Cyanobacteria have developed a photoprotective mechanism that decreases the energy arriving at the photosynthetic reaction centers under high-light conditions. The photoactive orange carotenoid protein (OCP) is essential in this mechanism as a light sensor and energy quencher. When OCP is photoactivated by strong blue-green light, it is able to dissipate excess energy as heat by interacting with phycobilisomes. As a consequence, charge separation and recombination leading to the formation of singlet oxygen diminishes. Here, we demonstrate that OCP has another essential role. We observed that OCP also protects Synechocystis cells from strong orange-red light, a condition in which OCP is not photoactivated. We first showed that this photoprotection is related to a decrease of singlet oxygen concentration due to OCP action. Then, we demonstrated that, in vitro, OCP is a very good singlet oxygen quencher. By contrast, another carotenoid protein having a high similarity with the N-terminal domain of OCP is not more efficient as a singlet oxygen quencher than a protein without carotenoid. Although OCP is a soluble protein, it is able to quench the singlet oxygen generated in the thylakoid membranes. Thus, OCP has dual and complementary photoprotective functions as an energy quencher and a singlet oxygen quencher.

INTRODUCTION

The orange carotenoid protein (OCP) is a soluble cyanobacterial protein that binds the keto-carotenoid 3’-hydroxyechinenone (hECN) (Kerfeld et al., 2003; Kerfeld, 2004a; Wilson et al., 2010). OCP is present in the vast majority of cyanobacteria containing phycobilisomes, a large extramembrane antenna formed by blue and red phycobiliproteins. Analysis of the 129 sequenced cyanobacterial genomes shows that 90 of these genomes contain at least one gene for a full-length OCP (Kerfeld and Kirilovsky, 2009). Only OCP is able to bind to phycobilisomes and to quench the energy and photoprotection via the quenching of energy and $1O_2$.

The active chromophore. Absorption of blue-green light by the carotenoid induces conformational changes in both the carotenoid and the protein, changes that are essential for its function in the photoprotective mechanism (Wilson et al., 2008). In darkness, OCP is orange (OCPo); upon illumination with strong blue-green light, it becomes red. The red form (OCPr) is the active form of the protein (Wilson et al., 2008; Punginelli et al., 2009). Only OCP is able to bind to phycobilisomes and to quench the energy they absorb (Giwizda et al., 2011). By increasing the thermal dissipation of the absorbed energy, OCP decreases energy arriving at the photosynthetic reaction centers (Wilson et al., 2006). This photoprotective mechanism is activated by blue-green light but not by orange or red light, which are not absorbed by OCP (Rakhimberdieva et al., 2004; Wilson et al., 2006). OCP has an $\alpha$-helical N-terminal domain (residues 15 to 165) and an $\alpha/\beta$ C-terminal domain (residues 190 to 317). The two domains are joined by a long ($\sim$25 amino acids) and flexible loop linker (Kerfeld et al., 2003; Wilson et al., 2010). OCP has a closed conformation, and OCP has an open conformation, allowing the interaction between the carotenoid and the bilin in the core of phycobilisome (Wilson et al., 2012).

In photosynthetic organisms, carotenoids solubilized in the membranes or attached to membrane proteins have three principal roles: to harvest light energy, to function as a light screen, and to quench the very reactive and damaging singlet oxygen ($1O_2$) (reviewed in Frank and Cogdell, 1996). In thylakoids, the carotenoids present in the membrane-embedded chlorophyll-containing antennae have a dual activity: harvesting light energy and photoprotection via the quenching of energy and $1O_2$. 

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4 Some figures in this article are displayed in color online but in black and white in the print edition.
5 Online version contains Web-only data. www.plantcell.org/cgi/doi/10.1105/tpc.114.123802
Carotenoids also have a protective role in nonphotosynthetic organisms, including humans, serving as a quencher of 1O2 and other reactive oxygen species induced by exogenous sensitizers or produced by metabolic processes (Stahl and Sies, 2003).

Cyanobacteria, like plants and algae, living in an oxygen-rich atmosphere are particularly exposed to oxidative stress provoked by 1O2 because their pigments, chlorophyll and bilins, act as photosensitizers. Especially under high-light conditions, in which the photosynthetic electron transport chain is mostly reduced and the rate of electron transport is saturated, chlorophyll triplets form at higher concentrations in the chlorophyll-containing membrane-bound antennae. Chlorophyll triplets can react with molecular oxygen, leading to the formation of 1O2 (reviewed in Krieger-Liszkay et al., 2008). Exposure of phycobilisomes to high light intensities also induces an increased accumulation of 1O2, most probably due to a reaction of molecular oxygen with triplet states of bilins (Rinalducci et al., 2008). In reaction center II, the formation of 1O2 increases under high irradiance due to an increase of charge recombination reactions and the formation of chlorophyll triplets (reviewed in Rutherford and Krieger-Liszkay, 2001; Vass, 2011, 2012). In the reaction centers, charge recombination reactions can increase even under nonsaturating light conditions when other stresses, like low CO2, low temperature, and nutrient starvation, induce the reduction of the electron carriers of the photosynthetic electron transport chain. Under high-light conditions, the OCP-related energy quenching mechanism is expected to diminish the accumulation of 1O2 by increasing the thermal dissipation of excess energy and thus decreasing the energy arriving at the reaction centers. However, this mechanism is not induced under low-light conditions, even if the photosynthetic electron transport chain becomes reduced due to other stresses. Other photoprotective mechanisms, like the synthesis of Flv2 and Flv4 proteins, which accept electrons from the second electron-accepting plastoquinone of photosystem II (QB) or the plastoquinone pool, are induced under these conditions (Zhang et al., 2009, 2012).

Since carotenoids in plants have dual activities as energy and 1O2 quenchers, we investigated if the carotenoid in OCP has 1O2 quenching activity in addition to its function as an energy quencher. In this work, we studied the ability of OCP to quench 1O2 produced by type II photosensitizers like rose bengal or methylene blue in vitro or by chlorophyll triplets in isolated photosynthetic membranes or whole Synechocystis cells. Our results clearly demonstrate that OCP has a photoprotective function as a 1O2 quencher in addition to its function as an energy quencher in cyanobacterial cells. We also show that isolated OCP is a very good 1O2 quencher. This quality is not a characteristic of all carotenoid proteins, since another soluble carotenoid binding protein, isolated from Anabaena sp PCC 7120, is not a good 1O2 quencher.

RESULTS

The OCP Protein Protects against Photodamage in Orange-Red Light When Its Nonphotochemical Quenching Activity Is Not Induced

In a previous work, we demonstrated that a Synechocystis mutant lacking OCP (∆OCP) was more sensitive to high intensities of white light than Synechocystis wild type (Wilson et al., 2008). Moreover, cyanobacterial strains lacking OCP were photoinhibited faster than strains containing OCP under fluctuating light conditions (Boulay et al., 2008). We attributed this to the fact that those cells lack the OCP-related nonphotochemical quenching (NPQ) mechanism that decreases the energy arriving at the reaction centers by increasing the thermal dissipation of excess energy.

Exposure of wild-type and ∆OCP Synechocystis cells to high intensities of red-orange light also induced photoinhibition, visualized as a decrease of photosystem II (PSII) variable fluorescence (Figure 1A). The variable fluorescence decreased faster in the ∆OCP mutant than in the wild type, suggesting a photoprotective function of OCP even under orange-red illumination. We also examined the effect of a larger concentration of OCP in the cells. We compared the decrease of variable fluorescence in a Synechocystis strain overexpressing OCP and in the ΔpsbA2 background strain; both strains lack the psbA2 gene, and this inactivation increases the sensitivity to photoinhibition compared with the wild type (Figure 1A). The overexpressing OCP strain contains the ocp gene expressed under the control of the strong psbA2 promoter and has OCP concentrations at least eight times higher than in the wild type (Punginelli et al., 2009). The variable fluorescence decreased more slowly in the mutant strain overexpressing OCP than in the ΔpsbA2 background strain and in the wild type (Figure 1A), showing a photoprotective effect of OCP.

In addition, we followed the decrease of PSII activity during the exposure to strong orange-red light by measuring oxygen evolution. The illumination conditions for this experiment differed from those used for the fluorescence measurements (see Methods for details). These samples experienced on average a lower light intensity and oxygen evolution decreased more slowly than fluorescence (Figure 1). Nevertheless, the much higher resistance to high intensities of orange-red light of the overexpressing OCP strain compared with the ∆OCP mutant was also observed when photoinhibition was followed by oxygen evolution measurements (Figure 1B). OCP is photoactivated only by strong blue-green light, which induces carotenoid and protein conformational changes converting OCPo to OCPc (Wilson et al., 2008). Only OCP is able to bind to phycobilisomes and induce energy and fluorescence quenching (Gwizdala et al., 2011). OCP illuminated with strong orange-red light remains orange (Supplemental Figure 1) and cannot bind to phycobilisomes. Thus, the experiments described in Figure 1 show that OCP can photoprotect Synechocystis cells also under conditions in which OCP is not photoactivated and does not induce light energy quenching.

OCP Decreases 1O2 Production in Intact Synechocystis Cells

Since 1O2 production can directly cause photodamage and 1O2 accumulation could be alleviated by a carotenoid-containing protein, we also tested whether OCP can quench 1O2. We measured the production of 1O2 in Synechocystis cells containing or lacking OCP during illumination with orange-red light, using a recently developed method (Rehman et al., 2013) for the detection of 1O2 in cyanobacterial cells, using His-mediated
OCP Is a Singlet Oxygen Quencher

Figure 1. Effect of OCP Amount on the Extent of Red Light–Induced Photodamage.

(A) Variable fluorescence calculated from the initial amplitude of flash-induced chlorophyll fluorescence transients. The data represent mean values obtained from three independent experiments, with the indicated SE values. Synechocystis cells were illuminated with strong red light in the presence of lincomycin, and changes in the activity of PSII were followed by measuring variable chlorophyll fluorescence. The measurements were performed using wild-type (squares) and ΔOCP (circles) cells as well as an OCP-overexpressing mutant (up triangles) and its OCP-containing background strain (down triangles).

(B) Decrease of oxygen evolution activity during exposure to strong red light. The oxygen evolution rate was measured in 1-mL aliquots by using a DW2 oxygen electrode (Hansatech) at 1000 μmol quanta m\(^{-2}\) s\(^{-1}\) visible light intensity in the presence of 0.5 mM 2,5-dimethyl-p-benzoquinone in wild-type (squares) and ΔOCP (circles) cells as well as an OCP-overexpressing mutant (up triangles) and its OCP-containing background strain (down triangles). The data are shown after normalization to their initial values, which was in the range of 170 to 190 μmol oxygen·mg\(^{-1}\) chlorophyll·h\(^{-1}\). The data represent mean values obtained from three independent experiments, with the indicated SE.

The illumination conditions differed for the experiments shown in (A) and (B) (see Methods for details).

chemical trapping. Rehman et al. (2013) previously showed that His penetrates the Synechocystis cells, getting close to the location of \(1\text{O}_2\) formation, and that it does not inhibit PSII activity. They also clearly demonstrated that the oxygen consumption observed during the illumination of Synechocystis cells in the presence of His corresponds to \(1\text{O}_2\) trapping (Rehman et al., 2013). While the oxygen evolution rate is saturated at around 1000 μmol quanta m\(^{-2}\) s\(^{-1}\), the rate of \(1\text{O}_2\) production increases linearly with light intensity without saturation (Rehman et al., 2013). Wild-type and mutant Synechocystis cells were illuminated with strong white light (2300 μmol quanta m\(^{-2}\) s\(^{-1}\)) (Figures 2A and 2B) or red-orange light (1400 μmol quanta m\(^{-2}\) s\(^{-1}\)) (Figure 2A) in the absence and presence of His, and the light-induced oxygen evolution was followed. From the differences in the rates, oxygen uptake and the production of \(1\text{O}_2\) were calculated as described (Rehman et al., 2013). The highest accumulation of \(1\text{O}_2\) was in Synechocystis cells lacking OCP under both illumination conditions (Figure 2). The lowest accumulation was observed in the strain overexpressing ocp. The measurements shown in Figure 2B were repeated in the presence of DCMU, which blocks oxygen evolution by binding to the Q\(_b\) site, or in the presence of DCMU and sodium azide, a strong \(1\text{O}_2\) quencher, to confirm that the His-mediated oxygen uptake is related to \(1\text{O}_2\) trapping (see Rehman et al. [2013] for details). The addition of sodium azide almost completely suppressed the accumulation of \(1\text{O}_2\) (Supplemental Figure 2). These results strongly suggested that OCP could have a \(1\text{O}_2\) quenching activity.

Isolated OCP Quenches \(1\text{O}_2\) in Vitro

To test if OCP can quench \(1\text{O}_2\), OCP was isolated from three different strains that produce high levels of OCP with different carotenoid compositions: Synechocystis wild-type cells overexpressing the slr1963 gene (ocp), Synechocystis ΔcrtR mutant cells overexpressing ocp, and Synechocystis ΔcrtO mutant cells overexpressing ocp. The composition of carotenoids in the OCP isolated from wild-type cells was 60% echinenone (ECN), 30% hECN, and 10% zeaxanthin (Punginelli et al., 2009). The ΔcrtR mutant strain lacks zeaxanthin and hECN, and the OCP isolated from this strain contained 95% ECN (Wilson et al., 2011). The ΔcrtO mutant strain lacks zeaxanthin and hECN, and the OCP isolated from this strain contained 95% zeaxanthin (Punginelli et al., 2009). We isolated OCPs following the previously described method (Wilson et al., 2008) and measured their \(1\text{O}_2\) quenching activity in vitro. Electron paramagnetic resonance (EPR) spin trapping was applied for \(1\text{O}_2\) detection using 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TEMPD-HCl). When this nitrone reacts with \(1\text{O}_2\), it is converted into the stable nitroxide radical, which is paramagnetic and detectable by EPR spectroscopy. The production of \(1\text{O}_2\) was induced by the illumination of two photosensitizers, methylene blue (Figure 3) and rose bengal (Supplemental Figure 2). Figure 3A shows the typical EPR signal of the nitroxide radical obtained after 3 min of illumination (1000 μmol quanta m\(^{-2}\) s\(^{-1}\)) of a solution containing methylene blue and TEMPD-HCl. When we incubated this reaction in the presence of 2 μM OCP, we detected almost no EPR signal, indicating that OCP acts as a \(1\text{O}_2\) quencher. We also observed a direct relationship between increases in the concentration of OCP and the
decrease of the EPR signal. Concentrations of 1.2 to 1.5 μM OCP decreased the EPR signal by 50% (I50) (Figure 3B; Supplemental Figure 2). Similar results were obtained with the three OCPs (wild-type, ECN, and Zea OCPs), although these OCPs have different carotenoids, strongly suggesting that the tertiary structure of the protein is more important than the carotenoid for the 1O2 quenching activity of OCP.

Illumination of Synechocystis thylakoid membranes with strong white light (2000 μmol quanta m⁻² s⁻¹) induced the formation of 1O2 (Figure 4A). This production was detected by EPR spin trapping using TEMPD-HCl. The presence of OCP

![Graph showing the effect of OCP content on the extent of 1O2 production as measured by His-induced oxygen uptake using visible or red light.](image)

**Figure 2.** Effect of OCP Content on the Extent of 1O2 Production as Measured by His-Induced Oxygen Uptake Using Visible or Red Light.

(A) 1O2 production was quantified by measuring the rate of His-mediated oxygen uptake in OCP-containing wild-type (right hatch) and OCP-deficient ΔOCP (left hatch) strains. The measurements were performed using either strong white light (2300 μmol quanta m⁻² s⁻¹) or red-orange light (1400 μmol quanta m⁻² s⁻¹) through a gelatin filter (Kodak Wratten 29). Each bar represents the mean of three independent experiments, with the indicated se.

(B) 1O2 production was quantified by measuring the rate of His-mediated oxygen uptake in OCP-containing wild-type (gray), OCP-deficient ΔOCP (left hatch), as well as OCP-overexpressing (OE) (crosshatch) strains. Since OCP overexpression was achieved in a mutant strain from which the psbA2 gene was deleted, the ΔpsbA2 strain (right hatch) was used as a control for OCP-overexpressing OCP. The measurements were performed using visible light (2300 μmol quanta m⁻² s⁻¹). Each bar represents the mean of three independent experiments, with the indicated se.

![Graph showing the quenching activity of OCP.](image)

**Figure 3.** 1O2 Quenching Activity of OCP.

1O2 was produced by illumination (1000 μmol quanta m⁻² s⁻¹) of 10 μM methylene blue during 3 min in the presence of TEMPD-HCl and in the absence (control) or in the presence of different concentrations of OCP. (A) EPR signals observed in the absence of OCP or in the presence of 2 μM OCP (16 scans).

(B) The percentage of EPR signal decrease versus OCP concentration. Symbols are as follows: ECN OCP (black circles), wild-type OCP (red triangles), and Zea OCP (violet squares). The curve represents the mean of three independent experiments, with the indicated se.

[See online article for color version of this figure.]
during membrane illumination largely decreased the EPR signal, indicating that OCP is able to quench $1{O}_2$ formed in membranes, although it is a water-soluble protein (Figure 4A). This result confirms that OCP could act as a $1{O}_2$ quencher in Synechocystis cells. OCP was also able to quench the $1{O}_2$ formed during the illumination of isolated spinach (Spinacia oleracea) PSII-enriched membrane fragments (Figure 4B). The concentration of $1{O}_2$ formed in this experiment was lower, since the membranes were illuminated for a short time with only 500 μmol quanta m$^{-2}$ s$^{-1}$ in order to avoid destruction of the sample, giving a low signal-to-noise ratio (Figure 4B). Nevertheless, the presence of OCP largely decreased the size of the EPR signal, indicating that OCP is able to quench $1{O}_2$ formed in the spinach PSII reaction center (Figure 4B).

OCP Quenches $1{O}_2$ More Efficiently Than Red Carotenoid Protein, Another Carotenoid Binding Protein

To test whether $1{O}_2$ quenching is a general property of carotenoid-containing proteins, we compared OCP activity with that of another carotenoid protein, red carotenoid protein (RCP), from Anabaena. In addition to the ocp gene, the genomes of several cyanobacterial strains contain one to four copies of genes encoding only the N-terminal domain of OCP (Kerfeld, 2004b; Kerfeld and Kirilovsky, 2013). The function of these genes, named rcp genes, is unknown. Anabaena contains four rcp genes. One of them, al1123, was amplified and cloned in the plasmid pPSBA2 (Lagarde et al., 2000) to express al1123 under the control of the strong psbA2 promoter in Synechocystis cells (see Methods for details). A His tag was added to facilitate the isolation of RCP. Synechocystis wild-type and ΔCrtR mutant cells (Wilson et al., 2011), lacking the β-carotene hydrolase CrtR, were transformed with this plasmid. From the Synechocystis mutants, we used a nickel column to isolate a His-tagged protein of 20 kD. The isolated proteins were red and were recognized by an antibody against the Arthrospira OCP (Supplemental Figure 4A). The absorbance spectrum of RCP presented peaks at 498 and 530 nm and a shoulder at 470 nm (Supplemental Figure 4B). This spectrum is different from those of the orange and red forms of OCP, presenting peaks at 476 and 496 nm and a large peak with a maximum at 510 nm, respectively (Polívka et al., 2005; Wilson et al., 2008; Chábera et al., 2011). These spectral differences relate (at least partially) to the different carotenoids bound to OCP versus RCP. OCP binds ECN and hECN, but the isolated RCPs largely bind myxoxanthophyll and deoxymyxoxanthophyll (Supplemental Figure 5). In addition, the two isolated RCP proteins have different carotenoids: the RCP isolated from the ΔCrtR strain bound 56 to 60% deoxymyxoxanthophyll, 15 to 20% ECN, 2 to 8% canthaxanthin, and 14 to 16% β-carotene, but the RCP isolated from wild-type cells bound 44 to 56% myxoxanthophyll, 28 to 40% zeaxanthin, 10 to 13% hECN, but the isolated RCPs largely bind myxoxanthophyll and deoxymyxoxanthophyll (Supplemental Figure 5). In addition, the two isolated RCP proteins have different carotenoids: the RCP isolated from the ΔCrtR strain bound 56 to 60% deoxymyxoxanthophyll, 15 to 20% ECN, 2 to 8% canthaxanthin, and 14 to 16% β-carotene, but the RCP isolated from wild-type cells bound 44 to 56% myxoxanthophyll, 28 to 40% zeaxanthin, 10 to 13% hECN, 2 to 7% ECN, and 10 to 13% β-carotene (Supplemental Figure 5). These RCP proteins attach almost all kinds of carotenoids present in the cells, with a slightly higher specificity for (deoxy)myxoxanthophylls (Supplemental Figure 5).

The $1{O}_2$ quenching activity of both RCPs was tested (Figure 5). When methylene blue (10 μM) was illuminated (1000 μmol quanta m$^{-2}$ s$^{-1}$) in the presence of TEMPD-HCl for 3 min, we detected the typical EPR signal of the nitroxide radical. This signal disappeared when 4 μM OCP was present during the illumination. By contrast, 10 μM RCP had no effect on the signal (Figure 5A). Even in the presence of 80 μM RCP, ~50% of the signal was still present (Figure 5B). Figure 5B shows that both RCPs have the same efficiency as $1{O}_2$ quenchers. This experiment shows that OCP is at least 40 times more active as a $1{O}_2$ quencher than RCP. The $1{O}_2$ quencher activity of the RCP was also compared with that of the fluorescence recovery protein. This protein of 14 kD, which does not bind any chromophore, is involved in the OCP-related photoprotective mechanism (Boulay et al., 2010). RCP showed the same efficiency of quenching $1{O}_2$ as the fluorescence recovery protein (Figure 5C). Thus, the presence of a carotenoid in the RCP does not affect its power as a $1{O}_2$ quencher.

OCP Quenches $1{O}_2$ by Physical and Chemical Mechanisms

Carotenoids quench $1{O}_2$ by physical and chemical mechanisms. In physical quenching, the carotenoid deactivates $1{O}_2$ by gaining energy from $1{O}_2$ and going to an excited triplet state that then rapidly loses its energy, returning to the ground state. In chemical quenching, the carotenoid is oxidized by $1{O}_2$, which prevents $1{O}_2$ from participating in further damaging reactions. This chemical process is considered to be a minor side reaction, which typically contributes less than 0.05% of the total $1{O}_2$ quenching rate by carotenoids (reviewed in Stahl and Sies, 2003). This reaction might also occur with the OCP carotenoid, since we
observed that under long illumination or in the presence of a high concentration of $^{1}$O$_{2}$, the activity of OCP was slightly reduced. To test this possibility, we followed the changes in the OCP absorbance spectrum when the protein was illuminated by strong white light (1000 $\mu$mol quanta $m^{-2}$ s$^{-1}$) or orange-red light (1000 $\mu$mol quanta $m^{-2}$ s$^{-1}$) in the presence of methylene blue (Figures 6A and 6B). These conditions mimic the conditions of the spin-trap experiments, in which a maximum activity of the OCP was observed after 3 min of illumination. We observed that during the first 3 min of illumination, the spectrum showed almost no variation, but at longer times the amplitude of the OCP absorbance spectrum decreased, indicating bleaching of the carotenoid (Figures 6A and 6B). This result suggests a possible oxidation of the OCP carotenoid when the concentration of $^{1}$O$_{2}$ is high. Since high $^{1}$O$_{2}$ quenching activity was observed already after 3 min of illumination, in which the carotenoid was almost fully intact, we hypothesize that OCP can deactivate $^{1}$O$_{2}$ by physical mechanisms. The carotenoid in the RCP was more resistant to $^{1}$O$_{2}$ attack, suggesting that the carotenoid in this protein could be less exposed than that in the OCP (Figure 6C).

**DISCUSSION**

**OCP Has a Double Role in Photoprotection, Quenching Excitation Energy and $^{1}$O$_{2}$**

We also compared the $^{1}$O$_{2}$ quenching activity of OCP with that of other chemicals well known as $^{1}$O$_{2}$ quenchers: the water-soluble Trolox (a soluble carotenoid derivative), His, and membrane-soluble carotenoids (Figure 7). The $I_{50}$ was 66 $\mu$M for His and 155 $\mu$M for Trolox. Isolated carotenoids are water insoluble, and their antioxidant activity is generally measured in organic solvents (homogeneous environment) in which their activity is maximal. In liposomes and micelles as models of the cell membrane, the $^{1}$O$_{2}$ quenching activity decreases due to the aggregation of carotenoids. This also occurs in water/alcohol mixtures. OCP is not soluble in organic solvents, but in a 50% water/50% ethanol mixture it still presented a rather high $^{1}$O$_{2}$ quenching activity, although less than that observed in water due to partial denaturation (Supplemental Figure 6). Although we are aware that the different solubility of OCP and isolated carotenoids in water and organic solvents complicates direct comparison, we used a mixture of 50% ethanol/50% water to compare the activity of OCP with that of hECN and astaxanthin. We are also aware that in these conditions, OCP and carotenoids have decreased $^{1}$O$_{2}$ quenching activity. The presence of ethanol largely increased the radical EPR signal in the absence of carotenoids or OCP due to a longer lifetime of $^{1}$O$_{2}$. Nevertheless, when rose bengal was illuminated in the presence of OCP or carotenoids, the EPR signal was smaller. The EPR signal was decreased by 65% in the presence of 2 $\mu$M OCP, 29 $\mu$M astaxanthin, or 35 $\mu$M hECN (Figure 7A). The $I_{50}$ for astaxanthin and hECN was 20 and 33 $\mu$M, respectively (Figure 7B).

**OCP is a Better $^{1}$O$_{2}$ Quencher Than Standard Water- or Membrane-Soluble Quenchers**

Figure 5. $^{1}$O$_{2}$ Quenching Activity of RCP.

$^{1}$O$_{2}$ was produced by illumination of 10 $\mu$M methylene blue for 3 min in the presence of TEMPD-HCl and in the absence (control) or the presence of different concentrations of RCP.

(A) EPR signals observed in the absence of RCP or OCP (control) or in the presence of 4 $\mu$M OCP or 10 $\mu$M RCP (16 scans).

(B) The percentage of signal quenching versus RCP concentration. Black circles represent the RCP isolated from the ΔCrtR strain, and triangles (in red online) represent the RCP isolated from wild-type cells. Each symbol represents the mean of three independent experiments, with the indicated SE.

(C) The percentage of signal quenching versus RCP and FRP concentration. Black circles represent the RCP isolated from the ΔCrtR strain, and triangles (in blue online) represent the FRP isolated from Escherichia coli cells. Each symbol represents the mean of three independent experiments, with the indicated SE. [See online article for color version of this figure.]
of $^{1}$O$_2$ is higher in a *Synechocystis* mutant lacking the OCP than in the wild type or in a mutant overexpressing the *ocp* gene, even under strong orange-red light. Under these conditions, OCP is not photoactivated and cannot act as an energy quencher. Thus, under strong orange-red light, OCP must protect the cells from photodamage by another mechanism. We also demonstrate that in vitro OCP is able to quench $^{1}$O$_2$ formed in the membrane in the reaction center II and the chlorophyll antennae, although it is a soluble protein. The strong interaction that OCP has with the photosynthetic membrane (Wilson et al., 2006) can help to quench $^{1}$O$_2$ formed in thylakoids. Our results strongly suggest that in the cells, OCP diminishes the concentration of $^{1}$O$_2$ by direct quenching. OCP activity as an energy quencher needs the photoactivation of the protein, since only OCP$^+$ is able to bind to phycobilisomes (Wilson et al., 2008).

![Figure 6](image1.png)

**Figure 6.** Changes in Absorption Spectra of OCP and RCP Illuminated in the Presence of Methylene Blue.

(A) and (B) OCP was illuminated for 20 min with 1000 μmol quanta m$^{-2}$ s$^{-1}$ white light (A) or orange-red light (filter cut on, 600 nm) (B) in the presence of 5 μM methylene blue (MB).

(C) RCP was illuminated with 1000 μmol quanta m$^{-2}$ s$^{-1}$ white light in the presence of 5 μM methylene blue for 20 min.

![Figure 7](image2.png)

**Figure 7.** Comparison of $^{1}$O$_2$ Quenching Activity of Different Carotenoids.

$^{1}$O$_2$ was produced by illumination (1000 μmol quanta m$^{-2}$ s$^{-1}$) of 10 μM rose bengal for 8 min in the presence of 2,2,6,6-tetramethyl-4-piperidone and 50% ethanol and in the absence (control) or the presence of different concentrations of carotenoids.

(A) EPR signals observed in the absence of carotenoids or in the presence of 2 μM OCP, 29 μM astaxanthin, and 35 μM hECN (32 scans).

(B) The $I_{50}$ of hECN, astaxanthin, water-soluble Trolox, and His.
contrast, the $^{1}{O_2}$ quenching activity is present in both forms of the protein, OCP$^\text{p}$ and OCP$^\text{a}$. We show in this work that the presence of OCP in Synechocystis cells decreases the accumulation of $^{1}{O_2}$, even under orange-red illumination. Under this illumination, OCP is in the OCP$^\text{p}$ state. On the other hand, in vitro, the measurements were done under relatively strong white illumination in order to induce the production of $^{1}{O_2}$ by the photosensitizers. Under these conditions, OCP was mostly orange in the first 1 min and then the concentration of OCP$^\text{p}$ increased with time, arriving at a steady state concentration containing about half of each form (Figure 6; Supplemental Figure 7).

The antioxidant activity of OCP is important to protect against oxidative stress at the level of the photosystems. OCP-related NPQ protects the cells by diminishing the energy arriving at the reaction centers and thereby the accumulation of $^{1}{O_2}$ only under high-light conditions. There exist other conditions in which the electron transport chain is rapidly reduced (like low CO$_2$, cold, etc.), and energy arriving at reaction center II can no longer be used for photosynthesis. Under these conditions, high concentrations of $^{1}{O_2}$ also accumulate, but OCP is not photo-activated. Although other photoprotective mechanisms, like the synthesis of Flv2 and Flv4 proteins, which accept electrons from Q$_b$ or the plastoquinone pool, are induced under these conditions (Zhang et al., 2009, 2012), the fact that OCP can quench $^{1}{O_2}$ largely helps protect the cell. This is also true under high-light conditions; although the OCP-related NPQ mechanism decreases the energy arriving at the reaction centers, they could still receive sufficient energy to produce some $^{1}{O_2}$.

**OCP Has Exceptional $^{1}{O_2}$ Quenching Efficiency in Comparison with Membrane- or Protein-Bound Carotenoids or Water-Soluble $^{1}{O_2}$ Quenchers**

Carotenoids in cyanobacteria and algae are mostly solubilized in membranes or associated with membrane proteins. Few water-soluble carotenoid proteins have been described and characterized (Pilbrow et al., 2012). Recent work reported the isolation of a water-soluble astaxanthin binding protein from a eukaryotic microalga exposed to prolonged desiccation or salt stress (Kawasaki et al., 2013). This protein presented antioxidant properties, but we cannot compare its activity with that of OCP (due to the lack of access to this protein). Here, we isolated another soluble carotenoid protein, RCP, whose gene is present in several cyanobacteria strains (Kerfeld and Kirilovsky, 2013; Kirilovsky and Kerfeld, 2013). The structure of this protein and the position of the carotenoid are still unknown. This protein was proposed to act as a $^{1}{O_2}$ quencher in cells (Chábera et al., 2011; Pilbrow et al., 2012). However, in our hands, its efficiency as a $^{1}{O_2}$ quencher is not higher than that of proteins without carotenoids. OCP shows high $^{1}{O_2}$ quenching activity independently of the bound carotenoid species (i.e., when it binds only ECN or zeaxanthin or a mixture of hECN, zeaxanthin, and ECN). By contrast, RCP did not show higher $^{1}{O_2}$ quenching activity than a protein without carotenoids independently of the carotenoid bound. Our results strongly suggest that differences in the tertiary protein structure have an important role in rendering OCP a better $^{1}{O_2}$ quencher than RCP. The role of the carotenoid species seems to be less important.

Our results also show that OCP is an excellent $^{1}{O_2}$ quencher, better than His or the soluble carotenoid homolog Trolox. To compare OCP activity with those of isolated carotenoids is difficult due to the differences in solubility and the fact that the activity of carotenoids largely varies depending on their environment. The best carotenoid activities as $^{1}{O_2}$ quenchers were obtained in pure organic solvents, while this activity decreased when measured in liposomes, membranes, or lipid vesicles or in water/alcohol mixtures (reviewed in Edge and Truscott, 2009). When the $^{1}{O_2}$ quenching activity is compared in a water/ethanol mixture in which carotenoids and OCP are soluble, the OCP activity is significantly better than those of isolated astaxanthin and hECN. Under this condition, the $^{1}{O_2}$ quenching activity of the OCP is lower than in water, most probably due to partial denaturation of the protein.

We can conclude that the association of a carotenoid with a protein could increase the $^{1}{O_2}$ quenching efficiency of the carotenoid. The relevance of these results to the situation in cells is not clear, and we cannot affirm that OCP is more effective in $^{1}{O_2}$ quenching than the membrane carotenoids. However, our results using *Synechocystis* mutants clearly show that the OCP activity as a $^{1}{O_2}$ quencher protects cells from oxidative stress.

The efficiency of OCP-induced quenching exceeds that of carotenoids, like astaxanthin, which are known to act via energy transfer (Di Mascio et al., 1989; Stahl and Sies, 2003). In this work, we show results suggesting that the carotenoid in the OCP also quenches $^{1}{O_2}$ by physical mechanisms: we observe high $^{1}{O_2}$ quenching activity after 3 min of illumination, when the carotenoid is almost not damaged. However, the oxidation of the hECN, ECN, or amino acids of the protein by $^{1}{O_2}$ could also play a role. Further study is necessary to understand the detailed molecular mechanism of $^{1}{O_2}$ quenching by OCP, especially the background of its very large quenching efficiency. RCP was more resistant to $^{1}{O_2}$ attack (Figure 6). The low efficiency of $^{1}{O_2}$ quenching by RCP is probably caused, at least partly, by the structure of the protein, which may decrease the accessibility of $^{1}{O_2}$ to the carotenoid moiety. Only the resolution of the tertiary structure of this protein can confirm this hypothesis. In the OCP, the carotenoid is not completely buried, and in addition, a channel for oxygen molecules may exist in the interface of the N- and C-terminal domains. This region contains plenty of water molecules in the crystal (Kerfeld et al., 2003; Wilson et al., 2010).

The work presented in this article provides a significant improvement of our understanding of the roles of OCP in cyanobacteria. Our results clearly demonstrate that OCP protects the photosystems and cells via two different mechanisms: by quenching of excess excitation energy (Wilson et al., 2006; Rakimberdieva et al., 2010; Tian et al., 2011), and thereby decreasing charge separations at the reaction centers, and by quenching $^{1}{O_2}$, which is formed during the light reactions that still occur in the reaction centers and in the antennae. These two protecting mechanisms complement each other. Indeed, excitation energy quenching cannot eliminate photochemical reactions completely, and $^{1}{O_2}$ can also be formed at low light intensities when other abiotic stresses are present, conditions in which the OCP energy quenching activity is not activated. Thus, in the cyanobacterial OCP, the carotenoid has a double
photon-protective activity (as energy quencher and as $^{1}\text{O}_2$ quencher), like zeaxanthin in the light-harvesting complex antennae of plants (Frank and Cogdell, 1996).

METHODS

His-Tagged Strain Constructions

The Anabaena sp PCC 7120 atf1123 gene (rcp gene) was amplified using the oligonucleotides NdeI-For and HpaI-Rev. The digested PCR fragment was cloned in the pPSBA2 ampicillin-resistant vector (Lagarde et al., 2000) between the NdeI and HpaI restriction sites. Nucleotides encoding the 6× His tag were added in the 3′ side of the atf1123 gene, and a kanamycin resistance gene of 1.3 kb was inserted. Synechocystis sp 6803 wild-type and ΔCrtR mutant cells were transformed by this plasmid. The construction of ocp-overexpressing strains was described previously (Wilson et al., 2008, 2011).

Purification of OCP, RCP, and FRP

In this study, OCP and RCP proteins were isolated using an Ni-ProBond resin and a Whatman DE-52 cellulose column, as described previously (Wilson et al., 2008). The FRP protein was isolated using the method described previously (Gwizdala et al., 2013).

Purification of Synechocystis Thylakoids and Spinach

PSII Complexes

Synechocystis thylakoids were isolated as described previously (Wilson et al., 2006), and PSII-enriched membrane fragments from market spinach (Spinacia oleracea) were isolated following the protocol described by Johnson et al. (1994).

$^{1}\text{O}_2$ Detection by EPR Spin Trapping

The formation of a nitroxide radical, which is a paramagnetic species arising from the interaction of 2,2,6,6-tetramethyl-4-piperidone with $^{1}\text{O}_2$, was measured by EPR in the absence or presence of different amounts of purified proteins or chemicals in 100 mM Tris-HCl, pH 8.0, 100 mM TEMPO-HCl, and 10 μM rose bengal (Supplemental Figure 2) or 10 μM methylene blue (Figures 3 and 5). The samples were illuminated for 3 min (Figures 3 and 5) or 8 min (Supplemental Figure 2) with strong white light (1400 μmol quanta m$^{-2}$ s$^{-1}$). The FRP protein was isolated using the method described previously (Gwizdala et al., 2013).

$^{1}\text{O}_2$ Detection by His-Induced Chemical Trapping

This method is based on the removal of oxygen from the suspension due to the oxidation of His by $^{1}\text{O}_2$. This effect can be detected and quantified by His-mediated oxygen uptake as described previously (Rehman et al., 2013). Briefly, cyanobacterial cells were washed once and resuspended in a final concentration of 5 μg chlorophyll/mL in fresh growth medium. The rate of white light-induced (2300 μmol quanta m$^{-2}$ s$^{-1}$) or red light-induced (1400 μmol quanta m$^{-2}$ s$^{-1}$) oxygen evolution was measured with and without 5 mM His using a DW2 oxygen electrode (Hansatech), and the difference between the oxygen rates measured with and without His was calculated. The rate of His-mediated oxygen uptake was used as a measure of $^{1}\text{O}_2$ production.

Photoinhibitory Treatments

Photoinhibitory treatment of Synechocystis wild-type and mutant cells was performed in the presence of 300 μg/mL lincomycin, which blocks the protein synthesis-dependent repair of PSII and allows determination of the rate of photodamage. Photoinhibition was performed by illuminating the cell suspension through a gelatin filter (Kodak Wratten 29), which cuts all light below 600 nm and transmits only orange-red light. The light treatment for variable chlorophyll fluorescence measurements was performed by illuminating a 2-mL cell suspension through the red filter directly in the chamber of a Hansatech oxygen electrode, resulting in a light intensity of 550 μmol quanta m$^{-2}$ s$^{-1}$. PSII activity was checked by measuring variable fluorescence in cells, which were moved from the chamber each 15 min into the fluorimeter and then back to the illumination chamber. The initial amplitude of variable chlorophyll fluorescence ($F_v - F_m$) induced by a 2-μs-long saturating light pulse was measured 150 μs after the saturating light pulse. The measurements were performed with the FL 3000 Fluorimeter (Photon Systems Instruments). The photoinhibitory treatment for oxygen evolution measurements (Figure 1B) was performed by illuminating a 100-mL cell suspension (5 μg chlorophyll/mL) through the red filter, resulting in a light intensity of 550 μmol quanta m$^{-2}$ s$^{-1}$ at the surface of the suspension. Due to the large volume compared with the 2-mL cuvette used in the fluorescence experiment, the cells absorbed less light energy and the decrease of the oxygen-evolving activity (Figure 1B) was slower than the decrease of variable fluorescence shown in Figure 1A. The oxygen evolution rate was measured in 1-mL aliquots using a DW2 oxygen electrode (Hansatech) at 1000 μmol quanta m$^{-2}$ s$^{-1}$ visible light intensity in the presence of 0.5 μM 2,5-dimethyl-p-benzoquinone.

Extraction and Analysis of Carotenoids

Samples of purified RCP were extracted with acetone at −20°C for 30 min. After centrifugation, the supernatant was incubated at −20°C for 30 min and then centrifuged. This procedure was repeated five times. The supernatant was evaporated to dryness, dissolved in methanol containing 0.1% NH₄OH, and filtered. Carotenoids from cyanobacteria cells were extracted with methanol containing NH₄OH as described (Punginelli et al., 2009). Liquid chromatography–UV–mass spectrometry analysis was conducted using a Quattro LC instrument (Micromass), MassLytx software, an Alliance 2695 separation module (Waters), and a Waters 2487 dual UV light detector as described (Punginelli et al., 2009). Carotenoids were identified by liquid chromatography–DAD–UV, mass spectrometry, and tandem mass spectrometry analyses and comparison with available standards, native cyanobacteria, previous experiments (Wilson et al., 2008, 2011), or literature (Britton, 1995). Relative quantification was calculated as described elsewhere (Punginelli et al., 2009).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: ocp (slr1963), NP_441508; frp (slr1964), NP_441509; and rcp (slr1123), NP_485166.

Supplemental Data

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

Supplemental Figure 1. Absorption Spectra of Dark Orange OCP and after 20 min of Illumination with Orange-Red Light.

Supplemental Figure 2. Effect of Sodium Azide on the Extent of Singlet Oxygen Production as Measured by His-Mediated Oxygen Uptake.

Supplemental Figure 3. $^{1}\text{O}_2$ Quenching Activity of OCP.

Supplemental Figure 4. Characterization of the All1123 RCP Protein.

Supplemental Figure 5. Carotenoid Analysis.

Supplemental Figure 6. Absorbance Spectra of OCP Wild Type in 50% Ethanol in Dark Conditions and under Illumination.
Supplemental Figure 7. Changes in the Absorption Spectra of OCP under Illumination.

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AUTHOR CONTRIBUTIONS


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The Cyanobacterial Photoactive Orange Carotenoid Protein Is an Excellent Singlet Oxygen Quencher

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