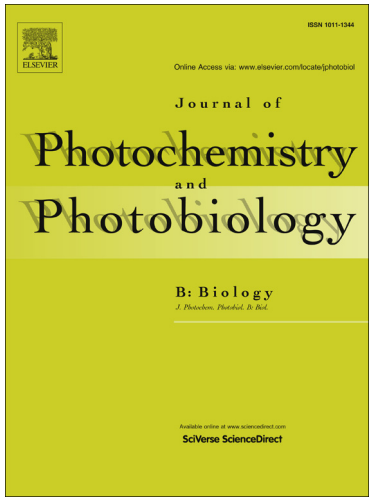

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Fluorescence quenching of the phycobilisome terminal emitter L_{CM} from the cyanobacterium *Synechocystis* sp. PCC 6803 detected *in vivo* and *in vitro*

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Abbreviations: APC, allophycocyanin; L_{CM} (ApcE), core-membrane phycobilisome linker and terminal emitter, or anchor protein; NPQ, non-photochemical quenching; OCP, orange carotenoid protein; PBS, phycobilisome; PC, phycocyanin.

A B S T R A C T

The fluorescence emission of the phycobilisome (PBS) core-membrane linker protein (L_{CM}) can be directly quenched by photoactivated orange carotenoid protein (OCP) at room temperature both *in vitro* and *in vivo*, which suggests the crucial role of the OCP-L_{CM} interaction in non-photochemical quenching (NPQ) of cyanobacteria. This implication was further supported (i) by low-temperature (77 K) fluorescence emission and excitation measurements which showed a specific quenching of the corresponding long-wavelength fluorescence bands which belong to the PBS terminal emitters in the presence of photoactivated OCP, (ii) by systematic investigation of the fluorescence quenching and recovery in wild type and L_{CM}-less cells of the model cyanobacterium *Synechocystis* sp. PCC 6803, and (iii) by the impact of dephosphorylation of isolated PBS on the quenching. The OCP binding site within the PBS and the most probable geometrical arrangement of the OCP-allophycocyanin (APC) complex was determined *in silico* using the crystal structures of OCP and APC. Geometrically modeled attachment of OCP to the PBS core is not at variance with the OCP-L_{CM} interaction. It was concluded that besides being a very central element in the PBS to reaction center excitation energy transfer and PBS assembly, L_{CM} also has an essential role in the photoprotective light adaptation processes of cyanobacteria.

1. Introduction

Light intensities that exceed the capacity of the photosynthetic apparatus are dangerous to photosynthetic organisms, as saturation of the electron transport chain facilitates the generation of harmful reactive oxygen species which disorganize the photosynthetic machinery, especially Photosystem II, and in extreme cases can also cause photobleaching, membrane damage and eventually cellular death. Photosynthetic organisms have developed several molecular mechanisms in order to optimize their photochemical efficiency on one hand, and to protect

themselves from excess of light on the other. The two dominant short-term light adaptation processes in oxygenic photoautotrophs are: (i) state transitions, i.e. dynamic changes in light energy distribution between the two photosystems, and (ii) non-photochemical fluorescence quenching (NPQ; regulated thermal dissipation of excess excitation energy), which protects photosynthetic organisms from damage upon exposure to intense light. In higher plants NPQ involves conformational changes of the light harvesting chlorophyll *a/b*-binding complex (LHCII) triggered by transmembrane proton gradient and modulated by several other factors including specific carotenoid molecules (for a review see [1]).

Instead of membrane-integral LHCII, the major light harvesting antennae in cyanobacteria are phycobilisomes (PBS), giant phycobiliprotein antenna complexes associated with the cytoplasmic surface of the thylakoid membranes. Hemidiscoidal PBS, the most common type, consist of (i) a central tricylindrical core composed of disk-shaped allophycocyanin (APC) trimers and (ii) six fanlike cylindrical rods emanating from the core and built up of stacked hexamers of phycocyanin (PC) and (if present) phycoerythrin(s) or phycoerythrocyanin [2]. Specific (colorless or chromophorylated) linker polypeptides are also associated with certain phycobiliprotein discs in order to optimize absorbance and in particular the excitation energy transfer characteristics of the PBS besides their evident structural roles [2,3]. As water-soluble PBS does not contain carotenoid molecules, it has long been assumed that cyanobacteria cannot dissipate excess energy at the antenna level [4]. However, as found recently [5-7], exposure of the model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) to strong light led to a reversible quenching of the PBS fluorescence emission. A very similar phenomenon was observed in the ancient cyanobacterium *Gloeobacter violaceus* that lacks thylakoids, thus, this photoprotective mechanism must be evolutionarily rather old [8]. The action spectrum of the quenching shows three bands characteristic for the carotenoid S_0-S_2 transition in the 450-500 nm spectral region as well as an additional "*trans*" peak at 290 nm [5,9] which is fully consistent with the absorption spectrum of the ketocarotenoid 3´-hydroxyechinenone, present as a single

noncovalently bound chromophore in the water-soluble orange carotenoid protein (OCP) [10,11]. Noticeably, although carotenoids apparently play an important role in the energy dissipation processes in cyanobacteria as well, these processes are quite different from those observed in higher plants.

OCP was first isolated more than three decades ago [10] and its 3D structure has been resolved [12,13]. OCP was shown to exist in two distinct conformational states: the inactive, dark-stable orange form (OCP_O) and the active, metastable red form (OCP_R), which can be generated by intense blue-green light [14]. OCP_R quenches the excited state very effectively: approximately 30-40% of the absorbed light energy can be thermally dissipated upon OCP_R induction [14,15]. It was proposed that OCP acts not only as a photosensor, but is also being an effector with a direct contact with PBS via electrostatic and/or hydrophobic interactions. Possible molecular mechanism of the quenching has extensively been discussed in recent literature (see e.g. [14-16]) including the putative interaction between OCP and the PBS. High affinity docking of OCP_R to PBS was shown by fluorescence spectroscopy using an *in vitro* reconstituted system of isolated OCP_O/OCP_R and PBS [17,18] and also by isolation of quenched PBS containing the OCP by sucrose gradient centrifugation [18].

While it is evident that OCP is responsible for the blue-green light induced fluorescence quenching in cyanobacteria, the exact binding site has not yet been established. Based on the large difference between the molecular masses of OCP and PBS (two orders of magnitude; 35 kDa vs. 3,000 kDa), respectively [2,10,12,19], and small OCP to PBS ratio (≤ 0.5) [18] the docking site seemed to be very specific and most likely involves a minor component inside the PBS. As PC-less *Synechocystis* mutant and PBS complexes isolated from this strain were still capable of OCP-induced quenching [7,14,18,20] the OCP binding site is apparently located within the PBS core. The tricilindrical core subcomplex (M ~ 1,200 kDa), consists of one upper and two basal cylinders; each of these is composed of four stacked $(\alpha\beta)_3$-APC trimers (Fig. 1A)

and bears altogether 24 phycocyanobilin molecules [2,19]. X-ray diffraction studies on APC crystals have revealed that each $(\alpha\beta)_3$ disk has a thickness of ~3 nm and a diameter of ~11 nm which involves a central cavity with a size of ~3.5 nm [21]. The boomerang-shaped monomeric ($\alpha\beta$) units form a threefold symmetry of the $(\alpha\beta)_3$ trimer (Fig. 1B). The phycocyanobilin chromophores which are covalently bound to cysteine residues α84 and β84 are packed rigidly in their respective pigment binding pockets. While the α84 chromophores are exposed to the outer surface of the disc, the β84 chromophores are partially exposed to the central channel (Fig. 1B).

In contrast to the assembled PBS (see above and [17,18,22]), purified APC trimers apparently do not interact with the OCP *in vitro* over a wide range of concentration [23] which makes docking of the OCP by bulk APC very unlikely. Nevertheless, the PBS core also possess three less abundant chromophorylated polypeptides which may serve as potential interacting partner for OCP: ApcD (α–subunit of allophycocyanin B), ApcF (β^{18} polypeptide), and L_{CM} (= ApcE or anchor protein) [2,3,19,24]. Similar to the APC α– and *β*–subunits, all of these three subunits bind one phycocyanobilin chromophore and each mediates the excitation energy transfer from the PBS core to the photosynthetic reaction centers. In accordance with this, the fluorescence emission peaks of ApcD, ApcF, and L_{CM} are red-shifted relative to the bulk population of APC [2,19,24]. While the upper cylinder is built up solely by bulk APC, one αAPC subunit is replaced by ApcD in one of the two terminal trimers in both basal cylinders. Similarly, in one of the two central trimers a βAPC subunit is replaced by ApcF, while the adjacent αAPC is substituted by the chromophorylated PB domain of L_{CM} (Fig. 1A). In addition, in all three APC cylinders the terminal trimers are stabilized by a small 7.8 kDa colorless linker protein, L_C [2,19]. Inactivation of the *apcD* and/or *apcF* genes does not prevent the PBS assembly as αAPC and βAPC subunits, respectively, can substitute the corresponding gene products [25]. This made it possible to study the role of these two subunits in *apcD*- and *apcF*-deficient cyanobacterial mutants with fully assembled PBS, which had shown that neither ApcD nor ApcF are required for OCP-induced fluorescence quenching [22,23,26]. Nevertheless, both polypeptides have an

influence on the other major short-term light adaptation process, the state transitions, in cyanobacteria [26,27].

Unlike ApcD and ApcF, the role of the large L_{CM} protein in the cyanobacterial NPQ cannot be elucidated by knockout mutagenesis as, in contrast to ApcD and ApcF, L_{CM} is essential for the PBS assembly [28-30]. However, its interaction with OCP can still be studied *in vitro* as was already demonstrated with isolated PBS and/or APC [17,18,22,23]. L_{CM}, like all other PBS linker polypeptides, is strongly basic, and, though it contains a considerable amount of hydrophilic amino acids, forms aggregates in aqueous solutions [2,3,19]. To prevent this, L_{CM} was purified at pH ~3 in the presence of 2 M urea, in which system its interaction with OCP was proven [23]. This proposes L_{CM} as the primary action site for OCP-induced quenching [23]. However, this assumption should be verified using intact cells and fully assembled PBS, which is not a trivial task. The low abundance of L_{CM} (two copies per PBS), the complex network of excitation energy transfer with forward and reverse processes, and overlapping fluorescence signals [31] makes this rather difficult. The goal of our current study is to further elucidate the role of L_{CM} in OCP-induced quenching. We have studied the interaction of OCP with purified L_{CM} and PBS *in vitro*, completed with *in vivo* examination of an L_{CM}-less *Synechocystis* mutant. At isolation of L_{CM} we have applied the mildest known method [32] that excludes the presence of the urea used before [23]. Our results clearly indicate that OCP directly quenches the L_{CM}-chromophore which event might have a crucial role in the NPQ of cyanobacteria. Moreover, the OCP docking site within the PBS was established by computer modeling using the crystal structures of OCP and APC trimers. Geometrically modeled binding site of OCP on the PBS core is not at variance with the OCP-L_{CM} interaction.

2. Materials and methods

2.1. Strains and culture conditions

The wild type strain of the cyanobacterium *Synechocystis* sp. PCC 6803 and its *apcE*-less mutant were routinely grown for 3-4 days at 30°C with constant shaking in standard liquid BG-11 medium under continuous white fluorescent light of 40 μmol photons $m^{-2}s^{-1}$. The medium of the mutant strain was supplemented with 5 mM glucose and 20 μg/ml erythromycin. The *apcE*-less light-tolerant *Synechocystis* strain [28] was a kind gift of Prof. W. F. J. Vermaas (Arizona State University).

2.2. Isolation procedures

2.2.1. Isolation of PBS

PBS's were isolated according to the method of Glazer [33]. *Synechocystis* cells were harvested, disrupted by French press at 20,000 p.s.i. and purified by 0.25-0.80 sucrose density gradient ultracentrifugation. The fraction of intact PBS which formed the lower narrow blue band in the gradient was collected in 0.8 M phosphate buffer at pH 7.0.

2.2.2. Purification of L_{CM}

The L_{CM} anchor polypeptide was dissociated from the isolated PBS (see 2.2.1) by 15 hours incubation in 60 mM formic acid (pH 3.0) in the presence of 1% (v/v) Triton X-100 and purified subsequently by gel filtration in the same formic acid solution using a Bio-Gel P-100 resin (Bio-Rad) as described in [32]. The isolation procedure did not involve the use of any detergents or denaturizing agents (e.g. urea) that supposedly preserved the conformation of the L_{CM}-

chromophore and its apoprotein close to their natural state. As L_{CM} easily forms an insoluble precipitate [34,35] the samples were used freshly for experimental purposes.

2.2.3. Purification of OCP

OCP was isolated from disrupted *Synechocystis* cells (see 2.2.1) by hydrophobic interaction chromatography using a reverse gradient of ammonium sulfate on Butyl-TSK followed by an ion-exchange chromatography on a TSK-DEAE column according to [12,17]. OCP fraction was stored in 5 mM Tris-HCl, 80 mM NaCl (pH 8.0) at –70 °C prior to use.

2.3. Dephosphorylation of PBS

For dephosphorylation of the PBS linker proteins, isolated PBS' were incubated for 30 min at room temperature in the presence of bovine alkaline phosphatase (Sigma) specific for *O*-phosphorylation of threonine and serine residues in a buffer containing 0.55 M phosphate, 0.1 M glycine, 1 mM $MgCl_2$, and 1 mM $ZnCl_2$ (pH 10.4) according to [36]. The reaction was stopped by adding of 20 mM EDTA in agreement with the original report [36].

2.4. Absorption spectra and sample concentrations

Purity and concentration of the samples were monitored using a Varian 2300 UV-Vis spectrophotometer. At the corresponding peak wavelengths, molar extinction coefficients of 93 $mM^{-1}cm^{-1}$ and 132 $mM^{-1}cm^{-1}$ were used for L_{CM} [35] and OCP [37], respectively. The molar PBS concentration was determined according to the equation

$$[PBS] = 1.6 \times 10^{-7}(1.16A_{652} - 0.23A_{620})$$

using the specific molar extinction coefficients for the two main phycobiliproteins of PBS, PC and APC, at 620 and 652 nm, respectively. (For more information regarding the determination of PBS-concentration, see [23,38]).

2.5. Fluorescence measurements

2.5.1. Steady-state fluorescence spectra

Fluorescence emission and excitation spectra were recorded using a Fluorolog-3 (Horiba Jobin Yvon) and an Aminco-Bowman Series 2 spectrofluorimeter. Prior to measurements, samples were either adapted to darkness for 20 min or – to induce the $OCP_O \rightarrow OCP_R$ phototransformation - illuminated for 5-15 min with intense, 1100 μmol photons $m^{-2}s^{-1}$, blue-green light (halogen white light filtered through a 430-540 nm band-pass filter). Fluorescence quenching with increasing OCP_R concentration was fitted by a routine based on the Stern-Volmer theory of quenching processes [39].

2.5.2. Fluorescence life-time determination

The fluorescence excited state dynamics with sub-nanosecond time resolution was recorded using a Simple Tau 140 single photon counting system described in [40]. Fluorescence of the samples was excited by a 635 nm diode laser (BHLP-700) delivering 100 ps pulses (FWHM) with 50 MHz repetition rate and was registered at 680 nm by an SPC-140 detector module. All components of the system were manufactured by Becker & Hickl, Berlin, Germany.

2.6. Building of a docking model

The geometrical attachment of OCP to the PBS core was explored using the Hex Protein Docking online service with a standard set of settings [41]. In the iteration process, proteins were considered as rigid bodies interacting through electrostatic and Van der Waals forces. The highest scored OCP-PBS complex structure represented the most likely arrangement for this protein-protein interaction. Homology-based molecular model of the PB domain of L_{CM} was created using the SWISSMODEL server (http://swissmodel.expasy.org/). The initial coordinates of OCP and APC trimers were obtained from the Protein Data Bank (1M98 for OCP from *Arthrospira maxima* [12] and 1ALL for APC from the same organism [42]). GROMACS 4.5.3. software package was used to determine the area of the OCP-PBS interaction interface by SASA algorithm with a probe particle radius equal to 1.4 Å [43]. The computed coordinates for both chromophores were used to determine the distance between the 3'-hydroxyechinenone in OCP and the nearest phycocyanobilin pigment molecule in the PBS core which probably participates directly in the quenching process [44].

3. Results

3.1. Quenching of L_{CM} by OCP in vitro

The fluorescence emission spectrum of isolated L_{CM} had a typical broadened shape with a maximum at 668 nm (Fig. 2A, black; [23,34,35]). Neither preillumination with intense blue-green light in the absence of OCP nor addition of OCP_O in the dark induced any change in the shape or amplitude of the spectrum (data not shown). However, when the dark-adapted sample was preilluminated for 5 min in the presence of the OCP_O with intense blue-green light which induced its phototransformation to OCP_R, the fluorescence yield of L_{CM} was significantly decreased (Fig. 2A, gray). This indicates that photoactivation of OCP is a prerequisite for

fluorescence quenching of L_{CM}, as was already reported for assembled PBS *in vivo* and *in vitro* [17,18]. A minor blue shift in the peak position from 668 nm to 665 nm suggests that the L_{CM}-OCP interaction may influence the apoprotein microenvironment around the phycobilin chromophore in L_{CM}. The degree of quenching, integrated over the entire 600-775 nm wavelength range was about 27% (Fig. 2A, bottom). These data are similar to those were obtained previously in the presence of the 2 M urea [23].

For a deeper insight, the concentration of added OCP was varied systematically over a wide range (almost one order of magnitude) while L_{CM} concentration was kept constant (Fig. 2B). In good accordance with the Stern-Volmer theory [39], the quenching of the L_{CM} fluorescence depended linearly on OCP concentrations and was fitted perfectly with the equation of

$$F_0/F = 1 + K[Q],$$

where F_0, F, K, and [Q] represent the initial fluorescence intensity, the intensity in the presence of quencher, the quenching constant, and the concentration of quencher, respectively (Fig. 2B). Generally, the linear concentration dependence is indicative of a single class of fluorophores accessible to the quencher [39]. Here, this may correspond to a single phycocyanobilin molecule covalently bound to the L_{CM} polypeptide [2,3]. The semi-logarithmic plot of the fluorescence transient of L_{CM} upon a 100 ps laser flash (Fig. 2C, black) shows a mono-exponential decay with an intrinsic lifetime of ~1.2 ns. In the presence of OCP the fluorescence decay and therefore the intrinsic lifetime was virtually unaffected (Fig. 2C, gray) which may indicate a static character of the quenching [39], i.e., L_{CM} forms a less-fluorescent complex with OCP.

3.2. Fluorescence quenching and energy migration from PBS to Photosystem I in wild type and L_{CM}-less Synechocystis

Figs. 3A and B show the impact of strong blue-green light on room temperature fluorescence emission spectra of wild type and L_{CM}-less *Synechocystis* cells, respectively, upon PBS excitation (λ_{ex} = 580 nm). Noticeably, the position of the major fluorescence peak in the mutant (Fig. 3B; 658 nm) was considerably blue-shifted as compared to that of the wild type (Fig. 3A; 667 nm) as a consequence of the absence of the long-wavelength L_{CM} emitter. In accordance with earlier observations [7,20,45], pre-illumination by intense blue-green light induced a significant (≥ 50%), reversible quenching in wild type *Synechocystis* (Fig. 3A). The original fluorescence yield was completely restored after 45 min dark incubation (Fig. 3A). In contrast, the quenching in the mutant cells was very small (less than 7%) and the initial fluorescence level has returned quickly (within 5 min; Fig. 3B). While absence of L_{CM} involves a partial loss of phycobiliproteins in *Synechocystis*, this deficiency does not imply changes in the cellular chlorophyll content and results only in a slight increase in the Photosystem II to Photosystem I ratio [30]. Nevertheless, the lack of L_{CM} leads to a partial decoupling of PC and APC from the reaction centers [28-30]. It is generally believed that PBS-absorbed excitation energy primarily transfers to Photosystem II, however, direct energy transfer from PBS to Photosystem I has also been reported [38,46,47] - low temperature (77 K) fluorescence excitation spectrum (λ_{em} = 740 nm) of wild type *Synechocystis* cells showing a PBS peak at 625 nm also clearly indicates this (Fig. 3C, black). Fluorescence excitation spectrum of the L_{CM}-less mutant was virtually the same (Fig. 3C, gray) which show that, in contrast to the energy transfer to Photosystem II, L_{CM} is not absolutely required for the energy transfer from - partially assembled - PBS to Photosystem I (see also [28]). In this case although Photosystem I in the mutant cells needs a photoprotection under high light conditions, the process, in absence of L_{CM}, is apparently not regulated via OCP-dependent energy dissipation (Figs. 3B and C).

3.3. Quenching of isolated PBS as revealed by low temperature fluorescence emission and excitation spectra

In contrast to room temperature fluorescence emission spectra of isolated PBS which displays a major peak at 660 nm originating from APC [17,18], low-temperature (77 K) fluorescence emission spectra are dominated by the emission of the PBS terminal emitters at 683 nm, while PC and APC exhibit a combined minor band at 657 nm ([24,48]; Fig. 4A). Illumination of the sample with intense blue-green light – prior to freezing – in the presence of OCP results in an about 40% quenching of the main fluorescence peak at 683 nm and only a minor change in the amplitude of the short-wavelength fluorescence band (Fig. 4A), in agreement with earlier observations with *Synechocystis* mutant cells lacking either both photosystems [49] or Photosystem II alone [6]. The data indicate the active involvement of the long-wavelength PBS emitters in NPQ, although the individual fluorescence of ApcD, ApcF, and L_{CM} are essentially indistinguishable on the emission spectra. Contrary to this, while light absorption of L_{CM} and ApcD are manifested as a small but well discernible band at 678 nm on the fluorescence excitation spectra (λ_{em} = 740 nm) [24], the fluorescence excitation of ApcF is blue shifted by about 5-7 nm [48]. Upon inducing OCP_R by blue-green light the faint 678 nm band has disappeared (Fig. 4B, inset), which, again, indicates the involvement of L_{CM} (and/or, less likely, ApcD) in the quenching process.

3.4. Enzymatic dephosphorylation of PBS influences the quenching

In *Synechocystis*, the main phycobiliproteins, i.e. PC and APC, are not phosphorylated under physiological conditions and the group of PBS constitutive phosphoproteins includes only linker polypeptides: L_{CM}, rod-core and rod linker proteins [36]. However, while the latter linkers are purely sensitive to a dephosphorylation treatment [36], L_{CM} and rod-core linker can exist in both phosphorylated and dephosphorylated forms and this feature may influence the quenching process. Indeed, our data shows that a large excess of alkaline phosphatase (which induced L_{CM}

dephosphorylation) involved a significantly higher quenching of the fluorescence of isolated PBS. This observation further supports the conclusion that L_{CM} is the primary site for OCP-induced fluorescence quenching.

3.5. A docking model for OCP-PBS interaction

Constructing of a homology-based 3D model of the chromophorylated PB-domain of L_{CM} based on its amino acid sequence [50] shows that the structure of PB-domain is very similar to that of αAPC (Fig. 5A; [21]). Thus, the structure of the $(\alpha\beta)_2$/ApcF/L_{CM}-PB trimer is most likely also very similar to structure of the APC trimer as determined by X-ray crystallography [51]. Regarding OCP, only the crystal structure of the dark-stable OCP_O form is known (Fig. 5B; [12,13]). However, by using a rigid body model one can draw conclusions regarding the OCP-PBS interaction. In spite of the altered conformational state of the photoactivated (metastable) OCP_R form, OCP is composed of two domains, the all α-helical N-terminal domain (Fig. 5B, yellow) and the α/β-composed C-terminal domain (Fig. 5B, orange); the carotenoid molecule spans both domains [12,13]. OCP has an irregular shape with several cavities and protuberances. It has a large (895 $Å^3$) surface cavity located in between the two structural domains and leading to the carotenoid binding cleft (Figs. 5B and C).

Due to the highly organized PBS superstructure, it is not *a priori* clear where and how the PBS core could attach OCP, and there are many constraints to be taken account, including the shape of both OCP and PBS. In the terminal APC trimers of the PBS core the chromophore-exposed central channel is occupied by the L_C-linker protein [52], therefore is inaccessible to OCP. In contrast, the lateral surface of the APC cylinders is not occupied by linker proteins and, therefore, could easily be accessed. The α-subunit of APC has characteristic tip jutting of its lateral surface (Figs. 1B and 5C). Modeling of the binding of OCP to the APC trimer using the Hex Protein Docking Server [41] provided a strong indication of OCP binding at this site. The

most likely structure of the OCP-APC complex is shown in Fig. 5C. Remarkably, the analysis also revealed the crucial role of the OCP's central surface cavity in the OCP-PBS interaction, facing towards the aforementioned tip. The contact area between the two proteins is about 800 $Å^2$. Seemingly, about 20 amino acid residues from αAPC/PB domain of L_{CM} and only 11 residues from the neighboring βAPC subunit take place in the docking which shows the potential role of the PB domain in the OCP-PBS interaction. According to the presented molecular model, the distance between the OCP carotenoid and the closest phycocyanobilin chromophore at position α84 in APC is 20.7 Å (Fig. 5C). Noticeably, based on the threefold rotational symmetry of APC, this latter pigment, except the PB-domain of L_{CM}, is technically interchangeable with the corresponding chromophore in ApcD but not with those of βAPC or ApcF as they are located close to the central channel of the APC trimers. In summary, the presented model is in agreement with the experimental results, and clearly shows the possibility of a direct OCP-L_{CM} interaction. If this is case, the PB loop of L_{CM} (Fig. 5A) with several phosphorylatable amino acid residues (i.e. serines) [50] and presumably located close to the middle of the interface region should play important role in OCP docking.

4. Discussion

In this work, we further examined the blue-light induced fluorescence quenching in cyanobacteria and provided experimental evidence that suggests a direct interaction between OCP and L_{CM} during this photoprotective process. L_{CM} is the largest chromopeptide within the PBS with a molecular mass of 72-128 kDa, depending on the organism [2,3]. Sequence analysis indicates that L_{CM} is a multifunctional protein [50]: It plays central roles in (i) formation of the PBS core substructure, (ii) anchoring PBS to the thylakoid membrane and (iii) excitation energy transfer, preferentially, to Photosystem II [2,3,19]. Besides these, as was shown in our very recent paper, L_{CM} serves as an interacting partner for OCP *in vivo* which suggests that L_{CM} is the

primary target for OCP binding within the PBS [23]. Like other linker polypeptides, isolated L_{CM} can easily form aggregates which make it difficult to handle. The relatively few biochemical and spectroscopic examinations of purified L_{CM} have also been hampered by its poor solubility (see [35] and references therein). To solve the stability problem, we have applied urea in our former study, which showed strong impact of OCP on the L_{CM} fluorescence yield [23]. We have confirmed this observation in our present study, using a very mild procedure for L_{CM} isolation without applying any denaturizing agents (e.g. urea), thus, most probably, the L_{CM}-chromophore and its apoprotein were preserved in their natural conformation [32]. The similar quenching (~30%) of isolated L_{CM} and fully assembled PBS *in vitro* strongly indicates that these two processes follow the same mechanism on the one hand, and attachment of OCP to L_{CM} present on the other. The facts that each PBS contains two L_{CM} subunits, and binding of only one OCP to the PBS is sufficient to induce fluorescence quenching, which implies a very specific binding *in vivo,* are in accordance with the latter conclusion [14].

Besides this, we provide here further evidence indicative of an OCP-L_{CM} interaction, and show also some qualitative data in respect of the quenching. We have shown that the quenching of L_{CM} fluorescence is not accompanied by diminishing of the intrinsic lifetime of the excited state (1.2 ns; Fig. 2C), which phenomenologically corresponds to a static type of quenching, according to the generally accepted classification [39]. This suggests that the L_{CM} PB-domain and OCP_R form a non-fluorescent complex, where a part of the excitation energy absorbed by PBS can be harmlessly dissipated into heat in the quenched L_{CM} state [6,15,20]. The lifetime of the S_1 state of the OCP-chromophore 3´-hydroxyechinenone (3.2 ps) is much (~400 times) shorter than of the excited phycobilins ([53,54], Fig. 2C). This extremely short lifetime, crucial for effective NPQ, cannot be detected in sub-nanosecond time resolution but is consistent with the static character of the quenching. In our former work [23], we have shown that, in the case of fully assembled PBS, the blue-green light induced NPQ was a combination of two different types of quenching mechanisms: a major dynamic one, in which the intrinsic lifetime of excited state

of chromophores (in the bulk APC) was shortened, and a minor static one, in which OCP might form a less- or non-fluorescent complex with an unidentified PBS subunit. Based on our current data, we can suggest that L_{CM} is responsible for the static part of the observed quenching, while PBS chromophores that transfer excitation energy to OCP *via* L_{CM} correspond to the dynamic part. However, this proposal is still to be verified by quantitative analysis of energy transfer pathways among the 72 phycocyanobilin chromophores of the three APC cylinders forming the PBS core [55].

For low temperature (77 K) fluorescence excitation measurements with isolated PBS, the fluorescence emission at 720 nm had contributions from both the bulk APC and long-wavelength emitters (Fig. 4B). The disappearance of the 678 nm-peak in the quenched state indicates the involvement of a minor long-wavelength PBS chromophore in the quenching process, which is further supported by the predominant quenching of the major 683 nm-band in the low temperature fluorescence emission spectra (Fig. 4A). Noticeably, isolated PBS require specific conditions (buffer, temperature, etc.) in order to maintain their functional and structural characteristics [33]. However, as shown here and elsewhere [15,20], blue-green light induced fluorescence quenching can be studied even in samples which were frozen immediately after inducing NPQ. Although the data obtained at low temperature do not certainly reflect the quenching developed under physiological conditions, they allow drawing solid conclusions in combination with other results.

Protein phosphorylation and dephosphorylation is one of the most common modes of regulation of cellular responses. Phosphorylation has already been suggested to contribute to PBS-related processes [56]. *In vivo* dephosphorylation of PBS linkers was also observed upon exposure to high light intensities [36] which also facilitates the photoactivation of OCP. The PB-domain of L_{CM} (see Results and Fig. 5A) includes a so-called PB-loop formed by 50 to 72 residues including potentially phosphorylatable amino acids [50]. Since deletion of this loop has no impact on PBS assembly or PBS to reaction centers excitation energy transfer, its role in

anchoring PBS to the thylakoid membrane was excluded [57], and the function of the loop remains unknown. As written above, L_{CM} is the only polypeptide within the PBS core which can (dynamically) be phosphorylated [36]. Based on our data showing that enzymatic dephosphorylation of PBS has an impact on OCP-induced quenching (Fig. 4C), one can speculate that the PB-loop plays role in OCP binding on the one hand, and that this binding is modulated *via* phosphorylation/dephosphorylation on the other.

The experiment with intact L_{CM}-less *Synechocystis* cells (Fig. 3) showed that besides binding to L_{CM}, OCP can also be bound to APC, where is can also induce NPQ. However, the small fluorescence quenching (< 7%) and fast recovery suggest that the binding of OCP to the partially assembled PBS [28,29] is unspecific and unstable. We conclude that OCP has much larger affinity to L_{CM} than to all other components of PBS.

The question whether OCP acts solely as a photosensor or also as a fluorescence quencher was open until direct PBS-OCP interaction was demonstrated *in vitro* [17,18]. Based on our *in silico* docking analysis, we propose an OCP binding site on PBS as presented in Fig. 5C (see also Results). According to our proposal the specific OCP-L_{CM} binding is realized between the αAPC lateral tip and central OCP aperture that results in a large contact surface of 800 $Å^2$. Previously, it was proposed that OCP interacts with PBS *via* its C-terminal domain; this suggestion was based on the structural similarity of this domain composed of α-helical and β-sheet motifs and the small L_C core linker protein [14]. Importantly, this model is in contradiction with the permanent presence of L_C in PBS and its direct contact with the β84 chromophores of APC, which would block the free access of OCP to PBS. Later, this original suggestion was reconsidered in favor of the N-terminal domain based on the assumption that the positively charged Arg155 inside the N-terminal OCP domain is involved in the interaction with PBS [58]. According to our novel model, both the N-terminal and C-terminal domains of OCP participate in docking to the PBS, in a sense that both domains take part in formation of the OCP central cavity with Arg155 being located in the interface between the N- and C-terminal domains. This

model also accounts for the position of the carotenoid that spans both OCP domains. This carotenoid molecule is almost completely buried within the protein matrix; only 3.4% of the pigment surface is accessible to the solvent [12,13]. However, as shown by our model (Fig. 5C), shielding of the carotenoid from the solvent itself does not hinder the interaction with PBS, since the distance to the nearest α84 phycocyanobilin chromophore is only about 20 Å which is compatible with the distance of two adjacent chromophores inside the APC trimers [21,42].

The structure of the PB domain of L_{CM} is rather similar to that of the αAPC subunit but not quite identical [19,21,59]. Also, the surface charge patterns of these two subunits, as well as the exact localization of the chromophore in the binding pocket, are slightly different [21]. These differences might be crucial for specific binding of OCP to L_{CM} and effective quenching. Carotenoid molecules are able to receive excitation energy from tetrapyrrole molecules *in vitro via* energy transfer to their optically forbidden S_1 state [60]. Three different molecular mechanisms for tetrapyrrole excitation quenching are distinguished: (i) tetrapyrrole-carotenoid electron transfer and recombination; (ii) excitonic coupling between tetrapyrrole and carotenoid excited states and ensuing internal conversion to the carotenoid ground state; (iii) tetrapyrrole singlet excited state to carotenoid S_1 state energy transfer and fast internal conversion to the carotenoid ground state [60,61]. The last of these three possibilities is implied by relatively large inter-chromophore distance (~20 Å) between the carotenoid and phycocyanobilin molecules in our docking model. The very recent data on the forbidden S_1 state of the OCP hydroxyechinenone in its photoactive and photoinactive states [62] might facilitate the determination of the exact molecular mechanism of blue-green light induced NPQ in cyanobacteria.

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Figure legends

Fig. 1. Schematic representation of the PBS core. A. A cartoon of the tricylindrical PBS-core model of *Synechocystis* according to refs. [2] and [19]. Each cylinder consists of four discs and each disc represents a trimer composed of six phycobiliprotein subunits - each of them contains one chromophore (see also B). Numbering refers the corresponding subunit composition of the disc. While the top cylinder contains only APC trimers - with or without the small L_C-linker - the two compositionally identical bottom cylinders, adjacent to the thylakoid membrane, also contain three terminal emitters, ApcD, ApcF and PB-domain of L_{CM} (ApcE) as indicated. B. Side view of an $(\alpha\beta)_3$-APC trimer, according to ref. [42]. The apoprotein is shown as a transparent gray matrix and the immersed phycocyanobilin chromophores are represented as black sticks. Designation of the chromophores in an ($\alpha\beta$) heterodimer is also indicated. For further details see text.

Fig. 2. A. Room temperature fluorescence emission spectra of isolated L_{CM} in the absence (black) and presence (gray) of OCP_R and their difference (bottom). The samples were excited at 580 nm. B. Fluorescence quenching of L_{CM} as shown in (A) as a function of OCP concentrations.

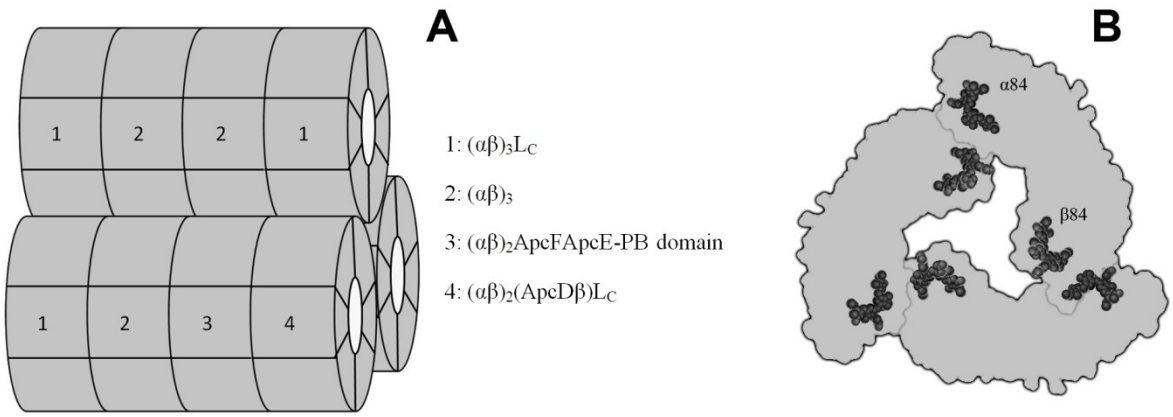
The experimental points were fitted by Stern-Volmer coordinates (see text). C. Semi-logarithmic plot of the normalized fluorescence transient of L_{CM} upon a 100 ps laser flash (λ = 635 nm) in the absence (black, τ_1) and presence (gray, τ_2) of 10-fold excess of OCP_R. The applied L_{CM} concentration was 2 x 10^{-8} M in each panel.

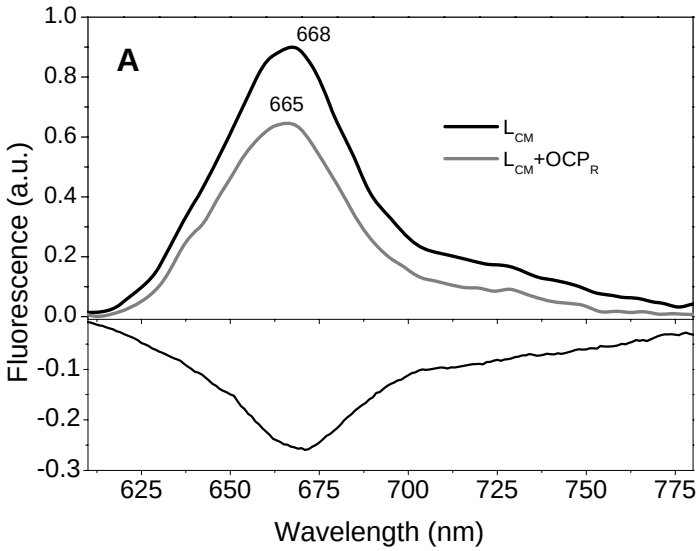
Fig. 3. Room temperature fluorescence spectra of intact wild type (A) and L_{CM}-less (B) *Synechocystis* cells before (black) and after (gray) the blue-green light induced fluorescence quenching and during the subsequent dark-recovery (dashed, dashed-dotted and dotted curves). The samples were excited at 580 nm; the recording time of the each spectrum was 15 s. (C) Low temperature (77 K) fluorescence excitation spectra of intact wild type (black) and L_{CM}-less (gray) *Synechocystis* cells. The fluorescence emission was registered at 740 nm.

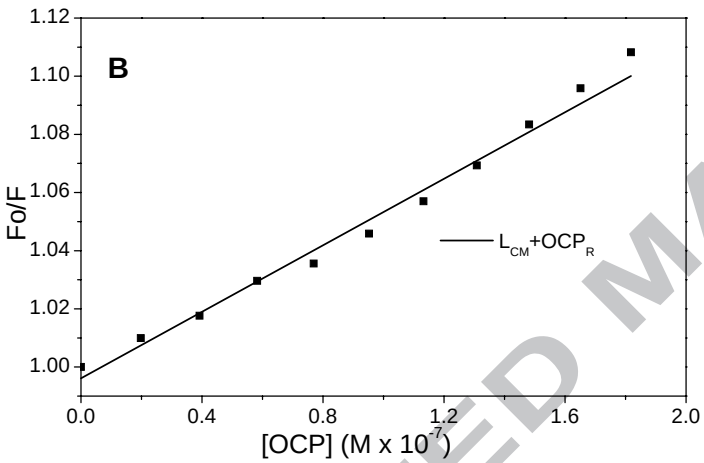
Fig. 4. A. Low temperature (77 K) fluorescence emission spectra (λ_{exc} = 580 nm) of isolated PBS in the presence of (dark-adapted, inactive) OCP_O (black) or OCP_R (gray) where formation of OCP_R was induced by intense blue-green light. Dichlorofluorescein diacetate was used as an internal standard as in ref. [15]. B. Low temperature fluorescence excitation (λ_{em} = 720 nm) spectra of isolated PBS in the presence of OCP_O (black) or OCP_R. Color coding and light treatment are as in panel (A). The spectra were normalized to the 625 nm peak. The inset shows the red part of the spectra magnified. C. Fluorescence quenching of isolated PBS dephosphorylated in the presence of OCP_R and increasing concentrations of bovine alkaline phosphatase after 5 min of blue-green light illumination. The height of the columns represents the average of three replicates. The PBS concentration was 10^{-9} M and 10-fold excess of OCP was applied at each probe.

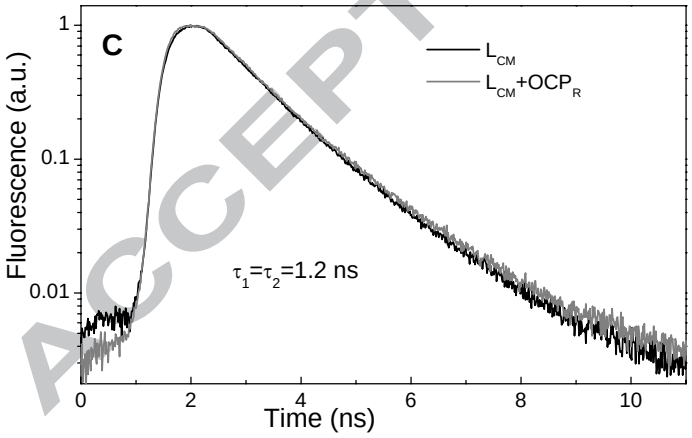
Fig. 5. A proposed 3D model for the OCP-PBS interaction. A. A homology-based stereo model demonstrating the high structural similarity between the PB domain of L_{CM} (red) and αAPC

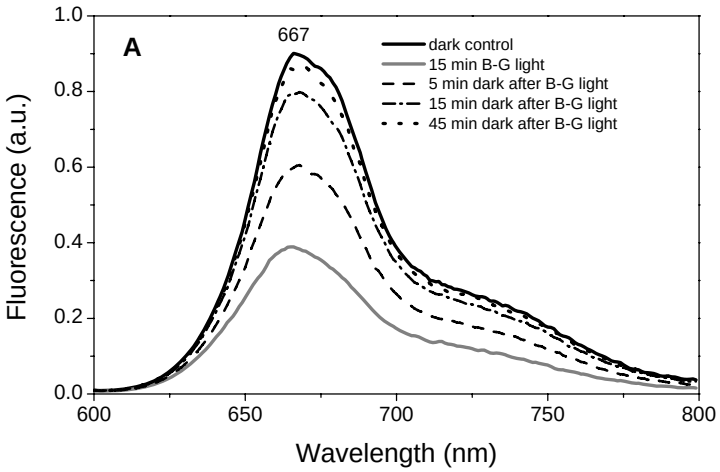
(blue). The phycocyanobilin chromophore of αAPC is shown in green. B. The 3D structure of OCP according to [11]. While the C-domain of the apoprotein is marked by orange, the N-domain is shown as yellow. The carotenoid is shown as a blue stick. C. OCP docking to the APC trimer. The OCP apoprotein is shown by orange, while its carotenoid chromophore is shown as red stick. The APC trimer is shown by blue, while the phycocyanobilin chromophores are shown as dark sticks. The distance between the center of masses of carotenoid and the neighbouring phycocyanobilin is also indicated.

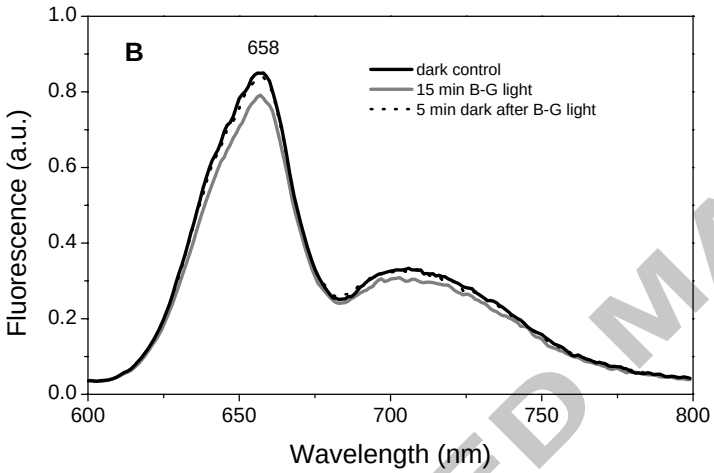


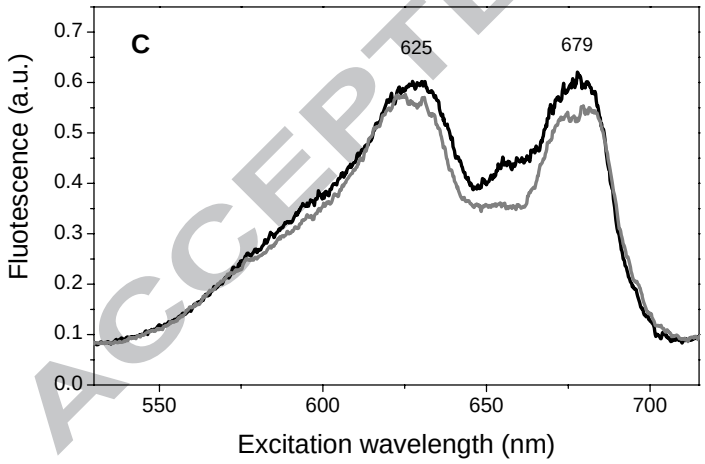


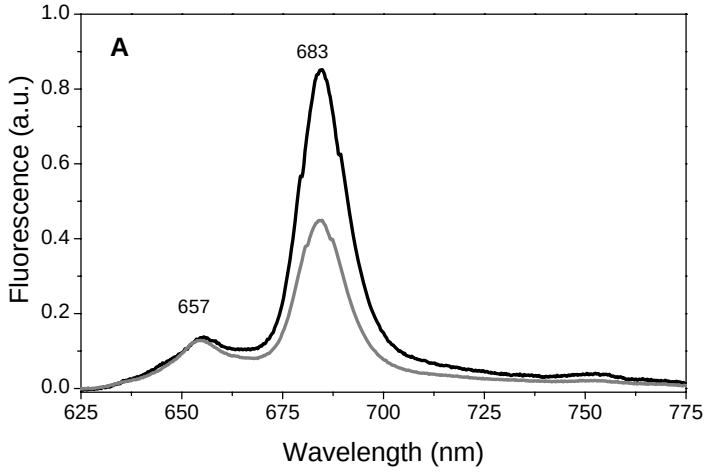


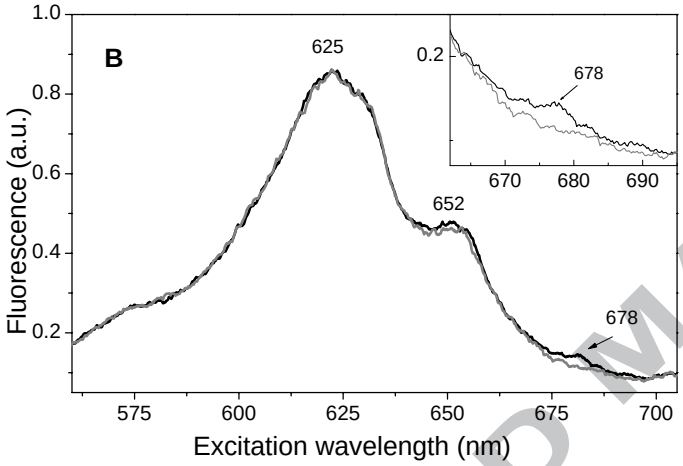


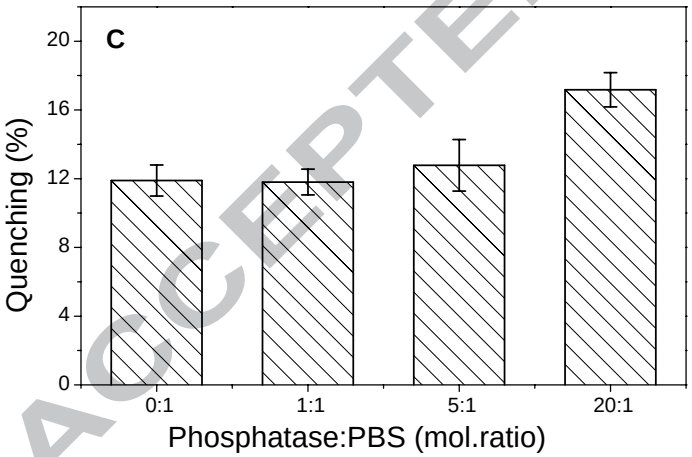


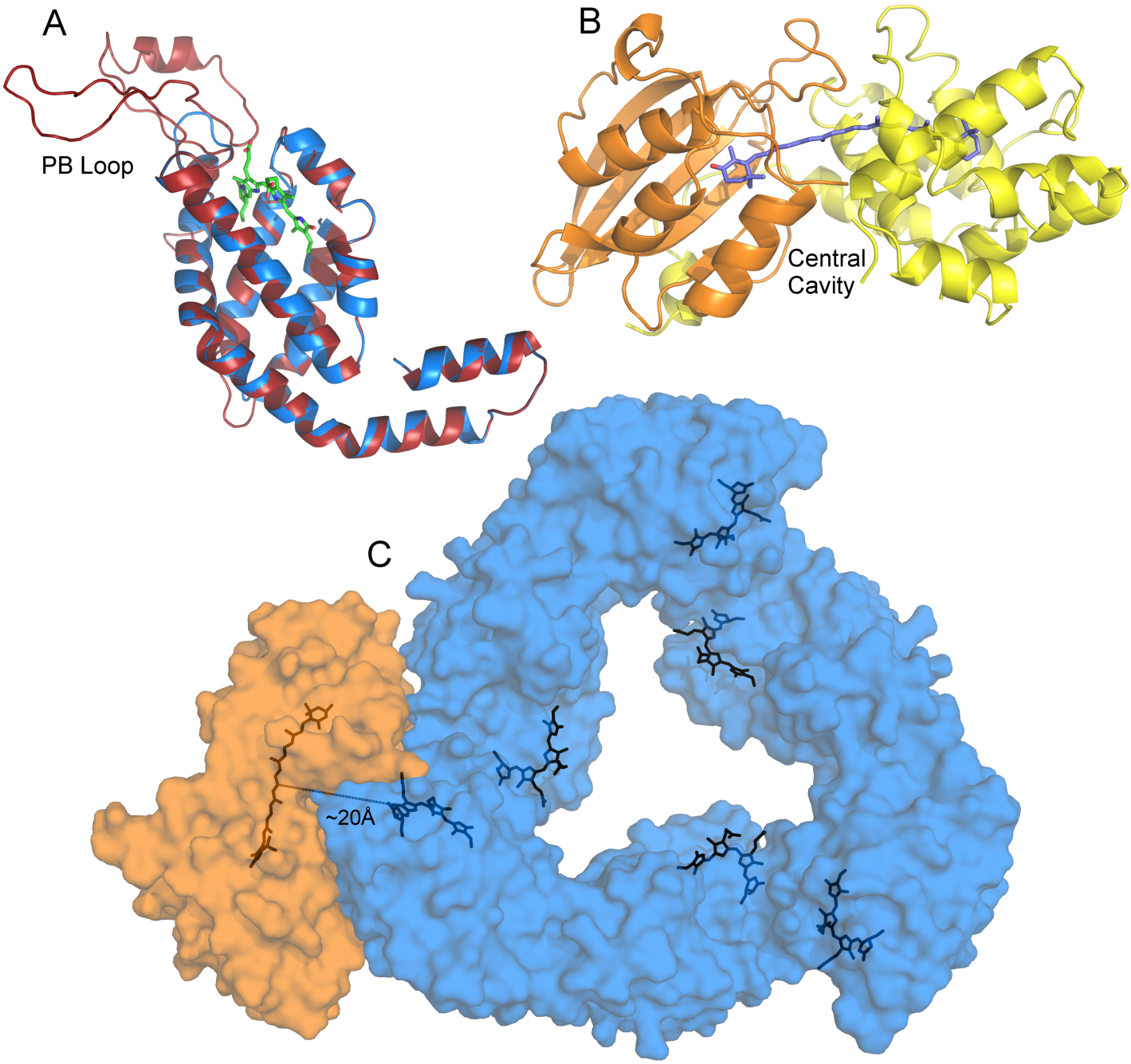




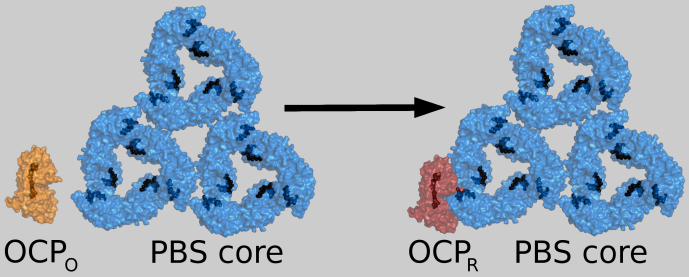








ABSTRACT. The fluorescence emission of the phycobilisome (PBS) core-membrane linker protein (L_{CM}) can be directly quenched by photoactivated orange carotenoid protein (OCP) at room temperature both *in vitro* and *in vivo*, which suggests the crucial role of the OCP-L_{CM} interaction in non-photochemical quenching (NPQ) of cyanobacteria. This implication was further supported (i) by low-temperature (77 K) fluorescence emission and excitation measurements which showed a specific quenching of the corresponding long-wavelength fluorescence bands which belong to the PBS terminal emitters in the presence of photoactivated OCP, (ii) by systematic investigation of the fluorescence quenching and recovery in wild type and L_{CM}-less cells of the model cyanobacterium *Synechocystis* sp. PCC 6803, and (iii) by the impact of dephosphorylation of isolated PBS on the quenching. The OCP binding site within the PBS and the most probable geometrical arrangement of the OCP-allophycocyanin (APC) complex was determined *in silico* using the crystal structures of OCP and APC. Geometrically modeled attachment of OCP to the PBS core is not at variance with the OCP-L_{CM} interaction. It was concluded, that besides being a very central element in the PBS to reaction center excitation energy transfer and PBS assembly, L_{CM} has also an essential role in the photoprotective/light adaptation processes of cyanobacteria.



Highlights

Fluorescence of L_{CM}-polypeptide of the phycobilisome is quenched by orange carotenoid protein.

L_{CM} quenching was studied by different methods *in vitro* and *in vivo*.

The 3D docking site of orange carotenoid protein and the phycobilisome core was demonstrated.