modes are typically quite low for a planar polyene but increase if local symmetry in the vicinity of the bond is broken (Eyring et al., 1980). Thus, we expect the intensity of this mode in OCP$^O$ to originate from torsional distortions to the polyene chain enhancing one or more of the A$_2$-type CH=CH HOOP modes. The observation of the reduced HOOP mode intensity in A. maxima OCP$^R$ is consistent with the Raman data presented for Synechocystis PCC 6803 OCP of Wilson et al. (2008) and their proposal of a twisted-to-planar conformational change for 3'-hECN in the OCP$^O$ → OCP$^R$ transition (Wilson et al., 2008). The interpretation of the Raman spectrum of the RCP is consistent with that of OCP$^R$: Even in the absence of the C-terminal domain, the 3'-hECN polyene of RCP is in a relatively planar and likely all-trans configuration (based on the similarity of the OCP$^R$/RCP spectra to that of trans β-carotene in solution; Koyama et al., 1988). This observation further underscores the specific
effect of the C-terminal domain protein environment on the carotenoid structure in OCP. Furthermore, the RCP Raman results suggest that the $\sim 980\text{-cm}^{-1}$ HOOP intensity observed in the OCP spectrum can be assigned to one or more of the torsionally distorted $\text{C}_7\text{H} = \text{C}_8\text{H, C}_11\text{H} = \text{C}_{12}\text{H, or C}_{15}\text{H} = \text{C}_{15}\text{H}$ combination HOOP wagging modes localized in the vicinity of the C-terminal domain of OCP. The striking similarity of the electronic absorption and Raman spectra of $3'$-hECN in RCP and OCP$\text{R}$ together suggests that $3'$-hECN chromophore is in a similar local environment in RCP and OCP$\text{R}$, an environment distinctly different than that of $3'$-hECN in OCP$\text{O}$.

**PB Fluorescence Quenching Assays**

Next, we investigated whether the RCP could bind to PBs and trigger quenching of their fluorescence. An in vitro reconstitution system allowed measuring the changes in the fluorescence of isolated *Synechocystis* PBs following addition of *A. platensis* OCP or RCP (Gwizdala et al., 2011). Figure 5A reveals that while *A. platensis* OCP induced very little PB fluorescence quenching (~10% in 300 s) in darkness, it induced a rapid fluorescence decrease (83% in 100 s) when mixed with *Synechocystis* PBs under continuous blue-green illumination. This process was accelerated by preconverting OCP$\text{O}$ to OCP$\text{R}$ prior to mixing (83% in 50 s). Similar experiments performed using *Synechocystis* OCP instead of *A. platensis* OCP gave very similar traces (see Figure 5 in Gwizdala et al., 2011), showing that both OCPs have a strong affinity for *Synechocystis* PBs.

The quenching activity of the *A. platensis* 17.5-kD RCP as well as that of the unfractionated proteolytic products from immobilized trypsin digests of the OCP (i.e., a 1:1 mix of the 17.5-kD N-terminal and 14.1-kD C-terminal fragments) was then assayed. Both samples were found to induce fast fluorescence quenching similar to that induced by full-length *A. platensis* OCP$\text{R}$. *Synechocystis* PB fluorescence decreased by 80 to 83% in only 50 s (Figure 5B). Thus, the 17.5-kD RCP strongly induced PB fluorescence quenching comparable to that of OCP$\text{R}$ (Figure 5A). PB fluorescence quenching by the RCP occurred under illumination and in darkness. The rates and amplitudes of fluorescence quenching were similar in both conditions (Figure 5B). Changing the blue-green actinic illumination conditions following the apparent completion of the RCP-PB reaction (illuminated to nonilluminated or vice versa) also had no observable effect on the final magnitude of fluorescence quenching (see Supplemental Figure 4 online). This result further confirms the actinic light–independent behavior of the RCP and its complex with the PB. Since quenching of PB fluorescence by the RCP did not require photoactivation and was not affected by the presence of the 14.1-kD C-terminal fragment, this indicates that quenching is the function of the constitutively active N-terminal domain alone.

![Figure 4. Spectroscopic Characterization of 3'-hECN in OCP and RCP.](image-url)

(A) UV-visible absorption spectra of *A. platensis* OCP$\text{O}$ (black), OCP$\text{R}$ (red), and 17.5-kD RCP (dotted purple). The inset shows OCP$\text{O}$, OCP$\text{R}$, and RCP each in a 1-cm path length cuvette. AU, absorbance units.

(B) Resonance Raman spectra of OCP$\text{O}$ (black), OCP$\text{R}$ (red), and RCP (blue). The frequencies (1 and 2) noted in parenthesis correspond to the $\sim 980\text{-cm}^{-1}$ in-phase HOOP wagging mode.

(C) Structure and C atom numbering of 3'-hECN in the $\text{C}_6$-$\text{C}_7$ s-trans configuration.
DISCUSSION

Formation of RCP by Native Proteolysis: A Probe of OCP Structural Change in Vitro

Comparative proteolysis of native OCP\textsuperscript{O} and OCP\textsuperscript{R} was used to probe for structural differences between the two forms of full-length OCP as well as to optimize conditions for preparative digests of native OCP to produce RCP. Comparison of the OCP\textsuperscript{R} and OCP\textsuperscript{O} digestions shows a remarkably enhanced susceptibility of OCP\textsuperscript{R} to proteolysis into its two component domains using a variety of serine proteases. The enhanced protease susceptibility of OCP\textsuperscript{R} provides clear evidence for structural rearrangement of the OCP holoprotein following light absorption.

The results of our proteolytic assays are also consistent with the results of analytical gel filtration of OCP\textsuperscript{R}, in which a higher apparent molecular mass (compared with OCP\textsuperscript{O}) suggests an increase in the Stokes radius of the protein. This change can be attributed to a change in overall tertiary protein structure following photoconversion. Proteolysis results in two intact domains and each, as demonstrated by CD, largely retains its native secondary structure. This, and the observation that the linker joining them is preferentially digested in OCP\textsuperscript{R}, suggests that the major changes to the protein structure result in increased accessibility of the linker to protease. Together, these data suggest that photoactivation minimally alters secondary structure but that the global tertiary structure and linker accessibility of the OCP changes significantly in the photoprotective OCP\textsuperscript{R} form. This is consistent with our previous proposal of an open form of OCP\textsuperscript{R}. A dissociation of the N- and C-terminal domains results in increased solvent exposure of the N-terminal domain surface containing Arg-155 and the carotenoid. Importantly, far-UV CD spectra collected on OCP\textsuperscript{D} and OCP\textsuperscript{R} (Figure 3C) are consistent with the proteolysis results; no major changes to secondary structure were observed between the two forms of full-length OCP. The enhanced rate of proteolysis in the OCP\textsuperscript{R} form is more likely to be due to a change in global tertiary structure affecting accessibility to the linker.

The OCP Is a Modular Protein with Specific Functional Roles for Its Structural Domains

The results of PB fluorescence quenching induced in vitro by the RCP clearly show that this protein, which contains only 3'-hECN and the OCP N-terminal domain, is an active quencher of PB fluorescence comparable to full-length OCPR\textsuperscript{R}. This result suggests that the C-terminal domain of the OCP is not absolutely required for either OCP-PB binding or quenching. While it is certainly possible that protein–protein interactions between the C-terminal domain and the PB core make minor contributions to the binding/affinity between full-length OCP and the PB, such interactions must be comparatively weaker. We conclude that the RCP, the carotenoid binding N-terminal domain of the OCP, is a constitutively active quencher of PB fluorescence.

The preferential retention of 3'-hECN by the N-terminal domain also suggests a relatively high affinity for this hydrophobic molecule; accordingly, the RCP is a remarkably compact soluble carotenoid binding protein. In addition, while select N-terminal domain residues (i.e., Tyr-44 and Trp-110) in the chromophore binding pocket have been shown to be critical for photosensory response (Wilson et al., 2010, 2011), the molecular basis for this relationship has yet to be explained. There is no reported evidence localizing structural dynamics during the photocycle to the N-terminal domain.

The function of the C-terminal domain is partly identified by our spectroscopic characterization of RCP and OCP; at most, it appears that the C-terminal domain minimally interacts, sterically or electrostatically, with 3'-hECN in full-length OCP\textsuperscript{R}. The observed differences in the steady state spectroscopic properties that distinguish OCP\textsuperscript{D} and RCP/OCP\textsuperscript{R} are modulated primarily by the
C-terminal domain. This is a provocative result since nearly all data pertaining to structural dynamics in the OCP photocycle have also been localized to possible changes in this domain. Specific proposals for structural changes implicating C-terminal domain dynamics include the following: (1) H-bonding changes in the Tyr-203/Tyr-290 H-bonds to the 3'-hECN carbonyl oxygen (Wilson et al., 2011), (2) Pro isomerization at a loop in the C-terminal domain β-sheet (Gorbunov et al., 2011), and (3) breaking of the Arg-155:Glu-244 salt bridge to form an open OCP (Wilson et al., 2012). Furthermore, the general requirement for a conjugated carotenoid group at the C$_x$ position of the carotenoid (Punginelli et al., 2009) and the absolute requirement of residues Tyr-203 and Tyr-290 for photoactivity (Wilson et al., 2011) also suggest the critical nature of this domain in photosensing. At the same time, the C-terminal domain appears to function in the deactivation of the OCP’s quenching activity: Recent structural modeling and coimmunoprecipitation results with fluorescence recovery protein (FRP), a protein responsible for catalyzing the OCP$^R$ to OCP$^D$ dark reaction (Boulay et al., 2010), have clearly identified the C-terminal domain as the site of OCP–FRP interaction (Sutter et al., 2013). These data collectively imply a regulatory role, through responses to light and to the FRP, for the C-terminal domain.

In addition to the dynamic protein structural changes listed previously, the C-terminal domain is also responsible for dynamic changes in the structure of the carotenoid. Removal of the C-terminal domain from the OCP$^D$ structure (Figure 1A) to form RCP must result in the solvent exposure of the 4-keto-β-ionylidene ring of 3'-hECN. Of course, since the C-terminal domain is entirely absent in RCP, the H-bonds to Tyr-203 and Trp-288 must also be broken. Our data suggest that the chromophore binding pocket in the C-terminal domain of OCP$^R$ must be loosened substantially or the chromophore is entirely unbound from the domain. Either is consistent with the broadening of the OCP$^R$/RCP absorption spectra, which can be attributed to increased conformational heterogeneity (i.e., unrestricted rotation around C$_6$–C$_7$ and other single bonds), as well as potential solvent polarity–induced effects resulting from H-bonding between the carbonyl group of the carotenoid and water. This is in stark contrast with the carotenoid–protein interactions in OCP$^D$, where Tyr-203 and Trp-290 are H-bonded to 3'-hECN’s carbonyl oxygen and carotenoid–protein interactions are largely responsible for locking the carotenoid in a twisted conformation (Kerfeld et al., 2003). This twisted conformation is apparent in the crystal structure (Figure 1B), where the 4-keto-β-ionylidene ring of 3'-hECN appears to adopt an unusually out-of-plane conformation to enable its H-bonding with Tyr-203 and Trp-290. This locked and twisted chromophore structure is further evidenced by the well-defined vibronic structure in the electronic absorption spectrum and the large ∼980-cm$^{-1}$ HOOP mode intensity in the Raman spectrum of OCP$^D$.

It appears that interactions between 3'-hECN and the C-terminal domain in OCP$^D$ are responsible for the out-of-plane distortions giving rise to the observed ∼980-cm$^{-1}$ HOOP intensity in the OCP$^D$.

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**Figure 6.** A Schematic Model of OCP N- and C-Terminal Domains and Their Roles in Cyanobacterial Photoprotection.

(1) In darkness/ambient light, OCP$^D$ is unbound from its binding site (X) on the PB core. The OCP$^D$ form is characterized by relatively strong protein–chromophore interactions in the C-terminal domain. (2) In high-light conditions, absorption of a photon by 3'-hECN triggers photochemistry primed by the C-terminal domain. A change in global tertiary structure and a weakening or loss of protein–chromophore interactions in the C-terminal domain accompanies the formation of OCP$^R$. This structural change exposes Arg-155 and presumably other N-terminal domain residues critical for PB binding. Concomitantly, the flexible linker between domains of the OCP becomes more exposed, resulting in increased protease sensitivity and the formation of RCP in vitro. (3) The N-terminal domain and carotenoid bind to the PB and form a quenched OCP$^R$:PB complex (with the binding site now labeled X$^*$ to emphasize the quenched state of the complex). An RCP:PB complex can also be formed in vitro as shown in this work. (4) To dissociate the OCP$^R$:PB complex, FRP interacts selectively with the C-terminal domain of OCP$^R$. (5) The FRP catalyzes the OCP's dark reversion to OCP$^D$, restoring the relatively compact tertiary structure of this form of OCP and dissociating it from the PB binding site.
Raman spectrum, which we propose to result from distortion around the $C_7=\text{C}_9$ region of the chromophore. While the unique H-bonding environment of the 3'-hECN’s carbonyl group in the C-terminal domain is known to be required for carotenoid binding and photoactivity, it is also appears that three highly conserved bulky residues, Leu-207, Leu-250, and Ile-305, form a bulky hydrophobic clamp that may additionally contribute to the out-of-plane twisting of the chromophore’s 4-keto-β-ionylidene ring in the OCP\textsuperscript{0} form.

We suggest that chromophore distortions resulting from protein–chromophore interactions in the C-terminal domain of OCP\textsuperscript{3} may prime isomerization about the $C_7=\text{C}_9$ bond and that $C_7=\text{C}_9$ trans-cis isomerization is a distinct possibility for primary photochemistry. While this result might seem to contradict the likely all-trans chromophore structure observed in the OCP\textsuperscript{3} Raman spectrum (Figure 4B), we note that a reisomerization to an all-trans structure may occur as a thermal/dark reaction on a faster time scale than slow protein structural changes implicated in the formation of the final OCP\textsuperscript{3} form. This proposal is thus consistent with both the OCP\textsuperscript{3} chromophore structure observed by x-ray crystallography, as well our spectroscopic data. Importantly, we would expect a cis $C_7=\text{C}_9$ chromophore to introduce a large amount of strain to the confined C-terminal domain chromophore binding pocket, an effect that would be expected to drive further structural changes in this domain. Protein structural changes driven through the relaxation of strained protein-chromophore intermediates are known to play a signaling role in other blue light sensors, such as photoactive yellow protein (Ihee et al., 2005).

A Revised Model for OCP Function

The emerging picture of the OCP as a modular protein with distinct structural and functional domains significantly advances the mechanistic understanding of the OCP’s photoprotective function. Given the constitutive quenching activity of the N-terminal domain RCP isolated and characterized here, we suggest that the primary role of OCP’s C-terminal domain is to regulate the accessibility, and hence activity, of the PB binding N-terminal domain. This occurs via a mechanism that effectively exposes the interdomain interface of the N-terminal domain containing Arg-155 following photochemical activation. Without such regulation, it seems that OCP’s carotenoid binding N-terminal domain could bind to the PB even in dim light or darkness. The PB binding interface of the N-terminal domain and the carotenoid are shielded by the C-terminal domain in OCP\textsuperscript{0} until required for photoprotection. FRP restores the ground state OCP\textsuperscript{2} structure by catalyzing the rate-limiting step of the OCP\textsuperscript{5} to OCP\textsuperscript{3} reaction through an interaction with the C-terminal domain, thus reforming the quenching inactive OCP\textsuperscript{0} state. Our updated domain-specific model for OCP activity is summarized in Figure 6.

The identification of the RCP as a constitutively active quencher of PB fluorescence raises the question as to the role of genes encoding N-terminal domain homologs in many cyanobacterial genomes: Do they play an OCP-like photoprotective role in vivo? Furthermore, the specialized functions of OCP’s discrete domains demonstrated in this work are also consistent with the speculation of modular assembly of homologs to the N- and C-terminal domains to form full length OCPs with unique photosensory and photoprotective properties (Kerfeld et al., 2003; Kirilovsky and Kerfeld 2013). This type of modular assembly is known for other photosensory proteins, in which blue light sensing and LOV domains are fused to various output modules (Crosson et al., 2003).

Finally, we note that the modular structure and function of the OCP differs from those of other photosensory proteins in that each domain of the OCP absolutely requires the 3’-hECN chromophore to perform its function. The C-terminal domain cannot function as a photosensor without a light absorbing chromophore. The N-terminal effector domain, when bound to the PB, requires 3’-hECN to directly dissipate energy in the OCP induced photoprotective mechanism (Tian et al., 2011, 2012; Stadnichuk et al., 2012). The OCP thus represents a remarkably minimized system in which sensor and effector domains share a single chromophore that is critical to both photosensory and photoprotective function.

METHODS

Purification of OCP from Arthrospira platensis

Frozen cells of A. platensis were a generous gift of Cyanotech and were stored as a wet paste at −80°C until needed. All purification steps were performed at 0 to 4°C in dim light or darkness. Cells were lysed by thawing wet cell paste in 100 mM Tris-HCl, pH 8.0 (4°C), 100 mM NaCl, 5% glycerol, 4 mM phenylmethylsulfanyl fluoride, 4 mM EDTA, and 2 mM EGTA (1 g wet cells/1 mL buffer). The cells were broken in 200-mL batches by beating with 0.1-mm glass beads for 15 s × eight cycles. Following lysis, the cellular extract was centrifuged at 10,000g to remove insoluble material. A saturated (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} solution was then slowly added to the decanted supernatant until the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} concentration of the extract reached 1.5 M. The extract was centrifuged at 40,000g to pellet remaining thylakoids and a small amount of precipitated phycobiliproteins. The supernatant was decanted and filtered (0.45 μm) prior to column chromatography.

A rapid separation of the OCP from the clarified soluble extract was performed using hydrophobic interaction chromatography (HIC). A detailed summary of HIC parameters can be found in Supplemental Table 1 online. HIC effectively separated the OCP from phycobiliproteins in the lysate. Visibly orange fractions containing OCP were reserved and desalted on a 2.5-cm diameter, 60-cm-high column of Superdex G-25 (GE Health Sciences) using 20 mM Tris-HCl, pH 8.5, and 1 mM EDTA as an elution buffer. Intermediate purification of the desalted OCP was performed using anion exchange chromatography (AEC; details in Supplemental Table 1 online). Following initial AEC, OCP-containing fractions with $A\text{_{496}}/A\text{_{280}} > 1.5$ were pooled and concentrated to approximately OD\textsubscript{280} = 50 cm using Amicon 15 Spin Concentrators (Millipore). These fractions were desalted and further polished using a Mono-Q HR 16/10 IEC column (GE Health Sciences). Following final SEC (details in Supplemental Table 1 online), the purity of the OCP was checked by absorption spectroscopy and SDS-PAGE. Absorption spectra of the OCP fractions reserved for further analysis all exhibited $A\text{_{496}}/A\text{_{280}} ≥ 1.8$. SDS-PAGE was performed using 12% Bis-Tris gels (Criterion XT; Bio-Rad) and MOPS or MES running buffer. Protein bands were stained with Coomassie Brilliant Blue G 250 (Thermo GelCode Blue).

Limited Proteolysis of Orange Carotenoid Protein

Proteolytic digests of OCP\textsuperscript{0} and OCP\textsuperscript{3} were performed using trypsin, chymotrypsin, and glu-C protease (Promega). Each digest was performed at 4°C with a protease:protein ratio of 1:100. For OCP\textsuperscript{5} digests, OCP\textsuperscript{0} was converted to OCP\textsuperscript{3} using strong, blue light-emitting diode (LED) illumination (≈470 nm, λ<sub>max</sub>, Philips Lumileds LXML-PB01-0030). Illumination was maintained throughout the duration of the digest following addition of protease to OCP\textsuperscript{3} sample. The OCP concentration was 0.3 mg/mL for all digests. Following addition of protease, aliquots were removed at specified
time points and the reaction stopped via addition of protease inhibitor (phenylmethylsulfonyl fluoride for trypsin and chymotrypsin) followed by freezing on dry ice. Digests with glu-C were stopped by flash-freezing alone. Control samples of OCPO and OCPR (with no protease added) were incubated for the full time course and treated identically to protease containing samples. After flash freezing, each sample was immediately transferred to −80°C for storage. Samples were quickly thawed in warm SDS buffer prior to analysis by SDS-PAGE. SDS-PAGE was performed using 12% Bis-Tris gels and MES running buffer. Proteins were visualized by staining with Coomassie Brilliant Blue G 250.

Preparative Isolation of RCP

RCP was initially isolated as a degradation product of OCP produced during purifications by a method similar to that of Holt and Krogmann (1981) with isoelectric focusing omitted. RCP produced by this method was initially separated from OCP during SEC, and protein isolated by this method was found to be homogeneous as indicated by SDS-PAGE (see Supplemental Figure 1 online). Thus, a more homogeneous N-terminal domain OCP fragment was produced by controlled proteolytic digestion of A. platensis OCP with immobilized trypsin (Pierce). Trypsin gel (washed and suspended according to the manufacturer’s instructions) was added to 0.6 mg/mL A. platensis OCP in 50 mM Tris-HCl, pH 7.4, and 200 mM NaCl at a ratio of 3:1 (v:v) of OCP:gel. The mixture was incubated with mixing for 24 h under blue light at 4°C. Trypsin gel was then removed by centrifugation, followed by 0.22-μm filtration of the supernatant. The native digestion products produced by this method were separated by AEC on a Mono-Q 5/50 GL column (GE Healthcare) or HiLoad 16/10 Q-Sepharose HP column (GE Healthcare) followed by final SEC as described previously. The purity of the isolated RCP was checked by absorption spectroscopy and SDS-PAGE.

Analytical SEC

Analytical SEC was performed on a Superdex 75 GL 10/300 gel filtration column (GE Healthcare) or HiLoad 16/10 Q-Sepharose HP column (GE Healthcare) using a linear gradient of 0 to 100 mM NaCl in 20 mM Tris-HCl, pH 7.5, followed by final SEC as described previously. The purity of the isolated RCP was checked by absorption spectroscopy and SDS-PAGE.

MALDI-TOF and N-Terminal Sequencing

MALDI-TOF mass determination of unfractonated fragments produced by trypsin digestion was performed at the Stanford Protein and Nucleic Acid facility using an Applied Biosystems Voyager DE-RI mass spectrometer. N-terminal sequencing of digest fragments was performed on solution-state samples (purified by IEC/SEC as described previously) using an Applied Biosystems 494-HT Precise Edman Sequencer at the University of California Davis Genome Center Proteomics Core Facility. A single unambiguous sequence was detected during N-terminal sequencing of both N-terminal and C-terminal OCP domain fragments produced during trypsin digests.

CD Spectroscopy

All protein samples were dialyzed into 20 mM potassium phosphate and 100 mM NaF prior to far-UV CD measurements. Far-UV CD spectra were acquired in a 1-mm path-length quartz cuvette using a Jasco J-815 spectropolarimeter with 1-nm bandwidth, 1-s time constant, 50-nm/min scan speed, and eight to 16 averages per spectrum. OCPR spectra were collected following 5 min of blue LED illumination prior to each individual scan. A buffer blank was subtracted from each CD spectrum, and no smoothing of the averaged data was performed. Protein concentrations were determined by quantitative amino acid analysis using a Hitachi L-8800 amino acid analyzer (University of California Davis Proteomics Core Facility), and the mean residue weight, MRW, of the protein is given by MRW = molecular mass/number of backbone amides (Greenfield, 2006).

UV-Visible Spectroscopy

Samples were buffer exchanged into 50 mM Tris-HCl, pH 7.4, and 200 mM NaCl prior to spectroscopic measurements. The UV-visible absorption spectrum of OCPR was collected using an Agilent 8453 spectrophotometer following 10 min of 470-nm LED illumination at 0°C. The OCPR spectrum was collected identically without actinic illumination. All other UV-visible absorbance spectra reported were collected on a Varian Cary Bio 100 spectrophotometer at room temperature.

Raman Spectroscopy

Raman spectra of OCP, OCPR, and RCP in 50 mM Tris-HCl, pH 7.4, 200 mM NaCl were collected using the 514.5 emission line of an Ar+ laser (Spectra Physics model 2020). The laser power was <2 mW for all measurements. The beam was spherically focused on a 0.8-mm i.d. glass capillary and perpendicular Raman scattering collected and subsequently imaged using a double subtractive spectrograph and LN2 cooled charge-coupled device. Protein concentrations were 0.10 to 0.30 mg/mL for all measured samples, and samples were flowed through the capillary using a peristaltic pump. OCP spectra were collected under rapid-flow conditions (50 mL/min) in order to minimize the effect of photolysis on the measured spectrum. The OCPR spectrum was collected under identical conditions, except with continuous blue LED illumination of the sample reservoir 10 min prior to beginning the measurement and throughout data acquisition. RCP spectra were collected in darkness with capillary flow techniques or (for volume limited samples) using a spinning cell in a backscattering geometry. All spectra were measured in triplicate using protein from three independent preparations and the reported frequencies, calibrated using a cyclohexane external standard, are accurate ±2 cm−1.

PB Fluorescence Quenching Assays

PB isolation from Synechocystis cells followed the protocol described by Gwizdala et al. (2011). At the beginning of every measurement, OCP or RCP was added to the isolated PBs (0.012 μM) kept in 0.8 M potassium-phosphate buffer, pH 7.5, giving an OCP to RCP-to-PB ratio of 40 or 20. Fluorescence was recorded at 23°C, using a pulse-amplitude modulated fluorometer (101-102-103-PAM; Walz). For the optional actinic treatment, samples were illuminated by blue-green light (halogen white light filtered with a Corion cutoff 550-nm filter (400 to 550 nm, 900 μmol photons m−2 s−1).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: A. platensis OCP, YP_005071727; Synechocystis PCC6803 OCP (str1963), NP_441508.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. UV-Visible Spectra and SDS-PAGE of RCP Isolated from Native Source OCP Purifications Performed by a Method Similar to That of Holt and Krogmann (1981).
Author Contributions

R.L.L. designed and performed the research, analyzed and interpreted the data, and wrote the article. C.A.K. designed the research, analyzed and interpreted the data, and wrote the article. D.J., M.L., R.A.M., and D.K. performed research and wrote the article. C.A.K. designed the research, analyzed and interpreted the data, and wrote the article. D.J., M.L., R.A.M., and D.K. acknowledge financial support from the Mathies Royalty Fund.

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Structural and Functional Modularity of the Orange Carotenoid Protein: Distinct Roles for the N- and C-Terminal Domains in Cyanobacterial Photoprotection
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