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Influence of zeaxanthin and echinenone binding on the activity of the Orange Carotenoid Protein

Claire Punginelli [a], Adjélé Wilson [a], Jean-Marc Routaboul [b], Diana Kirilovsky [a,*]

[a] Commissariat à l'Energie Atomique (CEA), Institut de Biologie et Technologies de Saclay (iBiTecS) and Centre National de la Recherche Scientifique, Bât 532, CEA Saclay, (CNRS), 91191 Gif sur Yvette, France

[b] Laboratoire de Biologie des Semences, Institut National de la Recherche Agronomique-AgroParisTech, Institut Jean-Pierre Bourgin, 78026 Versailles, France

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ABSTRACT

In most cyanobacteria high irradiance induces a photoprotective mechanism that downregulates photosynthesis by increasing thermal dissipation of the energy absorbed by the phycobilisome, the water-soluble antenna. The light activation of a soluble carotenoid protein, the Orange-Carotenoid-Protein (OCP), binding hydroxyechinenone, a keto carotenoid, is the key inducer of this mechanism. Light causes structural changes within the carotenoid and the protein, leading to the conversion of a dark orange form into a red active form. Here, we tested whether echinenone or zeaxanthin can replace hydroxyechinenone in a study in which the nature of the carotenoid bound to the OCP was genetically changed. In a mutant lacking hydroxyechinenone and echinenone, the OCP was found to bind zeaxanthin but the stability of the binding appeared to be lower and light was unable to photoconvert the dark form into a red active form. Moreover, in the strains containing zeaxanthin-OCP, blue-green light did not induce the photoprotective mechanism. In contrast, in mutants in which echinenone is bound to the OCP, the protein is photoactivated and photoprotection is induced. Our results strongly suggest that the presence of the carotenoid carbonyl group that distinguishes echinenone and hydroxyechinenone from zeaxanthin is essential for the OCP activity.

1. Introduction

Photosynthetic organisms have developed physiological mechanisms allowing acclimation and survival in a wide range of environmental conditions. Most of the mechanisms induced in response to changes in light quality and light intensity are controlled by photosynthesis-mediated changes in cellular redox potential and luminal pH. For example, the redox state of the plastoquinone pool (and/or the cyt b6f) regulates not only the energy distribution between the two photosystems by changing the relationship between the antennae and the reaction centers (state transitions [1,2]) but also regulates the transcription of photosynthetic genes [3–5]. In plants, the low luminal pH, generated during photosynthesis under high irradiance, induces a photoprotective mechanism involving energy dissipation as heat in the antenna (resulting in a detectable quenching of the chlorophyll fluorescence). This mechanism decreases the energy arriving at the Photosystem II reaction center, thus reducing the probability of damage of Photosystem II [6,7]. In cyanobacteria, prokaryotes performing oxygenic photosynthesis and playing a key role in global carbon cycling, a process decreasing the energy transfer between the antenna and the reaction centers has recently been observed [8–11]. However, this process is not triggered by a lowering of the luminal pH or a change in the redox state of the plastoquinone pool (PQ) [9–11]. Instead, the decrease of energy reaching the reaction centers and the quenching of the antenna fluorescence is induced by the light activation of the Orange-Carotenoid-Protein (OCP) [11], a soluble 35 kD protein containing a single-non covalently bound carotenoid [12–14]. The absorbance of blue-green light by the OCP induces structural changes in the carotenoid and the protein, converting its dark stable orange form into a relatively unstable active red form [15]. The presence of the red OCP form increases the thermal dissipation of the energy absorbed by the phycobilisomes, the external PSII antenna in cyanobacteria. This results in the decrease of the phycobilisome fluorescence emission and of the energy transfer from the phycobilisome to the reaction centers [9–11,15]. In the absence of OCP [11] or in the absence of the red OCP form [15], no fluorescence quenching is induced by strong white or blue-green light and cells are more sensitive to high light [11]. The OCP conversion from the orange to the red form occurs with a very low quantum yield (about 0.03); probably because the OCP protein is involved in a process that must be induced only under high light conditions [15].

In *Synechocystis* PCC6803 (hereafter called *Synechocystis*), the strain used for all the studies about the OCP-related-photoprotective mechanism, the OCP is encoded by the *slr1963* gene [14] and it is constitutively expressed. It is present even in *Synechocystis* mutants lacking phycobilisomes [16]. Stress conditions (high light, salt stress, iron starvation) increases the levels of OCP transcripts and proteins

* Corresponding author. Tel.: +33 1 69089571; fax: +33 1 69088717.
E-mail address: diana.kirilovsky@cea.fr (D. Kirilovsky).

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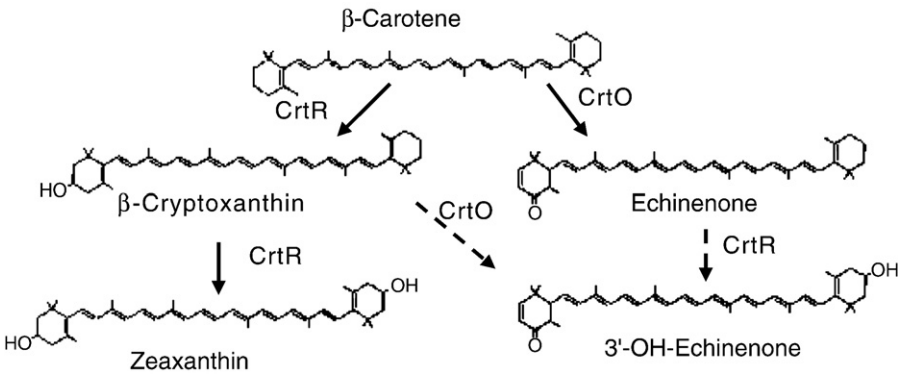

Fig. 1. Zeaxanthin, echinenone and 3′-hydoxyechinenone synthesis in *Synechocystis* PCC 6803. Dashed arrows indicated the two possible pathways for the synthesis of 3′-hydroxyechinenone.

[16–18]. Many cyanobacteria, within a large phylogenetic range and from diverse habitats, contain homologs of the *Synechocystis slr1963* gene. Seven of these strains have been studied showing that the whole OCP protein is present in each of them [19]. All of these strains are able to perform blue-green light induced fluorescence quenching indicating that the OCP related photoprotective mechanism is widespread in cyanobacteria [19]. In contrast, in the few OCP-lacking strains the mechanism appeared to be absent. These results strongly suggest that the blue-green light induced photoprotective mechanism is correlated to the presence of the OCP gene [19].

The structure of the OCP isolated from *Arthrospira maxima* was determined to 2.1 Å [20]. The OCP is a dimer in solution and the intermolecular interactions are largely mediated by hydrogen bonding among the N-terminal 30 amino acids. The OCP has two domains: an α-helical N-terminal domain and an α/β C-terminal domain. Both *A. maxima* and *Synechocystis* WT OCPs bind the keto carotenoid, hydroxyechinenone (hECN) [15,20] that is composed of a conjugated carbonyl group located at the terminus of a conjugated chain of 11 carbon-carbon double bonds. The carotenoid spans both protein domains with its keto terminus nestled within the C-terminal mixed α/β domain and it is in an all-*trans* configuration [20].

In *Synechocystis* the major carotenoids accumulated are β-carotene, myxoxanthophyll, zeaxanthin and echinenone [21]. Zeaxanthin and echinenone are derived from the same precursor, the β-carotene. The β-carotene hydroxylase [22] (encoded by the *crtR* gene) and the β-carotene monoketolase [23] (encoded by the *crtO* gene) catalyze the formation of zeaxanthin and echinenone respectively (for reviews see [24–26]). The hydroxyechinenone can be synthesized from the zeaxanthin (or cryptoxanthin) by CrtO or from echinenone by CrtR [27,28] (Fig. 1).

Since in both *Synechocystis* and *A. maxima*, the OCP binds the keto-carotenoid hydroxyechinenone, we investigated in this work whether other carotenoid can also bind to the OCP and induce the OCP-phycobilisome related photoprotective mechanism. For this purpose, two mutants were constructed and characterized: a mutant lacking the CrtO enzyme in which echinenone and hydroxyechinenone are absent and a mutant overexpressing the OCP gene (*slr1963*). In *Synechocystis* cells there is only a small quantity of hydroxyechinenone and that may not be sufficient to bind to the large amount of OCP present in the overexpressing OCP strain. Thus, in this mutant other carotenoids are likely to bind the OCP protein.

2. Materials and methods

2.1. Culture conditions

The mesophylic freshwater cyanobacteria *Synechocystis* PCC 6803 WT and mutants were grown photoautotrophically in a modified BG11 medium [29] but containing double the amount of sodium nitrate. Cells were kept in a rotary shaker (120 rpm) at 30 °C, illuminated by fluorescent white lamps giving a total intensity of about 30–40 μmol photons m^{-2} s^{-1} under a CO_2-enriched atmosphere. The cells were maintained in the logarithmic phase of growth and were collected at OD_{800}=0.6–0.8. For OCP isolation, cyanobacteria cells were grown in 3L Erlenmeyers in a rotary shaker under a light intensity of 90–100 μmol photons m^{-2} s^{-1}. The cells were harvest at OD_{800}=1.

2.2. The ΔCrtO mutant construction

A 2.5 kb region surrounding the *crtO* gene was amplified by PCR from genomic DNA from *Synechocystis* PCC 6803 using restriction sites-creating primers CrtO1 (5′-GCG CCT CGA GCG TTG CAA ATT TAC CAA G-3′) and CrtO3 (5′-GCG CAC TAG TAG CCG TGG TGC ATA TG-3′). The insert was then cloned into pBluescript SK (+) vector using the restriction sites *XhoI* and *SpeI* engineered by the amplifying primers. Disruption of the *crtO* gene was obtained by removing a 1.37 kb fragment, containing a large part of the *crtO* coding sequence including the initiation codon, by digestion with BclI. A 2.1 kb DNA fragment containing the aadA gene from Tn7 conferring resistance to spectinomycin and streptomycin (Sp/Sm resistance cassette) previously digested by *BamHI* was then inserted in the *BclI* sites destroying them both. The construction obtained was used to transform the *Synechocystis* 6803 WT, the *Synechocystis* His-tagged OCP strain and the *Synechocystis* strain overexpressing the OCP by double recombination. The construction of the His-tagged OCP and the overexpressing His-tagged OCP were described in [15].

2.3. Purification of the Orange Carotenoid Protein

In this study, we used OCP isolated from the *Synechocystis* mutants: Km-resistant His-tagged OCP [15], ΔCrtO-His-tagged OCP, overexpressing His-tagged OCP [15] and ΔCrtO-overexpressing His-tagged OCP. His-tagged OCP mutant cells (1 mg Chl ml^{-1}) in 0.1 M Tris–HCl pH=8 buffer were broken in dim light using a French Press. The membranes were pelleted and the supernatant was loaded on a column of Ni-ProBond resin. The OCP was further purified on a Whatman DE-52 cellulose column. More details of the purification are described in [15].

2.4. Absorbance measurements

Cell absorbance was monitored with an $UVIKON_{XL}$ spectrophotometer (SECOMAN, Alès). Chl content was determined in methanol using the extinction coefficient at 665 nm of 79.24 mg mL^{-1} cm^{-1}. The orange to red photoconversion was monitored in a Specord S600 (Analyticjena, France) spectrophotometer during illumination of the

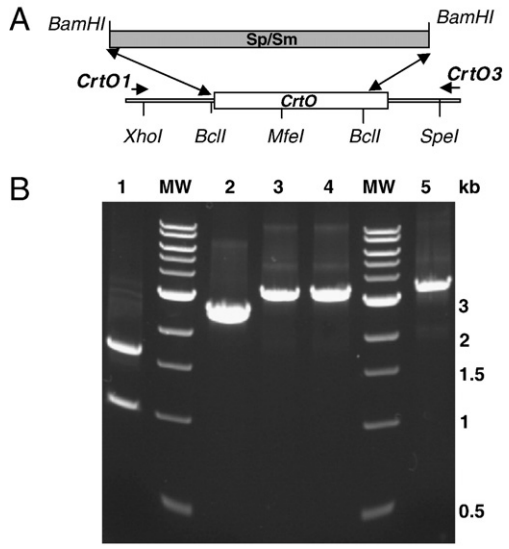

Fig. 2. ΔCrtO mutant construction. (A) Gene arrangement of the *crtO* gene and flanking regions. The primers used in PCR amplification are indicated by arrows. The sites of cut of the different restriction enzymes and the replacement of the *crtO* gene by the Sp/Sm resistance cassette are also indicated. (B) Amplification of genomic DNA containing the *crtO* gene isolated from WT (lane 2), ΔCrtO (lane 3) and ΔCrtO-overexpressing OCP (lane 4) using crtO1 and crtO3 oligonucleotides. MfeI digestion of the DNA amplified fragment from WT (lane 1) and from ΔCrtO-overexpressing OCP (lane 5).

OCP with 1200 μmol photons m^{-2} s^{-1} of blue-green light (400–550 nm) at 12 °C. For the kinetics experiments, the light intensity (350, 700, 1000 μmol photons m^{-2} s^{-1}) or the temperature (7, 15, 25, 32 °C) were modified.

2.5. Carotenoid characterization

Carotenoids were extracted from OCP proteins as previously described [30] and from *Synechocystis* cells with methanol–NH_4OH 0.01%. Liquid chromatography-mass spectrometry analysis was conducted on aliquots of the dried extract dissolved in 400 μl of acetonitrile/water (90:10, v:v) using a Quattro LC instrument with an ESI « Z-spray » interface (Micromass, Manchester, U.K.), MassLynx software, an Alliance 2695 separation module (Waters, Milford, MA), and a Waters 2487 dual UV detector set at 450 nm. Separation was achieved on two 100×4.6 mm and 150×4.6 mm, reverse-phase Adsorbosphere HS C18 3 mm columns in series (Alltech, Templemars, France) using a linear 20-min gradient of ethyl acetate (0 to 95%) in acetonitrile/water/triethylamine (9:1:0.01, v:v:v) at a flow rate of 0.5 ml/min and at a temperature of 30 °C. Pigments were further detected with a photodiode-array detector (Beckman-Coulter, http://www.beckmancoulter.com). Peak identification is based on comparison of retention times and absorption spectra to commercially available standards (Extrasynthèse, http://www.extrasynthese.com), or published values [31], comparison with native cyanobacteria and mass spectrometry spectra [15]. Relative quantification (% of total carotenoids) is based on response ratio of pigments measured at 450 nm and at λ max, and ε published in [31]. Mass analysis was conducted as previously published [15].

2.6. Fluorescence measurements

Cell fluorescence was monitored with a pulse amplitude modulated fluorometer (101/102/103-PAM; Walz, Effelrich, Germany). All measurements were carried out in a stirred cuvette of 1 cm diameter at growth temperature (32 °C). After illuminating dark-adapted cells with low irradiance of blue-green light (400–550 nm, 80 μmol photons m^{-2} s^{-1}) for about 1 min, fluorescence quenching was induced by 740 μmol photons m^{-2} s^{-1} of blue-green light for about 200 s and the cells were then shifted back to low blue-green light.

The minimal fluorescence level in dark-adapted samples (Fo) was determined by illuminating dark-adapted cells with a low intensity, modulated light that preferentially excited the major phycobiliprotein (1.6 kHz, 0.024 μmol photons m^{-2} s^{-1}, 650 nm). Saturating pulses (2000 μmol photons m^{-2} s^{-1}) were applied to measure the maximal fluorescence levels Fm_d and Fm' in dark-and light-adapted samples, respectively. Application of such pulses that transiently close all the PS II centers serves to distinguish between photochemical quenching and non-photochemical quenching.

2.7. Membrane-phycobilisome bound (MP) preparations and OCP immunodetection

Freshwater cyanobacteria cells (at a 1 mg Chl mL^{-1} concentration) were resuspended in a 0.7 M K-phosphate/0.3 M Na-Citrate (pH 6.8) buffer and broken in a mini-bead beater using glass beads. Beads and unbroken cells were discarded by mild centrifugation and the MP fractions were collected by centrifugation at 20,000 ×g and frozen at −80 °C until used for gel electrophoresis. MP fractions of freshwater cyanobacteria were analyzed by SDS-PAGE on a 12% polyacrylamide/2 M urea in a TRIS/MES system [32]. The OCP protein was detected using a polyclonal antibody raised against the OCP [16].

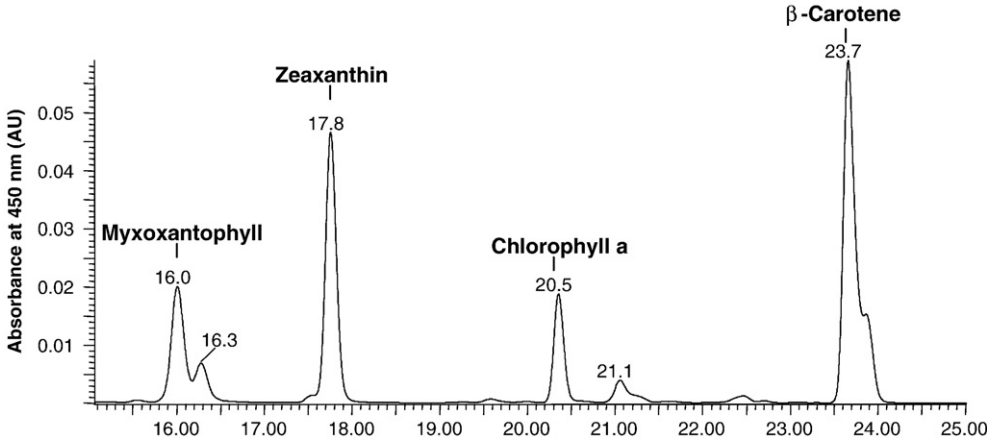

Fig. 3. HPLC analysis of carotenoids isolated from ΔcrtO cells.

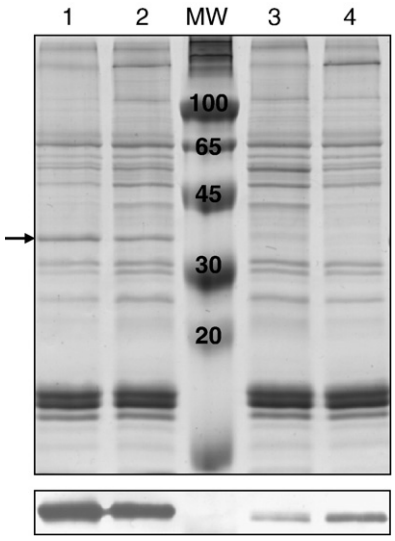

Fig. 4. Immunodetection of OCP. Coomassie blue-stained gel electrophoresis and OCP immunoblot detection (bottom panel) in membrane-phycobilisome fractions from over-expressing His-tagged OCP (lane 1), ΔCrtO-overexpressing His-tagged OCP (lane 2), ΔCrtO (lane 3) and WT (lane 4). The arrow indicates the OCP. Each lane contained 0.5 μg chlorophyll.

3. Results

3.1. Construction of mutants

To obtain *Synechocystis* mutants lacking the carotenoids echinenone and hydroxyechinenone, the genome region containing the *crtO* gene, which codes for the β-carotene monoketolase, was amplified by PCR and cloned. A plasmid in which the *crtO* gene was deleted and replaced by a Sp/Sm resistance cassette was then constructed (see Materials and methods and Fig. 2A). This plasmid was used to transform the wild-type (WT), a His-tagged OCP strain and an overexpressing His-tagged OCP strain. The construction of the His-tagged OCP and the overexpressing His-tagged OCP strains has been already described [15].

The construction allowed us to perform mutant selection by growing cells in the presence of antibiotics. To confirm the replacement of the *crtO* gene by the antibiotic resistance cassette and the complete segregation of the mutant, PCR analysis and DNA digestion by specific restriction enzymes were performed. Fig. 2B shows that amplification of the genomic region containing the *crtO* gene using the synthetic crtO1 and crtO3 oligonucleotides gave a fragment of 2.5 kb in the WT and of 3.2 kb in the mutants containing the spectinomycin/streptomycin cassette. No traces of the 2.5 kb fragment were detected in these mutants indicating complete segregation.

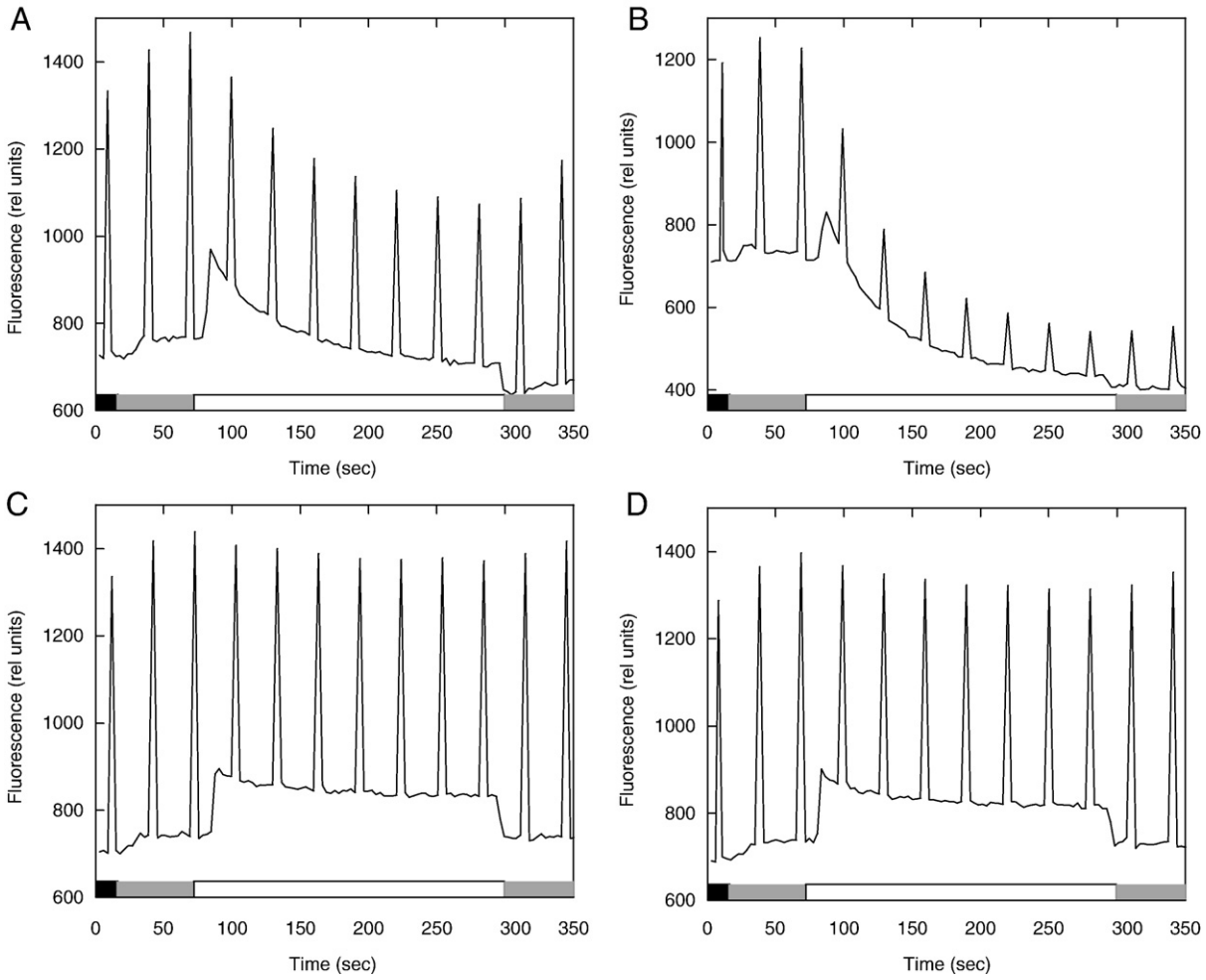

Fig. 5. Blue-green light induced fluorescence quenching. Dark-adapted cells (at 3 μg Chl mL^{-1}) of WT (A), overexpressing His-tagged OCP (B), ΔCrtO (C), and ΔCrtO-overexpressing His-tagged OCP (D) strains were illuminated successively with low (80 μmol photons m^{-2} s^{-1}) and high (740 μmol photons m^{-2} s^{-1}) blue green light (400–550 nm) and then again with low blue-green light. Fluorescence yield changes were detected with a PAM fluorometer and saturating pulses were applied to measure maximal fluorescence levels (Fm').

Moreover, while the 2.5 kb WT-fragment was completely digested by the *MfeI* restriction enzyme, the mutant-fragment was not digested at all (Fig. 2). These results indicated complete replacement of the *crtO* gene by the Sp/Sm resistance cassette in the mutant cells.

Carotenoid analysis was carried out on ΔCrtO mutant cells. Carotenoids were extracted from cells with methanol–NH_4OH 0.01% and identified by liquid chromatography-mass spectrometry based on their retention time, absorption spectra and mass spectrometry spectra. Fig. 3 shows the carotenoid content of the ΔCrtO strain. β-carotene, zeaxanthin and myxoxanthophyll are the principal carotenoids present in these cells and in those of the other mutants lacking crtO gene (data not shown). The peaks corresponding to hydroxyechinenone and echinenone (18.0 and 21.3 min, respectively) seemed to be absent in the ΔCrtO mutant cells (Fig. 3). A similar mutant deleted of the *crtO* gene has already been generated and characterized but no evident phenotype was observed in the echinenoneless strain [27,33].

3.2. The OCP content in the different mutants

We have already demonstrated that there is a relationship between the quantity of OCP present in the cells and the excitation energy quenching [15,16]. Western-Blot analyses were undertaken to compare the quantity of OCP present in the different mutants. Fractions containing the phycobilisomes attached to the membrane (MP) containing all the OCP present in the cell [11] were isolated from four strains, WT, ΔCrtO, His-tagged overexpressing OCP and the double mutant ΔCrtO/His-tagged overexpressing OCP. Their proteins were separated by SDS-PAGE and the OCP was detected using an anti-OCP antibody [16] (Fig. 4). The antibody reacts with a 35-kD polypeptide. As expected, in the overexpressing OCP strains, the amount of OCP observed was much higher than in the WT (about ten times) (Fig. 4 lower panel). The 35-kD OCP band was even visible in the Coomassie Blue staining of the gel in the overexpressing OCP strains while it was invisible in the WT (Fig. 4 upper panel). On the other hand, in the ΔCrtO strains, less OCP was visualised suggesting a destabilization of the protein in the absence of hydroxyechinenone (Fig. 4 lower panel).

3.3. The OCP-related photoprotective mechanism in the different mutants

The OCP-related photoprotective mechanism involves an increase of energy dissipation as heat in the phycobilisome. This results in a detectable decrease (quenching) of the phycobilisome fluorescence that can be followed by measurements in a PAM fluorometer. In a PAM fluorometer, the measuring light that has a maximum of excitation at 650 nm is not only absorbed by the chlorophyll (Chl) but also by the phycobilisomes. Thus, in cyanobacteria, the fluorescence detected by a PAM fluorometer, is emitted from Chl and phycobiliproteins [34]. Blue-green light, depending on its intensity, may induce an increase of the fluorescence due to a State 1 transition [34] or may induce a decrease of fluorescence related to the OCP-related mechanism [11,35].

The effect of the blue-green light was monitored using a PAM fluorometer in the four strains: the WT, the overexpressing OCP strain, the ΔCrtO mutant and the double mutant ΔCrtO-overexpressing OCP (Fig. 5). Illumination of dark-adapted WT cells by dim blue-green light (which preferentially excites PSI) increased fluorescence levels, indicating a transition to State I induced by the oxidation of the PQ pool (Fig. 5A). Subsequently, exposure of dim-light adapted cells to high blue-green light intensities (740 μmol photons m^{-2} $s^{-}1$) induced a quenching of all fluorescence levels (Fig. 5A). In the overexpressing OCP mutant (Fig. 5B), the increase of fluorescence observed upon illumination, associated to “State1” transition, was small. This is due to the fact that dim blue-green light induced fluorescence quenching in this strain (second flash lower than first one and data not shown). Then, a huge quenching of Fm' and Fs was observed under strong blue-green illumination; after 1 min, the level of Fm' was lower than that of the initial Fo (Fig. 5B). As already described by Wilson et al. [15] there is a correlation between the higher concentration of OCP present in the His-tagged overexpressing-OCP strain and the larger fluorescence quenching observed in this mutant. No fluorescence quenching was induced in the two mutants lacking CrtO (Fig. 5C and D), even under very high light intensities (1200 μmol photons m^{-2} s^{-1}, data not shown). The absence of the blue-light induced quenching in the ΔCrtO mutant could be justified by the low concentration of OCP in this mutant. However no fluorescence quenching was observed in the double mutant strain in which a massive amount of protein was present. Thus, the absence of the light induced photoprotective mechanism is not related to a low concentration of OCP in the ΔCrtO mutants.

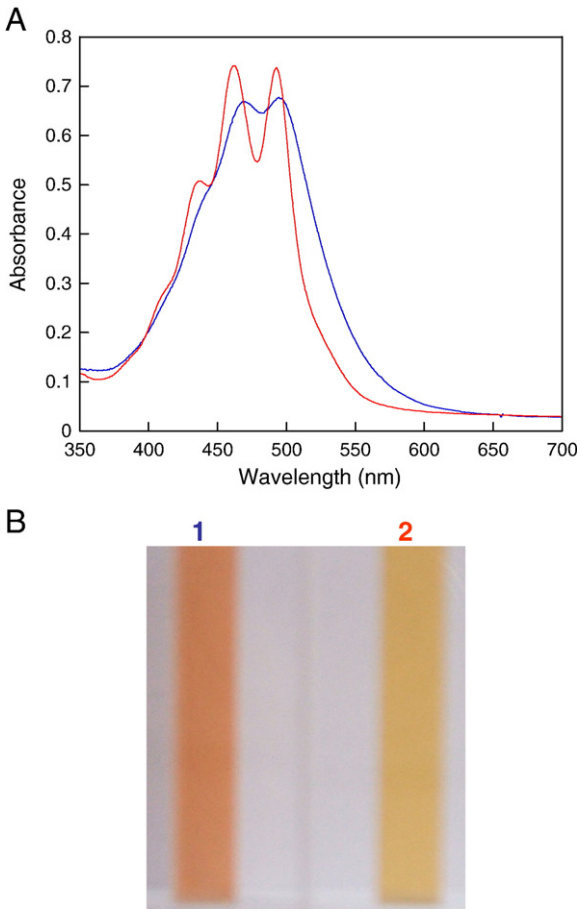

Fig. 6. The isolated OCP protein. Absorbance spectra and photograph of the OCP isolated from the overexpressing His-tagged OCP strain (blue, 1) and the ΔCrtO-overexpressing His-tagged (red, 2). The absorbance spectrum and the color of the OCP isolated from the His-tagged OCP strain (containing more than 95% hydroxyechinenone) [15] was identical to that of the OCP isolated from the overexpressing His-tagged OCP strain.

3.4. The isolated OCP protein

The OCP was isolated from the His-tagged OCP strains using affinity Ni-chromatography followed by an ion-exchange column [15]. In darkness or dim light, the OCP isolated from the His-tagged OCP and the overexpressing His-tagged OCP strains appeared orange and their absorption spectra were similar (Fig. 6). The OCP spectra of both strains, presented a typical carotenoid shape, reflecting the

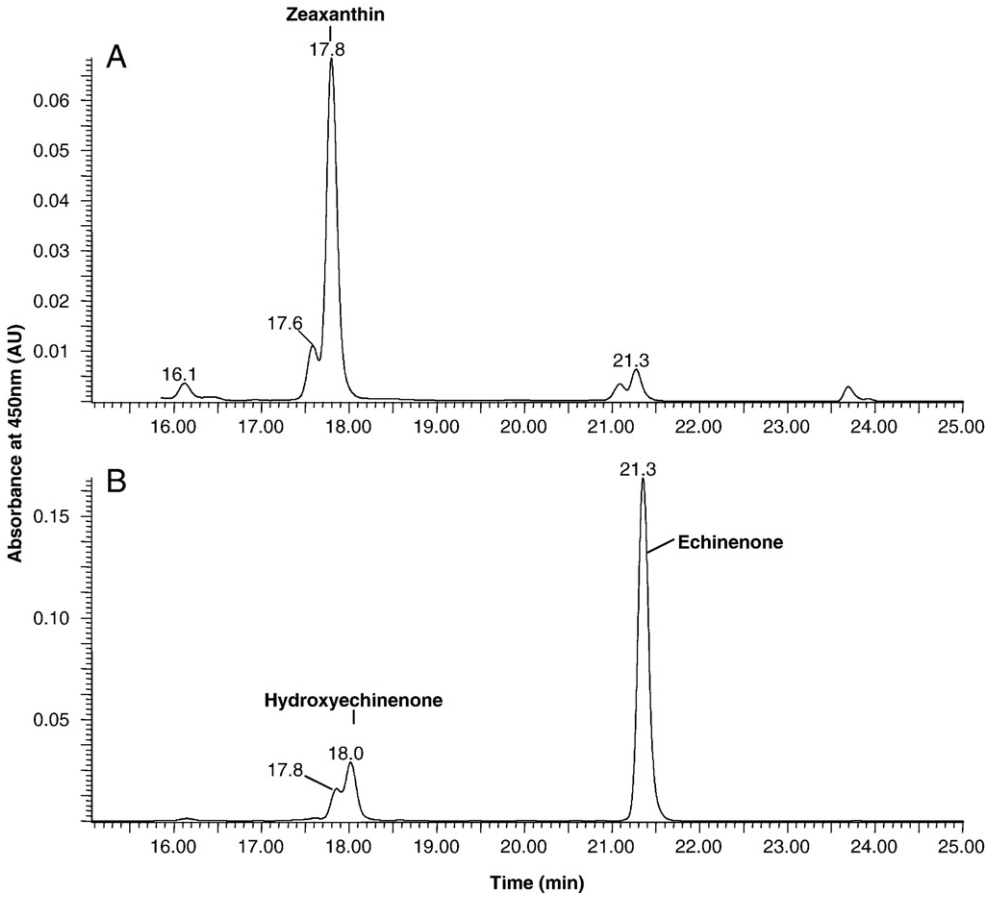

Fig. 7. HPLC analysis of carotenoids bound to the OCP isolated protein. ΔcrtO-overexpressing OCP strain (A) and to the OCP isolated from the overexpressing OCP strain (B). The ratio between hydroxyechinenone and echinenone changes in the different OCP samples isolated from the overexepressing OCP strain (see text for more details).

strongly allowed S_0–S_2 transition with three distinct vibrational bands; the 0–0 vibrational peak located at 496 nm [15,30]. In contrast, the OCP proteins isolated from the strains lacking CrtO, appeared yellow and their spectrum was completely different to that of the orange protein (Fig. 6). The resolution of the vibrational peaks was higher in the OCP isolated from the ΔCrtO strains and a blue-shift (2–3 nm) of the maximum of the three vibrational bands was visible (Fig. 6).

The carotenoid content of these different OCP proteins was analyzed (Fig. 7). While the WT His-tagged OCP contained mostly hydroxyechinenone with only traces of echinenone [15], the over-expressed His-tagged OCP contained large quantities of echinenone. In this strain, depending on the preparations, the echinenone content varied from 53 to 82% of total carotenoid, while the hydroxyechinenone content varied from 11 to 32%. Zeaxanthin was also present in the OCP preparation from overexpressing OCP strain with a content varying from 6 to 14%. The OCP isolated from the ΔCrtO strain contained mostly zeaxanthin (about 80%) but also other carotenoids (in much lower quantities) were bound to this OCP. Traces of a carotenoid that seems to be echinenone were found attached to this ΔCrtO-OCP suggesting that there is another enzyme that could synthesize very low quantities of echinenone or that the mutant was not 100% segregated (even though this was not detected by the DNA tests).

Upon illumination with blue-green light (400–550 nm) at 10 °C, the orange His-tagged OCP was completely photoconverted to a red form (Fig. 8A). The red-shifted spectrum of the red OCP with a maximum at 510 nm loses the resolution of the vibrational bands [15]. In contrast illumination of the OCP isolated from the ΔCrtO mutant did not change the color and the spectrum of the protein (Fig. 8B). Thus, the zeaxanthin-OCP is not photoactive; or at least, does not have a relatively stable light-form.

The His-tagged OCP isolated from the overexpressing strain was not completely coverted to the red form (Fig. 8C–E). The final spectrum was dependent on the zeaxanthin content. The spectra shown in Fig. 8C and E, which can be simulated by the combination of the absorbance spectrum of the OCP red form and of that of the zeaxanthin (94% red form/6% zeaxanthin and 83% red form/17% zeaxanthin respectively), corresponded to overexpressed-OCP samples binding 6% and 14% zeaxanthin respectively.

The spectra shown in Fig. 8C and D, which are similar, corresponded to overexpressed-OCP samples binding different quantities of echinenone: 82% echinenone, 11% hydroxyechinenone and 53% echinenone, and 32% hydroxyechinenone respectively. Therefore, the final spectrum was not dependent of the ratio echinenone to hydroxyechinenone found in the OCP preparations. Furthermore, the kinetics of the orange to red photoconvertion at different light intensities as well as the red to orange dark-conversion at different temperatures were similar in the WT-OCP and the over-expressed OCP (data not shown). This confirmed that the hydroxyechinenone can be functionally replaced by the echinenone in the OCP.

4. Discussion

We have previously demonstrated that the absorption of blue-green light by the 3′hydroxyechinenone induces changes in the carotenoid and the protein causing the conversion of the dark stable

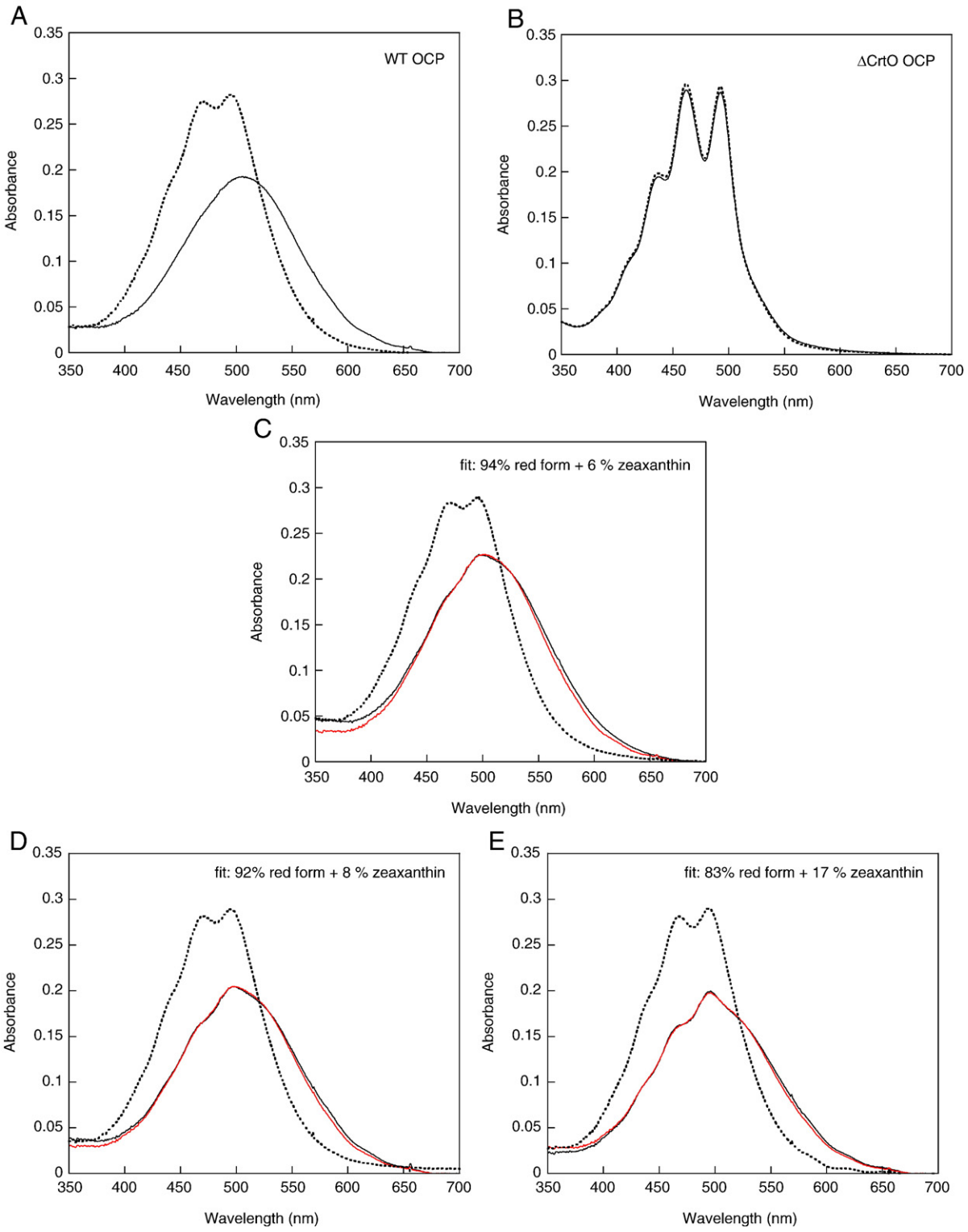

Fig. 8. Photoactivity of the isolated OCP. Absorbance spectra of the dark (dotted line) and light (solid line) forms of the OCP isolated from the His-tagged OCP strain (A), the ΔCrtO-overexpressing His-tagged OCP strain (B) and three different preparations of OCP isolated from the overexpressing His-tagged OCP strains (C–E). The fit of the light spectra using a combination of the spectrum of the red form and the zeaxanthin-OCP spectrum is shown in red. To obtain the spectrum of the light form, the isolated OCP was illuminated with 1200 μmol photons m^{-2} s^{-1}, at 12 °C, during 5 min.

orange OCP form to the metastable red OCP form [15]. The accumulation of this red form is essential for the induction of the photoprotective mechanism [15]. Here, we show that while echinenone can replace the hydroxyechinenone in its function in the photoprotective mechanism zeaxanthin cannot. The OCP can bind the zeaxanthin. However the stability of the binding seemed to be lower since less OCP protein was present in the ΔCrtO strains. Blue-green light had no effect on the absorbance spectrum (and the color) of the zeaxanthin-OCP indicating that this protein was unable to accumulate a metastable “red” zeaxanthin form. Moreover, in the

strains containing zeaxanthin-OCP, light was unable to induce any fluorescence quenching although the zeaxanthin-OCP absorbs very well the blue-green light. Therefore, the stabilization of the red form is essential for the induction of the fluorescence quenching. This is in agreement with previous results showing that in *Synechocystis* cells containing a point-mutated OCP that was unable to stabilize the red form, blue-green light did not induce fluorescence quenching and the photoprotective mechanism [15].

The three major carotenoids that were found bound to the OCP contain a central chain of 11 conjugated double bonds and differ in the oxygenated groups of their rings. The zeaxanthin has two hydroxyl groups, one in each ring (sites 3 and 3′). The echinenone and hydroxyechinenone have a carbonyl group (site 2). In addition, the hydroxyechinenone has an additional hydroxyl group. This group is not required for the activity of the OCP since echinenone behaves like hydroxyechinenone and both can generate the red OCP form. In contrast, the carbonyl group seems to be essential for the OCP activity. Zeaxanthin lacking the carbonyl group renders the OCP inactive.

We hypothesize that the lack of the activity of the zeaxanthin-OCP is associated to changes in the interaction and/or energy transfer between the phycobilisome and the OCP. This proposition requires testing in the coming years.

The carbonyl moiety at the keto terminus of hydroxyechinenone (or echinenone) forms hydrogen bonds with the absolutely conserved Tyr203 and Trp290 residues, belonging to the C-terminal domain of the OCP [20]. We had hypothesized that this part of the protein, by interacting with the centre of an allophycocyanin (APC) trimer in the phycobilisome core, may bring the carotenoid into proximity of the APC chromophores [15]. Modifications of the secondary structure of the protein could be induced by the binding of zeaxanthin due to the lack of the carbonyl group and avoid the required contact between the APC trimer and the OCP. In addition, if light induced changes in the carbonyl-protein interactions are required to induce energy transfer and dissipation, in the zeaxanthin-OCP these modifications are probably not happening. Finally, if alteration of the strength of the hydrogen bonds between the carbonyl and Tyr203 and/or Trp290 forms a signal propagation pathway from the carotenoid to the surface of the protein like in other photoactive proteins (PYP and LOV domains [36,37]), then this is not occurring in the zeaxanthin-OCP.

We hypothesized that the OCP could be the energy and fluorescence quencher and that the red shift of the hydroxyechinenone spectrum is necessary to tune the optically forbidden S1 state of hydroxyechinenone to a position allowing the energy transfer from the phycobilisome to the OCP [15]. The absence of the carbonyl group in the zeaxanthin may avoid the red-shift by preventing the increase of the apparent conjugation length of the carotenoid or the stabilization of a more planar structure observed in the hydroxyechinenone-OCP. As a consequence, the zeaxanthin-OCP due to the lack of a metastable “red” form is unable to absorb and quench the energy coming from the phycobilisome.

We conclude that the carbonyl group of the ketocarotenoids, echinenone and hydroxyechinenone, is essential for the photoactivation of the OCP and the induction of the phycobilisome-related-mechanism. Our results suggest that the hydrogen bond between the carbonyl and the Tyr203 and Trp290 could play a role in the photoconversion between the orange and the red forms.

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