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Essential role of two tyrosines and two tryptophans on the photoprotection activity of the Orange Carotenoid Protein

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ABSTRACT

Photosynthetic organisms have developed photoprotective mechanisms to protect themselves from lethal high light intensities. One of these mechanisms involves the dissipation of excess absorbed light energy into heat. In cyanobacteria, light activation of a soluble carotenoid protein, the Orange Carotenoid Protein (OCP), binding a keto carotenoid, is the key inducer of this mechanism. Blue-green light absorption triggers structural changes within the carotenoid and the protein, leading to the conversion of a dark orange form into a red active form. Here we report the role in photoconversion and photoprotection of individual conserved tyrosines and tryptophans surrounding the rings of the carotenoid. Our results demonstrate that the interaction between the keto group of the carotenoid and Tyr201 and Trp288 is essential for OCP photoactivity. In addition, these amino acids are responsible for carotenoid affinity and specificity. We have already demonstrated that the aromatic character of Tyr44 and Trp110 interacting with the hydroxyl ring is critical. Here we show that the replacement of Tyr44 by Ser affects the stability of the red form avoiding its accumulation at any temperature, while Trp110Ser is affected in the energy necessary to the orange to red conversion and in the interaction with the antenna. Collectively our data support the idea that the red form is essential for photoprotection but not sufficient. Specific conformational changes occurring in the protein seem to be critical to the events leading to energy dissipation.

1. Introduction

Excess light can be lethal for photosynthetic organisms because harmful reactive oxygen species are generated when the photochemical reaction centers cannot use the incoming energy fast enough. High light intensities induce irreversible inactivation of Photosystem II (PS II) due to damage and degradation of the D1 protein, an essential constituent of the PS II (for reviews, see [1,2]). In order to protect themselves from excess light, cyanobacteria, like plants, have developed one mechanism that decreases the energy arriving at the PS II reaction center, thus reducing the probability of damage of PS II [3–10]. The process is induced by the light activation of the Orange Carotenoid Protein (OCP) [9], a soluble 35-kDa protein containing a single-non-covalently bound carotenoid [11–13]. 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 [14]. The red OCP by interacting with the phycobilisomes, the external PSII antenna in cyanobacteria, increases the thermal dissipation of the energy absorbed by them. This results in a detectable decrease (quenching) of the phycobilisome fluorescence that can be followed by measurements in a pulse amplitude modulated (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 [15].

In *Synechocystis* PCC6803 (hereafter called *Synechocystis*), the strain used for most of the studies about the OCP-related-photoprotective mechanism, the OCP is encoded by the *slr*1963 gene [13] and is constitutively expressed. Many cyanobacteria, within a large phylogenetic range and from diverse habitats, contain homologs of the *Synechocystis slr1963* gene and present the OCP-related mechanism [16].

The structures of the OCP isolated from *Arthrospira maxima* [17] and from *Synechocystis* [18] were determined to 2.1 Å and 1.6 Å, respectively. The OCP that is a dimer in the crystals but a monomer in solution [18] 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) [14,17] that is composed of a conjugated carbonyl group located at the terminus of a conjugated chain of 11 carbon–carbon double

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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 [17]. The hydroxyl ring of the carotene interacts with Trp110 and Tyr44 (Fig. 1). We have already shown that changing the Trp110 and the Tyr44 to Ser abolishes the induction of fluorescence quenching [14,18]. In contrast, changing these amino acids by a Phe did not affect the OCP activity demonstrating the critical function of the aromatic rings of the side chains [18]. The keto-ring of the carotenoid interacts with the invariable Tyr201 and Trp288 (Fig. 1). The structure suggested the existence of hydrogen bonds between the keto group of the hECN and these aromatic amino acids [17,18]. The first results suggesting the importance of these hydrogen bonds came from studies using a strain lacking echinenone and hECN. In this mutant, OCP only binds zeaxanthin. In contrast, WT OCP binds only hECN. The over-expressing OCP strains contain around 8–10 times more OCP than the WT strain. Since hECN is a minor carotenoid in *Synechocystis*, in the overexpressing OCP cells, it is not sufficiently abundant to bind to all OCPs. In this strain, OCP binds also echinenone and zeaxanthin [19]. The binding of zeaxanthin is more pronounced with some mutated OCPs. For example, the isolated Tyr44Ser-OCP bound 30% zeaxanthin [18]. The zeaxanthin has two hydroxyl groups, one in each ring (sites 3 and 3′). The echinenone and hECN have a carbonyl group (site 4). In addition, the hECN has an additional hydroxyl group. This group is not required for the activity of the OCP since echinenone behaves like hECN and both can generate the red active OCP form [19]. In contrast, the carbonyl group seems to be essential for the OCP activity. Zeaxanthin lacking the carbonyl group renders the OCP inactive [19]. The carotenoids present in *Synechocystis* 6803 cells and their biosynthesis are shown in Fig. S1.

In this report, we further investigated the role of the amino acids Trp110, Tyr44, Trp288, and Tyr201 in the OCP using mutants lacking zeaxanthin in order to differentiate between effects of zeaxanthin binding to those related to point-specific mutations. We show that although the four amino acids are essential for photoactivity and photoprotection, they differently affect the OCP properties.

2. Materials and methods

2.1. Culture conditions

The mesophylic freshwater cyanobacteria *Synechocystis* PCC 6803 WT and mutants were grown photoautotrophically in a modified BG11medium [20] containing the double 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 3-L Erlenmeyer flasks 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. Construction of mutant strains

To obtain single and double OCP mutants overexpressing the mutated OCP, the plasmids containing the mutated *slr1963* gene under the control of its own promoter or under the control of the *psbAII* promoter were used to transform wild-type and single mutants of *Synechocystis* PCC 6803, respectively, by double recombination (Fig. S2). The construction of the plasmids was described in Ref. [14]. The point mutations W288 and Y201 were added in the *slr1963* gene in the normally expressing (OCP promoter) and the overexpressing plasmids (*psbAII* promoter) by site-directed mutagenesis using the Quickchange XL site-directed mutagenesis kit (Stratagene) and synthetic oligonucleotides (all of the oligonucleotides used in this work are described in Fig. S3). The construction of strains, which overexpress a C-terminus His_6-tagged WT-OCP gene (*slr1963*) or mutated Y44S and W110S OCP genes, was described earlier [14,18].

A 2.1 kb XhoI–SpeI fragment containing the *crtR* gene (*sll1468*) was amplified with oligonucleotides FCrtr and RCrtr and cloned into SK(+) ampicillin-resistant vector. A 114 pb fragment was removed from the *crtR* gene with restriction enzymes SmaI and BglII. A 1.1 kb chloramphenicol resistance cassette previously digested by SmaI was

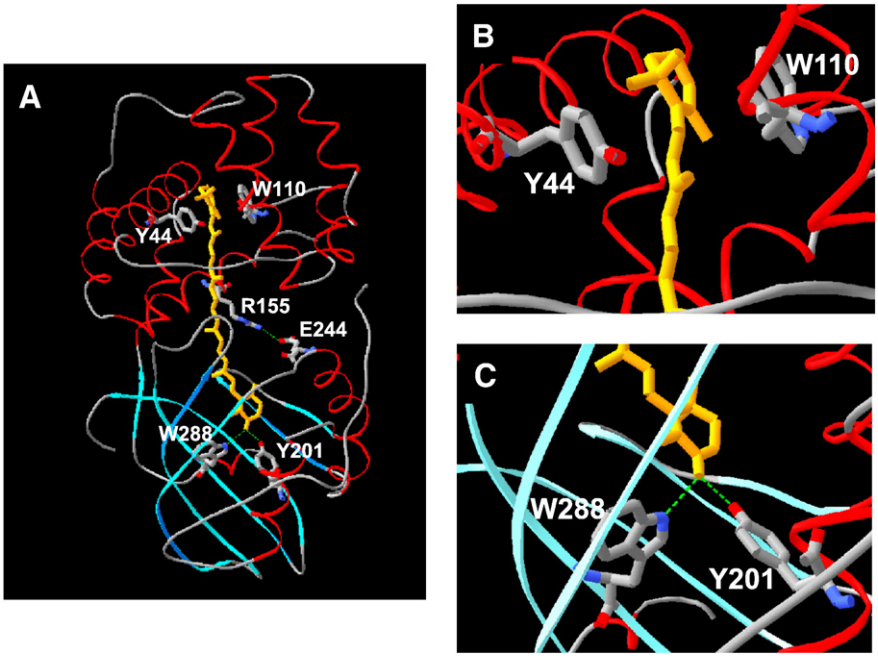

Fig. 1. Structure of *Synechocystis* OCP. Position of the mutated Tyrosines and Tryptophans. (A) Position of the carotenoid in the protein. (B) Interaction of Y44 and W110 with the hydroxyl ring of the hECN. (C) The hydrogen bonds between the carotenoid keto group and W288 and Y201. The α-helices are shown in red and the β-sheets in blue. The side chains are shown as grey sticks with oxygen in red and nitrogen in blue.

then inserted in the SmaI and BglII sites. The resulting plasmid was used to transform wild-type and overexpressing-OCP mutants of *Synechocystis* by double recombination (Figs. S2 and S4). The presence of the interrupted *crtR* gene in mutant cells was confirmed by DNA sequencing.

2.3. Purification of the Orange Carotenoid Protein

The purification of *Synechocystis* OCP was performed as previously described [14]. Briefly, mutant cells (1 mg Chl ml^{-1}) in 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 (Invitrogen). The OCP was further purified on a Whatman DE-52 cellulose column. Additional details of the purification are described in Ref. [14].

2.4. Carotenoid characterization

Carotenoids were extracted as previously described [19]. Liquid chromatography-mass spectrometry analysis was conducted on 5 µl 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, UK), 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 25-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. Peak identification is based on comparison of retention times and absorption spectra to commercially available standards (Extrasynthèse, http://www.extrasynthese.com), or published values [21], comparison with native or mutant cyanobacteria and mass spectrometry spectra [14]. Relative quantification (% of total carotenoids) is based on response ratio of pigments measured at 450 nm and at lambda max, and published in Ref. [21]. Mass analysis was conducted as previously published [14].

2.5. Absorbance measurements

Cell absorbance was monitored with an UVIKONXL 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 OCP with 5000 µmol photons m^{-2} s^{-1} of white light at 19 °C. For the pH experiments, the different buffer solutions were at 40 mM. Tris–HCl was used for pH 7 and 8; CHES was used for pH 8, 9, and 10, and CAPS was used for pH 8, 9, 10, 11, 11.5, and 12.

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 850 µmol photons m^{-2} s^{-1} of blue-green light for about 200 s. Saturating pulses (2000 µmol photons m^{-2} s^{-1}) were applied to measure the maximal fluorescence levels, Fm′, in light-adapted samples. Application of such pulses that transiently close all the PS II centers serves to distinguish between photochemical quenching and non-photochemical quenching.

2.7. OCP immunodetection

Total cell protein was analyzed by SDS-PAGE on a 12% polyacrylamide/2 M urea in a TRIS/MES system [22]. The OCP protein was detected by a polyclonal antibody against OCP. The blots were scanned and the density of bands was measured using the Image Mastertotal Lab v1.11 software (Amersham Pharmacia).

3. Results

3.1. Tyr 201 and Trp288 are essential for photoactivity and photoprotection

WT *Synechocystis* cells were transformed with plasmids containing the point-mutated *slr1963* gene in order to replace Tyr201 and Trp288, interacting with the carbonyl ring of the carotenoid, by Ser, Phe and His. Strong blue-green light was unable to induce fluorescence quenching in any of these mutants (Fig. 2A). This result contrasts to those obtained when Tyr44 and Trp110 (interacting with the hydroxyl ring of the carotenoid) were replaced by Phe. Phe was able to replace the Tyr and the Trp maintaining the photoprotection activity [18]. Even a double mutated Y44F/W110F OCP was still able to induce fluorescence quenching (Fig. 2A). We decided to further study the role of Tyr201 and Trp288 in photoactivity and photoprotection using the mutants in which these amino acids were changed by a His, which (theoretically) can form a hydrogen bond with the carotenoid carbonyl, or by a Ser, which is unable to form this bond. To this purpose, mutants overexpressing Y201S-OCP or Y201H-OCP or W288S-OCP or W288H-OCP were constructed. The mutated OCPs isolated from these strains contained between 60% to 85% zeaxanthin. Since zeaxanthin-OCP is not photoactive, it is difficult to differentiate the effect of point mutations from that of zeaxanthin. For this reason, the *crtR* gene coding for the β-carotene hydroxylase ([23]) was interrupted with an antibiotic resistant cassette in the mutants overexpressing WT and mutated OCPs (for the mutant construction, see Materials and methods and Figs. S2, S3, and S4). The interruption of the *crtR* gene leads to the lack of zeaxanthin and hECN in all mutant cells (Fig. S5). β-Carotene (a non-oxygenated carotenoid) is the principal carotene present in this mutant.

A common effect of the replacement of Tyr201 or Trp288 by Ser or His was the high instability of the mutated OCPs. Western blot analysis showed that these mutants accumulated much less amounts of OCP than the strain overexpressing WT-OCP: only 15–25% for Trp288 mutants and 23–29% for Tyr201 mutants (Fig. 3). However, in the cells overexpressing the mutated OCPs, there was at least the same quantity of OCP than in WT cells, in which strong blue light induces 25–30% quenching of maximal fluorescence. Strong intensities of blue-green light were unable to induce any fluorescence quenching in all the *Synechocystis* strains overexpressing the mutated OCPs (Fig. 2B).

The WT and the Y201S, Y201H, W288S, and W288H mutated OCPs were isolated from the overexpressing His-tagged point-mutated OCP-ΔCrtR strains using affinity Ni-chromatography followed by an ion-exchange column [14]. Very low quantities of the mutated OCPs were isolated : 3% to 5% of the quantity isolated from overexpressing WT cells. In addition, only 30% to 50% of the mutated OCPs eluted from the DE-52 column contained a carotenoid molecule. This was probably due to a loss of carotenoids during OCP isolation. These results indicated that both mutations decrease the affinity and/or the stability of the bound carotenoid and that the protein without carotenoid is very unstable.

The WT-OCP isolated from the ΔCrtR strains contained mostly echinenone (more than 95%) with only traces of other carotenoids. We have already shown that the orange WT-OCP is converted to a red active form by absorption of blue-green light [18,19]. However, this conversion is not complete when the OCP is isolated from

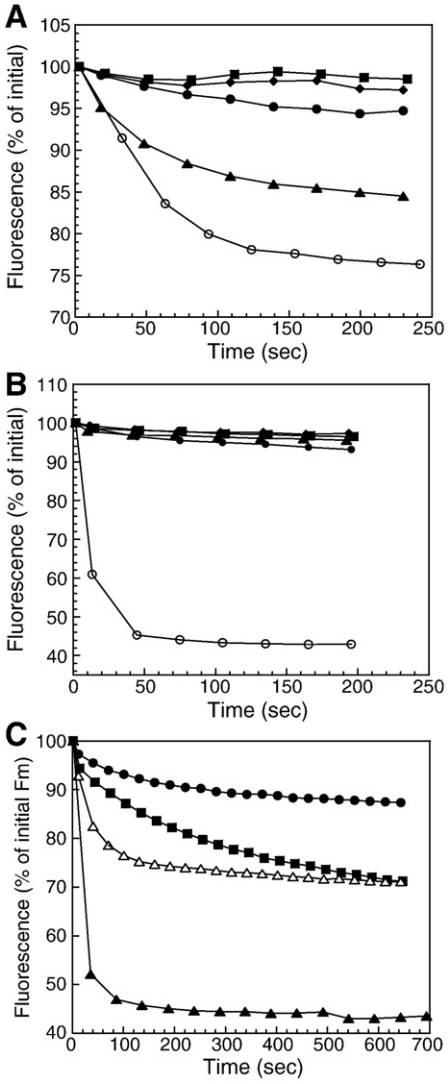

Fig. 2. Blue-green light-induced fluorescence quenching in OCP mutants. Low light (80 μmol photons m^{-2} s^{-1}, blue-green) adapted WT and mutant *Synechocystis* cells (at 3 μg Chl mL^{-1}) were illuminated with high intensities (850 μmol photons m^{-2} s^{-1}) of blue-green light (400–550 nm). Fluorescence yield changes were detected with a PAM fluorometer, and saturating pulses were applied to measure maximal fluorescence levels (Fm′). (A) WT (open circles), and mutated strains: Y44F/W110F OCP (triangles), Y201F OCP (closed circles), Y201H OCP (squares), and Y201S OCP (rhomboids). (B) Overexpressing strains WT-ΔCrtR OCP (open circles), Y201H-ΔCrtR OCP (closed squares), W288H-ΔCrtR (closed triangles), Y201S-ΔCrtR (closed circles), and W288S-ΔCrtC (closed rhomboids). (C) Overexpressing WT OCP-ΔCrtR (closed triangles), WT (open triangles), overexpressing Y44S-ΔCrtR (closed squares), and overexpressing W110S-ΔCrtR (closed circles).

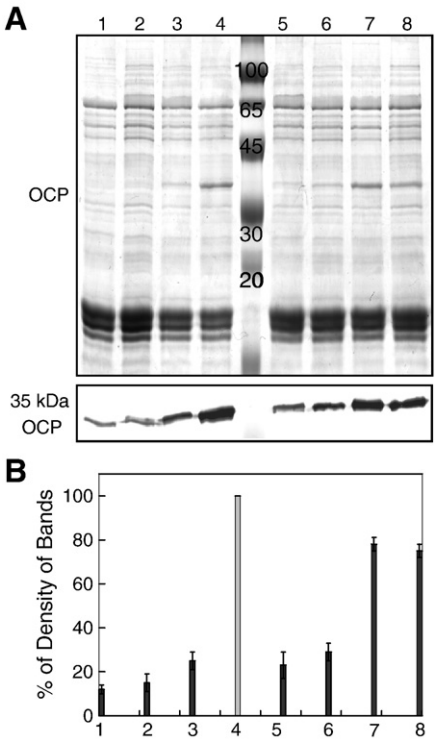

Fig. 3. OCP levels. (A) Coomassie blue-stained gel electrophoresis (top panel) and immunodetection of OCP (bottom panel). (B) Comparative densitometry of OCP levels in WT and different *Synechocystis* mutants. The results represent the average of three independent experiments. Each error bar shows the maximum and the minimum density of band values. 100% is the concentration of OCP in the overexpressing WT OCP. The samples are total proteins from strains lacking zeaxanthin (ΔCrtR): WT (1), overexpressing W288S OCP (2), overexpressing W288H OCP (3), overexpressing WT OCP (4), overexpressing Y201H OCP (5), overexpressing Y201S OCP (6), overexpressing Y44S OCP (7), and overexpressing W110S OCP. Each lane contained 0.5 μg chlorophyll.

overexpressing OCP strains, due to the presence of zeaxanthin-bound OCP, which is not photoactive. In contrast, the OCPs isolated from the overexpressing WT OCP-ΔCrtR strains were totally converted to the red form as the WT OCP [14] (Fig. 4F).

The Tyr201 and Trp288 mutated OCPs isolated from the ΔCrtR strains bound different kinds of carotenoids in addition to echinenone (Table 1). The Trp288 mutants bound principally keto-carotenoids but can also bind β-carotene especially when the Trp was replaced by a His. The relative quantity of the different carotenoids varied with the preparation (two examples for W288S are shown in Table 1). The UV-chromatogram of the carotenoids isolated from the W288S-OCP showed a big peak with retention time of 19.83 min and a small peak with retention time of 21.85 min in addition to the peak corresponding to echinenone. LC-MS spectra allowed us to identify the carotenoid at 19.83 min as canthaxanthin (having a keto group in each ring) (Fig. S6) and that at 21.85 as deoxymyxoxanthiphyll (Fig. S7). The isolated W288S and W288H OCPs appeared redder than the WT-OCP. Nevertheless, the spectra were slightly different depending on the concentration of the β-carotene present. Fig. 4 compares a preparation of W288H OCP binding 23% of β-carotene, 40% of echinenone, and 22% of canthaxanthin (Fig. 4E) with a preparation of W288S OCP binding 67% canthaxanthin and 21% echinenone (Fig. 4D).

The Tyr201 mutants principally bound β-carotene and echinenone (Table 1). They also bound other carotenoids like canthaxanthin and deoxy-myxoxanthophylls (Table 1). As observed for the W288 mutants, the relative quantity of each carotenoid varied with the preparation. The isolated Y201S and Y201H-OCPs appeared more yellow than the WT-OCP probably due to the presence of β-carotene (around 50%) in a high number of proteins (Table 1). During elution of the DE-52 ion-exchange column, the first mililiters of the eluted Y201S OCP was enriched in non-keto carotenoids (deoxymyxoxanthophyll or β-carotene) and the last mililiters, in keto carotenoids. This suggested that the external charge of the protein is slightly different depending on the bound carotenoid. Fig. 4B shows the spectrum of a small fraction of the Y201S OCP containing more than 70% echinenone and only 10% of β-carotene eluted at the end of the elution fraction in the DE-52 column. This fraction was redder than the WT. The spectra of the mutated OCPs depended on the carotenoid content. The spectrum of mutated OCPs containing high quantities of β-carotene presented maxima at 496 and 467 with shoulders at 450 and 530 nm. The shoulder at 530 nm decreased with increasing concentrations of β-carotene (Fig. 4). The spectrum of the Y201S containing high quantities of echinenone presented a maximum at 500 nm with shoulders at 467 and 530 nm (Fig. 4B). This spectrum was almost identical to that of the W288S-OCP

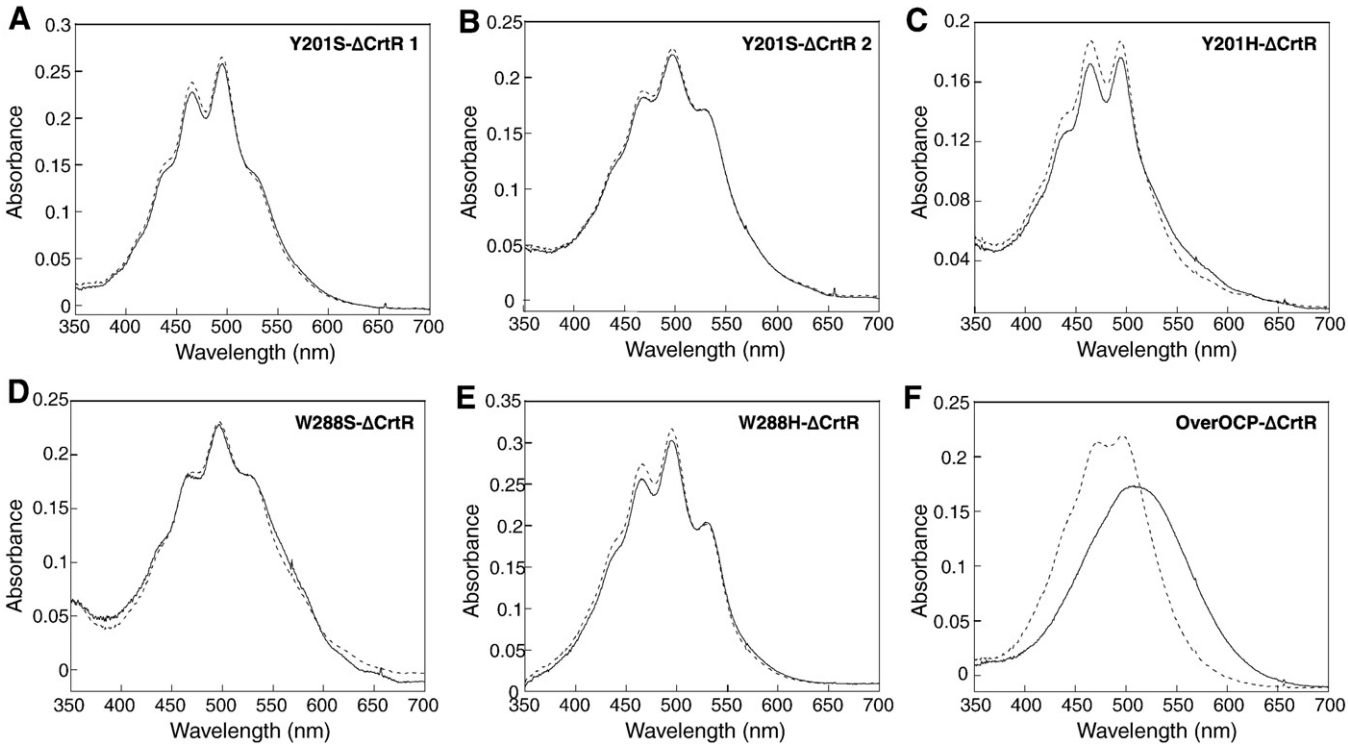

Fig. 4. Photoactivity of isolated OCP preparations. Absorbance spectra of the dark (dashed line) and light (solid line) forms of the OCP isolated from the strains lacking zeaxanthin (ΔCrtR) overexpressing Y201S (A and B), Y201H (C), W288S (D), W288H (E), or WT (F). The protein was illuminated 5 min with strong white light (5000 μmol photons m^{-2} s^{-1}) at 19 °C.

mostly binding canthaxanthin. Thus the red shift of the spectra and the important shoulder at 530 nm are most probably related to the position of the carotenoid in the protein and not to the longer conjugation of the canthaxanthin compared to the echinenone. None of the Tyr201 and Trp288 mutated OCPs were photoactive. Dark and light spectra were similar (Fig. 4).

Table 1
Carotenoid content of WT and mutated OCPs isolated from overexpressing OCP strains lacking zeaxanthin and hECN. The relative quantity of echinenone, canthaxantin, and β carotene changed in each preparation. In the table are shown the examples discussed in the text. The phenotype of the mutated OCPs is also described.

Strains	Carotenoid[a] content (%)				Phenotype[b]
	Echinenone	β-Carotene	Canthaxanthin	Deoxymyxo-xanthophyll	
Over WT	99	–	1	–	Orange Q, P Stable
Over W110S	91	–	9	–	Orange NoQ, P (≥30 °C) Less stable
Over Y44S	88	–	6	–	Orange NoQ, NoP Stable
Over W288H	40	23	22	10	Reddish NoQ, NoP Unstable
Over W288S (1)	21		67	–	Reddish NoQ, NoP Unstable
Over W288S (2)	51	2	47		Reddish NoQ, NoP Unstable
Over Y201H	34	60	6	–	Yellowish NoQ, NoP Unstable
Over Y201S Fraction 1	34	40	5	10	Yellowish NoQ, NoP Unstable
Over Y201S Fraction 2	71	11	13	–	Reddish NoQ, NoP Unstable

[a] See Supp. Fig S1 for carotenoid structures.
[b] Phenotype: Q, fluorescence quenching observed; NoQ, no fluorescence quenching observed; P, photoconvertion of orange to red form; NoP, does not convert to red form. The color of the dark form and the stability of the protein are also indicated.

Although the W288S and W288H OCPs proteins were redder than the WT OCP in darkness and they absorbed light of wavelengths similar to those absorbed by the red WT OCP, the mutant *Synechocystis* cells were not quenched (Fig. 5). At the same chlorophyll concentration, Fm and Fv (Fm–Fo) were similar in strains overexpressing WT-OCP and W288S-OCP. This was observed in darkness and under low intensities of blue light indicating that the W288S-OCP containing cells were not quenched before exposure to strong blue light. Illumination of the low-light adapted *Synechocystis* cells overexpressing WT-OCP induced a decrease of all levels of fluorescence (Fig. 5A). The same type of illumination had no effect on cells overexpressing W288S-OCP (Fig. 5B). These results suggested that the presence of a red carotenoid is not sufficient to induce the photoprotective mechanism.

The involvement of the hydrogen bond between Tyr201 and the carotenoid carbonyl on OCP photoactivity was also suggested by experiments measuring the kinetics of the orange to red conversion under different pH conditions. Fig. 6A shows the initial rates of the orange to red conversion at pHs going from 7 to 12. Tris–HCl, CAPS, and CHES buffers (40 mM) were used to perform these measurements. The initial rates measured in CAPS were slightly lower than those measured in Tris–HCl or CHES indicating an influence of the nature of the buffer. Nevertheless, a strong pH effect was observed. The initial rates of conversion were similar at pH 7, 8, and 9 and then, at higher pH levels, they increased (Fig. 6A). At pH 12, the initial rate was about 8 times faster than that at pH 8. Moreover, red OCP slowly accumulated even in darkness (at 19 °C, Fig. 6B). The pK of the Tyr being around 10, our results could suggest that the de-protonation of a Tyr favored the OCP conversion to the red form.

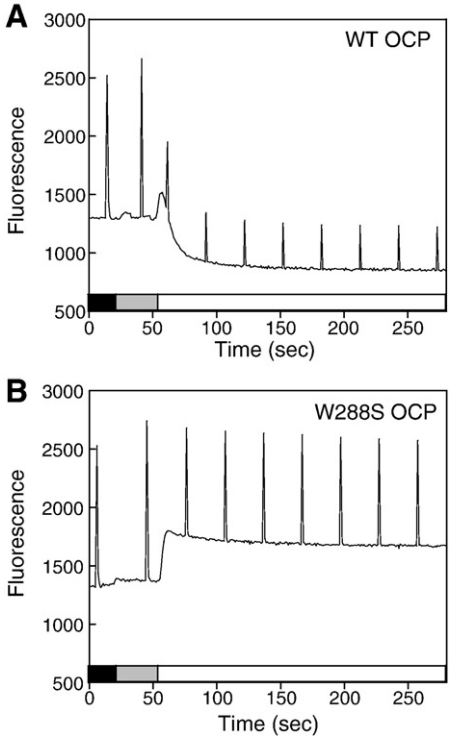

Fig. 5. Blue-green light-induced fluorescence quenching in OCP mutants. Dark adapted *Synechocystis* cells lacking zeaxanthin and overexpressing WT OCP (A) or W288S OCP (B) (at 3 μg Chl mL^{-1}) were successively illuminated with mild (80 μmol photons m^{-2} s^{-1}) (grey) and strong blue-green light (850 μmol photons m^{-2} s^{-1}, 400–550 nm) (white). Fluorescence yield changes were detected with a PAM fluorometer, and saturating pulses were applied to measure maximal fluorescence levels (Fm′).

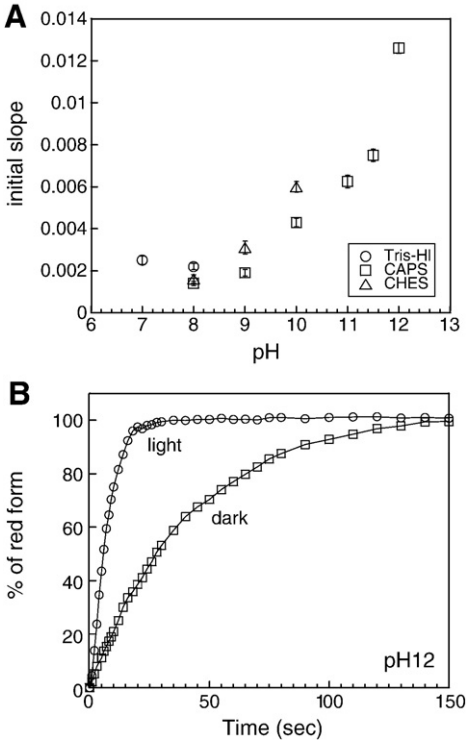

Fig. 6. Orange to red OCP conversion at different pHs. (A) Initial slope of red OCP accumulation (measured as the absorbance increase at 550 nm) at different pHs. The measurements were done using different buffers: Tris–HCl (circles), CAPS (squares), and CHES (triangles). The OCP was illuminated with white light (5000 μmol photons m^{-2} s^{-1}) at 19 °C. Average of 3 independent experiments is shown. (B) Red OCP accumulation in light (5000 μmol photons m^{-2} s^{-1} white light) (circles) and darkness (squares) at pH 12 at 19 °C.

3.2. The Y44S and W110S ΔCrtR strains: interaction of hydroxyl ring of the carotenoid with the protein

The W110S-OCP and particularly the Y44S-OCP contained high concentrations of zeaxanthin when they were isolated from the zeaxanthin-containing strains [18]. The isolation of these mutated proteins from a strain lacking zeaxanthin and their characterization allowed us to differentiate the roles of Tyr44 and Trp110 in photoactivity. The Y44S-OCP and W110S-OCP isolated from the ΔCrtR mutant strains contained mostly echinenone with traces of canthaxanthin (Table 1). Both mutated proteins were present at high concentration in the overexpressing strains (75–78% of that present in the overexpressing WT-OCP) (Fig. 3). Thus mutations in these amino acids had low effect on the affinity of echinenone. Nevertheless, a smaller quantity of isolated W110S-OCP than of isolated Y44S was obtained suggesting a lower stability of W110S-OCP.

Accumulation of red OCP was measured at different temperatures, going from 2 °C to 35 °C. It was already described that for the WT OCP, at a fixed light intensity, the steady-state concentration of the red OCP form largely depends on the temperature: higher concentrations at lower temperatures. This is due to the fact that the half-time of the back-reaction (red to orange) varies from 30 s at 32 °C to more than 1 h at 8 °C while the initial rate of the light photoconversion is temperature-independent [14]. Under our light conditions (5 min, 5000 μmol photons m^{-2} s^{-1} of white light), 100% of the OCP was converted to the red form at temperatures lower than 20 °C, while almost no accumulation was observed at 32 °C (Fig. 7A). Almost no redY44S-OCP was accumulated from 2 to 32 °C (Fig. 7B). Nevertheless, *in vivo*, blue-green light slowly induced fluorescence quenching (Fig. 2C). After 10 min of illumination (850 μmol photons m^{-1} s^{-1}), the maximal fluorescence was quenched almost 30% (Fig. 2C). In WT

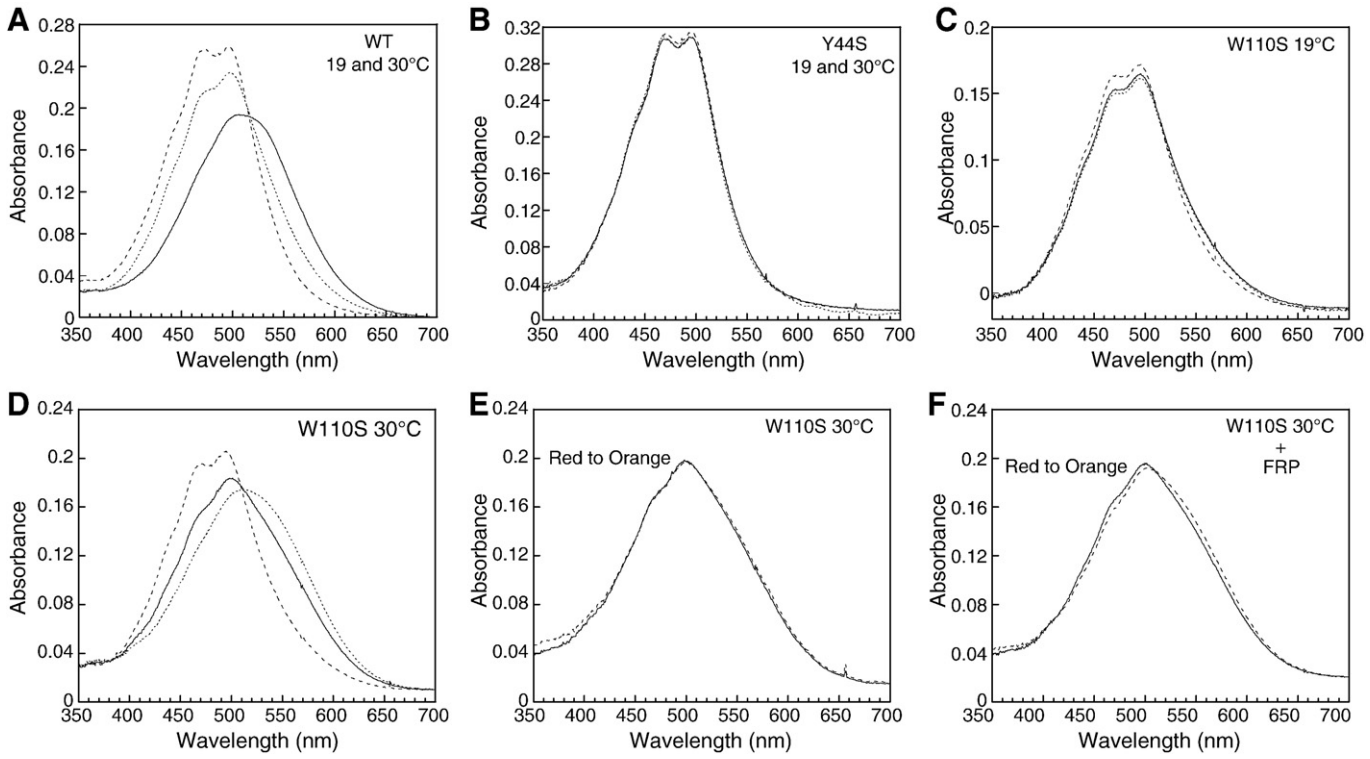

Fig. 7. Photoactivity of isolated OCP preparations. (A and B) Absorbance spectra before (dashed line) and after illumination (5 min, 5000 μmol photons m^{-2} s^{-1}) at 19 °C (solid line) and 30 °C (dotted line) of the OCP isolated from the strains lacking zeaxanthin (ΔCrtR) overexpressing WT or Y44S. The Y44S-OCP had the same color and spectrum before and after illumination from 2 to 32 °C. (C and D) Absorbance spectra before (dashed line) and after illumination of W110S-OCP (5 min, solid line; 10 min dotted line) at 19 °C (C) and 30 °C (D). (E and F) The 5 min illuminated over W110S (solid line) was then incubated in darkness during 20 min in the absence (E) or presence of FRP (F) (dashed line).

cells the maximal fluorescence quenching (25 to 30%) is attained after 2-min illumination.

In vitro, illumination of the dark orange W110S OCP induced a very small change of the dark absorbance spectrum when performed at temperatures lower of 30 °C ([14] and Fig. 7C). Surprisingly, when the illumination was done at temperatures equal to or higher than 30 °C, a large accumulation of a red form was observed (Fig. 7D). The concentration of the red form increased if the OCP was illuminated longer times (Fig. 7D). Moreover, this red form was very stable (Fig. 7E). After 20-min darkness, almost no recovery was observed while red WT-OCP is converted to the orange form in seconds at this temperature [14].

The presence of the Fluorescence Recovery Protein (FRP) accelerates the conversion of the red OCP form to the orange form [24]. The effect of FRP on the stability of the red W110S form was tested. Fig. 7F shows that the presence of FRP did not help the red to orange conversion of the W110S OCP suggesting that FRP did not interact with this mutated protein.

Although *in vitro* the W110S OCP was completely converted to a red stable form at 30 °C, in *Synechocystis* cells grown at 32 °C, blue-green light induced only 10% of fluorescence quenching after 10-min illumination measured by a PAM fluorometer at 30 °C (Fig. 2C). These results supported the hypothesis that the presence of a red carotenoid is not sufficient to induce photoprotection.

4. Discussion

In this work, we studied the role of 100% conserved OCP Tyrs and Trps around the carotenoid rings in photoactivity and photoprotection. Our results showed that W110, Y44, W288, and Y201 are essential to induce the OCP changes necessary to induce photoprotection. However, the effect of replacing each of these amino acids in photoactivity and photoprotection was different.

4.1. Interaction of hydroxyl ring of the carotenoid with Tyr44 and Trp110

In the hydroxyl ring of the carotenoid, the hydroxyl group has not an important role in the activity of the OCP. hECN and echinenone containing OCPs are photoactive and induce large fluorescence quenching. Moreover, no differences in the orange to red and red to orange kinetics were observed suggesting a similar stability of the red form in both proteins [19].

When the Tyr44 and the Trp110 were replaced by Phe, the photoactivity was maintained and the mutated proteins were able to induce the photoprotective mechanism. In contrast, replacement of these amino acids by a Ser resulted in loss of activity. These results indicated that the interaction between the aromatic amino acids and the hydroxyl ring of the carotenoid is essential for OCP activity. However, the mutations affected in a different way the photoactivity of the OCP. *In vitro*, the W110S-OCP mutant is photo-inactive at temperatures lower than 25 °C but photoactive at 30 °C. Moreover, at this temperature, the red form was relatively stable. However, *in vivo* no induction of the photoprotective mechanism was observed. In contrast, although *in vitro* the Y44S-OCP mutant was photo-inactive from 8 to 35 °C, *in vivo* it was able to induce some fluorescence quenching with slow kinetics. Thus, substitution of the Tyr44 by a Ser seemed to destabilize the red form which in the cell could be partially stabilized by interaction with the phycobilisomes or other proteins. On the other hand, substitution of Trp110 by a Ser seemed to affect the energy needed to form the red form. In addition, the red form became more stable at higher temperatures. Nevertheless, the red W110S was not able to induce fluorescence quenching suggesting that this form is

not able to correctly interact with the phycobilisomes. Indeed, our results showed that the presence of FRP did not accelerate the red to orange conversion of the W110S-OCP suggesting a poor interaction between this mutated OCP and FRP.

4.2. The hydrogen bonds between the keto group of the carotenoid and Tyr201 and Trp288

The structure of the WT-OCP from *Arthrospira maxima* [17] and *Synechocystis* 6803 [18] showed that the keto group of the hECN hydrogen bonds with the absolutely conserved Y201 and W288 of the central strand of the C-terminal β-sheet. The hydrogen bond distances are 2.6 and 2.9 Å, respectively, the first one being a relatively short hydrogen bond. The orientation of Y201 seems to be stabilized via aromatic interactions with two absolutely conserved Phe side chains (217 and 290 residues), whereas the orientation of W288 could be stabilized by the interaction with the sulfur of Met 202 [18].

The importance of the hydrogen bonds in the photoactivity of the OCP was first suggested by studies on the zeaxanthin-containing OCP; the zeaxanthin lacks the keto group involved in the hydrogen bond and the zeaxanthin-OCP is photo-inactive [19]. Here, the hydrogen bond was avoided by replacement of the Tyr or the Trp by another amino acid. The mutated OCPs, although binding keto carotenoids (echinenone and canthaxanthin), were photo-inactive. Moreover, the orange to red conversion of the WT-OCP is about 8 times faster at pH 12 than at pH 8 or 7 suggesting that the de-protonation of a Tyr destabilizes the orange form. At pH 12 the orange form is so unstable that the red form is accumulated even in the absence of light. We hypothesize that in the red form, the hydrogen bond between Y201 (and W288) and the carotenoid carbonyl is weaker than in the orange form or it is broken.

The OCP specificity for keto carotenoids was also diminished by replacing the Tyr201 and the Trp288 by other amino acids. The Tyr201 and Trp288 mutants bound more or less important quantities of β-carotene, a non-oxygenated carotene, while the WT and Tyr44 and Trp110 mutants bound only echinenone (in the absence of zeaxanthin). Moreover, the fact that the concentration of Tyr201 and Trp288 mutated OCPs was largely lower in cells containing these mutated OCPs than in WT cells (or Y44S-OCP or W110S-OCP mutant cells) suggested that the attachment of the carotenoids to these specific mutated OCPs was less stable. Nevertheless, the presence of His at the place of the Trp288 or the Tyr201 seemed to slightly stabilize the binding of β-carotene.

Raman and FTIR studies indicated that absorption of light by the hECN induces conformational changes of the carotenoid and the protein [14]. Most probably the changes in the protein are induced by the conformational changes in the carotenoid. In the red active form, the carotenoid has a more planar configuration and its apparent conjugation length was increased. The echinenone (canthaxanthin) binding W288 and Y201-OCPs were redder than the WT-OCP suggesting that both hydrogen bonds are essential to maintain the carotenoid in its orange position. However, this redder protein was unable to induce fluorescence quenching.

4.3. The existence of a red OCP is not sufficient to induce photoprotection

Previously, we have shown that changing the Arg155 to Leu suppresses the induction of fluorescence quenching without loss of photoactivity [18]. This mutant provided the first evidence that photoconversion and photoprotection are discrete events in the OCP's mechanism. Thus, it is not sufficient that the OCP becomes red. Here, we described another mutant (W110S), which is able to accumulate the red OCP form at cell growth temperatures but unable to induce fluorescence quenching. Two other mutated OCPs that already in darkness are redder than the WT OCP are also unable to induce the photoprotective mechanism (W288S, W288H). These mutations are present in different parts of the protein, but they are all situated around the carotenoid. They could be involved in the cascade of protein conformational changes induced by the modification in the position of the carotenoid upon illumination. These specific conformational changes in the protein must be essential to allow a good interaction between the OCP and the phycobilisomes to induce fluorescence quenching and photoprotection. In the mutants, they are most probably modified or partially inhibited avoiding a good interaction OCP-phycobilisome.

For example, variation of the hydrogen bond distances and/or of the position of the Tyr201 and Trp288 could induce a cascade of other conformational changes in the protein and/or in a network of hydrogen bonds leading to a higher affinity of the OCP for the phycobilisome. In the Trp288 and Tyr201 mutants, in which one of the hydrogen bonds does not exist, the modifications required to induce energy transfer and dissipation are probably not induced. These residues belong to the C-terminal domain of the OCP, which is structurally similar to the phycobilisome 7.8 kDa core linker protein [14]. We had hypothesized that the C-terminal OCP domain, by interacting with the center of an allophycocyanin trimer in the phycobilisome core, may bring the carotenoid into proximity of the allophycocyanin chromophores [14]. Modifications of the secondary structure of the protein, especially in the C-terminal domain, or in the light-induced changes in the carbonyl–protein interactions, could possibly modify the interaction between the carotenoid and the allophycocyanin chromophores avoiding energy transfer.

In the past, we have suggested that one of the causes for the lack of the photoprotective activity of some OCP mutants could be related to the energy level of the S1 state of the orange form. The results obtained with the Arg155Leu, Trp110Ser, Trp288Ser, and Tyr201Ser mutants strongly suggest that the most important cause for the loss of photoprotective activity in all these mutants is an incorrect interaction of the mutated OCPs with the phycobilisome. *In vitro* OCP–phycobilisome interaction studies must be realized to confirm this hypothesis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2010.12.009.

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