Hybrid coupling of R-phycoerythin and the orange carotenoid protein supports the FRET-based mechanism of cyanobacterial photoprotection

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ABSTRACT

To regulate the effectiveness of photosynthesis and photoprotection cyanobacteria utilize a system consisting of only few components. Photoactivation of the orange carotenoid protein (OCP) enables its interaction with a specific, yet controversial site in the core of the light-harvesting antenna, the phycobilisome (PBS). The resulting delivery of a quenching carotenoid molecule to the antenna pigments leads to thermal dissipation of the excitation energy absorbed by the latter, and, consequently, to depression of the photosynthetic activity. The nature of the OCP-induced PBS fluorescence quenching mechanism remains debatable, however, specific protein-protein interactions between PBS and photo-activated OCP should provide a unique environment for interactions between the excitation energy donor and acceptor. Here we questioned whether the Förster theory of resonance energy transfer can explain PBS quenching by OCP even at their very small spectral overlap and whether in model systems, the absence of specific protein-protein interactions of OCP with a donor of energy can be compensated by a better spectral overlap. Hybridization of algal R-phycoerythin with cyanobacterial OCP by chemical crosslinking results in a significant decrease of R-phycoerythin fluorescence lifetime, irrespective of the OCP photoactivation status. Supported by structural considerations, this indicates that FRET may be the essence of cyanobacterial photoprotection mechanism.

1. Introduction

All photosynthetic organisms have to balance between the efficiency of photosynthesis and photoprotection [1,2], since photoexcitation of light-harvesting pigments in the presence of molecular oxygen may lead to the formation of extremely harmful reactive oxygen species. Mechanisms of photopadaptation include both, non-photochemical quenching of excited states (i.e. the dissipation of absorbed excess excitation energy into heat) [3–6] and chromatic adaptation (i.e., the adjustments of pigment composition of the light-harvesting complexes, depending on the light conditions) [7]. Phycobilisomes (PBS), the megadalton light-harvesting complexes [8] of cyanobacteria and red algae, consist of hundreds of phycobiliproteins (PBPs), which are organized in terms of an “energy funnel” delivering the excitation energy from blue- to red-absorbing pigments, and, eventually, to chlorophylls of the photosynthetic reaction centers [9–14] by Förster resonance energy transfer (FRET) [11].

To regulate the effectiveness of primary photosynthetic and photoprotective reactions cyanobacteria utilize a system consisting of only three components [15]. Besides water-soluble PBS it requires a quencher of excitation energy. Similar to higher plants, which use carotenoids and a well-known gear-shift mechanism [5]
to switch between light harvesting and energy dissipation. Cyanobacteria utilize carotenoids to quench PBS. In both cases, such choice of a quencher molecule is explained by an extremely short lifetime (1–10 ps) of the carotenoids in the excited state [16]. However, since carotenoids are not soluble in water, in order to quench water-soluble PBS, cyanobacteria developed a variety of water-soluble proteins, which can bind keto-carotenoids [17–21]. One of such proteins is a 35 kDa photoactive Orange Carotenoid Protein (OCP), which acts as a sensor of light intensity [22]. High levels of insolation trigger conversion of the basal orange OCP form (OCP(β)) into the physiologically active, red form (OCP(α)). The active red OCP form delivers the quenching molecule to the PBS core, through which passes excitation energy from thousands of antenna pigments to the chlorophylls of photosynthetic reaction centers. The location of a single carotenoid molecule in the “neck of the energy funnel” causes the fluorescence quenching of all pigments of PBS, dissipating the excitation energy into heat with high efficiency [23,24]. This is necessary in conditions of high light stress, whereas to restore the photosynthetic activity the so-called Fluorescence Recovery Protein (FRP) is required, which accelerates the detachment of OCP from PBS and its inactivation [25–28]. These mechanisms underlie the successful adaptation of cyanobacteria to various environmental conditions and, accordingly, determine the production of biomass.

However, the site of interaction of OCP with PBS and the exact nature of the quenching process has not yet been established unequivocally. The situation is complicated by the enormous size of PBS compared to OCP and the lack of a convenient model system for studying non-photochemical quenching. Isolation of PBS from cells for in vitro experiments is associated with a disturbance of their structure, the stabilization of which requires the use of ~1 M phosphate buffer, which affects the photoactivity of OCP [29] and significantly reduces the effectiveness of its interaction with PBS. For example, in vitro, a 100-fold excess of OCP per 1 PBS is required to achieve maximum quenching efficiency [15], although it is now known that each PBS is capable of simultaneously interacting with only two OCP molecules [30]. Such experimental difficulties causing underestimation of quenching rates alongside with the fact that PBS core-associated emission peaks at ~660 nm, where absorption of OCP is very low forced some authors to consider virtual excited levels of carotenoids and unexpected for significantly different types of pigments excitonic coupling [31]. Application of modern single-particle time-resolved spectroscopy solves at least part of these problems, unambiguously demonstrating that one PBS has only two sites for interactions with OCP and OCP causes at least 94.5% reduction of PBS fluorescence lifetime [30]. Such estimates show that the rate of PBS-to-OCP energy transfer is at least two times faster than PBS-to-chlorophyll, indicating that OCP can effectively compete with chlorophylls of reaction centers for the excitation of PBS pigments. But if OCP is such a good quencher why does it require a specific site in the PBS core to induce quenching?

Here, we questioned whether OCP can play a role of the quencher of a PBS component lacking the specific OCP binding site but presenting more pronounced spectral overlap. We apply Förster formalism to support the FRET-based mechanism not only in the selected hybrid system but, effectively, also in PBS-OCP interactions.

2. Materials and methods

2.1. Proteins

The His-tagged orange OCP from Synechocystis PC6803 was obtained in a carotenoid-producing Escherichia coli strain [32]. R-PE was isolated from Porphyra yezoensis obtained from the seaside of Yantai. Freeze-dried samples (5 g) were resuspended in 50 ml phosphate buffer (0.02 M, pH 6.8) and supplemented with ammonium sulfate (60% saturation) to precipitate R-PE. The precipitate (1 g) was resuspended in 10 ml phosphate buffer (0.02 M, pH 6.8) and loaded on a Phenyl-Sepharose 6 Fast Flow (GE, USA) column. After washing at 1 M ammonium sulfate, the elution of R-PE was done with a phosphate buffer (0.02 M, pH 6.8) at a flow rate of 2 ml/min. Protein concentration was determined by spectrophotometry using molar extinction coefficients equal to 65000 M⁻¹ cm⁻¹ at 500 nm for OCP [27] and 1.96 · 10⁶ M⁻¹ cm⁻¹ at 565 nm for R-PE [33].

2.2. Analytical size-exclusion spectochromatography

R-PE was loaded on a Superdex 200 Increase 5/150 column (GE, USA) equilibrated with a 20 mM Tris-HCl buffer (pH 7.6) containing 150 mM NaCl and operated using a Varian Prostar chromatography system with multiparametric detection consisting in full-spectrum absorbance (PS335 detector) and tunable fluorescence (PS363 detector). The column was calibrated by protein standards: bovine serum albumin monomer (66 kDa), bovine serum albumin dimer (132 kDa), ovalbumin (43 kDa) and α-lactalbumin (15 kDa) to estimate the apparent molecular mass of the R-PE sample.

2.3. Glutaraldehyde (GA) crosslinking

The individual R-PE and OCP samples were dialyzed overnight at 4 °C against 60 mM phosphate buffer, pH 7.2, and subjected to crosslinking. The reaction (final GA concentration 0.25%) lasted 15 min at room temperature and was terminated by the addition of an excess of 1 M Tris solution.

2.4. Steady-state absorption and fluorescence measurements

Absorption spectra and time-courses were recorded by a spectrometer based on Maya 2000PRO (Ocean Optics, USA) CCD with 25 μm entrance slit and stabilized deuterium UV-VIS light source (SL S204, Thorlabs, USA). In all experiments, OCP photoconversion was triggered by a blue LED (SOLIS-445C, Thorlabs, USA) which passed through the FB450-40 bandpass filter (Thorlabs, USA). All steady-state fluorescence and fluorescence excitation spectra were recorded on a Lumina fluorescence spectrophotometer (Thermo Fisher Scientific). Wavelengths are indicated in figures; the slits width was 2 nm, absorbance at the excitation wavelength was less than 0.1 to exclude the effect of the inner filter. Steady-state spectra were used for the calculations of the overlap integral between the emission of donor and absorption of energy acceptor.

2.5. Time-resolved fluorescence measurements

Fluorescence decay kinetics were recorded using time- and wavelength-correlated single photon counting setup based on SPC-150 module and HMP-100-07C detector (Becker&Hickl, Germany) with excitation by 30 ps pulses of 510 nm laser (InTop, Russia), driven at a repetition rate up to 50 MHz. A 550 nm longpass filter (Thorlabs, USA) was used to block excitation light. During the experiment temperature of the sample was controlled by a cuvette holder Qpod 2e (Quantum Northwest, USA).

3. Results and discussion

In order to test the reliability of Förster theory and further study the mechanism of OCP-mediated energy dissipation, we decided to...
try if OCP can quench the fluorescence of R-phycoerythrin (R-PE), which we purified from *Porphyra yezoensis* and characterized by analytical size-exclusion spectrochromatography. The chromatographic profile, absorbance and fluorescence spectra indicated high sample monodispersity and spectral purity — see Fig. 1. Such choice of the energy donor was dictated by two reasons. First, although the overall protein structure of R-PE is very similar to other PBPs it probably does not have a specific site to bind *Synechocystis* OCP individually, especially because these proteins are from different organisms. Second, the emission of R-PE is significantly blue shifted (maximum at 577 nm), comparing to the emission of PBS (~672 nm), which results in a higher value of the overlap integral between the emission of the donor (R-PE) and absorption of energy acceptor (OCP, see Fig. 2). Thus, we expected to see no complexes of OCP and R-PE in solution, but in the frames of Förster theory [34], if the distance between the donor and acceptor would be lower than 80 Å we would detect a reduction of R-PE fluorescence lifetime.

Mixing of R-PE even with a ~1000-fold molar excess of OCP has no effect on the fluorescence decay of R-PE (Fig. 3), which indicates that no stable complexes are formed. In order to trap complexes, we used an approach which was previously used to study the PBS-OCP complex [35], namely, protein crosslinking by glutaraldehyde (GA). Incubation with GA causes crosslinking mainly between lysines, which demonstrated no spectroscopic effects on the individual R-PE, but in the presence of OCP, we observed significant quenching of R-PE fluorescence. Analysis of R-PE fluorescence decay revealed a decrease of the lifetime of the main (over 60%) component from 2.7 to 0.4 ns. We assume that this effect is due to energy transfer from R-PE to OCP. The efficiency of this process reaches 85%, which is equivalent to 34.8 Å distance between a R-PE bilin and carotenoid of OCP, or 40.4 Å if OCP was converted into the red form upon GA crosslinking.

Why do we provide distances for both types of complexes with different states of carotenoid in OCP? First, in our experimental conditions, GA causes at least 20% conversion of OCP into the red form in the presence of R-PE. Chemical activation of OCP was shown previously [36,37]. Second, GA does not prevent photoactivation of the remaining orange fraction of OCP, which means that excitation of carotenoid induced by energy transfer from R-PE can convert OCP into OCPR if GA is present and OCP is converted into the red form upon GA crosslinking. Likewise, a sensitized OCP activation may also happen in native PBS-OCP complexes, which should probably prevent OCP from spontaneous detachment, and, therefore, the restoration of full antenna capacity requires FRP. Of note, we were able to track carotenoid translocation from the C- into N-terminal domain by monitoring the changes in TMR fluorescence lifetimes [38], which was well described by the Förster theory due to strong quenching properties of OCP’s carotenoid. Recently, we produced a fusion of OCP and red fluorescent protein (OCP-TagRFP), in which energy transfer between TagRFP chromophore and carotenoid in OCP was also successfully described by Förster formalism, revealing the details of such complex structural organization [39].

![Fig. 1. Characterization of the R-PE sample by analytical size-exclusion spectrochromatography.](image)
the effects observed in complexes of OCP and different energy donors (quenching of R-PE ~ 85%, TMR up to 71% [38], TagRFP up to 75% [39]) are striking, in all cases OCP carotenoid quenches fluorescence of the donor with efficiency which depends on the spectral characteristics (overlap integral between emission of the donor and S0–S2 absorption of carotenoid) and donor-acceptor distance. Considering such similarity, we postulate that Förster theory is suitable for the description of energy transfer in PBS-OCP complexes, which results in non-photochemical quenching of PBS fluorescence and energy dissipation into heat.

Since the most red-shifted phycobiliproteins are located in basal cylinders of the PBS core and represent the “neck of the energy funnel”, OCP-mediated delivery of quenching carotenoid to the core seems more than reasonable. The apparent problem is that PBS core-associated emission peaks at ~660 nm (at room temperature), where absorption of OCP is very low. Values of Förster radii \( R_0 \) for PBS and OCP in the orange and red states are equal to 27.0 and 33.5 Å, respectively (Fig. 2). Now there are several working models of OCP-PBS interactions. First was obtained by molecular docking of APC trimer and OCPO [31]. Distance between carotenoid and phycocyanobilin (PCB) was estimated to be equal to 24.7 Å, however, the 12 Å photoactivation-induced translocation of carotenoid into the N-terminal domain [40] was not taken into account. Another model was obtained by using crosslinking mass-spectrometry. Using 11.4 Å long crosslinkers, authors found that the N-terminal domain of the OCP is closely involved in the association with a site formed by two APC trimers in the basal cylinders of the PBS core [41]. Although this model shows significant steric clashes and carotenoid translocation upon the photoactivation was not considered due to the chronology of these discoveries, the distance between the OCP carotenoid and closest PCB was estimated as 25.8 Å [41]. We find both of these estimates fair enough for the minimum possible distance between the carotenoid and PCB, since it follows from the linear dimensions of proteins. Considering the 25 Å distance, we can estimate the efficiency of energy transfer (Fig. 2) and PBS quenching by OCP if energy transfer occurs via

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**Fig. 2.** (A) — absorption spectra of OCP in orange and red forms and fluorescence spectra of PBS and R-PE. The values of overlap integral between the emission of the donor and absorption of the energy acceptor and corresponding Förster radii were used to plot (B) - a dependency of calculated FRET efficiency on the distance between the donor and acceptor of energy. In all cases orientation of the transition dipoles (\( \chi^2 \)) was considered to be random. Vertical dotted line shows the estimated distance between the phycobilin in PBS and carotenoid in photoactivated OCP, considering the efficiency of energy transfer observed in Ref. [30]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Fig. 3.** (A) — fluorescent decay kinetics of R-PE in the presence of OCP before (red line) and after crosslinking (black) by GA. A control sample of R-PE which was incubated with GA without the addition of OCP is shown in blue. Concentration of OCP was equal to 8.3 μM, R-PE to 12.8 nM, which is equivalent to 22 carotenoids per 1 bilin. (B) — schematic representation of the R-PE/OCP hybrid crosslinked by GA. The efficiency of excitation energy transfer and corresponding distance between the chromophore of R-PE and carotenoid were calculated from the changes in the fast component of R-PE fluorescence decay and overlap between the emission spectrum of R-PE and absorption of OCPO (see Fig. 2). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
FRET. Our calculations show that PBS fluorescence should decrease by 85% if OCP6 is bound, and by 61% if OCP absorption corresponds to the orange, physiologically inactive state. Note that our estimations are based on specific values of donor-acceptor distance and spectral overlap, while the orientation of transition dipoles in all cases was set to random. Thus, underestimation of orientation factor can lead to lower efficiency of FRET. And it is very reasonable to assume that, in a specifically organized PBS–OCP complex, orientation of transition dipoles is not random. Anyway, 85% PBS quenching is very close to effects observed in experiments [15], which proves that Förster theory is suitable for description of OCP induced quenching of PBS fluorescence. Another outcome of such simple calculations shows that to prevent unregulated PBS quenching by OCP it should not be able to come closer than 60 Å to the energy donor (PCB), which is almost the size of the whole OCP.

In this work, we questioned if Förster theory is suitable for the description of energy transfer between the pigments of PBS and carotenoid of OCP. By comparison of the effects observed upon the energy coupling of OCP and different energy donors, we conclude that Förster resonance energy transfer is a reliable model. This conclusion has several outcomes which are important for the control of novel materials based on OCP.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.06.098.

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