Fluorescent Labeling Preserving OCP Photoactivity Reveals Its Reorganization during the Photocycle

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ABSTRACT Orange carotenoid protein (OCP), responsible for the photoprotection of the cyanobacterial photosynthetic apparatus under excessive light conditions, undergoes significant rearrangements upon photoconversion and transits from the stable orange to the signaling red state. This is thought to involve a 12 Å translocation of the carotenoid cofactor and separation of the N- and C-terminal protein domains. Despite clear recent progress, the detailed mechanism of the OCP photoconversion and associated photoprotection remains elusive. Here, we labeled the OCP of *Synechocystis* with tetramethylrhodamine-maleimide (TMR) and obtained a photoactive OCP-TMR complex, the fluorescence of which was highly sensitive to the protein state, showing unprecedented contrast between the orange and red states and reflecting changes in protein conformation and the distances from TMR to the carotenoid throughout the photocycle. The OCP-TMR complex was sensitive to the light intensity, temperature, and viscosity of the solvent. Based on the observed Förster resonance energy transfer, we determined that upon photoconversion, the distance between TMR (donor) bound to a cysteine in the C-terminal domain and the carotenoid (acceptor) increased by 18 Å, with simultaneous translocation of the carotenoid into the N-terminal domain. Time-resolved fluorescence anisotropy revealed a significant decrease of the OCP rotation rate in the red state, indicating that the light-triggered conversion of the protein is accompanied by an increase of its hydrodynamic radius. Thus, our results support the idea of significant structural rearrangements of OCP, providing, to our knowledge, new insights into the structural rearrangements of OCP throughout the photocycle and a completely novel approach to the study of its photocycle and non-photochemical quenching. We suggest that this approach can be generally applied to other photoactive proteins.

INTRODUCTION

Orange carotenoid protein (OCP) is a specific 35 kDa watersoluble protein that plays an essential role in non-photochemical quenching (NPQ) of the cyanobacterial light-harvesting antennae, phycobilisomes (1–4). OCP acts as a blue-green light intensity sensor (5). In vivo under strong light, OCP is converted to its active signaling state, which interacts with phycobilisomes and quenches the excess of excitation energy (3,6–9), thereby decreasing the effective absorption cross section of the photosystems and preventing the formation of potentially damaging reactive oxygen species (10). The mechanism of NPQ and its regulation is still unclear due to the number and size of protein complexes involved. It is now generally accepted that initiation of NPQ requires absorption of blue-green light by OCP harboring the specific keto-carotenoid echinenone (ECN), or 3′-OH-echinenone (hECN), as a cofactor. Absorption of light by (h)ECN triggers a red shift of the OCP absorption and causes significant rearrangement of the protein’s tertiary structure. Such a transition is often referred to as a photoconversion of the orange (OCPD) into the red (OCPB) form, and it has been attentively studied in vitro by different absorption techniques, due to pronounced changes of the S0–S2 absorption of (h)ECN upon photoconversion (11–14).

Although the structure of the red form of OCP is still unknown, recent efforts provided valuable information about photoinduced rearrangements of OCP (15–20). It appeared that the red carotenoid protein (RCP), which is a spectral and functional analog of OCPB, could be obtained by partial proteolysis of OCP upon removal of its C-terminal domain.
(CTD) (15). This fact raised the idea of functional modularity of the CTD and N-terminal domain (NTD). At the same time, it was shown that an active red form could be obtained in the dark by a so-called chemical activation induced by high concentrations of NaSCN (21) or by introducing point amino acid mutations (22). Indeed, very recently we demonstrated that the purple W288A mutant of OCP, which shows a remarkable structural similarity to OCP\(^R\) (22), is capable of constitutive phycobilisome fluorescence quenching in vitro with no need of photoactivation (23). This indicates that the absorption spectrum characteristic for the orange form is a result of the specific protein-chromophore interactions provided by a distinct protein conformation. OCP photoconversion leads to reduction of the protein order (21), partial unfolding (17,19,20), or formation of the so-called molten globule state (24), which is typical for some other photoactive proteins (25). By definition, a molten globule has an increased protein volume (26), which was demonstrated for OCP\(^R\) by small-angle x-ray scattering (SAXS) and size-exclusion chromatography (17,22). On the basis of the SAXS data (17), it was proposed that the CTD and NTD become completely separated upon photoconversion. Simultaneously, it was assumed that ECN not only stays in the NTD during photoconversion, but may also be translocated within the protein, as indicated by the 12-Å shift in chromophore position between the x-ray crystal structure of OCP\(^O\) and that of RCP (18,27,28), which could be an important step for the formation of the active quenching state. Thus, the OCP structure is significantly rearranged upon the photoconversion, and protein-chromophore interactions play an important role in spectral tuning of this photoactive protein. However, since RCP (a truncated OCP coinciding with its NTD) is obliged to house the whole carotenoid, normally situated in the interdomain cavity formed by the NTD and CTD of OCP\(^O\), the position of the chromophore in the context of full-length OCP\(^R\) and mutual structural rearrangements of protein domains remains to be experimentally demonstrated.

OCP photophysics has previously been accessed mostly by means of absorption spectroscopy in a wide range of timescales. This requires relatively high protein concentration, which may entail non-physiological association or oligomerization of OCP in the orange state. In our previous work, we showed that the photocyclic transitions of OCP could be monitored by intrinsic tryptophan fluorescence and by fluorescence of non-specifically bound hydrophobic dyes, e.g., Nile Red (24). Overall, we found that the information about the rate constants of OCP\(^O\) → OCP\(^R\) → OCP\(^O\) conversion obtained via fluorescence techniques is in a good agreement with the data obtained by absorption spectroscopy. Intrinsic and/or extrinsic fluorescence reporters also allow for the application of such powerful techniques as picosecond time-resolved fluorometry, fluorescence anisotropy measurements, and differential scanning fluorimetry (DSF), which provide additional information about protein rearrangements and the local environment of the fluorophore, as well as the stability and size of the protein (22), whereas absorption only allows monitoring of the spectral shift of the carotenoid. However, measurements of OCP transitions via Trp fluorescence in such complex systems as living cells is complicated because of the fluorescence background caused by other proteins containing these amino acid residues. On the other hand, interpretation of the fluorescence of hydrophobic dyes, which are non-covalently bound to OCP, is also difficult, since the exact binding sites are unknown.

The goal of the current research was to establish a system that allows monitoring by Förster resonance energy transfer (FRET) of ECN translocation upon OCP photoconversion. This required us to develop site-specific fluorescence labeling of OCP using a dye, which appeared to be energetically coupled with ECN via excitation energy transfer (EET) and thus sensitive to changes of ECN absorption and/or the conformation of OCP in orange and red states. Here, we took advantage of all three endogenous caroteines of OCP to construct a photoactive fluorescent protein and to characterize its photophysical properties in relation to the protein structure. We found that tetramethylrhodamine-5-maleimide (TMR) is suitable for our goals. Fortunately, covalent derivatization of OCP by TMR resulted in the formation of two distinct donor sites for EET to the ECN cofactor, thus forming two distinct donor-acceptor pairs within the structure of a single OCP molecule. EET in these pairs was sensitive to OCP photoconversion, and this feature allowed us to estimate the relative distances and distance changes between the TMR labels in the CTD, NTD, and ECN cofactor. Our results confirm that in the full-length OCP protein, an increase in the distance between the domains and the carotenoid shift indeed occurs in a dynamic and reversible fashion. In addition, we show that the obtained OCP-TMR complexes are sensitive to the light intensity, temperature, and viscosity of the solvent, which makes them attractive for creating future biosensors.

**MATERIALS AND METHODS**

cDNA constructs and cloning

For the expression of the OCP apoprotein from *Synechocystis* sp. PCC 6803, we designed a plasmid harboring the cDNA of OCP according to the published amino acid sequence (PubMed/UniProt: P74102) optimized for codon usage in *Escherichia coli* by an artificial gene synthesis (GeneOptimizer algorithm (29), GeneArt, Life Technologies, Carlsbad, CA; sequence available upon request). The cDNA was excised by *Bam*HI and *Nol* enzymes and ligated in frame into a modified pQE81L vector (Qiagen, Hilden, Germany) harboring an engineered *Nol* restriction site and carrying an ampicillin resistance gene. These DNA manipulations resulted in the following amino acid sequence at the N-terminal 6xHis tag preceding the first methionine of OCP: MRGSHHHHHHTDPAT. The complete open reading frames of a construct were verified by sequencing (Eurofins MWG Operon, Frankfurt, Germany).

To ensure the insertion of a carotenoid cofactor into OCP after its expression in *E. coli*, NEB turbo cells (New England Biolabs, Ipswich, MA) were co-transformed with a 1:1 mixture of pQE81L-Amp plasmid carrying the
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OCP sequence and 1) pACCAR16ΔcrtX plasmid (harbors the contiguous gene cluster consisting of \(\text{crtY}, \text{crtI}, \text{crtB}, \text{and} \text{crtE}\) genes from \text{Erwinia uredovora}) for the accomplishment of \(\beta\)-carotene biosynthesis in \text{E. coli}; or 2) pACCAR25ΔcrtX plasmid (harboring the contiguous gene cluster consisting of \(\text{crtY}, \text{crtI}, \text{crtB}, \text{crtZ}, \text{and} \text{crtE}\) from \text{Erwinia uredovora}) for the accomplishment of zeaxanthin biosynthesis, as described previously (30). The \(\text{crtE}\) gene product (a geranylgeranyl-pyrophosphate synthase) forms geranylgeranyl-pyrophosphate from isopentyl-pyrophosphate and farnesyl-pyrophosphate. The latter two precursors are natively synthesized in \text{E. coli}. The \(\text{crtB}\) gene product (a pythoeose synthase) forms pythoese from two geranylgeranyl-pyrophosphate molecules. The \(\text{crtI}\) gene product (a phytoene desataturase) forms lycopene from pythoese; and the \(\text{crtE}\) gene product (a lycopene cyclase) forms \(\beta\)-carotene from lycopene. The \(\text{crtZ}\) gene product (a \(\beta\)-carotene hydroxylase) converts \(\beta\)-carotene into zeaxanthin in two hydroxylation steps at the 3 and 3’ positions of the \(\beta\) rings. Synthesis of the echinone cofactor was accomplished by means of the pACCAR25ΔcrtXZcrtO plasmid, in which the \(\text{crtZ}\) gene was inactivated by cloning the \(\text{crtG}\) gene encoding a \(\beta\)-carotene ketolase (31,32) into the \text{Hpol} cloning site of the \(\text{crtZ}\) gene. After transformation, cells were selected on plates containing 50 \(\mu\text{g/mL}\) ampicillin and 34 \(\mu\text{g/mL}\) chloramphenicol and incubated overnight at 37°C. Starter cultures of 10 mL Luria-Bertani (LB) medium with the corresponding antibiotics were inoculated from single colonies and grew overnight at 37°C at 200 rpm in an orbital shaker (in- for HT, Bottmingen, Switzerland).

Protein expression and purification

For protein expression, we used our procedure described previously (22,23). Briefly, two flasks with 500 mL lysisogeny broth supplemented with 34 \(\mu\text{g/mL}\) chloramphenicol and 50 \(\mu\text{g/mL}\) ampicillin each were inoculated from a starter culture to optical density (OD) equal to 0.1 and grown at 37°C in an orbital shaker at 200 rpm until the OD reached 0.8. After induction with 500 \(\mu\text{M}\) isopropyl-\(\beta\)-D-1-thiogalactopyranoside, cells were grown for 48 h at 25°C. Cells were harvested by centrifugation, yielding reddish pellets, indicating carotenoid biosynthesis. Cell pellets were resuspended in phosphate buffer with 100 mg lysozyme and Complete (Roche, Basel, Switzerland) protease inhibitor and lysed by three to four freeze/thaw cycles on dry ice with absolute ethanol. After removal of the cell debris by centrifugation (12,000 \(\times\) g at 4°C), the clarified and intensively colored supernatant was purified by affinity chromatography with an A\(\text{KTA}^{\text{FPLC}}\) purification system (GE Healthcare, Little Chalfont, England) to remove the excess of dithiothreitol. Immediately after, the sample (1.2 mL) was mixed with 12 mL of 20 mM TMR stock solution in dimethylsulfoxide and left initially for 30 min at room temperature and then overnight at +4°C for labeling. The excess of label was thoroughly removed by six to seven consecutive rounds of 10-fold dilution with buffer L followed by concentration on Amicon units with a 10 kDa cut-off (Merck Millipore) and the final buffer exchange to buffer L with NAP-10 columns to obtain 1 mL of TMR-labeled OCP solution. The colored flow-through containing no protein was used as a reference for free TMR.

Absorption measurements

Absorption spectra were recorded using a Perkin Elmer (Waltham, MA) Lambda-25 spectrophotometer as described before (24). In all experiments, an M455L3 (Thorlabs, Newton, NJ) 900 mW light-emitting diode with maximum emission at 455 nm was used for blue-green illumination of the samples (antic light for OCP\(^\text{D} \rightarrow \text{OCP\text{E}}\) photoconversion).

Steady-state fluorescence measurements

The steady-state fluorescence measurements were performed using a FluoroMax-4 spectrofluorimeter (Horiba Jobin Yvon, Kyoto, Japan). Samples were diluted to OD ~0.01 units at 510 nm to avoid inner filter effects and reabsorption.

Fluorescence emission of TMR (Sigma-Aldrich) was measured at excitation wavelength set to 510 nm, and the emission was recorded in the 550–680 nm region. The temperature of the sample was stabilized by a Peltier-controlled cuvette holder Qpod 2e (Quantum Northwest, Liberty Lake, WA).

Picosecond time-resolved fluorescence and anisotropy measurements

In this work, we used the TMR dye covalently bounded to OCP to follow the structural transitions of the protein. Simultaneously, as TMR was attached to the protein, we measured anisotropy kinetics of the TMR fluorescence to estimate the correlation time, which is related to a protein size and shape. Fluorescence-decay kinetics with picosecond time resolution data were collected by time- and wavelength-correlated single-photon counting setup (Becker and Hickl, Berlin, Germany). Excitation was performed at 510 nm (InTop, Russia), driven at a repetition rate up to 50 MHz. A 550 nm longpass filter (Thorlabs) was used to block excitation light. Fluorescence decay curves were approximated by a sum of exponential decay functions with the SPCImage (Becker and Hickl) software package. To compare different decay curves, we calculated the average decay time according to the expression

\[
\tau_{av} = \sum_{i}^{n} \tau_{i}a_{i},
\]

where \(\tau_{i}\) and \(a_{i}\) are the lifetime and amplitude (normalized to unity: \(\sum_{i}^{n} a_{i} = 1\)) of the \(i\)th fluorescence-decay component, respectively.

To measure fluorescence anisotropy kinetics, we used a filter-based system with a set of two ultrabroadband wire-grid polarizers WP25M-UB (Thorlabs). Fluorescence-decay kinetics \(I(t)\) was measured at different positions of the emission polarizer—in parallel (//) and perpendicular (\perp) orientation to the excitation polarizer, and the anisotropy kinetics \(r(t)\) was calculated as

\[
r(t) = \frac{I(t)_{//} - I(t)_{\perp}}{I(t)_{//} + 2I(t)_{\perp}},
\]

The obtained anisotropy decay kinetics were approximated by the sum of decaying exponents.
\[ r(t) = \sum_{m} r_{0m} \exp\left(-t/\theta_m\right), \]  

\[(3)\]

where \(r_{0m}\) is the amplitude and anisotropy of the \(m\)th component at \(t = 0\) and \(\theta_m\) is the correlation time of the \(m\)th component.

**RESULTS AND DISCUSSION**

**Absorption spectra of the TMR-labeled OCP**

Absorption spectra of the OCP and TMR-labeled OCP (hereafter OCP-TMR) are presented in Fig. 1. OCP^\(\text{O}^\text{R}\) absorption is characterized by a pronounced vibronic structure with multiple peaks. By contrast, the spectrum of photoconverted OCP^\(\text{R}\) is red-shifted and lacks vibronic structure. This fact is often interpreted as a consequence of increased heterogeneity of the chromophore environment in OCP^\(\text{R}\). TMR labeling causes significant changes in the absorption spectrum of OCP. Although the vibronic bands centered at \(\sim 440\) and \(470\) nm are still present in the OCP^\(\text{O}^\text{O}^\text{P}\)-TMR absorption spectrum, the most intense band is shifted from \(496\) nm, with new bands appearing in the OCP^\(\text{O}^\text{O}^\text{P}\)-TMR absorption spectrum at \(520\) and \(552\) nm. Although the \(552\) nm band undoubtedly originates from the TMR absorption maximum, the intensity of the \(520\) nm band is a novel spectral feature that appears to be significantly higher in OCP-TMR compared to the absorption of TMR in ethanol.

Indeed, subtraction of the OCP^\(\text{O}^\text{O}\) absorption spectrum from that of OCP^\(\text{O}^\text{O}^\text{P}\)-TMR resulted in a spectrum with two peaks at \(552\) and \(520\) nm (OD\(_{530}\)/OD\(_{552}\) = 0.75). Such a feature of the OCP-TMR absorption can be explained by formation of TMR dimers that are known to be characterized by increased absorption at \(520\) nm and significantly lower fluorescence quantum yield compared to TMR monomers (33–35). Since the effect of TMR dimerization is crucial for further interpretation of our results, we examined this process in more detail. To address this issue, we labeled the OCP apoprotein (Apo-OCP), which had been isolated separately from *Escherichia coli* cells lacking the carotenoid-producing machinery, with TMR (Apo-TMR) under the same experimental conditions. Because Apo-OCP does not contain the ECN chromophore, its absorption in the visible region should be solely due to the absorption of TMR. Notably, the Apo-TMR complex also displayed an increased absorption at the \(520\) nm band, although slightly lower than for OCP^\(\text{O}^\text{O}^\text{P}\)-TMR (Fig. 1 B). We assume that formation of a dimer-like structure is plausible, because there are two cysteines (Cys84 and Cys95 in PDB: 3MG1) in the NTD located close to each other (Fig. 1 D) and both of them are accessible for labeling by TMR. Another fact indirectly supporting this assumption is a fast component in the fluorescence decay kinetics of both OCP-TMR and Apo-TMR, which could be attributed to the emission of dimers; this phenomenon will be discussed in detail below. Finally, by using the reported ability of trypsin protease to cleave OCP into 17 and 14 kDa fragments corresponding to the NTD and CTD, respectively (15), we were able to directly confirm the presence of TMR labels on the main tryptic fragments of the OCP-TMR, which had been separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized upon ultraviolet illumination. By doing so, we were able to prove near-stoichiometric derivatization of OCP cysteines by TMR, a finding that was additionally supported by mass spectrometry.

Very importantly, illumination of OCP^\(\text{O}^\text{O}\)-TMR by actinic blue-green light causes a red shift and disappearance of the vibronic structure in the blue region of the OCP spectrum, indicating that after TMR labeling, OCP remains photoactive. Difference spectra of the orange and red forms of OCP and OCP-TMR are very similar, with the exception

![FIGURE 1 Absorption spectra of OCP (A) and OCP-TMR complexes (B) in the dark-adapted state (1) and after 2 minutes of actinic illumination (2) by a 150 mW blue LED. The difference (3) between absorption of the samples in photoactivated and dark-adapted states. The black line (4) shows absorption spectra of the TMR-labeled Apo-OCP, the dashed line (5) represents absorption of free TMR in ethanol. Absorption was measured at 2°C in order to reduce the rate of OCP^\(\text{O}^\text{O}^\text{P}\)-OCP^\(\text{O}^\text{O}\) conversion. (C) Chemical structure of tetramethylrhodamine-5-maleimide (TMR) with maleimide group, used for its attachment to OCP, looking upwards. (D) Crystal structure of OCP (PDB: 3MG1). The protein is shown in cartoon representation, the ECN molecule is shown as blue spheres and Cys residues are shown as green sticks and are labeled corresponding to *Synechocystis* sp. PCC 6803 amino acid numbering. To see this figure in color, go online.](image)
of a shoulder at 586 nm in the OCP-TMR difference spectrum (Fig. 1, A and B, blue lines). The similarity of the difference spectra in the 400–550 nm region indicates that photoconversion affects only the state of ECN, but has no substantial effect on TMR and does not trigger dissociation or formation of TMR dimers. Collectively, these results show that there is no significant non-linear interference between the absorption of ECN and TMR in the form of monomers or dimers, which allows us to use absorption spectra of OCP in the orange and red form for calculations of the overlap integral between the donor (TMR) and acceptor (ECN in OCP) of excitation energy.

**Steady-state fluorescence and time-resolved measurements of OCP-TMR fluorescence**

Fig. 2A shows the fluorescence excitation spectrum of OCP-TMR, which is very similar to the absorption of TMR in ethanol. The low fluorescence intensity at 520 nm excitation is consistent with the reported low quantum yield of TMR dimer (33). The fluorescence emission spectrum of the dark-adapted OCP-TMR sample and the spectrum after illumination by actinic light have a similar band shape, but the overall fluorescence intensity of OCP-TMR is significantly lower compared to OCP-TMR (Fig. 2A). Importantly, photo-induced TMR fluorescence quenching is reversible, and it gradually recovers after the actinic light is switched off, reaching the initial level (Fig. 2B). The observed time-course of the OCP-TMR fluorescence shares several properties with photo-induced transitions observed by us previously by monitoring ECN absorption, Trp fluorescence, and fluorescence of the hydrophobic Nile Red non-covalently bound to OCP (24). The initial stage corresponds to the transition of OCP from the dark-adapted orange state to the active red state under actinic light, which is followed by OCP→OCP conversion (Fig. 2B). The changes in the amplitude of fluorescence depend on the relation of rate constants of these forward and backward processes and could be described by a simple model, which was introduced for the description of transitions followed by absorption (24). It should be noted that the contrast between OCP-TMR fluorescence in the orange and red states is the most pronounced compared to any such differences that have been measured by other methods to date. Therefore, the fluorescence of the TMR label offers the most sensitive tool for studying the OCP photocycle and probably can be generally applied to other photoactive proteins. In addition, this approach makes it possible to reduce protein concentration by a factor of $10^2$–$10^4$ relative to the concentrations generally applied to other photoactive proteins. In addition, the fluorescence measurements can be performed even at relatively low protein concentrations (>1–2 mg/mL) (23,36), which would have dramatically complicated interpretations of the TMR fluorescence and FRET data.

**Characterization of photocyclic transitions of OCP-TMR fluorescence under different environmental conditions**

Since OCP-TMR fluorescence is sensitive to the protein state, it was interesting to delineate how different experimental conditions may affect the rates of the transitions, as it is well known that protein structure and dynamics depend on temperature, viscosity, and other environmental factors.

![FIGURE 2](image-url) Fluorescence of the OCP-TMR complex. (A) Excitation (1) and emission spectra of OCP-TMR before (2) and after (3) photoactivation by actinic blue-green light. (B) Characteristic photocyclic transitions of OCP-TMR, measured as changes of fluorescence intensity at 580 ± 5 nm at 33°C. Arrows indicate the moment when actinic blue light was switched on (↑) and off (↓). Approximated TMR fluorescence decay kinetics (C) and fluorescence anisotropy decay kinetics (D) in ethanol (3), in the Apo-OCP sample (4 on C), in dark-adapted OCP-TMR (1) and after 2 minutes of actinic illumination (2), measured at 2°C in order to reduce the rate of OCP→OCP conversion. TMR fluorescence was excited by 3 ps 510-nm laser pulses driven at a repetition rate of 25 MHz. To see this figure in color, go online.
High sensitivity of the rates of OCP conversion to external factors, such as ionic strength and temperature, has been revealed in previous absorption measurements (5,24), suggesting that the fluorescent OCP-TMR complex reported here could be a promising reporter of local environmental parameters. To this end, we performed measurements of the OCP<sup>O</sup>→<sub>C</sub>OCP<sup>R</sup> and OCP<sup>R</sup>→OCP<sup>O</sup> conversion rate constants by monitoring the TMR fluorescence at different powers of actinic light, temperature, and viscosity of the solvent (Fig. 3).

It was found that the OCP<sup>O</sup>→OCP<sup>R</sup> conversion rate depends linearly on the power of actinic light, whereas the backward OCP<sup>R</sup>→OCP<sup>O</sup> conversion was almost insensitive to this factor (Fig. 3 A). An increase in the ambient temperature from 20° to 42°C caused an increase of both conversion rates. At temperatures >36°C (<i>1/T</i> < 3.236 (K<sup>-1</sup>) × 10<sup>3</sup>), however, the rates were less temperature dependent, resulting in lower activation energies for both transitions from Arrhenius plots (Fig. 3 B). The latter fact could be explained by destabilization of the protein structure induced by high temperatures. Previously, we observed similar effects monitoring OCP absorption transients in vitro (24).

Since OCP photoconversion is correlated with substantial domain rearrangements and increased hydrodynamic radius, we questioned whether changes in solvent viscosity would change the transition rates. Indeed, the dependence of protein folding rates on solvent viscosity is described by the Kramers theory, which predicts an inverse relation of the transition rate on viscosity and can formally be applied to the specific case of OCP photoconversion (37,38). Hence, we measured the dependence of OCP transition rates on solvent viscosity that was varied by changing glycerol content in the buffer solution (39,40).

Upon an increase of glycerol concentration up to 80%, we observed a decrease of the OCP<sup>R</sup>→<sub>C</sub>OCP<sup>R</sup> and OCP<sup>R</sup>→<sub>C</sub>OCP<sup>O</sup> conversion rate constants (Fig. 3 C). The characteristic times of the conversions showed a linear dependence on viscosity in double-logarithmic plots, in line with the Kramers theory. Similar results were obtained with sucrose as a modulator of viscosity (data not presented). Thus, the OCP<sup>R</sup>→<sub>C</sub>OCP<sup>O</sup> conversion is extremely sensitive to changes in ambient temperature and viscosity and probably can be used as a specific and local indicator for estimation of these environmental parameters.

### Excitation energy transfer from TMR to ECN in a single OCP molecule

To estimate the mechanism of TMR fluorescence quenching in the OCP-TMR complexes, we used picosecond time-resolved fluorescence spectroscopy. To achieve a high signal/noise ratio in the time-correlated single photon counting (TCSPC) regime, it is necessary to collect ~10<sup>8</sup> photons, which takes minutes at count rates of ~10<sup>4</sup> photons/s. Since the typical OCP<sup>R</sup>→OCP<sup>O</sup> conversion rate at room temperature is ~0.01 s<sup>-1</sup> (24), the mentioned limitation of TCSPC does not allow us to register fluorescence decay of OCP<sup>R</sup>-TMR at the desired precision (without application of complex pump-probe techniques). To obtain a high and stable concentration of the red form and to reduce the rate of OCP<sup>R</sup>→OCP<sup>O</sup> conversion, we performed measurements of OCP-TMR at 2°C, where the backward conversion rate is almost negligible (Fig. 2 C). Evaluation of fluorescence lifetimes revealed a multi-exponential decay of OCP-TMR fluorescence in both the O and R states and demonstrated that TMR fluorescence in the investigated proteins was significantly quenched compared to free TMR in ethanol. Namely, OCP-TMR complexes exhibited a fast component with a lifetime of several hundred picoseconds that is absent in ethanol solution of TMR. The presence of this fast component can be explained by 1) highly efficient EET from TMR to the ECN cofactor that is possible due to spatial proximity and a significant spectral overlap, and 2) dimerization of TMR molecules in the NTD of OCP. This fast (~860 ps) component is also present in the fluorescence decay of the Apo-OCP sample labeled with TMR (Fig. 2 C), which, along with the characteristic absorption spectrum of Apo-TMR (pronounced shoulder at 520 nm; see Fig. 1 B), suggests that dimerization of TMR is responsible for the fast decay component.

The most intriguing fact is that upon OCP-TMR photoconversion, the lifetime of the fast component decreases (from 570 to 250 ps), but the lifetime of the slow component changes in the opposite direction (from 1.98 ns in the orange state to 2.54 ns in the red state). To quantify these changes in the framework of the Förster theory...
was calculated as

\[
E = 1 - \frac{\phi_{DA}}{\phi_D} = 1 - \frac{\tau_{DA}}{\tau_D}
\]

where \(\phi_D\) and \(\phi_{DA}\) are fluorescence quantum yields, and \(\tau_D\) and \(\tau_{DA}\) are the corresponding fluorescence lifetimes of a donor in the absence and presence, respectively, of an acceptor. For the calculations of EET efficiency related to the slow-component \(E_M\), we used \(\tau_D\) values corresponding to the slow decay component of the Apo-TMR complex (3.35 ns), which is fairly close to the lifetime of TMR in ethanol (3.5 ns; Fig. 2 C). As mentioned before, dimerization of TMR causes a significant reduction of its fluorescence quantum yield (33). To estimate the proper value of EET efficiency related to the fast component, we used the \(\tau_D\) value of 860 ps, which corresponds to the fast decay component of the Apo-TMR complex.

Assuming that the slow component is related to single TMR molecules (M) in the CTD, whereas the fast component originates from the dimer-like forms (D) of TMR in the NTD of OCP due to the close proximity of Cys84 and Cys95, the observations are consistent with an increase of EET efficiency to ECN from the TMR dimer located in the NTD and a simultaneous decrease of EET from the single TMR molecule bound to Cys245 in the CTD (Table 1).

Table 1: Characterization of EET in Dark-Adapted and Photoactivated States of the OCP-TMR Complex

<table>
<thead>
<tr>
<th>Condition</th>
<th>(J) (cm(^{-1}))</th>
<th>(R_D) (Å)</th>
<th>(R_A) (Å)</th>
<th>(E_D) (%)</th>
<th>(E_A) (%)</th>
<th>(\tau_D) (ps)</th>
<th>(\tau_A) (ps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCP**∆**</td>
<td>4.77 \times 10^{-14}</td>
<td>34.8</td>
<td>42.9</td>
<td>33.7</td>
<td>41.0</td>
<td>39.0</td>
<td>45.6</td>
</tr>
<tr>
<td>OCP**∆**</td>
<td>1.62 \times 10^{-13}</td>
<td>42.7</td>
<td>52.6</td>
<td>70.9</td>
<td>24.1</td>
<td>36.8</td>
<td>63.6</td>
</tr>
</tbody>
</table>

The dark-adapted and photoactivated states correspond to the orange and red forms of the OCP-TMR complex, respectively. The overlap integrals, \(J\), and corresponding Förster radii, \(R_0\), were calculated using the absorption spectra of different OCP forms (Fig. 1) and the emission spectra of TMR (Fig. 2) \(\phi_D\) values for the dimeric and monomeric states were set to 0.2 and 0.7, respectively) using Eqs. 1–4. \(\kappa^2\) was assumed to be equal to 2/3. Fluorescence decay kinetics presented in Fig. 2 C were used for the calculation of EET efficiency (\(E_D\) and \(E_A\)) and the corresponding distances (\(R_D\) and \(R_A\)) between the donor and acceptor of energy, where subscripts D and M refer to dimeric and monomeric TMR states, respectively.

where \(f_D(\lambda)\) is the donor emission spectrum normalized to an area of 1, and \(e_A(\lambda)\) is the molar extinction coefficient of the acceptor. According to our calculations, the \(J\) value of the OCP-TMR complex in the red state is ~3.4 times larger compared to the one in the orange state (Table 1), which is due to the red shift of the ECN absorption upon photoconversion, i.e., to the region of larger overlap with the TMR emission spectrum. The obtained values of overlap integrals, \(J\), were used to estimate the Förster radii:

\[
R_0 = \sqrt[3]{\frac{9 \ln 10}{128 \pi^5 N_A}} \frac{\kappa^2 \phi_D}{n^2} J.
\]

Here, \(\kappa^2\) is the dipole orientation factor, \(n\) is the refractive index of the medium, and \(N_A\) is Avogadro’s number. It is important to note that the \(R_0\) values for OCP-TMR in the orange and red states are different not only because of the spectral overlap integrals, \(J\), but also due to the differences in the quantum yield of the donor (TMR) in the monomeric and dimeric states.

Usually, \(\kappa^2\) is assumed to be equal to 2/3, which is correct when both donor and acceptor molecules are freely rotating and can be considered to be isotropically oriented during the lifetime of the excited state. The results of OCP-TMR anisotropy decay measurements (Fig. 2 D) indicate that there are two distinct correlation times, which are characteristic for segmental motions of the small dye molecules bound to the protein. The slow rate (~0.1 ns\(^{-1}\)) of TMR fluorescence depolarization corresponds to the rotation of the protein and depends on its size and shape. The observed increase of the protein rotation correlation time upon photoactivation (compare red and orange curves in Fig. 2 D) is in good agreement with the concept of the CTD and NTD separation and the increase of the protein hydrodynamic radius according to (17) and our data (22). The fast rotation of the attached TMR dye (~2 ns\(^{-1}\)) probably corresponds to the rotation of TMR around its -C-C- bonds and is characteristic for both free and bound dye. It should be noted that such rotation rates are of the same order of magnitude as the lifetime of the fast component of TMR fluorescence decay, so the real values of the dipole orientation factor, \(\kappa^2\), could be quite different from 2/3. Another fact that follows from anisotropy decay measurements and relatively fast protein rotation rates is that, under the conditions used, in both the orange and red form OCP remains in its monomeric protein state. This is a crucial point, because the ability of OCP to form dimers at high concentrations is well known (36), and if dimerization had been induced by TMR labeling, it would have resulted in formation of multiple donor-acceptor pairs, thus making subsequent analysis of EET changes extremely complicated.

The distances, \(R\), between the ECN (acceptor) and TMR (donor) in the CTD and NTD and their relative changes upon photoconversion were estimated as
Results of the analysis of EET changes in the OCP-TMR complexes, presented in Table 1, show that upon photoactivation, ECN moves closer to the TMR dimer at the NTD while at the same time moving farther away from TMR bound to the CTD, as dictated by the positions of cysteines in the Synechocystis OCP structure.

To prove that the changes of fast and slow components of TMR fluorescence decay are associated with the changes of protein state (in other words, that they are reversible and not related to photodegradation/bleaching of TMR), we performed measurements of picosecond fluorescence decay kinetics during the course of the OCPR/OCPO conversion. To achieve better time resolution, we reduced the conversion rate by increasing the solvent viscosity and setting the temperature to 15°C. Fig. 4A shows how TMR fluorescence intensity gradually increases upon OCPR/OCPO conversion; however, after normalization of the fluorescence to its maximal level, the decrease of the slow component becomes clearly visible (Fig. 4B).

Profile plots (Fig. 4, C and D) indicate the changes of a fast component (at 1 ns) and a slow one (at 7.5 ns) in time. The rates of these opposite changes are almost identical and correspond to the rate of OCPr→OCPO conversion.

These results demonstrate that fluorescence techniques make it possible to precisely follow the concerted reorganization of the OCP molecule in the course of the R→O transition: TMR labeling resulted in the formation of the two donor/acceptor pairs, i.e., TMR monomer to ECN in the CTD and TMR dimer to ECN in the NTD, each exhibiting distinct decay profiles. Based on the Förster theory, one can use the obtained construction as a double spectroscopic ruler to map the mutual translocation of the CTD, the NTD, and ECN upon photoconversion.

CONCLUSIONS

In this work, we determine the intramolecular distance changes during photocyclic transitions of OCP using highly sensitive fluorescence techniques, which required construction of cysteine-labeled, photoactive, and fluorescent orange carotenoid protein, OCP-TMR. We demonstrated that TMR

\[ R = R_0 \sqrt{1 - \frac{1}{E}} \]  

(7)
fluorescence intensity and lifetimes are informative indicators of the protein conformation state. The labeling of a spatially neighboring pair of cysteines, Cys84 and Cys95, resulted in formation of a TMR dimer, the fluorescence of which is easily distinguishable from the fluorescence of a TMR monomer due to significant differences in their quantum yields and fluorescence lifetimes. This allowed us to monitor changes of TMR fluorescence bound to different domains of the protein throughout the photoconversion. Assignment of multiexponential fluorescence decay components required construction of the ECN-lacking Apo-TMR complexes to estimate the fluorescence lifetimes of TMR in the monomeric and dimeric states without EET to ECN so that we could apply the formalism of the Förster theory. The observed changes in efficiency of EET from TMR to ECN upon photoconversion revealed an 18 Å increase of the distance between the TMR monomer in the CTD and ECN, which is in good agreement with the idea of separation of OCP domains as the result of photoactivation (17). Simultaneously, a 2.2 Å decrease of the distance between the TMR dimer in the NTD and ECN was observed (Fig. 5), which is in line with the data considering that ECN in the red form stays in the NTD and even penetrates deeper into the protein core (15,18). It should be noted that the Förster theory considers donor and acceptor as point-like electromagnetic oscillators, i.e., objects without size and shape. Thus, the calculated distances between the donor and acceptor characterize relative distances between the transition dipoles that may be significantly different from the actual positions and sizes of molecules and should be compared with structural data with caution. Due to several assumptions, the results obtained on the basis of the Förster theory are more qualitative in nature, but they allow further elucidation of the rearrangements of the NTD, the CTD, and ECN based on changes in their mutual positions. According to SAXS data (17) and the crystal structure of OCP, both forms of OCP could be considered as prolate rotational ellipsoids with different aspect ratios and approximate dimensions of 63 × 36 Å for OCP\textsuperscript{O} and 90 × 27 Å for OCP\textsuperscript{R}. Our estimation of distances between the different donors and ECN at least do not exceed the linear length scales of the protein. We found that the distance between the TMR dimer in the NTD and ECN increases from 45.6 Å in the orange to 63.7 Å in the red form, the latter representing the length of OCP\textsuperscript{O} itself, providing additional evidence for significant rearrangements of the protein structure and changes of the protein size. This is complementarily supported by the time-resolved anisotropy decay of TMR fluorescence (Fig. 2 D), the latter also indicating the non-aggregated and monomeric protein state of the OCP-TMR samples, which is important, because any oligomerization would have dramatically complicated the interpretations of FRET data. This was another advantage of the fluorescence-based approach, because it permitted utilization of very low protein concentrations, well below those required by absorbance spectroscopy.

Thus, the collected data suggest that, in the context of full-size OCP, photo-induced isomerization of ECN, triggered by the absorption of light quanta, results in translocation of ECN from the CTD and separation of the CTD and the NTD (see Fig. 5).

Further experiments with cysteine labeling of OCP by fluorescent dyes may benefit from point mutations and sequential reduction of the number of cysteines, with a caveat that site-directed mutagenesis itself can unpredictably affect properties of the rather fragile photoactive OCP, where a single mutation can dramatically change the behavior of the protein on different levels (22,23,42). Nevertheless, such a strategy may make it possible to obtain two different types of OCP-TMR complex, the first with TMR bound only at the NTD, for which fluorescence will be quenched upon photoconversion, and the second with TMR bound exclusively to the CTD, for which fluorescence will be enhanced in the red form of OCP. In combination with the structural data of the orange and red forms of OCP, such constructions may provide more detailed information about the protein-chromophore interactions, which are of paramount importance for the photoconversion process. Apart from this,

![Diagram](https://example.com/diagram.png)
OCP labeled by fluorescent dyes could be useful for future studies of non-photochemical quenching, since both OCP photoactivation followed by quenching of phycobilisome fluorescence and the subsequent fluorescence recovery could be studied simultaneously by fluorescence spectroscopy. Another perspective is the development of OCP-based biosensors, since the obtained fluorescent OCP-TMR complexes could be used as novel molecular sensors of light intensity, temperature, and viscosity, which would obviate the need for complex instrumentation, such as fluorescence correlation spectroscopy, to measure these parameters. The first intracellular temperature mapping methods based on fluorescent thermometers and fluorescence-lifetime imaging microscopy were developed recently (43–45). The observed dependence of the OCP conversion rate on temperature and viscosity is consistent with the general behavior expected for protein folding: whereas temperature dependence followed the Arrhenius law, the dependence on viscosity was in line with the Kramers theory. The high activation energy barrier for OCP back conversion in the dark results in an ~4-fold enhancement of the conversion rate upon temperature increase from 20°C to 36°C, whereas the dependence of the back conversion time on viscosity in the physiologically relevant range is linear, with a slope of 22 s/GPa. The obtained characteristics suggest that the OCP-TMR complex is a promising reporter of local environmental parameters. It is noteworthy that the working principle of the proposed OCP-TMR biosensor is different from that of conventional molecular rotors (46). In the latter case, fluorescence dependence on viscosity is due to fast processes of intramolecular rotation on the scale of an excited-state lifetime, whereas for the OCP-TMR complex, the parameter conferring sensitivity to viscosity is the rate of the global conformational change of the protein, which occurs on a timescale of seconds and thus does not require fluorescence instrumentation with extremely high time resolution. Therefore, the OCP-TMR biosensor would offer coverage of different timescales relevant to many biological applications. Thus, by constructing a proper fluorescent complex with OCP, one can assess the microenvironment parameters in the desired spectral region.

**AUTHOR CONTRIBUTIONS**

E.G.M. performed research and wrote the paper; N.N.S. performed research and wrote the paper; K.S.M. performed research; E.A.S. wrote the paper; KEK – performed research, GVT – performed research, MM – performed research, TF – designed research and wrote the paper, DAL - designed research, SIA - designed research, VZP - designed research, ABR - designed research.

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**REFERENCES**

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