Oligomerization processes limit photoactivation and recovery of the Orange Carotenoid Protein.

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Abstract (155 words)

The Orange Carotenoid Protein (OCP) is a photoactive protein involved in cyanobacterial photoprotection, by quenching of the excess of light harvested energy. The photoactivation mechanism remains elusive, in part due to absence of data pertaining to the timescales over which structural changes in the protein take place. It also remains unclear whether or not oligomerization of the dark-adapted and light-adapted OCP could play a role in the regulation of its energy quenching activity. Here, we probed photo-induced structural changes in OCP by a combination of static and time-resolved X-ray scattering and steady-state and transient optical spectroscopy in the visible range. Our results suggest that oligomerization partakes in the regulation of OCP photocycle, with different oligomers slowing down the overall thermal recovery of the dark-adapted state of OCP. They furthermore reveal that upon non-photoproductive excitation, a numbed-state forms, which remains in a non-photoexcitable structural state for at least $\sim 0.5 \mu\text{s}$ after absorption of a first photon.

Significance Statement (120 words):

The orange carotenoid protein (OCP) is a photoactivatable protein involved in cyanobacterial photoprotection. Upon photoactivation, OCP becomes able to quench the excess of energy uptaken by the light-harvesting antennae, thereby evading damage to the cells. It remains unclear, however, what is the exact OCP photoactivation mechanism, and whether or not oligomerization partakes in the regulation of the OCP function. Here, we investigated these two issues by combining static and time-resolved (TR) X-ray scattering and optical spectroscopy. Our results show that OCP oligomerizes both in the dark-adapted inactive and light-adapted active states, suggesting a role for oligomerization. TR scattering data furthermore reveal that the first large-scale conformational changes associated with the photoactivation of OCP take place on the μs time scale.

Introduction (1457 words)

Photosynthetic organisms have evolved to make use of up to 99 % of light harvested energy (1). In most cyanobacteria, the main light harvesting antenna is the phycobilisome (PBS), a soluble complex capable of directly funneling its harvested photon energy into thylakoid-membrane-embedded reaction-centers. In the event of an energy overflow into photosystem II reaction center, charge recombination can occur that will lead to the production of $^1\text{O}_2$, which in turn may damage the photosynthetic apparatus (2, 3). The main function of the soluble two-domain photoactive orange carotenoid protein (OCP) is to quench the excessive energy absorbed by the PBS, enabling the dissipation of this energy into heat. This is accompanied by a decrease in the PBS fluorescence. For its energy-quenching function to be elicited, OCP needs to be photoactivated by the absorption of a blue-green photon, which triggers the transition from the dark inactive orange state (OCP^{O}) to the active red state (OCP^{R}) (4–11) capable of quenching up to 80 % of the PBS fluorescence (12). As the photoactivation yield of OCP is extremely low (0.2 %) (13), interactions between PBS and OCP^{R} only occurs when irradiance threatens cell survival. Additionally, OCP can also quench $^1\text{O}_2$, and is thus one of the central players in cyanobacterial photoprotection (14, 15). Phylogenetic investigations have allowed classification of OCP sequences into three clades, viz. OCP1, OCP2 and OCPX (16).

Most research endeavors on OCP (including the present work) have been conducted on the *Synechocystis* PCC 6803 OCP1 variant featuring echinenone (ECN) as the functionalizing carotenoid (17, 18). Nonetheless, the first crystallographic structure of a dark-adapted OCP^{O} was from the cyanobacteria *Limnospira* (*Arthrospira*) *maxima*, and featured 3'-hydroxy-ECN as the natural pigment (14). The structure revealed that OCP folds into two domains separated by a ~20 residue linker (Fig. 1). The N-terminal domain (NTD), comprised of residues 1–165, is the effector domain binding to PBS, while the C-terminal domain (CTD) is comprised of residues 190–317 and serves as the regulator of OCP energy-quenching activity (19). The keto-carotenoid pigment binds in a ~35 Å long hydrophobic tunnel that spans the two domains. The NTD is fully α -helical (αA – αJ), featuring a fold unique to cyanobacteria, whereas the CTD is a seven-stranded β -sheet (β1 – β7) sandwiched between two sets of α -helices, viz. αK , αL and αM , on one side (referred to as the F-side), and the terminal αA (residues 1–19; also referred to as the N-terminal extension or NTE) and αN (residues 304–317; also referred to as the C-terminal tail or CTT), on the opposite side (referred to as the A-side). The two latter have been shown to play important roles in the regulation of OCP photoactivation and recovery. Stabilization of the OCP^{O} state is achieved at two main interfaces, viz. the NTD/CTD interface, burying 677 Å² of surface area and featuring two strictly-conserved H-bonds (*i.e.*, R155 to E244, N104 to W277), and the αA /CTD interface, burying 790 Å² and featuring six H-bonds (buried surface areas calculated from the *Synechocystis* OCP structure) of which two to the C-terminal αN (L16(O)–A308(N) and A18(N)–L307(O)). Thus, the αA helix is one of the main secondary structure elements to promote the stabilization of the OCP^{O} structure. Additionally, the carotenoid buries ~990 Å² of surface area

across the two domains (~ 545 and $445 \text{ \AA}^2$ in the NTD and the CTD, respectively), establishing two H-bonds with Y201(OH) and W288(N ϵ 1) in the CTD. The pigment is therefore also essential in stabilizing OCP^O.

All available crystal structures of OCP^O feature a dimer, wherein monomers are associated through contacts between facing α A, α B and α H helices, burying $\sim 1090 \text{ \AA}^2$ at the dimerization interface. Due to the high concentration of OCP^O within crystals (20-30 mM, depending on the crystal structure considered), the prevalence of OCP^O in its dimeric form was, until recently, regarded as a crystallization artifact and therefore overlooked (17). Recent results obtained using native mass spectrometry have since shown that OCP^O dimers can form in solution at concentrations as low as $3 \text{ \mu M}$ (0.1 mg/ml) (20). The presence of OCP^O dimers was also observed by size exclusion chromatography (SEC) and in small-angle X-ray/neutron scattering (SAXS/SANS) experiments (21, 22), which furthermore identified the crystallographic dimer as that which naturally occurs. Additionally supporting the hypothesis that OCP^O dimers could form *in vivo* is the report that the mutation of a single (R27) of the three highly-conserved residues (D19, R27, S134) among the 22 making up the dimerization interface (17) results in a constitutively monomeric mutant (10). Further adding to this complexity is the question as to whether or not OCP^R can also oligomerize, which has been supported by recent SAXS/SANS data (21, 22) but not by native mass spectrometry (10, 20, 23, 24). Altogether these results raise cogent questions as to if and how oligomerization participates in the regulation of OCP function.

The exact photoactivation mechanism of OCP – meaning, accumulation of OCP^R, not the mere appearance of a red spectrum – remains elusive. Indeed, despite the use of different experimental approaches to probe the OCP^O to OCP^R transition with a high resolution in either time (25–27) or space (18, 28–30), uncertainties remain regarding the exact sequence of events leading to photoactivation – in part due to the absence of a high resolution structure of metastable OCP^R and because a consensus remains to emerge regarding the events occurring on the ultra-fast timescales (15, 25), but also because data pertaining to the (long) timescales over which (large-scale) structural changes take place are lacking. All investigators agree on the fact that upon photon absorption, the carotenoid pigment transitions to an excited S_2 state, which decays within $\sim 0.1 \text{ ps}$ into multiple ps-lived excited states (S_1 , ICT, S^*). Only one of these presumably leads to the minute-lived OCP^R state after the formation of at least three different intermediates (photoproducts P_1 , P_2 , P_3) over the ps- μ s timescale (25). Thus, the formation of OCP^R is mainly limited by picosecond (ps) timescale excited state dynamics, with $\sim 99 \%$ of carotenoids relaxing back to the ground state along non-productive pathways. It was suggested that photoproduct P_1 is characterized by rupture of the H-bonds between the carotenoid and the protein; P_2 by a repositioning of the β 1-ring in the CTD, putting its carbonyl oxygen at a distance incompatible with H-bonding to Tyr201(OH) and W288(N ϵ 1); and P_3 by the

translocation (12 Å) of the carotenoid into the NTD. The dissociation of helix α A and α N from the A-side of the CTD was suggested to await the millisecond (ms) time scale (24), and to be followed by dissociation of the two domains yielding OCP^R. This hypothesis has since been supported by SEC and SAXS (28). By specific labeling of accessible carboxylate groups, mass spectrometry further pointed to a signal transduction mechanism whereby, upon photoactivation and rupture of H-bonds between the carotenoid and the CTD, disorder is propagated across the CTD β -sheet resulting in a detachment of the N-terminal helix α A and subsequent dissociation of the dimer through destabilization of helix α B. Direct structural evidence for the existence of these steps is yet still lacking. Most recently, experiments carried out on an OCP mutant wherein H-bonding of the carotenoid to the CTD is modified supported the existence of two additional intermediate states between P₃ and OCP^R, namely P_M and P_X, proposed to be characterized by dissociation of the α A and α N helices, respectively (27). It yet remains unclear whether these states also exist in the wild-type (wt) protein.

In the present work, we used a combination of mutagenesis, transient spectroscopy in visible range, and static and time-resolved X-ray solution scattering to clarify (i) the oligomerization states of OCP^O and OCP^R in solution, (ii) the timescale over which large-scale structural changes take place during photoactivation, and (iii) whether or not pulsed-illumination permits the formation of OCP^R. By conducting static SAXS experiments on the wt OCP and on a stable monomeric R27L mutant (10), wherein the conserved R27-D19 salt-bridge at the dimerization interface is suppressed, we obtained confirmation that both OCP^O and OCP^R can oligomerize in solution, suggesting a role for oligomerization in the regulation of OCP activity. Using TR-SAXS, we obtained evidence that the 'red'-shifted state generated by pulsed illumination, which forms and decays within ~10 μ s and ~10-200 ms (depending on the his-tag location), respectively, differs from the OCP^R state accumulated under stationary illumination conditions, which forms and decays within ~1 ms and ~1-30 min (depending on concentration), respectively. Our data furthermore reveal that upon a non-productive pulsed photoexcitation – *i.e.* in the 99.7 % of the cases where OCP^R does not form – OCP remains in a non-photoexcitable structural state for at least ~ 0.5 μ s, despite the carotenoid returning back to the electronic ground state within tens of ps and the protein featuring a spectrum characteristic of the OCP^O state (25).

Materials and Methods (2499 words)

Protein expression and sample preparation.

OCP expression and extraction were carried out as described in Gwizdala et al. (8) and Bourcier de Carbon et al. (31). Briefly, expression in the holo form of the N-terminally his-tagged *wild-type* OCP (OCP_{wt-Ntag}) and the N-terminally his-tagged monomeric mutant (OCP_{R27L-Ntag}) was achieved by respective transformation of the *pCDF-NtagOCPSyn* or *pCDF-NtagOCPSynR27L* plasmids into ECN-producing BL21 (DE3) *E.coli* cells – *i.e.* cells previously transformed with the pAC-BETA and pBAD-CrtO plasmids described in Bourcier de Carbon et al. (31). Expression in *Synechocystis* cells of the holo form of C-terminally his-tagged *wild-type* OCP (OCP_{wt-Ctag}) was achieved using the protocol described in Gwizdala et al. (8). The proteins were purified in three steps including affinity chromatography on a Ni-NTA column (Ni-Probond resin, Invitrogen), hydrophobic chromatography on a phenyl-sepharose column (HiTrap Phenyl HP, GE Healthcare) and size exclusion chromatography on an analytical HiLoad 16/60 Superdex 75 (HiLoad 16/600 Superdex 75 pg, Sigma Aldrich). Proteins were eluted from the latter at a flow rate of 1 ml/min using a buffer composed of 50 mM Tris-HCl pH 7,4 and 150 mM NaCl. To assert the molecular weight of eluted species, the column was previously calibrated using the following standard proteins: γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.35 kDa). OCP concentrations were estimated from the absorbance signal at 490 nm, using the extinction coefficient of 63000 M⁻¹ cm⁻¹ recently proposed by Sluchanko et al. (32). We note that an uncertainty remains as to the exact extinction coefficient that should be used to derive OCP concentration from absorbance measurement. Protein concentration is nowadays generally estimated using the absorbance signal at 280 nm and a calculated extinction coefficient (e.g. obtained from <https://web.expasy.org/cgi-bin/protparam/protparam> (33, 34)), which in the case of *Synechocystis* OCP amounts 34 659 M⁻¹.cm⁻¹. This value agrees well with the extinction coefficient at 490 nm proposed by Sluchanko et al., as the ratio between the 490 and 280 nm peaks is ~2 for the pure protein, but it neglects the potential absorption of the ketocarotenoid pigment at 280 nm. Thus far, most of the papers published on OCP have accordingly used an extinction coefficient of 110 000 M⁻¹ cm⁻¹ at 490 nm, based on the debatable assumption that the ketocarotenoid pigment has similar absorption spectrum when bound to OCP or dissolved in organic solvents. Indeed, if there is no absorbance of the carotenoid at 280 nm, then one expects a ratio of ~3 between the absorbances peak at 490 nm and 280 nm. But if we consider absorption of the carotenoid at 280 nm, and assume the same extinction coefficient in the protein cage as in organic solvents – *i.e.* roughly 15 % of the absorption peak at 490 nm (measured by us as well as others) – then the measured ratio of ~2 between the absorbance at 490 and 280 nm can be rationalized since $A_{280\text{ nm}} = (0.33+0.15) \cdot A_{490\text{ nm}} = 0.48 \cdot A_{490\text{ nm}}$. At present, we cannot favor either hypothesis. However, as our current aim is to report on oligomerization processes, we believe it is preferable that we underestimate – rather than overestimate – dissociation constants. Hence, the concentrations reported in the present paper were estimated using the conservative extinction coefficient of 63 000 M⁻¹ cm⁻¹ (32). The actual

concentrations and dissociation constants could yet be 1.7 times lower if the ketocarotenoid absorbs at 280 nm and the real extinction coefficient of OCP at 490 nm is 110 000 M⁻¹.cm⁻¹.

Static SAXS measurements.

Static X-ray scattering data were collected at 21°C on an EigerX-4M detector at the SWING beamline of the Soleil Synchrotron (Saint-Aubin, France). X-ray solution scattering patterns in the SAXS region ($q = 0.02 - 0.5 \text{ \AA}^{-1}$) were collected for OCP_{wt-Ctag} and OCP_{R27L-Ntag} at various concentrations using a polychromatic X-ray beam centered at 16 keV and an EigerX-4M (Dectris) detector. Two sets of measurements were carried out, before and after 30 min irradiation of OCP solutions with a 430 nm LED source (~ 500 mW/cm²), allowing collection of X-ray scattering signals in the dark-adapted (OCP^O) and light adapted (OCP^R) states, respectively. For OCP_{wt-Ctag}, data were acquired at 0.7, 3.5, 10.5 and 23 mg/ml for the OCP^O and 0.7, 3.5 and 10.5 mg/ml for the OCP^R state, while for OCP_{R27L-Ntag}, data were recorded at 0.7, 3.5, 10.5 and 14 mg/ml for the OCP^O state and at 0.7, 3.5 and 10.5 mg/ml for the OCP^R state. For each concentration, 30 X-ray scattering patterns (each registered after 990 ms of X-ray exposure) were collected with 30 analogous patterns of the buffer before and after each protein measurement. Each 2D pattern was converted to a 1D scattering pattern by azimuthal integration using the Foxtrot-3.5.2 software available at the SWING beamline. Corresponding scattering patterns were averaged and protein signals were obtained by subtraction of buffer scattering. The distance distribution functions were computed using GNOM (35) and the parameters reported in Table S1 were determined from the reduced data using relevant programs from the ATSAS suite (35, 36). Low-resolution molecular envelopes were computed using the ATSAS reconstitution tool DAMMIF (37, 38). To construct the OCP^R dimer and tetramer models, we used the published structures of paralogs of the isolated N-terminal and C-terminal OCP domains, viz. the crystal structures of *Anabaena Nostoc* PCC 7120 and *Fremyella diplosiphon* HCP (pdb ids: 6MCJ and 5FCX, respectively) (39, 40) and of *Anabaena Nostoc* PCC 7120 CTDH (pdb id: 5FEJ) (41), respectively. Our modeling strategy is presented in the Results section.

Spectroscopic monitoring of the OCP^R to OCP^O thermal recovery after accumulation of OCP^R by a prolonged continuous illumination.

Thermal recovery was investigated as a function of concentration on the three OCP variants, using a JASCO V-630 UV/Vis spectrophotometer (Easton, USA). Purified proteins in OCP_{R27L-Ntag} and OCP_{wt-Ctag} were assayed at 0.1, 1.7, 3 and 16 mg/ml, and OCP_{wt-Ntag} at 0.1, 3, 10 and 16 mg/ml. Specifically, the absorption at 467 nm was monitored at room-temperature (21 °C) in the dark following 30 minutes of continuous blue light irradiation with a 430 nm LED light collimated to 1 cm (~ 500 mW/cm²) to maximize accumulation of OCP^R and reproduce the conditions used in the static SAXS experiments. Measurements at 0.1 mg/ml were carried out in a PMMA cuvette with 1 cm

pathlength, those at 1.7, 3 and 10 mg/ml in a quartz cuvette with 0.1 cm pathlength, and that at 16 mg/ml in an Infrasil cuvette with 0.05 cm pathlength.

To facilitate comparison of data collected at various concentrations, the absorption difference at 467 nm was normalized using $A_{\text{Norm}} = [A(t) - A(t_0)]/[A(t_{\text{max}}) - A(t_0)]$, where $A(t)$, $A(t_0)$ and $A(t_{\text{max}})$ are the absorption values measured at a generic time t after switching off the 430 nm light; at time t_0 (i.e., just after illumination at 430 nm was switched off, when the concentration of OCP^o is at its minimum); and at the time t_{max} when the starting OCP^o state has recovered, respectively. The non-linear least-squares optimization and parameter extraction were performed using LMFIT (42). After individual fits of each curve data pointed to the necessity to use at least three exponential components to fit the recovery data, we opted for a per-OCP variant global-fitting of the normalized absorption data. For each sample, the recovery data measured at four concentrations were jointly fitted using either a triple or a quadruple exponential function,

$$A(t) = a_{ci-1} \times \left[1 - \exp\left(\frac{-t}{\tau_{ci-1}}\right) \right] + a_{ci-2} \times \left[1 - \exp\left(\frac{-t}{\tau_{ci-2}}\right) \right] + a_{ci-3} \times \left[1 - \exp\left(\frac{-t}{\tau_{ci-3}}\right) \right] + a_{ci-4} \times \left[1 - \exp\left(\frac{-t}{\tau_{ci-4}}\right) \right]$$

where a_{ci-1} , a_{ci-2} , a_{ci-3} and a_{ci-4} are concentration-dependent pre-exponential factors, τ_{ci-1} , τ_{ci-2} , τ_{ci-3} and τ_{ci-4} are process-dependent characteristic times, and a_{ci-4} is fixed to zero in the case of a triple exponential fit. A better fit was obtained with four exponential components for the OCP_{wt-Ctag} recovery data, but improvements were marginal for the data collected on OCP_{wt-Ctag} and OCP_{R27L-Ntag}, with amplitudes for the fourth component consistently inferior to 0.1 (see Table S2 and Table S3). Hence, we retained results from the four exponential fit for OCP_{wt-Ctag}, but from the three exponential fit, for the two N-tagged variants. We nonetheless present results for both types of fits in Fig. 3, Supplementary Fig. S5 and S6, and Tables S2 and S3.

Spectroscopic monitoring of the spectral evolution after ns-pulsed excitation at 470 nm.

Measurements were performed using the custom apparatus described previously (43). OCP^o photo-excitation was achieved using a 470 nm nanosecond laser delivering 5 mJ energy per 8 ns pulse at a repetition rate of 0.05 Hz. The probing beam was filtered using a bandpass filter, so that only 440 nm (FWHM: 9 nm) or 565 nm (FWHM: 12 nm) light passed through the sample, avoiding putative probe-induced effects from the white spectrum of the xenon lamp. Scattered pump light was reduced by a notch filter set installed upstream of the monochromator which precedes the photomultiplier tube. All measurements were carried out at a sample absorbance of ~ 0.7 at 470 nm. For the lowest concentration, a 10 x 10 mm cuvette was used, which was set with a right-angle configuration between the pump and the probe beams. For higher concentrations, 2 mm and 500 μ m flat cuvettes were used,

affording to increase the concentration whilst keeping the absorbance in the optimal range. Use of these cuvettes required a quasi-collinear ($\approx 5^\circ$ angle) configuration between the pump and probe beams. Measurements were performed at different temperatures ranging from 8 to 36° C. Stirring was not applied to avoid sample displacement from the probed volume at longer time delays. All experiments were repeated 100 times on each of 4 partially-overlapped time-windows, altogether covering the 50 ns - 1 s time-range. Recorded data were merged and projected on a logarithmic grid. Stability of the protein was checked by its steady-state absorbance after each experiment. The quantum yield formation of photo-products was determined using ruthenium as actinometer (32, 44) and molar absorption coefficient of OCP^0 at 490 nm = $63000 \text{ cm}^{-1} \cdot \text{M}^{-1}$ (32).

Time-resolved X-ray scattering.

Time-resolved WAXS experiments were performed at the ID09 beamline (European Synchrotron Radiation Facility (ESRF), Grenoble, France) (45). X-ray solution scattering patterns in the WAXS region ($q = 0.01 - 2.5 \text{ \AA}^{-1}$) were collected at 23 mg/ml (620 μM) for $\text{OCP}_{\text{wt-Ntag}}$ and $\text{OCP}_{\text{wt-Ctag}}$ using a polychromatic X-ray beam centered at 15 keV and a Rayonix MX170-HS detector placed 350 mm from the sample. The protein samples, $\text{OCP}_{\text{wt-Ntag}}$ and $\text{OCP}_{\text{wt-Ctag}}$, were photoactivated with circularly-polarized laser pulses from two different laser systems: either an EKSPLA NT342B laser or a COHERENT Evolution laser. While the first laser is a tunable laser generating laser pulses with a duration of 5 ns (full width half maximum, FWHM) at a maximum repetition rate of 10 Hz, the second laser runs at 1 kHz, has a fixed wavelength (527 nm) and has a pulse duration that can be modulated by changing the current of its diode pump. The use of the COHERENT Evolution laser allowed us to perform a set of experiments with pulse durations longer than those of the EKSPLA laser (in particular, experiments with 300 or 450 ns long pulses were performed); moreover, it allowed us to excite the protein sample with either a single laser pulse or a burst of several laser pulses (up to 30) at 1 kHz. For each experiment, the required number of photoexcitation laser pulses were extracted from the train of pulses generated by the Evolution laser by means of a pair of synchronized fast mechanical shutters (Lambda SC, Sutter). By changing the relative delay of the first shutter opening and of the last shutter closing, it was possible to cleanly select either a single laser pulse or several. In all experiments, the laser beam was focused with cylindrical lenses to an elliptical spot approximately $2.5 \times 0.25 \text{ mm}^2$ (FWHM) and the energy was regulated so as to have an energy density of $\sim 3 \text{ mJ/mm}^2$ at the sample position, corresponding to ~ 75 absorbed photons per chromophore.

To maximize the overlap between the pump- and probe-illuminated volumes, an orthogonal pump–probe geometry was employed, with the X-ray beam ($0.06 \times 0.1 \text{ mm}^2$, FWHM) hitting the sample capillary 0.3 mm from its edge. In order to both minimize X-ray radiation damage and allow the recovery of the OCP^0 state, the protein solution ($\sim 5 \text{ ml}$) was circulated with a peristaltic pump (Gilson Minipuls 3) through a 2 mm X-ray quartz capillary in a closed loop. During the flow, most of the sample

was contained in a small reservoir kept in the experimental hutch, thermalized at 22 °C. The flow speed was set according to both the optical-laser pump – X-ray probe time delay and the repetition rate, allowing the sample to be kept in the pump–probe intersection area during a pump–probe sequence while refreshing it between two consecutive pump pulses. Single X-ray pulses were selected from the synchrotron pulse train by means of a high-speed chopper and a millisecond shutter. Up to 30 scattering patterns per time delay were acquired and averaged to improve the signal-to-noise ratio. Laser-off (dark) images were also acquired with the X-ray pulse arriving 50 ms before the laser pulse and used as a reference to compute the difference patterns after normalization to the water peak ($2 - 2.2 \text{ \AA}^{-1}$) (46). Difference patterns were azimuthally averaged and the peak of the undulator spectrum ($\sim 0.83 \text{ \AA}^{-1}$) was used as the reference to convert the scattering angle to the momentum transfer q . Independent TR-WAXS measurements on a solution of 4-amino-1,1'-azobenzene-3,4'-disulfonic acid monosodium salt with an optical density comparable to the OCP sample were used to correct the water heating scattering signal of the collected OCP data (46). TR-WAXS was also used to follow the OCP^R to OCP^O thermal recovery with a direct structurally sensitive technique. In particular, X-ray scattering patterns were collected over 30 minutes from a sample of $\text{OCP}_{\text{wt-Ctag}}$ that had been exposed for 30 minutes to continuous illumination by a 430 nm LED light.

The TR-X-ray scattering dataset was further analyzed by a singular value decomposition (SVD) analysis using a custom-written Python script. The time-dependent difference patterns form a $j \times k$ matrix, with j the number of q values and k the number of time delays. The SVD algorithm calculates the matrices U and V and the vector S , so that $A = U \times S \times V^T$. The columns of matrix U are called left singular vectors, or basis patterns, the rows of V^T are called right singular vectors, or amplitude vectors, and the elements of S are called singular values. The basis patterns are ordered following the high-to-low sorting of singular values. The SVD analysis of kinetic data provides a subset of time-independent patterns containing the relevant information out of the random noise. The dataset can be then reproduced as a linear combination of such time-independent patterns. Inspection of the shape of basis patterns, amplitude vectors and singular values together with the autocorrelation analysis of both basis patterns and amplitude vectors (47) indicates that only one component contains significant information. Hence, a two-step model (with growing and decaying exponentials) was used to fit the evolution of the integrated time-resolved X-ray scattering signal.

Results (5901 words)

Soluble OCP⁰ can form dimers in solution.

We used small-angle X-ray scattering to investigate the oligomerization state of dark-adapted OCP (OCP⁰) in solution. For this purpose, we worked on two proteins: (i) a wt OCP, functionalized by ECN and featuring a six-histidine tag (6Xhis-tag) at the C-terminus (OCP_{wt-Ctag}); and (ii) a R27L monomeric mutant, functionalized by ECN and featuring a 6Xhis-tag at the N-terminus (OCP_{R27L-Ntag}). Use of the latter, wherein the conserved D19 – R27 salt bridge is suppressed, afforded a control for the X-ray scattering signal of an OCP monomer (10).

Data were first collected on dark-adapted OCP_{wt-Ctag} and OCP_{R27L-Ntag} at increasing protein concentrations, informing on the OCP⁰ state. The buffer-subtracted scattering patterns of dark-adapted OCP_{wt-Ctag} and OCP_{R27L-Ntag} are shown in Fig. 2A and 2B. For OCP_{R27L-Ntag} at all concentrations, the radii of gyration (R_g) derived from the Guinier region are similar (Fig. 3A), and the derived pairwise distance distribution functions P(r) overlay (Fig. 2C). Accordingly, the low-resolution molecular envelopes calculated by the DAMMIF algorithm (48) from the dark-adapted OCP_{R27L-Ntag} data collected at 0.7, 3.5, 10.5 (Fig. 4) and 14 mg/ml (Fig. S1) are comparable, both matching the crystallographic OCP⁰ monomer. Hence, the OCP⁰_{R27L-Ntag} sample remains in the same monomeric state at all tested concentrations and is therefore a good control for monomeric OCP⁰. Contrastingly, examination of the P(r) for dark-adapted OCP_{wt-Ctag} samples (Fig. 2C) reveals an increase in pairwise distances when the concentration exceeds 3.5 mg/ml and, accordingly, an increase is observed in the radii of gyration derived from the Guinier region (Fig. 3A). These two observations indicate that the average size of dark-adapted OCP_{wt-Ctag} increases with concentration. The low-resolution molecular envelopes calculated by DAMMIF (37) from the dark-adapted OCP_{wt-Ctag} data are consistent with the presence of a predominant OCP⁰ dimer (Fig. 4 and Fig. S1) at concentrations $\geq$ 3.5 mg/ml, and a mixture of monomers and dimers at 0.7 mg/ml. It is of important note that the above assignments of molecular envelopes to monomers and dimers agree with the molecular weight estimates from I(0) (49) (see Table S1 (*i.e.*, the intensity extrapolated at $q=0$)) after scaling data on absolute units.

We used the program OLIGOMER (38) to estimate the relative abundance of monomers and dimers in the dark-adapted OCP_{wt-Ctag} samples prepared at different concentrations (Fig. 3B). Calculated X-ray scattering curves for dimeric and monomeric OCP⁰ were generated from the crystal structure of *Synechocystis* PCC 6803 OCP (pdb id: 3MG1) (17) (Fig. 1) and used for deconvolution of the experimental scattering patterns measured at increasing concentrations (see Materials and Methods). The fit suggests that at 0.7 mg/ml ($\sim$ 20 μ M), dark-adapted OCP_{wt-Ctag} is present as a 1:1 mixture of

monomers and dimers. The latter attain dominance at ~3.5 mg/ml (~ 100 μ M) with a dissociation constant of around 0.5 mg/ml (~ 14 μ M).

Illumination induces a dissociation of the dark OCP^O dimer and the formation of OCP^R dimers and tetramers.

To provide details on the large-scale conformational changes accompanying the OCP^O $\rightarrow$ OCP^R transition, including possible change(s) in oligomeric state, we also collected X-ray scattering data on light-adapted OCP_{wt-Ctag} and OCP_{R27L-Ntag} at 0.7, 3.5 and 10.5 mg/ml (Fig. 2D and 2E). To assess the time required for the accumulation of photoactive OCP_{wt-Ctag} and OCP_{R27L-Ntag} states, we carried out preliminary pre-illumination experiments at ESRF-ID09. A highly-concentrated protein solution (30 mg/ml) of OCP_{wt-Ctag} was used, which was presented to the X-ray beam in a 2 mm thick glass capillary. Firstly, a reference pattern was collected on the dark-adapted protein, after which the protein was illuminated by exposure to a 430 nm LED. The distance between the light source and the sample was such that the diameter of the illumination spot was ~ 1 cm at sample position. Scattering patterns were collected every 15 s, each with 750 ms X-ray exposure, and two illumination powers were consecutively tested, viz. 250 and 500 mW. Only after tens of minutes of illumination at 500 mW was accumulation of OCP^R found to reach a plateau, in our experimental conditions (Fig. S2). Therefore, all subsequent experiments aimed at characterizing the active OCP^R state involved ~ 30 minutes illumination by the 500 mW continuous LED source emitting at 430 nm prior to data collection.

A concentration series was recorded for OCP_{R27L-Ntag} and OCP_{wt-Ctag}, each in their light adapted state, at the SWING bio-SAXS beamline (Synchrotron SOLEIL). Absolute X-ray scattering patterns for OCP_{wt-Ctag}^R and OCP_{R27L-Ntag}^R are shown in Fig. 2D and 2E, strongly differing from those obtained, at the same concentrations, for the corresponding proteins in their dark state. The patterns exhibit a dependence on protein concentration, with the low-resolution signal increasing as the latter augments. The derived R_g accordingly increases with concentration, being 32.5 and 29 Å for OCP_{R27L-Ntag}^R and OCP_{wt-Ctag}^R at 0.7 mg/ml, 34 Å for both OCP_{R27L-Ntag}^R and OCP_{wt-Ctag}^R at 3.5 mg/ml, and 44.9 and 43.8 Å for OCP_{R27L-Ntag}^R and OCP_{wt-Ctag}^R at 10.5 mg/ml, respectively (Fig. 3A and Table S1). These values are far larger than those observed for the dark-adapted counterparts, but notably similar for the two proteins at each of the three tested concentrations (0.7, 3.5 mg/ml and 10.5 mg/ml). The increase in the size of OCP_{R27L-Ntag} and OCP_{wt-Ctag} upon illumination, the dependence of this size to concentration, and the similarity between OCP_{R27L-Ntag}^R and OCP_{wt-Ctag}^R at comparable concentrations can also be deduced from the derived P(r) (Fig. 2F) and from the MW derived from I(0) estimation (49) (Table S1). Thus, our results not only indicate an overall expansion of the protein upon illumination, but also a change in the OCP^R quaternary structure as a function of concentration. Similar results (notwithstanding a missing data point for 2 mg/ml concentration) were obtained with OCP_{wt-Ntag}^R (not shown). For each protein, a light minus dark difference scattering pattern $\Delta S(q)$ was

calculated at the highest tested concentration (10.5 mg/ml), offering a signature of the change in scattering signal upon illumination (Fig. 3C). The $\Delta S(q)$ for OCP_{wt-Ctag} is characterized by the presence of a negative peak at $\sim 0.05 \text{ \AA}^{-1}$ and a positive peak at $\sim 0.12 \text{ \AA}^{-1}$, whereas for OCP_{R27L-Ntag}, a negative peak is observed at $\sim 0.07 \text{ \AA}^{-1}$ and a positive peak at $\sim 0.14 \text{ \AA}^{-1}$ (Fig. 3C). Since OCP_{R27L-Ntag} and OCP_{wt-Ctag} adopt similar states at 10.5 mg/ml, the displacement of negative and positive peaks presumably emanates solely from the difference between dark-adapted structures, viz. OCP^O_{R27L-Ntag} and OCP^O_{wt-Ctag}.

We used DAMMIF to compute the low-resolution molecular envelopes of OCP_{R27L-Ntag} and OCP_{wt-Ctag} at the three tested concentrations (0.7, 3.5 mg/ml and 10.5 mg/ml). At 0.7 and 3.5 mg/ml (20 and 100 μM), we obtained for both OCP_{wt-Ctag} and OCP_{R27L-Ntag} an envelope consistent with the presence of a dimer formed by two elongated OCP molecules wherein the NTD and CTD are separated (Fig. 4). While at odd with the report of Gupta et al. (28), these results agree with the findings of Golub et al. (21) who earlier reported a similar structure for OCP_{wt-Ctag} at 1 and 65 mg/ml (*i.e.* 28 μM and 1.8 mM) based on SAXS and SANS data. The molecular envelopes computed for OCP_{R27L-Ntag} and OCP_{wt-Ctag} at 10.5 mg/ml (300 μM), however, are both consistent with the presence of up to four elongated OCP molecules, suggesting that oligomers of higher-order than dimers can form at increased concentrations – presumably, OCP^R tetramers (Fig. 4). Again, the assignment of these molecular envelopes to tetramers is supported by the molecular weights derived from $I(0)$. Differences between our findings and those of Golub et al. (21) could arise from the difference in ionic strength of buffer solutions, as these investigators used 10 mM phosphate buffer at pH 7.4 whereas here, we used 50 mM Tris-HCl pH 7.4 complemented with 150 mM NaCl. Presence of NaCl was intended to reproduce the osmolarity of cyanobacterial cells and could have favored the formation of tetramers.

We attempted to model the OCP^R structures assuming that : i) OCP^R dimers and tetramers are related, *i.e.* tetramers form from the coalescence of two OCP^R dimers; ii) interactions between OCP^R monomers in a dimer and between OCP^R dimers in a tetramer should be symmetrical, *i.e.* they should involve the same interface in the two interacting proteins; iii) functional OCP^R dimers should assemble via their CTD so that their two NTD are exposed and free to interact with PBS via R155 (50); consequently, OCP^R tetramers would form from an interaction between two NTD in the OCP^R dimer, yielding a larger entity with as many exposed – per say functional – NTD moieties as the OCP^R dimer; iv) the interfaces allowing the assembly of OCP^R dimers and tetramers should not involve R27 (and by extension, the dark dimerization interface contributed by helix αB) since the R27L mutant – unable to form OCP^O dimers – is capable of forming OCP^R dimers and tetramers; v) rather the CTD-CTD and NTD-NTD interfaces involved in OCP^R oligomerization should feature residues that are not exposed in the dark OCP^O dimer, nor present at symmetrical crystal contacts in the OCP^O structure, otherwise OCP^O tetramers and higher oligomers would form in the dark-adapted state; presumably, these would

be residues present at the NTD-CTD interface in the dark OCP⁰ – an assumption which echoes the earlier proposal of Moldenhauer et al. 2017 (51) and Muzzopappa et al. (10, 51, 52) that the interface between CTD domains in an OCP^R dimer should overlap with the CTD surface that interacts with the NTD in OCP⁰; vi) the interfaces allowing the assembly of OCP^R dimers and tetramers may already have been observed in available crystal structures of OCP and isolated OCP domains, e.g. in the structure of the isolated holo-NTD of *wt* OCP (18), or in those of NTD and CTD paralogs, respectively coined helical carotenoid proteins (HCP) and CTD-homologues (CTDH) in the literature (39–41). Indeed, the study of Harris et al. (41) has shown that the apo-*Anabaena* CTDH – a homologue of the CTD of OCP involved in carotenoid transport and wherein the C-terminal helix is not apposed onto the β -sheet – is capable of forming two types of dimers, associating either through back-to-back (A-type) or head-to-head (F-type) interactions between two CTDHs. The F-type dimer, wherein CTDH residues equivalent to those contributing to carotenoid binding in the CTD of OCP⁰ face one another reconstituting a carotenoid tunnel, was proposed to be involved in carotenoid uptake (41). In absence of the carotenoid, however, the A-type interface can form between CTDH molecules, and it was demonstrated by SAXS that the isolated apo-CTD of OCP⁰ dimerizes by this interface. Golub et al. accordingly proposed that the two OCP^R monomers in a dimer also interact by this interface (10, 22). Like them, we hypothesize that OCP^R dimers form through interaction at this interface, which fulfills our above requirements for a sound modeling of OCP^R oligomers. It was further verified that only the A-type interface can fit the extended molecular envelope derived from our data. Indeed, the F-type interface would result in a “Z”-shaped molecular envelope. In the dark OCP⁰, interaction by the A-interface is prevented by the presence of helices α N (CTT, corresponding to residues 304-317) and α A (or NTE, corresponding to residues 1-17) which appose aside on the external face of the β -sheet but detach upon photoactivation. These helices were therefore considered as disordered in OCP^R and not included in our OCP^R dimer model. To avoid clashes, and account for the known exposure, in OCP^R, of the face of the NTD that interacts with the CTD in OCP⁰ – and notably R155, which is required for the interaction of OCP^R with PBS, and stabilizes the NTD/CTD interface of OCP⁰ by hydrogen bonding to E244 – the NTD was rotated by 180° and translated by 10 Å. The OCP^R dimer was then used as a starting point for modeling the tetramer, by assembly of two NTD in a fashion reminiscent to that observed in the HCP crystal structures from *Anabaena* Nostoc PCC 7120 and *Fremyella diplosiphon* (pdb ids: 5FCX and 6MCJ, respectively) (39, 40). In these, two molecules are found in the asymmetric unit, forming a dimer assembled through a symmetrical interface, featuring at its center the residue equivalent to R155 in OCP. Hence, in our model, the two dimers in an OCP^R tetramer interact by the same face known to bind the PBS, leaving two NTD available for binding to the latter. This tetramer model fits the *ab initio* envelopes computed for OCP^R_{R27L-Ntag} and OCP^R_{wt-Ctag} at 10.5 mg/ml (300 μ M) (Fig. 4).

Kinetics of $\text{OCP}^{\text{R}} \rightarrow \text{OCP}^{\text{O}}$ thermal recovery after accumulation of OCP^{R} by continuous illumination.

The recoveries of the OCP scaffold and the carotenoid structure could be uncoupled. Hence, with aim to split a difference between the structural recovery (informing on the protein structure) and the spectral recovery (informing on the electronic structure and environment of the carotenoid pigment), we monitored the kinetics of thermal OCP^{R} to OCP^{O} recovery using SAXS ($\text{OCP}_{\text{wt-Ctag}}$) and optical spectroscopy at 467 nm ($\text{OCP}_{\text{R27L-Ntag}}$, $\text{OCP}_{\text{wt-Ntag}}$ and $\text{OCP}_{\text{wt-Ctag}}$).

For the structural monitoring, accumulation of the OCP^{R} state was achieved by illuminating a 23 mg/ml (620 μM) solution of $\text{OCP}_{\text{wt-Ctag}}$ for ~ 30 minutes at 430 nm with a 500 mW continuous LED source collimated to ~ 1 cm (*i.e.* the same illumination protocol used in SAXS experiments described in the previous section), providing the starting point of the kinetics, and scattering data were thereafter recorded every ~ 12 s. It was established in our static SAXS measurements that formation of $\text{OCP}_{\text{wt-Ctag}}^{\text{R}}$ state is characterized by the presence, in the $\Delta S(q)$ difference pattern, of a negative peak at $\sim 0.05 \text{ \AA}^{-1}$ and a positive peak at $\sim 0.12 \text{ \AA}^{-1}$ (Fig. 3C). Hence, we used as a proxy to evaluate the structural recovery of the starting $\text{OCP}_{\text{wt-Ctag}}^{\text{O}}$ state the $\Delta S = S(t) - S_0$ difference signal in the $0.05 - 0.5 \text{ \AA}^{-1}$ range, where $S(t)$ and S_0 are the scattering patterns at a generic time (t) after switching off the 430 nm light and before the 430 nm irradiation procedure, respectively (Fig. S3 and Table S2). These data show that structural recovery of the dimeric $\text{OCP}_{\text{wt-Ctag}}^{\text{O}}$ state occurs on a time scale of tens of minutes, when $\text{OCP}_{\text{wt-Ctag}}^{\text{R}}$ is accumulated at high concentrations by prolonged illumination.

The spectroscopic recovery of $\text{OCP}_{\text{R27L-Ntag}}$, $\text{OCP}_{\text{wt-Ntag}}$ and $\text{OCP}_{\text{wt-Ctag}}$ was monitored in the 0.1 - 16 mg/ml concentration range, after accumulation of OCP^{R} by the same illumination protocol used in the static SAXS measurements. Just after switching-off the 430 nm light at time t_0 (*i.e.* when the concentration of OCP^{R} is at its maximum), the time evolution of the normalized absorption difference $[A(t) - A(t_0)]/[A(t_{\text{max}}) - A(t_0)]$ at 467 nm was monitored, where $A(t)$ and $A(t_{\text{max}})$ are the absorption values measured at a generic time t during the thermal recovery and at the time $t_{\text{max}} = 5000$ s, where the initial OCP^{O} state has been 95-99 % recovered (Fig. S4), respectively. Normalization was performed to enable direct comparison of data (Fig. S4) and of the fits at the various tested concentrations, viz. 0.1, 1.7, 3 and 16 mg/ml (Fig. S5). Alike the structural recovery, spectroscopic recovery occurs on a time scale of tens of minutes, with recovery slowing down as the protein concentration increases. Regardless of the concentration, however, $\text{OCP}_{\text{R27L-Ntag}}$ recovers faster than $\text{OCP}_{\text{wt-Ntag}}$, which itself recovers faster than $\text{OCP}_{\text{wt-Ctag}}$. As $\text{OCP}_{\text{R27L-Ntag}}$ and $\text{OCP}_{\text{wt-Ntag}}$ differ only in their capacity to form OCP^{O} dimers, and $\text{OCP}_{\text{wt-Ntag}}$ and $\text{OCP}_{\text{wt-Ctag}}$ only in the location of the his-tag, these observations suggest that (i) the re-association of OCP^{O} monomers into dimers is the limiting step in the recovery of $\text{OCP}_{\text{wt-Ntag}}$; and (ii) presence of a his-tag at the C-terminus negatively impacts dark-state recovery. We attempted fitting the recovery data using constrained (same set of characteristic times for the four

concentrations) mono-to-quadruple exponential functions (see Materials and Methods), where (i) a_{ci-1} , a_{ci-2} , a_{ci-3} and a_{ci-4} are pre-exponential factors depending on the protein variant and the concentration, (ii) T_{ci-1} , T_{ci-2} , T_{ci-3} and T_{ci-4} are characteristic times describing up to four separate molecular steps. A minimum of three exponentials are needed to account for the recovery kinetics of the three OCP variants. The characteristic times derived from the OCP_{R27L-Ntag} data ($T_{ci-1} = 59.8 \pm 0.3$ s; $T_{ci-2} = 159.1 \pm 0.6$ s; $T_{ci-3} = 641.7 \pm 1.1$ s, Table S2) are nearly half of those derived from the OCP_{wt-Ntag} data ($T_{ci-1} = 94.5 \pm 0.2$ s; $T_{ci-2} = 356.5 \pm 1.2$ s; $T_{ci-3} = 1327.5 \pm 4.3$ s, Table S2), and no better fits are obtained by accounting for a fourth component (similar χ^2 for either dataset; Tables S2 and S3), precluding extraction of a time constant for dimerization of OCP_{wt-Ntag}. Nonetheless, it is presumable based on the evolution of pre-exponential factors as a function of concentration that our data inform on the contribution to recovery of various oligomeric OCP^R species, namely the monomers (a_{ci-1} ; T_{ci-1}), dimers (a_{ci-2} ; T_{ci-2}) and tetramers (a_{ci-3} ; T_{ci-3}). Indeed, the populations associated to the first and second components intersect at 0.5, at low concentration, while those of the second and third components do the same, at higher concentration (Fig. 3D, E, F). A three-exponential fitting procedure applied to the OCP_{wt-Ctag} (Table S3) data yields longer characteristic times ($T_{ci-1} = 76.7 \pm 0.2$ s; $T_{ci-2} = 593.1 \pm 2.1$ s; $T_{ci-3} = 9517.4 \pm 75$ s), in line with the observed slower recovery, yet the data are better fitted (~ 4 times smaller χ^2 , i.e., a value close to those obtained for OCP_{R27L-Ntag} and OCP_{wt-Ntag} regardless of whether three or four exponential components are considered) by a four-exponential model ($T_{ci-1} = 51.5 \pm 0.2$ s; $T_{ci-2} = 209.9 \pm 1.3$ s; $T_{ci-3} = 946.0 \pm 3.7$ s; $T_{ci-4} = 14057.7 \pm 68.8$ s; Table S2). Presumably, the fourth component informs on OCP^O dimerization, given the comparatively low contribution of this component to the recovery signal and the expectation that presence of a his-tag at the N-terminus will challenge dimer formation in OCP_{wt-Ntag}, but not OCP_{wt-Ctag}. Alternatively, this component could underlie a step that becomes rate-limiting in the OCP_{wt-Ctag}, e.g. repositioning of the CTT on the CTD β -sheet. A global four exponential-fit of the three data sets, whereby the characteristic recovery times for OCP_{R27L-Ntag}, OCP_{wt-Ctag} and OCP_{wt-Ntag} were constrained to be identical and the simpler kinetics of OCP_{R27L-Ntag} (no dimerization of OCP^O) accounted for by constraining to zero the population parameter a_{ci-4} , corresponding to the longest characteristic time, was unsuccessful. Our data thus point to at least three, and in the case of OCP_{wt-Ctag} four independent molecular steps being involved in the OCP^R to OCP^O recovery.

Kinetics of OCP^R formation and OCP^R $\rightarrow$ OCP^O thermal recovery on the ns to s time-scale.

To investigate short-live intermediate states which exist along the OCP^O $\rightarrow$ OCP^R and OCP^R $\rightarrow$ OCP^O transitions, we used ns-s timescale transient spectroscopy whereby 8 ns pulses from a 470 nm laser (5 mJ pulse energy) were employed to trigger photoactivation, and difference absorbance (ΔA) signals at 440 and 565 nm were monitored. The 440 nm probe wavelength is located at the blue edge of OCP^O absorption spectrum, and therefore informs on the depletion and recovery of this state upon

and after excitation by the 470 nm pulse, respectively. Conversely, the 565 nm probe is at the red edge of the OCP^R absorption spectrum and thus serves as an indicator for the formation of the red absorbing states, including the first probed photoproduct P₂ ($\lambda_{\Delta A \max}$ = 565 nm, with occupancy proportional to the ΔA signal), OCP^R ($\lambda_{\Delta A \max}$ = 550 nm) and all OCP^R intermediate states between these.

We first investigated OCP_{wt-Ctag}, OCP_{R27L-Ntag} and OCP_{wt-Ntag} at 12 μ M concentration, *i.e.* $\sim$ 0.18 mg/ml (Fig. 5A), where nearly as many OCP_{wt-Ctag}^O and OCP_{wt-Ntag}^O are present in the monomeric form (54 %, *i.e.* 6.5 μ M) and in the dimeric form (46 %, *i.e.* 2.79 μ M (Table S4)), assuming a similar dissociation constant of 14 μ M for the two variants. Given this starting concentration, the pulsed nature of the excitation and the low quantum yield of photoactivation, OCP^R cannot accumulate in these experiments, hence the formation of OCP^R dimers and tetramers by OCP_{wt-Ctag}^O and OCP_{wt-Ntag}^O is extremely unlikely. We examined the temperature dependence (8°-36° C) of the ΔA signals at 440 nm and 565 nm in OCP_{wt-Ctag}, with the aim to identify key intermediate states and determine their associated enthalpy and entropy of activation. Experiments revealed that irrespective of temperature, all kinetics recorded at 440 nm start from the same ΔA value, *viz.* - 2 mOD (Fig. S7), indicating that depletion of the initial OCP_{wt-Ctag}^O state (with $\sim$ 0.7 % yield, as determined by Ruthenium actinometer; see Materials and Methods) hardly depends on the temperature. Notwithstanding, the initial ($\sim$ 50 ns) ΔA signal at 565 nm decreases with increasing temperature, amounting to 3 mOD at 8° C but only 1.5 mOD at 36° C (Fig. S7A and S7E). A possible interpretation is that the increased temperature results in a lower yield of the red states, including the final OCP_{wt-Ctag}^R, due to non-adiabatic processes occurring earlier than our first data point ($\sim$ 50 ns) which do not repopulate directly the dark OCP_{wt-Ctag}^O state (monitored at 440 nm). Another interpretation could be that the molar extinction coefficient of the OCP^R-bound carotenoid decreases with increasing temperature.

The ΔA signal for OCP_{wt-Ctag} at 565 nm appears triphasic in the 8-36 °C range (Fig. S7A – S7F), and likewise for the 440 nm ΔA signal in the 8-22 °C range (Fig. S7C – S7F). The two sets of difference signals at 440 and 565 nm probe the same molecular events, since similar characteristic times and activation energies can be derived from the two sets of data (Table S4 and Fig. 5E, respectively). Hence, we opted for a global tri-exponential fitting of the 440 and 565 nm data. Note that an additional fourth component intrudes into kinetics after 100 ms at 29° C and 36° C (Fig. S7D and S7E), which presumably lives longer than our time window at lower temperatures. Characteristic times were extracted for each step (Table S4), and Eyring plots generated from the measurements carried out at different temperatures (Fig. 5E). The fastest component ($\sim$ 2 μ s) displays an adiabatic behavior and is associated with a negative change in entropy, pointing to a reorganization process that reduces the complexity of the system, does not involve rupture of H-bonds or salt-bridges, and is fully fueled by conformational changes that have occurred before our first time-delay, *i.e.* $\sim$ 50 ns. The two other

components, characterized by characteristic times in the 0.2 - 5 and 10 - 250 ms range, respectively, are both characterized by increase of the entropy (11-18 cal.mol⁻¹.K⁻¹), indicating irreversibility, and display similar enthalpies of activation, viz. 18 kcal/mol (Fig. 5E).

The extent of recovery of the ΔA signal at 440 nm is slightly higher at increased temperature, with $\approx$ 50 % of the initially-depleted OCP^O state (0.7 % at 50 ns) having recovered after 1 s at 8 °C, but $\sim$ 75 % at 36 °C (Fig. S7). Briefly, overall OCP^R yields of 0.35, 0.26, 0.25, 0.20 and 0.18 % are found at 8, 15, 22, 29 and 36 °C based on the 440 nm data at 500 ms, respectively. Hence, the recovery of the OCP^O state somehow benefits from increased thermal energy, but is not completed after 1 s (*i.e.* the ΔA signal at 440 nm is not null (Fig. S7), regardless of temperature. We interpret this data as a confirmation that the limiting step in the monomeric OCP^R_{wt-Ctag} $\rightarrow$ OCP^O_{wt-Ctag} transition occurs on a time scale longer than 1s (observed in SAXS data, Fig. S3 and Table S2 and S3). The 565 nm kinetics also decay faster at higher temperatures (yields at 500 ms are 0.8, 0.44, 0.18, 0.02, 0.16 % at 8, 15, 22, 29 and 36 °C, respectively), indicating a lower yield for the red states (including OCP^R_{wt-Ctag}). Surprisingly, however, the ΔA signal at 565 nm is null after 1 s at 29 °C (and negative at 36 °C), which would suggest that no OCP^R_{wt-Ctag} remains. These diverging results illustrate the usefulness of probing photoactivation and recovery at the two extremes of OCP^O and OCP^R absorption spectra to obtain meaningful insights into the complex underlying mechanisms.

We repeated the above-described experiments on the constitutively monomeric OCP_{R27L-Ntag} mutant. The 565 nm kinetics, shown in Fig. 5, are overall similar for OCP_{wt-Ctag} and the OCP_{R27L-Ntag} mutant. From the multiexponential fits, we accordingly derive comparable characteristic times, with the main difference between the two proteins differing in a two-times shorter τ_3 component for OCP_{R27L-Ntag}, as compared to OCP_{wt-Ctag} (Table S4). Thus, only the last component shows a putative influence of the presence of dimers (recall that 46 % of OCP^O_{wt-Ctag} is present in the form of dimers at 12 μ M). Accordingly, the Eyring plots for OCP_{R27L-Ntag} (Fig. 5F) reveal analogous trends for the two first components, while the τ_3 component is characterized by larger activation enthalpy and entropy. To determine whether or not these differences could arise from the position of the his-tag at the N- or C-terminus of the protein, we further investigated OCP_{wt-Ntag} at 22 °C, and compared derived characteristic times with those obtained from OCP_{R27L-Ntag} and OCP_{wt-Ctag} (Table S4). We again observed similar dynamics, with derived characteristic times and relative contributions for the three steps being almost identical in OCP_{wt-Ntag} and OCP_{wt-Ctag}. An interesting difference is, however, that OCP_{wt-Ntag} displays a nearly two-times increased OCP^R yield at 1 s, suggesting that dissociation of the NTE from the CTD, facilitated by adjunction of an his-tag at the N-terminus, is a limiting step in photoactivation of OCP. This event is yet silent in terms of changes in the absorption of the carotenoid, since none of the components identifiable in the 440 and 565 nm kinetics are affected by the change in tag position in OCP_{wt-Ntag} and OCP_{wt-Ctag}.

Transient spectroscopy data presented so far were acquired in conditions where $\text{OCP}^{\text{O}}_{\text{wt-Ctag}}$ and $\text{OCP}^{\text{O}}_{\text{wt-Ntag}}$ monomers are slightly prevalent (54 %), and differences with the constitutively monomeric $\text{OCP}^{\text{O}}_{\text{R27L-Ntag}}$ were seen only for the τ_3 component. To further verify whether or not these changes arise from dimerization of the protein, we repeated experiments on $\text{OCP}_{\text{wt-Ctag}}$ and $\text{OCP}_{\text{R27L-Ntag}}$ at 50 and 250 μM , where dimers account for 78 and 95 % of the OCP^{O} population, respectively (Fig. 5C and 5D). Only the ΔA signal at 565 nm was probed. Both the initial P_2 yield at 50 ns and the overall OCP^{R} yield at 0.1 s increase with concentration for $\text{OCP}_{\text{wt-Ctag}}$, but not for $\text{OCP}_{\text{R27L-Ntag}}$, indicating that stabilization of the closed conformation of OCP^{O} through dimerization favors the initial steps of photoactivation (Fig. S8A and Table S4). The characteristic times derived for the $\text{OCP}_{\text{R27L-Ntag}}$ sample are only marginally affected by the increase in concentration, whereas a nearly two-fold increase in τ_3 lifetime is seen for $\text{OCP}_{\text{wt-Ctag}}$ at 50 and 250 μM . Thus, these experiments confirm that the τ_3 component, although present also in the monomer, is affected by dimerization. Specifically, a plot of τ_3 lifetime as a function of OCP dimers concentration, assuming the average τ_3 lifetime of $\text{OCP}_{\text{R27L-Ntag}}$ for zero dimer concentration, reveals a logarithmic behavior with half-maximum reached at ~ 10 μM dimer, corresponding to ~ 25 μM $\text{OCP}^{\text{O}}_{\text{wt-Ctag}}$ (Fig. S8B). It is interesting to note that regardless of the sample and concentration, the τ_2 component (~ 1 ms) has an almost constant value and relative contribution in all kinetics measured at 22 °C.

Time-resolved X-ray scattering.

Time-resolved X-ray scattering measurements were carried out using a pump-probe scheme with view to investigate the large-scale structural changes occurring in OCP_{wt} upon photo-activation, and the associated time scales. We used two variants of OCP_{wt} , viz. $\text{OCP}_{\text{wt-Ntag}}$ and $\text{OCP}_{\text{wt-Ctag}}$, enabling to test whether or not presence of a histidine-tag at the N- or C- terminus influences pulsed photoactivation kinetics. Indeed, it has been observed that both OCP^{R} accumulation under prolonged illumination and the subsequent thermal recovery are faster for $\text{OCP}_{\text{wt-Ntag}}$ than $\text{OCP}_{\text{wt-Ctag}}$ (Wilson et al., in review.; Nizinski et al., in review). From our ns-s transient absorption characterization of photoactivation and recovery in $\text{OCP}_{\text{wt-Ntag}}$ and $\text{OCP}_{\text{wt-Ctag}}$, it was expected that at 22° C, the two steps involving large scale motions are characterized by characteristic times of ≈ 1 and ≈ 80 ms (Fig. 5). Hence, time delays (Δt) between the pump optical-laser pulse and the probe X-ray pulse ranged from 15 μs to 200 ms. Experiments were performed at 23 mg/ml (620 μM) $\text{OCP}_{\text{wt-Ntag}}$ and $\text{OCP}_{\text{wt-Ctag}}$, i.e. a concentration at which dimers account for ~ 98 % of the protein.

The experimental setup designed for this experiment is described in the Experimental Section and involves the circulation of a large volume of sample across the X-ray beam to minimize cumulative X-ray damage during the experiment. The circulation speed was set so as to ensure that the sample is

kept in the pump–probe intersection area during a pump–probe sequence, but refreshed between two consecutive pump pulses. We verified that with this setup, the same difference scattering signal observed in static SAXS experiments could be obtained upon continuous illumination of the sample reservoir by a 500 mW LED source emitting at 430 nm. The LED illumination was prolonged until the photoinduced change in scattering signal was stable. The resulting difference scattering pattern is similar to that produced from our static measurements, *i.e.*, it features a negative peak at $0.05 \text{ \AA}^{-1}$ and a positive peak at $0.12 \text{ \AA}^{-1}$ (Fig. 3C). This control indicates that the use of a different continuous illumination setup at a different beamline does not result in shifts in the difference scattering signal (Fig. 6A).

With intention to determine the extent to which photoactivation can be increased by successive re-excitations within the pulse, we first measured the ‘light’ (laser-on) minus ‘dark’ (laser-off) difference scattering signal at $\Delta t = 1 \text{ ms}$, using two different laser pulse durations, viz. 5 and 450 ns. This required using two different lasers, emitting at 430 and 527 nm, respectively. At these wavelengths, the extinction coefficients of OCP^O are similar ($\approx 35\,000 \text{ M}^{-1} \text{ cm}^{-1}$), suggesting that the OCP^R formation quantum yield should be similar and that an increase in the difference signal at 527 nm would stem from successful re-excitation within the 450 ns pulse. Both pulse durations are indeed longer than the time necessary to recover the electronic ground state ($\sim 20 \text{ ps}$) (25), (53). A pulse power density of 3 mJ.mm^{-2} was chosen, affording the delivery of ~ 75 photons per chromophore across the pulse, corresponding to a photon every 0.06 and 5 ns for the 5 and 450 ns pulse durations, respectively. The two ‘light’ (laser-on) minus ‘dark’ (laser-off) difference scattering patterns at $\Delta t = 1 \text{ ms}$ both feature a negative peak in the small angle region up to $\approx 0.1 \text{ \AA}^{-1}$, but the amplitude of the peak does not vary significantly, indicating that stretching the pulse up to 450 ns does not improve the photoactivation extent (Fig. 6A). Thus, OCP molecules that have not engaged towards formation of a red state (P_1) and are back to the ground state (25) cannot be re-excited within the 450 ns pulse. Given the fact that these molecules do not show a difference signal in transient absorption spectroscopy experiments – meaning that the carotenoid is back in the orange state and therefore presumably H-bonded to the protein scaffold – their inability to re-enter a photocycle could be rooted in the fact that some protein residues, which are important for photoactivation but do not influence the spectrum, have not returned yet to their initial conformation after 450 ns. We refer to this *hitherto* undescribed intermediate state, which accounts for 99.3 % of the OCP^O population upon pulsed illumination, as the OCP “numbed-state”, abbreviated OCP^{NS}. To estimate an upper boundary for the lifetime of this intermediate, the repetition rate (1 kHz) of the 527 nm laser, coupled with a synchronized chopper/shutter system, was used to measure time-resolved patterns at a time delay of 100 ms excitation by an increasing number of consecutive pulses spaced by 1 ms. The scattering difference signal (laser-on minus laser-off) was then integrated in the $0\text{--}0.15 \text{ \AA}^{-1}$ range and plotted as a function of the number of pulses (Fig. 6B). The signal increases linearly with the number of pulses up to 5 pulses for OCP_{wt-Ntag} and up to 10

pulses for OCP_{wt-Ctag}, after which a plateau is observed, presumably due to concomitant recovery of the dark state structure (see below). Thus, the lifetime of the non-re-excitable OCP^{NS} can be estimated to lie between 450 ns and 1 ms. Irrespectively, comparison of the two time-resolved difference signals with the static difference scattering signal shows that the shape of the difference pattern generated by pulsed excitation does not coincide with that obtained by stationary illumination. In particular, the position of the negative peak differs, and the positive peak at $\sim 0.12 \text{ \AA}^{-1}$ is absent (Fig. 6B).

A complete time-resolved dataset was measured at time delays between 15 μs and 200 ms. Data for OCP_{wt-Ctag} are shown in Fig. 6C, while analogous results are obtained for OCP_{wt-Ntag}. Fig. 6C demonstrates that substantial changes in the scattering patterns are (i) already visible after 15 μs , (ii) maximized before 1 ms, and (iii) substantially damped by 200 ms. To further investigate the kinetics of the scattering signal changes, the time-resolved scattering dataset was analyzed by the singular value decomposition (SVD) method (see Materials and Methods), which revealed that the entire dataset can be described by a single time-independent basis pattern (continuous line in Fig. 6C). In other words, no other intermediate state than that responsible for this basis pattern is visible in our data. It must be noted that the difference between the time-resolved and static difference signals, *i.e.* the shift in q -value of the negative peak and the absence of a positive peak, is observed for all recorded time delays (15 μs -200 ms). Notwithstanding a rough estimation of the photoactivation extent by ns excitation can be obtained from the amplitude of the time-resolved difference signal compared to the static signal, which gives a value of $\sim 1.8 \%$ (Fig. 6A). Difference patterns in (Fig. 6C) were integrated in the $0 - 0.15 \text{ \AA}^{-1}$ range and the results as a function of time are shown in (Fig. 6D) for both OCP_{wt-Ntag} and OCP_{wt-Ctag}. The integrated intensity starts decaying between 1 and 10 ms in OCP_{wt-Ntag}, and between 10 and 100 ms in OCP_{wt-Ctag}, indicating a recovery of the dark state in these timescales, in full agreement with the results in Fig. 6C. Thus, large scale conformational changes start occurring as of $\sim 10 \mu\text{s}$, maximize at 1 ms, and revert with apparent characteristic times (*i.e.* time at half-maximum amplitudes) of ~ 37 and ~ 150 ms for OCP_{wt-Ntag} and OCP_{wt-Ctag}, respectively.

It is tantalizing, but difficult, to propose an interpretation for our observations. It has been proposed earlier that the first large-scale conformational change (~ 1 ms time scale) occurring in OCP post-excitation is the detachment of the NTE from the CTD β -sheet, possibly concomitant with a repositioning of the CTT, followed by dissociation of the two domains (25, 27). Given the quantum yield of OCP and the fact that our TR-SAXS experiment was carried out at 23 mg/ml (620 μM), where dimers account for $\sim 98 \%$ of the sample, it is yet extremely unlikely that two monomers in the same dimer would have been photoexcited by the same pulse (probability is $\approx 1e^{-5}$). Furthermore, since the NTE is involved in the dimerization interface, domain dissociation would have to await monomerization. Hence, it is most probable that the conformational change probed in our experiment

701 is neither dimer or domain dissociation, but rather a subtle conformational change occurring in one of
702 the two monomers in a dimer.

703

Discussion (4273 words)

In the present work, we used TR visible spectroscopy, as well as static and TR X-ray scattering to investigate the structural changes – including changes in quaternary structure – accompanying photoactivation of the orange carotenoid protein (OCP). It was repeatedly observed that OCP crystallizes as a dimer, yet it had remained elusive whether or not this interface is functional given the high concentration of the protein in crystals, viz. ≈ 23 mM. Mutation of a single conserved residue at the crystalline dimerization interface results in the loss of the ability to dimerize, suggesting that the dimer could indeed be physiological (10). Collection and analysis of static X-ray scattering patterns of dark-adapted OCP_{wt-Ctag} as a function of concentration confirm the *in vitro* existence, already suggested in the literature (20, 21, 23, 24, 28, 54), of a dimer which becomes predominant at concentrations ≥ 1 mg/ml (Fig. 3B, Fig. 4 and Fig. S1B). Our SAXS titration data ($K_d \sim 14$ μ M) is in accordance with (i) previous results from native mass spectrometry experiments, reporting the presence of OCP^O dimers at concentration as low as 3 μ M (20); (ii) the recent assertion based on SAXS and SANS data that OCP^O resides in the same state at 1 and 65 mg/ml (*i.e.* 28 μ M and 1.8 mM) (21, 22); and (iii) the dissociation constant that can be derived from size exclusion chromatography results presented in Fig. 3 of Muzzopappa et al. (10), *i.e.*, ≈ 0.6 mg/ml (17 μ M), provided that we use the same extinction coefficient at 490 nm to estimate protein concentrations.

Analogous concentration-dependent analyses of static X-ray scattering patterns collected after accumulation of OCP^R_{wt-Ctag} and OCP^R_{R27L-Ntag} by 30 min irradiation at 430 nm suggest that they are also prone to oligomerization. Indeed, for both proteins, not only is the scattering signal different between the dark-adapted (OCP^O) and light-adapted (OCP^R) states, but this difference evolves as a function of concentration. Remarkably, OCP^R_{R27L-Ntag} and OCP^R_{wt-Ctag} display similar $P(r)$ (Fig. 2F) and R_g (Fig. 3A) at the various tested concentrations, suggesting that they form the same OCP^R state(s). The high-concentration (10.5 mg/ml) difference-scattering patterns between the light- and dark-adapted states, *i.e.* $\Delta S(q)_{R27L-Ntag}$ and $\Delta S(q)_{wt-Ctag}$, are characterized by the presence of a negative peak (at 0.07 and 0.05 $\text{\AA}^{-1}$, respectively) and a positive peak (at 0.14 and 0.12 $\text{\AA}^{-1}$, respectively). These peaks respectively signal the loss and gain of interatomic distances in the OCP samples, upon illumination and their transition from the OCP^O to the OCP^R state. Given that OCP^R_{R27L-Ntag} and OCP^R_{wt-Ctag} form the same OCP^R species, the observation that the negative and positive peaks are found at different q -values in the $\Delta S(q)_{R27L-Ntag}$ and $\Delta S(q)_{wt-Ctag}$ difference patterns can be rationalized by the fact that their dark-adapted states differ – OCP^O_{R27L-Ntag} being present as a monomer, whereas OCP^O_{wt-Ctag} features a dimer.

The molecular envelopes reconstructed from the OCP^R data suggest that OCP^R dimers form as of 0.7 mg/ml (20 μ M), while tetramers form above 3.5 mg/ml (100 μ M), becoming predominant at concentrations greater than 10.5 mg/ml (300 μ M) (Fig. 3B and 4). The observation that the R27L

mutant also forms OCP^R dimers and tetramers suggests that the dimerization interfaces of OCP^O and OCP^R do not overlap. Based on this hypothesis, and on knowledge from the literature, we were able to build tentative models for OCP^R dimers and tetramers. The models notably assume that : i) OCP^R tetramers form from the coalescence of two OCP^R dimers; ii) interactions between OCP^R monomers in a dimer and between OCP^R dimers in a tetramer involve the same interface in the two interacting proteins; iii) residues involved in assembly of OCP^R into dimers and tetramers are not exposed in the dark OCP^O dimer (51, 52); and iv) OCP^R dimers assemble via their CTD, so that their two NTD are exposed and free to interact with PBS via R155 (50), while the OCP^R tetramers form from an interaction between two NTD in the OCP^R dimer, as seen in the crystal structures of HCP homologues (39, 40), (41, 50). Our model for the OCP^R_{wt-Ctag} dimer is in overall agreement with that proposed earlier (21), featuring two monomers associated by their CTD at an interface reminiscent of that observed at the A-type interface in the apo-*Anabaena* CTDH crystal structure. Of note, helices αN (or CTT) and αA (or NTE), which sit aside one another on the external side of the β-sheet but detach upon photoactivation (9), were considered as disordered in OCP^R and therefore not included in our OCP^R_{wt-Ctag} dimer model. To account for the known exposure, in OCP^R, of the face of the NTD that interacts with the CTD in OCP^O, each NTD was rotated by 180° and further translated by a few Å to avoid clashes with the CTD. The exposed face of the NTD features R155, which tethers the NTD to the CTD via H-bonding to E244 in OCP^O, but is essential for the interaction of OCP^R with the PBS. To fit the elongated density of the tetramer, and account for the unavailability for interaction of the CTD in the OCP^R_{wt-Ctag} dimer, we assembled two such dimers by their NTD, using as a template the dimerization interface observed in all three currently available crystal structures of the homologous Helical Carotenoid Proteins (HCPs) (pdb ids: 5FCX and 6MCJ (39, 40)). This interface features the residue equivalent to R155 in OCP; hence, in our model, the two dimers in an OCP^R tetramer interact by the same face known to bind to the PBS. In other words, in all OCP^R tetramers, only two NTD faces are available to bind and quench the PBS.

We used a combination of optical spectroscopy and X-ray scattering to investigate OCP^R to OCP^O thermal recovery and determine whether or not it is influenced by oligomerization processes. First, we monitored the structural recovery of the OCP^O_{wt-Ctag} state, following accumulation of the OCP^R_{wt-Ctag} state by prolonged illumination at 430 nm. The difference scattering pattern features a positive and a negative peak (Fig. 3C), which together signs for the disappearance of the starting OCP^O_{wt-Ctag} state and the concomitant appearance of OCP^R_{wt-Ctag} states. Hence, the time evolution of the difference signal in the 0.05 -0.5 Å⁻¹ q-range was used to follow the overall recovery, and is best fitted by a single exponential with a characteristic times τΔS(q) overall of ~ 4160 s (Fig. S3). The fact that this kinetics can be fitted by just a single exponential can be explained by technical and physical considerations: (i) the low temporal resolution (≈ 20 s) conceals to reveal characteristic times shorter than hundreds of seconds; (ii) the comparatively short time range explored obscures the full

description of the recovery kinetics; (iii) the low-resolution structural probe used in our study is not sensitive to the presence of intermediates localized at the chromophore level.

Hence, further insights in the OCP^R to OCP^O thermal recovery were attained by resorting to spectroscopy at various protein concentrations. First, we accumulated OCP^R by prolonged illumination at 430 nm, and monitored recovery of OCP^O by following absorption at 467 nm. Regardless of the sample, we found that the recovery time markedly increases as a function of concentration, in full agreement with the indication from static X-ray scattering data that both OCP^O ($OCP_{R27L-Ntag}$, $OCP_{wt-Ntag}$ and $OCP_{wt-Ctag}$) and OCP^R ($OCP_{R27L-Ntag}$, $OCP_{wt-Ntag}$ and $OCP_{wt-Ctag}$) oligomerize. Indeed, the more oligomeric states present, the higher the overall energy barrier for a full recovery of the starting dark OCP^O to occur, and therefore the longer it will take for full recovery to be befall. Irrespective of the assayed concentration, the spectroscopic recovery of $OCP^O_{wt-Ntag}$ was found to be slower than that of $OCP^O_{R27L-Ntag}$, indicating that the reassembly into dimers of OCP^O monomers is the step that limits $OCP^O_{wt-Ntag}$ recovery. Likewise, the recovery of $OCP^O_{wt-Ctag}$ was slower than that of $OCP^O_{wt-Ntag}$, indicating that presence of the his-tag at the C-terminus adversely affect recovery of the dark-adapted state. We plotted the pre-exponential factors as a function of concentration to follow the evolution of the associated populations (Fig. 3D-F and Fig. S6). Focusing first on results from the three-exponential fits, we observe a similar evolution as a function of concentration in the three samples: (i) the amplitude of the fastest component (a_{ci-1} , T_{ci-1}) decreases; (ii) the amplitude of the intermediate component (a_{ci-2} , T_{ci-2}) initially increases but then decreases; (iii) the amplitude of the slowest component (a_{ci-3} , T_{ci-3}) steeply increases. The observation of intersections at 0.5 population between amplitudes of rising and declining components is suggestive of a link between their relative populations. The populations of the first and second components feature such an intersection at concentrations of ~ 0.7 and 1.8 , in $OCP_{R27L-Ntag}$ (Fig. 3D) and $OCP_{wt-Ntag}$ (Fig. 3E), and at 6 mg/ml, in $OCP_{wt-Ctag}$ (Fig. S6C). Assuming that the first and second components inform on the contribution to recovery of OCP^R monomers and dimers, a possible explanation is that presence of the his-tag at the C-terminus challenges dimerization of OCP^R in $OCP_{wt-Ctag}$, elevating the apparent dissociation constant. For $OCP_{R27L-Ntag}$ and $OCP_{wt-Ntag}$, we furthermore see that the population of third component intersects those of the first (at ~ 1.7 and ~ 7 mg/ml, respectively) and second components (at ~ 9.5 and ~ 14.5 mg/ml, respectively), with the latter occurring at a population of 0.5 . Assuming that the slowest component informs on the contribution of OCP^R tetramers to the recovery signal, this observation would be suggestive of a facilitated accumulation of OCP^R tetramers in $OCP_{R27L-Ntag}$, compared to the $OCP_{wt-Ntag}$, possibly due to elimination of a competition between the dark-adapted dimerization and light-adapted tetramerization interfaces. Indeed, both are localized in the NTD of $OCP_{wt-Ntag}$. In the $OCP_{wt-Ctag}$ sample, we do not see intersections of the slowest component amplitude with those of others components, when results of the three-exponential fit are considered. However, a much better agreement is obtained when the $OCP_{wt-Ctag}$ data are fit with four-exponentials (Tables

S2 and S3), yielding a χ^2 comparable to that obtained with three exponentials for OCP_{R27L-Ntag} and OCP_{wt-Ntag}. Plotting the four amplitudes as a function of concentration, the same observations can be made for the OCP_{wt-Ctag} (Fig. 3F), *i.e.*, intersections at 0.5 population can be seen for the first and second component at ~ 1.7 mg/ml, and for the second and third component at ~ 10 mg/ml. The population of the additional fourth component, characterized by a characteristic time in the order of ~ 4 hours (14057.7 ± 68.8 s), varies from ~ 0.04 to 0.1 with increasing concentrations, indicating that the contribution of this rate-limiting step to the recovery signal is small. We see two possible explanations for the presence of this additional component. A first hypothesis is that it informs on the dimerization of OCP^O_{wt-Ctag} monomers, which due to absence of a tag at the N-terminus would reassemble faster than OCP^O_{wt-Ntag}, explaining that a characteristic time could be extracted for the former, but not for the latter, in our experiments ($t_{\max} = 5000$ s). Another hypothesis is that the additional component informs on a step that is dramatically slowed down in OCP_{wt-Ctag}, compared to OCP_{wt-Ntag}, *e.g.* the reattachment of the CTT to the F-side of the CTD β -sheet. Indeed, the CTT is longer and presumably more flexible in OCP_{wt-Ctag}, compared to OCP_{wt-Ntag}. Our data do not allow to favor one hypothesis over the other. However, the doubling of the characteristic times extracted for OCP_{wt-Ntag}, compared to OCP_{R27L-Ntag}, provides circumstantial evidence that dimerization of the dark-adapted OCP^O_{wt-Ntag} is the limiting step in its recovery. Therefore, we favor the first hypothesis. We accordingly propose that our recovery data inform on four molecular steps (Fig. 7) involved in the concentration-dependent OCP^R to OCP^O thermal recovery: (i) the transition from monomeric OCP^R to OCP^O, characterized by a characteristic time $\tau_{ci-1} = 50$ - 100 s ; (ii) the dissociation of OCP^R-dimer into OCP^R-monomers, characterized by a characteristic time $\tau_{ci-2} = 1$ 60 - 600 s and a dissociation constant in the order of ≈ 1 - 2 mg/ml, depending on the considered OCP variant (≈ 30 - 40 μ M); (iii) the dissociation of OCP^R-tetramers into OCP^R-dimers, characterized by a characteristic time $\tau_{ci-3} = 640$ - 950 s and a dissociation constant in the order of ≈ 10 mg/ml (~ 280 μ M); (iv) the re-association of OCP^O-monomers into OCP^O-dimers, characterized by a characteristic time $\tau_{ci-4} > 14$ 057 s and a dissociation constant in the order of ≈ 0.5 mg/ml (~ 14 μ M). That OCP^O dimer formation is slowed down in OCP_{wt-Ntag}, compared to OCP_{wt-Ctag}, could result from presence of a his-tag at the N-terminus, *i.e.*, in the vicinity of the OCP^O dimerization interface.

To obtain additional insights into molecular events occurring on shorter time scales, we used pulsed ns illumination to generate OCP^R (with a yield of ≈ 0.7 %), and monitored the ns-s timescale evolution of the difference absorption signal (ΔA) at the blue and red edges of the OCP^O (440 nm) and OCP^R (565 nm) absorption spectra, respectively. The use of the two wavelengths was justified by our need to obtain insights into both the recovery of the OCP^O state (ΔA_{440} nm) and the formation and disappearance of OCP^R and preceding red intermediates (ΔA_{565} nm). The wild type OCP_{wt-Ntag} and OCP_{wt-Ctag} were investigated, as well as the constitutively monomeric OCP_{R27L-Ntag} mutant. Regardless of the OCP sample, we could identify three intermediate states with characteristic times of ~ 2 μ s, $\sim$

1 ms and ~ 40-200 ms, respectively, at 22° C. The characteristic times derived from OCP_{wt-Ntag} and OCP_{wt-Ctag} data are nearly identical, indicating that the presence of the his-tag does not influence the dynamics probed at the carotenoid level on the ns-s time scale. Nonetheless, a higher OCP^R yield was found for OCP_{wt-Ntag}, demonstrating influence of the tag on a step that is not rate-limiting, and whose contribution to photoactivation and recovery is silent from the spectroscopic perspective. In these experiments, carried out at 12 μM concentration where the dimeric form accounts for 46% of the wt proteins, the first and second components were found to not vary as a function of the protein variant, while a two-times reduced characteristic time was found for the third component in the constitutively monomeric OCP_{R27L-Ntag}. Additional experiments carried out at 50 and 250 μM concentrations of OCP_{R27L-Ntag} and OCP_{wt-Ctag}, where the prevalence of OCP_{wt-Ctag} dimers are 78 and 95 %, respectively, revealed a marginal influence of concentration on the characteristic times determined for OCP_{R27L-Ntag}, but a two-fold increase in the lifetime of the third component τ_{ci-3} for OCP_{wt-Ctag}. A plot of the τ_3 characteristic time as a function of dimer concentration, using for zero concentration the average value from OCP_{R27L-Ntag} measurements at the 12, 50 and 250 μM (*i.e.* 54.8 ± 7.7 ms), shows a logarithmic shape, with a dimer concentration of ~ 10 μM (corresponding to an overall OCP_{wt-Ctag} concentration of ~ 20 μM) at half maximum (Fig. S8B). This result establishes that the presence of dimers influences component τ_3 in a concentration dependent fashion, but neither τ_1 or τ_2 . The data also show that the presence of dimers does not benefit the overall OCP^R_{wt-Ctag} yield, despite that of the initial P₂ intermediate rising with increased OCP_{wt-Ctag} concentration (Fig. S8A). To obtain further insights into the enthalpic and entropic contributions to the formation of each of the three states, we furthered our analysis by carrying out experiments at various temperatures in the 8-36 °C range (Fig. 5B and Fig. S7). We found that in both OCP_{wt-Ctag} and OCP_{R27L-Ntag}, the first intermediate – whose associated characteristic time hardly varies among proteins and concentrations – is adiabatically formed, *i.e.* with no exchange of heat (Fig. 5E and 5F). Thus, all the energy necessary for the formation of this intermediate is funneled into the protein scaffold before 50 ns, *i.e.* the first data point in our ns-s time span. The fact that no enthalpic contribution is associated with this component indicates that no H-bond or salt-bridge forms nor breaks, upon accumulation of this intermediate, while the observation of a negative entropic contribution suggests a reduction in the complexity of the system. Hence, component τ_1 could underlie the multi-step translocation of the carotenoid from the NTD/CTD interface into the NTD. As to the two other components, τ_2 and τ_3 , they are associated with similar enthalpies of activation (~ 18 kcal.mol⁻¹) in OCP_{wt-Ctag}, whereas a slightly larger value is found for τ_3 (~ 26 kcal.mol⁻¹) as compared to τ_2 (~ 20 kcal.mol⁻¹) in OCP_{R27L-Ntag}. This difference in enthalpy could be rooted in the absence of dimers in the OCP_{R27L-Ntag} sample, as we have seen that the τ_3 component – but not the τ_2 component – is affected by the presence of these. It is of note that the activation energy associated to the monomeric OCP^R to OCP^O transition after prolonged illumination is 38-42 kcal.mol⁻¹ (10, 55), *i.e.* roughly the sum of the activation energies determined for the τ_2 and τ_3 components.

Time-resolved SAXS/WAXS on the 15 μ s – 200 ms timescale allowed us to shed partial light on the structural changes associated with the OCP photocycle. Pulsed illumination results in a negative peak at $\sim 0.03 \text{ \AA}^{-1}$ in the ‘light’-minus- ‘dark’ $\Delta S(q)$ pattern, with this peak being already present at 15 μ s, maximizing before 1 ms, and starting to decrease as of 10 ms and 100 ms in OCP_{wt-Ntag} and OCP_{wt-Ctag}, respectively. Hence, our data demonstrate that the first large scale conformational changes associated with OCP photoactivation take place on the tens of μ s time scale (15 μ s), and that reversion of these changes is influenced by the localization of the his-tag in the protein. Both transient absorption spectroscopy and TR-SAXS required to work at comparatively high OCP concentration, with ~ 46 % of the protein in the dimeric state at the lowest concentration tested in spectroscopy experiments, and more than 98 % dimers at the highest concentration tested in spectroscopy experiments or used in TR-SAXS/WAXS experiments. Thus, the two techniques probe the same events occurring in similar OCP samples from the structural and the spectral perspectives, thereby informing independently on the fate of the protein scaffold and of the carotenoid during photoactivation, respectively. Caution must be taken when trying to jointly interpret such data hence reader discretion is advised as other interpretations that those proposed herein could exist to explain the two sets of data. The TR-SAXS/WAXS data is sensitive to structural changes regardless of where they occur in the protein, but is too low-resolution to afford information on the carotenoid position and on non-predominant species. Given that the probability that two monomers in a dimer can be activated by the same pulse is $\approx 1e^{-5}$, this means that our TR-SAXS experiment cannot probe the formation of OCP^R, but only that of the predominant non-productive intermediate state. This is at variance with our spectroscopy data which informs on the formation of all red-shifted intermediates – productive (OCP^R, offset at 0.5 s) and unproductive alike – but the spectroscopy is blind to structural changes that do not affect the local environment of the carotenoid. Hence, structural and spectroscopic transitions may be asynchronous, and they should be considered independently. With keeping this in mind, we can propose the following sequence of events for the OCP photocycle based on ours as well as others results (Fig. 8). It is known from the literature that upon absorption of the actinic photon, a S_2 state forms which decays within ps into three states: the S_1 and ICT excited states and S^* , which could either be an excited state (25) or a vibrationally-hot ground state (56). It has been proposed that S^* is the precursor of P_1 , a photoproduct characterized by rupture of the H-bonds between the carotenoid and the protein scaffold and which displays a lifetime of ≈ 50 ns. Thereafter, the carotenoid debuts its migration from the NTD/CTD interface into the NTD, first forming photoproduct P_2 , after repositioning at the NTD/CTD interface, and then P_2' , after completing migration across the NTD. Clearly, and as noted already by Konold et al. (25), this migration of the carotenoid must be accompanied by changes in the relative orientation of helices αC , αE and αG , which harbor the carotenoid-tunnel bottleneck-residues L37, M83 and M117. Eventually, these changes will propagate to the αG - αH loop and to E34, at the kink between αB and αC , resulting in an opening of the carotenoid tunnel across the NTD wall and in the formation of P_3 , wherein the $\beta 2$ ionone ring of the carotenoid is exposed to the solvent

– possibly as in the structure of the isolated NTD with canthaxanthin (18). We suppose that the first step identified in our transient absorption spectroscopy ($\sim 2\text{-}3\ \mu\text{s}$) and TR-SAXS ($< 15\ \mu\text{s}$) experiments corresponds to the P_2' to P_3 transition, in line with (Konold et al. (25). Failed bridging of the bottleneck (L37, M83, M117) is sanctioned by rapid back-migration and re-binding to H-bonding partners in the CTD, *i.e.* partial recovery of OCP^0 from P_2' , as evidenced by the fact that the decrease in $\Delta A_{565\text{ nm}}$ is mirrored by an increase in $\Delta A_{440\text{ nm}}$ (Fig. S7). The next spectroscopically-visible step occurs on the $\sim 1\text{ ms}$ time scale, and is characterized by a large decrease in $\Delta A_{565\text{ nm}}$, that is not accompanied by a similarly-extended increase in $\Delta A_{440\text{ nm}}$. Hence, only limited recovery of the OCP^0 state occurs on this time-scale, where TR-SAXS data furthermore show that no large structural changes occur. Therefore, we presume that this spectroscopic step can be attributed to a back-migration, in a fraction of P_3 intermediates, of the carotenoid into the tunnel. The back-migration would only be partial because of the opening at both end of the carotenoid tunnel in the NTD. Our TR-SAXS data suggest that depending on whether the his-tag is present at the N- or C-terminus, the dimeric P_3 intermediate reverts to the OCP^0 state, with characteristic times of 37 and 150 ms. This structural recovery could ensue the back-migration of the carotenoid on the 1 ms time scale, *i.e.*, the two steps would be decoupled. What is clear is that we do not see a large conformational change, such as detachment of the NTE or CTT, occurring on the $\sim 1\text{ ms}$ time scale, as proposed earlier (25); this could either indicate that these structural steps occur latter in the photoactivation mechanism, or that TR-SAXS is insensitive to structural changes occurring in only a sub-percent fraction of probed molecules. The last spectroscopic transition visible in our data occurs on the 200 ms time scale, and is again characterized by a large drop in $\Delta A_{565\text{ nm}}$ that is not mirrored by a similarly-scaled increase in $\Delta A_{440\text{ nm}}$. Recalling that this step displays the same activation energy as that occurring on the $\approx 1\text{ ms}$ time scale and that its characteristic time increases with the OCP concentration, we propose that it as well signs for the back migration of the carotenoid into the carotenoid tunnel, yet this time in an intermediate wherein the NTE (and possibly the CTT) has (have) detached from the CTD. Indeed, since the NTE is involved in the dimerization interface, the probability that it detaches will decrease (and therefore the overall time taken for the detachment to happen in an ensemble of photoactivated OCP molecules, increase) as the amount of OCP dimers augments. Accordingly, we further pose that NTE detachment is a prerequisite to dimer dissociation, which itself must precede separation of the two domains. In other words, for OCP^0 dimers to yield monomeric OCP^R , formation of P_3 must occur in each of the two monomers and the dimeric P_3 fall apart into its monomeric counterpart, before the reaction can further proceed. Given the low quantum yield, and our finding that P_3 starts recovering the OCP^0 state on the time scale of 37 and 150 ms in N-tagged and C-tagged dimers, respectively, photoactivation of dimers would thus be achievable only in experiments where one photon is delivered per chromophore every few ms. This is for example the case in our continuous illumination experiments where, by illumination of a 23 mg/ml (620 μM) solution of OCP with a 500 mW LED emitting at 430 nm, we estimate that 1 photon is delivered per chromophore every 10 - 30 ms. In this

context, it is interesting to note that the initial steps of photoactivation (specifically, the formation of P_2) are favored by dimerization, possibly due to an increased stiffness of monomers in dimers that would benefit productive excitation (formation of P_1 , and thence P_2) and migration along the carotenoid tunnel (P_2). The overall OCP^R yield, however, is not drastically improved, as expected given the above considerations.

The most unexpected finding from our TR-SAXS/WAXS experiment concerns the existence of a 'numbed' OCP intermediate, OCP^{NS}, formed upon non-productive laser excitation and decay to the ground-state, which remains non-re-excitable up to the μ s time scale (Fig. 6B and 8). Evidence for the existence of this state was obtained serendipitously as we attempted to increase the extent of photoactivation by distribution of actinic photons in a longer pulse. This attempt was infructuous, indicating that the ground state OCP^O structure formed after excitation remains non-photoactivatable for at least 0.5 μ s, in line with the previous report that increasing the power of ns pulses does not result in a higher photoactivation yield (13). By use of a multi-pulse approach, whereby re-excitation was triggered every 1 ms for up to 30 pulses reveals an increase of the difference scattering signal, we were able to estimate the lifetime of OCP^{NS} to lie between 450 ns and 1 ms. Hence, in the case of OCP^O dimers, the time window for absorption of an actinic photon by the second monomer in a dimer is 0.5 μ s – 100 ms. Any photon that arrives before 0.5 μ s is set to be unproductive, while any photon that arrives after 100 ms will hit a dimer wherein both monomers are structurally back in the starting state (Fig. 8).

Conclusion (264 words)

The emerging picture from our study suggests that oligomerization partakes in the regulation of the OCP photocycle, both at the level of OCP^O and OCP^R. Whether or not these findings have implications regarding the biological function depends on the local concentration of OCP in the vicinity of cyanobacterial thylakoid membranes; yet the observation that the mutation of a single conserved amino-acid at the OCP^O dimerization interface results in monomerization of the protein is a strong argument in favor of a functional role for OCP^O dimers. The dissociation constant of OCP^O was estimated to be 14 μ M, and that of OCP^R dimers to be similar, whereas the dissociation constant of OCP^R tetramers is likely to fall in the 3.5 mg/ml – 10.5 mg/ml concentration range. Models based on our static SAXS data suggest that in OCP^R dimers and tetramers alike, only two NTD are exposed and thus capable of binding to the PBS to perform their energy-quenching function. Should these models be correct, one could infer that oligomerization is a means by which a plateau is set for OCP-induced quenching of PBS excessive energy – and this, regardless of irradiance levels. We also show that the first large-scale conformational changes occurring in photoactivated OCP take place on the μ s time scale, likely corresponding to the P₂-P₃ transition. We note that ours is the first study to offer time-resolved structural insights into the OCP photocycle with a non-resonant structural technique, and as well the first TR structural study to be carried out on a protein with such a low quantum yield. We hope our work attracts the attention of structural biologists on the possibilities offered by time-resolved SAXS/(WAXS).

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Author Contributions: J.-P.C. coordinated the project. E.A.A., G.S. and A.T. performed static X-ray scattering. E.A.A. analyzed static X-ray scattering data and performed thermal recovery assays. S.N., M.S. and G.B. performed time-resolved spectroscopy. S.N. analyzed time-resolved spectroscopy data. A.W. and D.K. performed protein expression and purification; E.A.A., N.Z. and R.M. prepared protein samples. E.D.Z. performed fitting analysis of thermal recovery data. J.-P.C., G.S. and M.L. designed the TR-SAXS experiment. E.A.A., S.N., M.L., F.M., G.B., M.S., G.S., J.-P.C. performed the TR-SAXS experiment. G.S. analyzed time-resolved X-ray scattering data; E.A.A. and J.-P.C. wrote the paper with input from other coauthors.

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Data and materials availability: All data are available in the main text or the supplementary materials. Raw SAXS data can be made available upon request.

Figures captions (1688 words)

FIGURE 1. OCP crystallize as a dimer. (A, B) Structure of the dark-adapted *Synechocystis* PCC 6703 OCP^O dimer (PDB id: 3MG1). The dimerization interface is supported by conserved H-bonds between facing D19, R27, N134 (shown as sticks). Within the dimer, each OCP monomer features two domains: (i) an N-terminal domain (NTD; coloured in shades of orange) comprising residues 1–165; (ii) a C-terminal domain (CTD; coloured in shades of grey) comprising residues 193–317. The two domains are attached by a ~ 20 residue linker (coloured in shades of cyan). The keto-carotenoid pigment, ECN (coloured in slate) binds in a ~ 35 Å long hydrophobic tunnel spanning the two domains. In the CTD, ECN establishes two H-bonds with Y201 and W288. The N-terminal extension (NTE) and the C-terminal tail (CTT) are shown in pink and black, respectively.

FIGURE 2. Static X-ray scattering reveals changes in the OCP structure as a function of concentration and upon illumination. (A, B) X-ray scattering patterns of (A) dark-adapted OCP^O_{wt-Ctag} and (B) OCP^O_{R27L-Ntag} at various concentrations. The insets show overlays of the low-resolution data in the Guinier region. their corresponding overlays in the Guinier region (insets) at various concentrations. (C) Pair distance distribution function P(r) of dark-adapted OCP^O_{wt-Ctag} and OCP^O_{R27L-Ntag} at increasing concentrations. (D, E, F) X-ray scattering patterns of (D) light-adapted OCP^R_{wt-Ctag} and (E) OCP^R_{R27L-Ntag}, and (F) pair distance distribution function P(r) of light-adapted of OCP^R_{wt-Ctag} and OCP^R_{R27L-Ntag} at increasing concentrations.

FIGURE 3. Probing OCP oligomerization in dark and light-adapted states. (A) Evolution of the radii of gyration (Rg) of dark-adapted and light-adapted OCP_{wt-Ctag} and OCP_{R27L-Ntag} as a function of concentration. Error bars: standard deviation. (B) Estimation of the oligomerization state of OCP^O_{wt-Ctag} as a function of concentration. (C) Light minus dark SAXS difference patterns ($\Delta S(q)$) for OCP_{R27L-Ntag} at 10.5 mg/ml and OCP_{wt-Ctag} at 10.5 mg/ml (signal recorded on the bio-SAXS SWING beamline, Synchrotron SOLEIL) and 23 mg/ml (signal recorder at ID09 beamline, ESRF). (D, E, F) Plots of the retained pre-exponential factors retrieved from fits of recovery data as a function of concentration. (D) OCP_{R27L-Ntag}, (E) OCP_{wt-Ntag} and (F) OCP_{wt-Ctag}. The OCP_{R27L-Ntag} and OCP_{wt-Ntag} data are best fit by a model accounting for three exponential components, whereas the OCP_{wt-Ctag} data require inclusion of a fourth exponential component. Fitting parameters are reported in Table S2 and S3.

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envelopes are better fitted by the crystallographic OCP^O dimer (PDB id: 3MG1). At each concentration, the molecular envelopes determined for light-adapted OCP_{R27L-Ntag} and OCP_{wt-Ctag} are similar, and consistent with the presence of an OCP^R dimer, at 0.7 and 3.5 mg/ml, and an OCP^R tetramer, at 10.5 mg/ml.

FIGURE 5. Transient absorption spectroscopy enables identification of three intermediate states on the 50 ns – 0.5 s time scale. The figure shows the time evolution of the difference absorption signal at 565 nm. (A) Nanosecond transient absorption data for OCP_{wt-Ctag}, OCP_{wt-Ntag} and OCP_{R27L-Ntag} at 12 μ M. **(B)** Nanosecond transient absorption data for OCP_{R27L-Ntag} at 12 μ M and various temperatures. **(C and D)** Comparison of nanosecond transient absorption data between OCP_{wt-Ctag} and OCP_{R27L-Ntag} at 50 μ M, and OCP_{wt-Ctag} and OCP_{R27L-Ntag} at 250 μ M, respectively. **(E-F)** Eyring plots used to extract enthalpy and entropy of activation for OCP_{wt-Ctag} **(E)** and OCP_{R27L-Ntag} **(F)**, ($\kappa = 1$ is assumed). See Figure S7 for complementary data.

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FIGURE 7. OCP photoactivation and recovery are limited by oligomerization processes. OCP monomers and dimers are represented schematically with their N-terminal (coloured in orange and red for OCP^O and OCP^R, respectively) and C-terminal domains (coloured in white) represented by two circles. The ground state keto-carotenoid is shown in grey. Ticks and crosses in the CTD respectively indicate presence and absence of H-bonds between the carotenoid and CTD residues Tyr201 and Trp288. The presence of ticks in the NTD indicate closure of the carotenoid tunnel at its NTD end and presence of π -stacking interactions between the carotenoid and NTD residues Tyr44

1265 and Trp110. Our data show that dark-adapted OCP_{wt} forms dimers, with a dissociation constant of ~
 1266 14 μ M. The dimerization interface at play is likely that which has been repeatedly observed in the
 1267 OCP crystal structures, since substitution for a leucine (L) of R27, involved in a conserved salt-bridge
 1268 to D19 at this interface, fully abrogates dimerization. In the event where only one of the two monomers
 1269 in a dimer is activated, it may undergo all photoactivation steps until formation of P₃, but will promptly
 1270 revert to the dark state unless the second monomer is as well photoactivated (OCP^O/OCP^R). Indeed,
 1271 domain dissociation may occur after dimer disassembly, which itself can only occur after NTE
 1272 detachment, but the latter depends on the carotenoid achieving a successful transit across the NTD,
 1273 *i.e.*, formation of P₃. In practice, this means that reaching the OCP^R state in a pulsed illumination
 1274 experiment probing OCP_{wt} dimers is extremely unlikely unless re-excitation can be achieved within
 1275 the lapse of the excitation pulse. Indeed, the probability that two monomers in a dimer can be activated
 1276 by the same pulse is ~ 1e⁻⁵. Nonetheless, under continuous illumination, the OCP^R state can be
 1277 accumulated, which first coalesces into dimers, with a dissociation constant similar to that displayed
 1278 by dark-adapted OCP (~ 14 μ M), but may as well form tetramers, with a nearly 20 times higher
 1279 dissociation constant (~ 286 μ M). Existence of these oligomers elevate the overall energy barrier for
 1280 a full recovery of the starting dark OCP^O to occur, and therefore delay the upturn of the OCP^O state,
 1281 as each OCP^R oligomer presents a different lifetime. It is yet the reassociation of dark monomers into
 1282 dimers that constitute the limiting step in the recovery.

1283 **FIGURE 8. Proposed model for OCP photoactivation and recovery upon pulsed excitation.**
 1284 OCP is schematically represented with its N-terminal (coloured in orange for OCP^O, pink for P₁,
 1285 magenta for P₂, red for P₂', P₃, OCP^R, Int.1, Int.2 and Int.3) and C-terminal domains (coloured in white),
 1286 represented by two circles. The ground state keto-carotenoid is shown in grey. Ticks and crosses in
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 1288 residues Tyr201 and Trp288. Ticks and crosses in the NTD respectively indicate presence and
 1289 absence of π -stacking interactions between the carotenoid and NTD residues Tyr44 and Trp110. For
 1290 clarity, only the OCP monomer is shown. Complete migration of the carotenoid across the NTD tunnel
 1291 requires the unsettling of these interactions and passage of a bottleneck contributed by NTD residues
 1292 Leu37, Met83 and Met117, further down the tunnel. We posit that upon photoexcitation, the
 1293 carotenoid of OCP forms multiple excited states, only one of which is parent to the first photoproduct
 1294 P₁. Non-productive photoexcitation yet does not allow recovery of a photo-excitable state; instead, a
 1295 numbed state forms that displays all spectral characteristics of the ground state but cannot be
 1296 reactivated before a time delay estimated to lie between 0.5 μ s and 1 ms. We can only presume that
 1297 before then, important residues for the photoactivation mechanism (*e.g.* Y44 and W110) have not
 1298 returned to their ground state positions, despite the carotenoid being at its H-bonding position and
 1299 the protein therefore displaying an orange spectrum. Productive photoexcitation yields P₁, after
 1300 rupture of H-bonds to the carotenoid; past 50 ns, the carotenoid repositions at the NTD/CTD interface

(P₂), before bridging the above-mentioned bottleneck at 500 ns (P₂'). Conformational changes occur in the NTD domain, enabling the carotenoid to pass across the NTD wall and its β₂ ionone ring to become solvent-exposed (P₃). At all steps before P₃, back-migration of the carotenoid to its dark-state position can occur with rapid recovery of the orange state, as the tunnel remains shielded from the bulk. After P₃, however, recovery follows another pathway that does not enable rapid recovery of the orange state spectrum. In the fraction of P₃ that does not recover, the NTE will eventually detach from the CTD β-sheet, however this step was not visible in our data. Either before or after detachment of the NTE, back-migration of the carotenoid can occur, with characteristic times of 1 and 50-200 ms, respectively. Separation of the two domains may yet take place before occurrence of carotenoid back-migration and NTE reattachment, yielding the minute-lived OCP^R state, after repositioning of the CTT over the empty CTD half of the carotenoid tunnel. P₃ could thus serve as an intermediate along the OCP^R to OCP^O recovery.

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OCP monomers and dimers are represented schematically with their N-terminal (coloured in orange and red for OCP^O and OCP^R, respectively) and C-terminal domains (coloured in white) represented by two circles. The ground state keto-carotenoid is shown in grey. Ticks and crosses in the CTD respectively indicate presence and absence of H-bonds between the carotenoid and CTD residues Tyr201 and Trp288. The presence of ticks in the NTD indicate closure of the carotenoid tunnel at its NTD end and presence of π -stacking interactions between the carotenoid and NTD residues Tyr44 and Trp110. Our data show that dark-adapted OCP_{wt} forms dimers, with a dissociation constant of $\sim 14 \mu\text{M}$. The dimerization interface at play is likely that which has been repeatedly observed in the OCP crystal structures, since substitution for a leucine (L) of R27, involved in a conserved salt-bridge to D19 at this interface, fully abrogates dimerization. In the event where only one of the two monomers in a dimer is activated, it may undergo all photoactivation steps until formation of P₃, but will promptly revert to the dark state unless the second monomer is as well photoactivated (OCP^O/OCP^R). Indeed, domain dissociation may occur after dimer disassembly, which itself can only occur after NTE detachment, but the latter depends on the carotenoid achieving a successful transit across the NTD, *i.e.*, formation of P₃. In practice, this means that reaching the OCP^R state in a pulsed illumination experiment probing OCP_{wt} dimers is extremely unlikely unless re-excitation can be achieved within the lapse of the excitation pulse. Indeed, the probability that two monomers in a dimer can be activated by the same pulse is $\sim 1e^{-5}$. Nonetheless, under continuous illumination, the OCP^R state can be accumulated, which first coalesces into dimers, with a dissociation constant similar to that displayed by dark-adapted OCP ($\sim 14 \mu\text{M}$), but may as well form tetramers, with a nearly 20 times higher dissociation constant ($\sim 286 \mu\text{M}$). Existence of these oligomers elevate the overall energy barrier for a full recovery of the starting dark OCP^O to occur, and therefore delay the upturn of the OCP^O state, as each OCP^R oligomer presents a different lifetime. It is yet the reassociation of dark monomers into dimers that constitute the limiting step in the recovery.

FIGURE 8. Proposed model for OCP photoactivation and recovery upon pulsed excitation. OCP is schematically represented with its N-terminal (coloured in orange for OCP^O, pink for P₁, magenta for P₂, red for P₂', P₃, OCP^R, Int.1, Int.2 and Int.3) and C-terminal domains (coloured in white), represented by two circles. The ground state keto-carotenoid is shown in grey. Ticks and crosses in the CTD respectively indicate presence and absence of H-bonds between the carotenoid and CTD residues Tyr201 and Trp288. Ticks and crosses in the NTD respectively indicate presence and absence of π -stacking interactions between the carotenoid and NTD residues Tyr44 and Trp110. For clarity, only the OCP monomer is shown. Complete migration of the carotenoid across the NTD tunnel requires the unsettling of these interactions and passage of a bottleneck contributed by NTD residues Leu37, Met83 and Met117, further down the tunnel. We posit that upon photoexcitation, the carotenoid of OCP forms multiple excited states, only one of which is parent to the first photoproduct P₁. Non-productive photoexcitation yet does not allow recovery of a photo-excitable state; instead, a numbered state forms that displays all spectral characteristics of the ground state but cannot be reactivated before a time delay estimated to lie between 0.5 μ s and 1 ms. We can only presume that before then, important residues for the photoactivation mechanism (e.g. Y44 and W110) have not returned to their ground state positions, despite the carotenoid being at its H-bonding position and the protein therefore displaying an orange spectrum. Productive photoexcitation yields P₁, after rupture of H-bonds to the carotenoid; past 50 ns, the carotenoid repositions at the NTD/CTD interface (P₂), before bridging the above-mentioned bottleneck at 500 ns (P₂'). Conformational changes occur in the NTD domain, enabling the carotenoid to pass across the NTD wall and its β 2 ionone ring to become solvent-exposed (P₃). At all steps before P₃, back-migration of the carotenoid to its dark-state position can occur with rapid recovery of the orange state, as the tunnel remains shielded from the bulk. After P₃, however, recovery follows another pathway that does not enable rapid recovery of the orange state spectrum. In the fraction of P₃ that does not recover, the NTE will eventually detach from the CTD β -sheet, however this step was not visible in our data. Either before or after detachment of the NTE, back-migration of the carotenoid can occur, with characteristic times of 1 and 50-200 ms, respectively. Separation of the two domains may yet take place before occurrence of carotenoid back-migration and NTE reattachment, yielding the minute-lived OCP^R state, after repositioning of the CTT over the empty CTD half of the carotenoid tunnel. P₃ could thus serve as an intermediate along the OCP^R to OCP^O recovery.

Supplementary Figures captions (599 words)

FIGURE S1. Molecular envelopes and models fitted thereof for dark-adapted OCP_{wt-Ctag} and OCP_{R27L-Ntag} at the highest tested concentration. The molecular envelopes determined based on static SAXS data are shown for (A) dark-adapted OCP_{R27L-Ntag} and (B) OCP_{wt-Ctag} at 14 and 23 mg/ml, respectively. Proposed models are overlaid on the molecular envelopes. For the dark-adapted OCP_{R27L-Ntag}, the molecular envelope is consistent with the presence of an OCP^O monomer, whereas for the dark-adapted OCP_{wt-Ctag}, it is better fitted by the crystallographic OCP^O dimer (pdb id: 3MG1).

FIGURE S2. Photoactivation of OCP at 30 mg/ml requires tens of minutes of illumination by a 430 nm LED operating at 500 mW. The figure shows X-ray scattering difference signals of OCP_{wt-Ctag} at 30 mg/ml in a 2 mm thick quartz capillary produced by continuous illumination with a 430 nm collimated LED source. The LED nominal power was first set at 250 mW, switched to 500 mW after ~750 s and kept on during 750 s after which saturation of the signal change was reached. Data was collected at the ID09 beamline (ESRF, Grenoble).

FIGURE S3. Structural recovery of OCP^O_{wt-Ctag}, following accumulation of OCP^R_{wt-Ctag}. The plot shows the time evolution of the integrated intensity in the 0.05-0.5 Å⁻¹ region of the X-ray difference pattern $\Delta S = S(t) - S^0$, with $S(t)$ and S^0 the scattering patterns at a generic time t after switching off the 430 nm light and before the 430 nm irradiation procedure, respectively. The continuous line corresponds to a fit of the data by single exponential function.

FIGURE S4. The illumination procedure used to investigate OCP photoactivation and recovery does not damage the protein sample. (A-C) Absorption spectra of OCP before (black) and after (green) 30 minutes illumination plus 16 hours of thermal recovery. (A) OCP_{wt-Ntag} at 3 mg/ml, (B) OCP_{R27L-Ntag} at 1.7 mg/ml and (C) OCP_{wt-Ctag} at 1.7 mg/ml.

FIGURE S5. Global fitting of the recovery data for the different OCP variants. (A, B) For each of the three OCP variants, thermal recovery was monitored at four concentrations and data were globally fitted using a model accounting either for three (A) or four (B) exponential components. The upper, middle and lower rows show fitting results for OCP_{R27L-Ntag}, OCP_{wt-Ntag} and OCP_{wt-Ctag}, respectively. Fitting parameters are reported in Table S2 and S3.

FIGURE S6. Plots of the non-retained pre-exponential factors retrieved from fits of recovery data as a function of concentration. (A) OCP_{R27L-Ntag}, (B) OCP_{wt-Ntag} and (C) OCP_{wt-Ctag}. The OCP_{R27L-Ntag} and OCP_{wt-Ntag} data are best fit by a model accounting for three exponential components, whereas the OCP_{wt-Ctag} data require inclusion of a fourth exponential component. Fitting parameters are reported in Table S2 and S3.

1347 **FIGURE S7. Nanosecond transient absorption kinetics for OCP_{wt} C-tag at different temperatures.**
 1348 (A-E) Difference absorption was monitored at both 440 and 565 nm, enabling to probe photoactivation
 1349 and recovery at the two extremes of OCP^O and OCP^R absorption spectra, respectively. (F)
 1350 Nanosecond transient absorption data for OCP_{wt} C-tag at various temperatures, from 8 to 36 °C. See
 1351 Table S4 for complementary data.

1352 **FIGURE S8. OCP dimerization increases the yields at shorter time delays and delays the**
 1353 **occurrence of the last recovery step in the ns-s spectral evolution.** (A) Estimated yields of OCP_{wt}-
 1354 Ctag red-states at various time delays and concentrations. The plot demonstrates that dimerization
 1355 benefits the P₁ and P₃ yields. The OCP^R yield, however, only improves slightly. (B) The characteristic
 1356 time for the third component used to fit the ns-s spectral evolution of OCP_{wt}-Ctag increases as a function
 1357 of OCP-dimer concentration. The average characteristic time extracted from fits of the OCP_{R27L}-Ntag
 1358 data at various concentrations was used for zero dimer concentration.

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1361 **Supplementary Tables:**

1362 TABLE S1. Data collection and structural information pertaining to static SAXS analysis on dark-
1363 adapted (OCP^O) and light-adapted (OCP^R) OCP_{wt-Ctag} and OCP_{R27L-Ntag}.

1364 Table S2: Retained fitting parameters for the structural (SAXS) and spectroscopic (absorbance at 467
1365 nm) recovery of OCP_{R27L-Ntag}, OCP_{wt-Ntag} and OCP_{wt-Ctag}, following accumulation of OCP^R by continuous
1366 illumination.

1367 Table S3: Non-retained fitting parameters for the spectral (absorbance at 467 nm) recovery of
1368 OCP_{R27L-Ntag}, OCP_{wt-Ntag} and OCP_{wt-Ctag}, following accumulation of OCP^R by continuous illumination.

1369 TABLE S4. Fitted parameters extracted from nanosecond transient absorption data. Percentage
1370 contributions in the table are calculated as a given pre-exponential factor divided by $a_1 + a_2 + a_3 + a_4$.
1371 Measurements on OCP_{wt-Ctag} at 22 °C were repeated twice to confirm repeatability (note replicated
1372 row in the table).

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