Here, we propose a possible photoactivation mechanism of a 35-kDa blue light-triggered photoreceptor, the Orange Carotenoid Protein (OCP), suggesting that the reaction involves the transient formation of a protonated ketocarotenoid (oxocarbenium cation) state. Taking advantage of engineering an OCP variant carrying the Y201W mutation, which shows superior spectroscopic and structural properties, it is shown that the presence of Trp201 augments the impact of one critical H-bond between the ketocarotenoid and the protein. This confers an unprecedented homogeneity of the dark-adapted OCP state and substantially increases the yield of the excited photoproduct $S^*$, which is important for the productive photocycle to proceed. A 1.37 Å crystal structure of OCP Y201W combined with femtosecond time-resolved absorption spectroscopy, kinetic analysis, and deconvolution of the spectral intermediates, as well as extensive quantum chemical calculations incorporating the effect of the local electric field, highlighted the role of charge-transfer states during OCP photoconversion.
In cyanobacteria, carotenoid-dependent non-photochemical quenching is mediated by a unique class of water-soluble carotenoid-binding proteins, the homologs of the Orange Carotenoid Protein (OCP). OCP was identified in 1981, however, its structure and function were established only decades later. OCP is a 35-kDa photoreceptor triggered by blue light and coordinates a single ketocarotenoid molecule required for photosactivity. When the protein is purified from native OCP-containing cyanobacteria it is 3'-hydroxyechinomene, but OCP is also fully functional with either echinone (ECN) or canthaxanthin (CAN) instead. In the compact dark-adapted orange state (OCPD), the ketocarotenoid is enclosed by two protein domains of about equal size: an all α-helical N-terminal domain (NTD) and a mixed α-helical/β-sheet C-terminal domain (CTD). Upon photoactivation, OCP converts into the physiologically active red state (OCPR) via the formation of numerous intermediates, and the quantum yield of OCPR formation is extremely low (about 0.2%). During the conversion into OCPR, the structure of OCP undergoes global rearrangement: the domains separate from each other, after the carotenoid moves 12 Å deeper into the NTD13-15. These events lead to the exposure of sites for protein-protein interactions with the light-harvesting antenna complex, the phycobilisome (PBS), enabling quenching of its photodamage of the photosynthetic apparatus. Under conditions in the absence of PBS, OCPR spontaneously backconverts into OCPD in the dark, which is strictly dependent on temperature11,12. Termination of the OCP-dependent PBS fluorescence quenching in vivo is usually promoted by the Fluorescence Recovery Protein (FRP), which forces detachment of OCPR from PBS and promotes relaxation of OCPR back to OCPD21-23. Despite recent advances in characterization of protein-protein interactions of OCP with FRP and PBS24,25, two crucial questions remain unanswered: (i) how does OCP manage to dissipate excitation energy of PBS pigments, and (ii) how does OCP dependent PBS pigments photoconversion (less than 100 femtoseconds) and vibrational cooling in the S1 state38,39. For linear carotenoids, the lifetime of S1 is inversely proportional to the number of π-electrons in the conjugated system of C=C bonds, which is essentially the consequence of the energy gap law40. This correlation is also observed in cyclic carotenoids such as β-carotene. However, since the end rings can be twisted relative to the plane of the conjugated chain, non-integer values are observed for the effective conjugation length (N), which indicates that double bonds in the end rings partially contribute to the π-conjugated system. In polar solvents, and especially when embedded in protein matrices, ketocarotenoids show additional features in femtosecond transient absorption spectra which are attributed to intramolecular charge transfer (ICT) states occurring farther red-shifted from the S1-S0 spectral signatures. ICT signatures are valuable indicators for the contribution of the carbonyl group(s) to the conjugated system, and might inform on the configuration of the carotenoid end rings41. These effects are of particular interest for the chromophores embedded in OCP since the only specific carotenoid-protein interactions in the OCPD state are two short and strong hydrogen bonds between the 4-keto oxygen of the carotenoid and two hydrogens in the CTD, one belonging to the Tyr-201 hydroxyl group and another to the N-H group of Trp-288 (numbering corresponds to the OCP sequence of Synechocystis sp. PCC 6803). These hydrogen bonds affect the positioning of the terminal ring and decouple it from the conjugated polyene chain41.

Amino acid substitutions of both Tyr-201 and Trp-288 lead to destabilization of the compact OCPD state and result in permanently red-shifted OCP forms (e.g., OCPD21A/W288A, further designated using the one letter code OCPAA, as we focus on substitutions of Tyr-201 (first superscript letter) and Trp-288 (second superscript letter) in this work). Such red forms with separated protein domains are capable of inducing PBS fluorescence quenching without prior light activation21. However, many other amino acid substitutions lead to the same result due to destabilization of carotenoid-binding abilities of OCP, which increases conformational and thus spectral heterogeneity of the sample20,45,46. Spectral heterogeneity can also be increased due to the embedding of different carotenoids into OCP47. For example, the OCPD288A variant (according to our nomenclature described above, OCPDTA) expressed in ECN/CAN-producing E. coli strains preferentially bind CAN, appears red-purple and is not photostable, although WT OCP with CAN is orange (in the dark-adapted state) and photostable45. At the same time, even in the dark-adapted OCP several experimental approaches revealed spectral heterogeneity and a contribution from red-like forms confined in the compact protein structure36,41,48. Consistent with this, modelling of the linear absorption spectrum of dark-adapted WT OCP requires consideration of a red-shifted OCP-like component. This suggests that the carotenoid can principally occur in several distinct configurations in the compact OCP state. Sample heterogeneity together with the low quantum yield of OCP photoconversion makes it difficult to spot the formation of photoproducts in pump-probe experiments. Only recent developments of ultrafast spectroscopic approaches revealed the existence of so-called S* features40 in OCP with a yield of about 5%, which may represent either a structurally distorted form of the S1 carotenoid state or a hot ground state with extended lifetimes39,49,50. Femtosecond pump-probe fluorescence spectroscopy of an OCP variant with only one critical tryptophan residue (Trp-288) left in place revealed that the hydrogen bond with the carotenoid disappears with a time constant (~20 ps) corresponding to the S* state
lifetime, suggesting that the S\(^8\) state might be related to breakage of the hydrogen bond(s) and formation of the very first intermediate of the OCP photocycle\(^{10}\). Since the breakage of the hydrogen bonds between the keto oxygen and Tyr-201/Trp-288 residues is considered to be a crucial step towards the activation of OCP, a detailed analysis of this reaction is necessary for understanding the mechanism of OCP photoconversion.

In this work, we present our approach to elucidate the impact of the aforementioned hydrogen bond donors on the spectroscopic properties and photoactivity of OCP and infer structural determinants of spectral heterogeneity. After selection and atomic structure determination of the most spectrally homogeneous OCP sequence variant available, a construct termed OCP\(^{WW}\), we carried out femtosecond transient absorption spectroscopy experiments. The OCP\(^{WW}\) construct exhibits striking differences compared to wild-type OCP regarding excited state dynamics with accentuated occurrence of S\(^8\) signatures. Based on experimental data, computational modeling and quantum chemical studies, we propose the mechanism of hydrogen bonds breakage upon photoexcitation of OCP.

Results and discussion

Spectral heterogeneity of OCP preparations is associated with the H-bond donors in positions 201 and 288. It is known that interruption of protein-carotenoid interactions by amino acid substitutions destabilizes the compact OCP state, facilitating the formation of additional protein forms and thereby increasing spectral heterogeneity\(^{12,22,46,51}\). Since photoactivity is an exclusive feature of the compact orange OCP state, this state should be considered a starting point of the photocycle. The increased hydrodynamic size of the apoprotein and of red forms with separated protein domains allows for a complete chromatographic separation of these expanded forms from the compact holoform of OCP\(^{21,22}\). In order to exclude other intermediates from consideration, we sought for spectrally homogenous OCP variants, which ideally exhibit only OCP\(^{0}\) features in the dark-adapted state, without any signatures of the red forms.

In previous work\(^{12}\), we demonstrated that the purple CAN-containing OCP\(^{YA}\) variant could be converted into the orange photoactive state by kosmotropic agents (e.g., 0.8 M phosphate), which promoted compaction of the species with separated domains by reinforcing domain interactions. Of note, this was accompanied by the emergence of discernible vibronic structure in the carotenoid absorption spectrum of OCP\(^{YA}\). We suggested that this effect might be due to the reduction of the number of hydrogen bond donors in the CTD. This assumption was supported by the facts that (i) kosmotropes do not affect absorption of WT OCP and that (ii) a minor orange photoactive fraction of OCP\(^{YA}\) binding ECN exhibits intense vibronic bands even at low (0.2 M) concentrations of phosphate\(^{12}\). Therefore, it is safe to conclude that the cause of spectral heterogeneity resides within the CTD of OCP, and, more specifically, concerns the carotenoid-contacting residues in positions 201 and 288.

Spectral heterogeneity of the compact orange OCP state might be related to the competition of Trp-288 and Tyr-201 for H-bond formation to the keto oxygen of the carotenoid because of steric effects. Therefore, our strategy was to survey substitutions of these residues for their ability to affect the stability and spectral properties of OCP in the compact state (see Fig. 1). Importantly, a compact state could not be formed in the absence of both H-bond donors (in the OCP\(^{AA}\) sequence variant carrying Y201A/W288A substitutions). In all other cases, an orange photoactive species could in principle be obtained, albeit with a considerably different yield (Fig. 1A). The OCP\(^{YY}\) (W288Y substitution) and OCP\(^{AW}\) (Y201A substitution) variants expressed in E. coli strains bound exclusively CAN and showed no features of orange photoactive states in the absence of kosmotropes, which indicates that the stability of the compact state in these constructs is low. The OCP\(^{WW}\) (Y201W substitution) and OCP\(^{YA}\) (W288A substitution) variants were able to bind ECN as well, and, therefore, the orange compact state (with ECN bound) could efficiently be separated from the apoprotein and from purple, CAN-binding forms by size-exclusion chromatography (SEC). While the stability of the compact state of OCP\(^{YA}\) was still low compared to WT, the unprecedentedly stable OCP\(^{WW}\) variant was seen as a very fortunate object for the purpose of our investigation.

OCP\(^{WW}\) is capable of forming almost exclusively the compact orange state and is the most stable among all variants investigated. Its compact state is spectrally and structurally almost free from expanded OCP\(^{R}\)-like states (Fig. 1C), which makes it the best candidate from our portfolio (Fig. 1A) for spectroscopic and structural studies. However, careful inspection of the absorption spectra of different OCP variants also reveals heterogeneity of their extreme orange states. In particular, the vibronic structure is least pronounced in WT OCP and most pronounced in OCP\(^{YA}\) (in which the compact state is not stable, note the ~16% contamination by OCP\(^{R}\)-like states), while the absorption spectrum of OCP\(^{WW}\) is intermediate between these two (Fig. 1A and Supplementary Fig. 1). Therefore, we questioned if the tryptophan residue introduced in position 201 actually forms a hydrogen bond with the ketocarotenoid in the OCP\(^{WW}\) variant.

Atomic structure of OCP\(^{WW}\) reveals peculiarities of protein-pigment interactions. To elucidate possible reasons behind the spectral heterogeneity of OCP in its compact orange state, we determined the atomic structure of OCP\(^{WW}\). It crystallized at three different pH values in the same space group P3\(_2\)1,21, giving three structures with resolutions from 1.37 Å (pH 6.5) to 1.49 Å (pH 5.5) (Table 1 and Supplementary Table 1). We would like to note that OCP is stable and photoactive even at the lowest pH used for crystallization (4.6, see Supplementary Fig. 7 and description). All three structures confirm binding of exclusively ECN, are well superimposable, and reveal a protein fold that is barely distinguishable from that of WT OCP (Ca RMSD is 0.15–0.19 Å upon overlay with the PDB ID 4XB5 structure\(^{15}\), Supplementary Fig. 2). The position and conformation of Trp-288 are identical in WT and OCP\(^{WW}\), indicating the presence of a strong hydrogen bond between the keto oxygen and Trp-288 (the distance between the carotenoid’s keto oxygen and the nitrogen of Trp-288 is 2.9 Å). The electron density for the residue 201 reveals two alternative conformations of the engineered Trp in this position with nearly identical occupancies, nicely supported at this high level of spatial resolution. One Trp-201 conformation has the nitrogen oriented towards the keto carotenoid (‘IN’ conformation), presumably enabling the formation of a weaker hydrogen bond with a distance of 3.2 Å. In the second Trp-201 conformation (‘OUT’), the side chain is rotated by 180° roughly in the same plane, which is incompatible with a hydrogen bond to the keto oxygen of ECN (Fig. 2C). Analysis of \(F_o-F_c\) difference maps shows that IN and OUT Trp-201 rotamers have identical occupancy (within experimental error) each at all probed pHs. The distribution of dihedral angles (\(\chi_2\) along the obtained molecular dynamics (MD) simulation trajectories shows similar mobility of Trp-201 conformation ‘IN’ and ‘OUT’ states on the ns time-scale (Fig. 2D). MD simulations of the OCP\(^{WW}\) structures starting from these two distinct orientations of the Trp-201 side chain (‘IN’ or ‘OUT’) revealed no transitions between these two conformations in two independent 100 ns-long trajectories (Fig. 2D). This hints at a low rate of transitions between these forms at least in the basal dark-adapted OCP\(^{WW}\).
Considering the similarity of the absorption spectra of OCP variants having only one hydrogen bond donor (i.e., the OCPYA and OCPAW mutants, see Fig. 1A and Supplementary Fig. 1) to that of OCPWW, we associate the manifestation of the vibronic features of ECN S0-S2 absorption in OCPWW with the ‘OUT’ Trp-201 conformation, in which only one hydrogen bond is left (between ECN = O and Trp-288). If two hydrogen bonds are present simultaneously, in OCPWW with the ‘IN’ Trp-201

**Table 1 OCPWW X-ray data collection and refinement statistics.**

| PDB ID  | pH 6.5 | pH 5.5 | pH 4.6 |
|---------|--------|--------|--------|
|         | 6T6K   | 6T6M   | 6T6O   |
| Data collection |        |        |        |
| Space group | P 3 2 1 | P 3 2 1 | P 3 2 1 |
| Cell dimensions: a, b, c (Å) | 82.726, 82.726, 88.044 | 83.149, 83.149, 87.488 | 82.996, 82.996, 88.123 |
| α, β, γ (°) | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 |
| Resolution range (Å)* | 44.02-1.37 | 41.57-1.49 | 41.50-1.40 |
| [44.02-7.38] (1.39-1.37) | [41.57-8.16] (1.52-1.49) | [42-7.67] (1.42-1.40) |
| Wavelength (Å) | 0.976250 | 0.976250 | 0.976250 |
| Rmerge | 0.046 | 0.038 | 0.033 |
| [0.038] (1.49) | [0.032] (1.21) | [0.031] (1.16) |
| Rmean | 0.048 | 0.04 | 0.034 |
| [0.041] (1.57) | [0.034] (1.268) | [0.033] (1.193) |
| <I/σ> | 29.1 (2.3) | 37.3 (3.3) | 41.4 (3.5) |
| CC1/2 | 0.999 (0.816) | 0.999 (0.880) | 1.00 (0.882) |
| Completeness (%) | 100 (100) | 100 (100) | 100 (100) |
| Redundancy | 20.2 (20.2) | 20.2 (20.2) | 20.0 (20.2) |
| Refinement |        |        |        |
| Resolution range, (Å) | 37.54-1.37 | 41.57-1.49 | 41.53-1.40 |
| No. of reflections: total | 69781 | 54626 | 65885 |
| ‘free’ set | 3654 | 2826 | 3461 |
| Rwork (%) | 13.5 | 12.84 | 12.84 |
| Rfree (%) | 16.7 | 16.72 | 16.65 |
| Average B-factor (overall Å²) | 32.7 | 35.8 | 33.1 |
| No. of non-H atoms: protein/ligands/solvent | 2431/86/322 | 2438/51/343 | 2448/58/401 |
| R.m.s.d. bond lengths (Å)/angles (°) | 0.010/1.30 | 0.013/1.48 | 0.013/1.46 |
| Ramachandran favored/outliers (%) | 100/0.0 | 99.7/0.0 | 99.4/0.0 |
| Molprobity score/Clash score | 1.45/7.5 | 1.43/7.9 | 1.42/7.7 |

*Statistics for the lowest and highest resolution shells are indicated in square brackets and parentheses, respectively. The IN and OUT Trp-201 rotamers have 50% occupancy (within experimental error) each at all probed solution pH values.
conformation, we assume that absorption of this form should be similar to that of WT OCP (i.e., with the less pronounced vibronic structure), with the caveat that the length of the ECN = O—Trp-201 bond (3.2 Å) is appreciably longer than in the case of ECN = O—Tyr-201 in WT OCP (2.6 Å, see Supplementary Fig. 2). Although we noticed that a minor fraction of OCPWW indeed has absorption similar to WT OCP, a major part of the sample has very profound vibronic features (Fig. 1A and Supplementary Fig. 1). Thus, due to the long H-bond (3.2 Å), which in addition is formed only in half of the observed situations, the interaction of the carotenoid with Trp-201 in OCPWW appears to be weak and likely has limited effects on ECN absorption. This suggests that the single H-bond between ECN = O and Trp-288 is dominating and is responsible for the profound vibronic structure in the absorbance spectrum. Following this logic, we assume that the orange state with only one hydrogen bond is present also in OCPYA, because Tyr in place of Trp-288 would not be suitable for the formation of a strong hydrogen bond with ECN = O. We assume that the absence of the second, weak donor of the hydrogen bond in position 201 accentuates the role of Trp-288 in protein-pigment interactions in OCPWW.

Additionally, our structures also revealed heterogeneity of the Tyr-44 conformation (Fig. 2B). It is known that substitution of this residue by serine affects photoactivity of OCP, making photoinduced accumulation of the physiologically active red state ineffective, most likely due to an increased rate of back conversion. Therefore, besides characterization of the uniquely homogeneous absorption spectrum of OCPWW, it was necessary to analyze OCPWW in terms of photoactivity.

Photoactivity of OCPWW reveals a reduced number of hydrogen bonds with ketocarotenoid in solution. To test the ability of OCPWW to form the red state upon photoactivation, we performed a series of photoconversion kinetics experiments with relatively long (10 s) exposure to actinic light (see for description) at different temperatures, for comparison with WT OCP and OCPYA. Upon illumination of the dark-adapted OCPWW sample by actinic blue light (450 nm, LED 200 mW), we observed a gradual increase of the optical density at 550 nm, which was completely reversible in the dark, although back-conversion was considerably slower compared to WT OCP (Supplementary Fig. 3). Such a reduction of the back-conversion rate in OCPWW, which is also observed in the OCPYA sample, can be explained by a reduced number of residues involved in the anchoring of the ECN keto group upon the translocation of carotenoid back into the CTD. Since the activation energies (see Table 2) for the back-conversion of photoactivated mutants (OCPWW and OCPYA) are comparable to the one of WT OCP, we assume that the lack of one hydrogen bond donor results in an increased number of spontaneous conformational motions during achievement of the basal conformational state. These extended carotenoid and protein configurational dynamics decrease the probability of reformation of the compact orange state and, therefore, reduces the corresponding rate of the red state relaxation. Using a kinetic model proposed earlier, we determined the apparent activation energy necessary for the transition from the orange into the red state and found that in OCPWW this reaction requires only 2.3 kcal/mol which is approximately 5 kcal/mol less compared to WT OCP (Table 2 and Supplementary Fig. 3). Remarkably, the corresponding apparent activation energy for the accumulation of the red state was also reduced in the OCPYA sample compared to WT OCP. Since one hydrogen bond is also absent in the OCPYA sample, we assume—due to the similarities of the observed effects on activation energies and the clearly monoexponential decay of the red state (Supplementary Fig. 3)—that the second H-bond is also partially absent in OCPWW. This is in line with our structural data (Fig. 2) showing that Trp-201 in the “OUT” orientation is
Activation of the S0-S2 transition causes depopulation of the OCP reveal several distinct characteristic regions (Fig. 3A). Laser pump pulses at 520 nm, the transient absorption spectra of S in OCP Excited-state dynamics and photochemistry of the carotenoid photoactivation. Thus, in solution OCPWW represents a unique OCP variant state in OCP was standard deviation 5%37,53. Notably, the presence of the S* state in OCP was first reported only in 2019.9 We assume that this is due to the complexity of the experimental approach and the low yield of S* in WT OCP. Very recently, it was reported that S* is not a S1-like state in OCP, but it is a S0-like state.8 Since members of the OCP2 clade have a more profound vibronic structure of the S0-S2 spectra and faster accumulation of the red active state compared to OCP1 variants, it was concluded that the S0 state could be necessary for the photoactivation37. Since (i) the steady-state absorption spectrum of the OCPWW mutant exhibits one of the most pronounced S1 features, which is necessary to explain the relatively slow accumulation of S1, ICT, and S* signals after the rapid decay of the S2 state (see Fig. 3B). The excited state absorption (ESA) S1-SN transition of ECN in WT OCP and OCPWW is centered at approximately 650 nm. Since the S0-S2 transition is forbidden, S1 is formed by the decay of the S2 state with a lifetime of approximately 60–85 fs. Notably, the accumulation of ESA in the visible region occurs not only at the typical lifetime of S2, but also within 300–400 fs after the excitation, suggesting the presence of an intermediate state, which may decay into different long-living excited states (like S1). The S2-SM transition dominates in ESA at wavelengths above 800 nm (up to 1200 nm, according to reference 37), which is only covered up to 910 nm by our present setup, thus we see only the blue flange as the positive band at 860–900 nm in the decay-associated difference spectra (DADS) component (Fig. 3D, gray curve), which is enough to estimate the lifetime of S2. Thus, during its short lifetime, S2 populates other excited states, and according to global analysis (Fig. 3C, D), ESA of OCP represents a superposition of at least four distinct components with different lifetimes. We assign the DADS component with approximately 3.0 ps lifetime, which has positive amplitude in the 600–800 nm region of the transient absorption spectra to the mixture of the S1 state and a so-called Intramolecular Charge Transfer (ICT) state, which is characteristic of asymmetric keto- and ketocarotenoids in a polar environment, being especially pronounced in OCP at wavelengths above 700 nm.32–37,53–55 The most general explanation of the ICT phenomenon is related to substantial transfer of the electron density to the excited states induced by the keto oxygen. It should be noted that for many carotenoids, which do not show ICT signatures, ESA of S1 vanishes completely above 750 nm, which is not the case for the ~3.0 ps DADS component observed in our experiments (Fig. 3C, D, black curves). Thus, we assume that for WT OCP and OCPWW, ICT features are mixed with the S1 state. In addition to presumable S1/ICT states in OCPWW, we observed a substantial contribution of a compound decaying with a characteristic time constant of about 340 fs (Fig. 3D). This component has positive amplitude in DADS above 700 nm, and negative below 550 and 700 nm. Due to the pronounced positive ESA signal above 700 nm, we assign this component to an ICT-like state, which, as suggested by DADS, gives rise to the component with ~9.3 ps lifetime (Fig. 3D). Due to the aforementioned formalism, we assigned components with the longest lifetime to the S* state. The yield of the S* state is particularly high in OCPWW (~25%), with spectral signatures dominating around 550 nm, while in WT OCP, the yield of components with lifetimes longer than 5 ps is relatively low. We would like to note an interesting feature in the S* spectrum—the high resolution of vibrational bands in the GSB region (Fig. 3C, D, blue lines). This strongly indicates that the S* signal is associated with a subset of OCP molecules with some special ground state configuration of ECN which is more pronounced in OCPWW than in WT OCP. This might be related to a single hydrogen bond between ECN and protein in OCPWW in ‘OUT’ configuration (see Fig. 2C).

Further, we conducted a target analysis of ESA in OCPWW, since it has the most pronounced S* features. The most suitable and explanatory kinetic model proposed (Fig. 3E) considers five states, including the so-called ‘Mixed State’ (or the hot S1/ICT/S* state), which is necessary to explain the relatively slow accumulation of S1, ICT, and S* signals after the rapid decay of the S2 state (see Fig. 3F). This model allowed us to reconstruct spectra of the individual states (Fig. 3G), separating features of S1, ICT, and S* that are superimposed in DADS. Absorption of the ‘Mixed State’ represents a broad group of bands, indicating that exited states S1, ICT and S* are formed already within the first 100 fs after excitation of the sample.

| Table 2 Apparent activation energies (Ea) for the rate of accumulation (k0,a) and subsequent relaxation (k,a) of the OCP\(\text{R}\) state in WT OCP and two sequence variants studied. |
|-----------------|-----------------|-----------------|
|                 | Ea (k0,a), kcal/mol | Ea (k,a), kcal/mol |
| WT OCP          | 7.5 ± 0.3        | 31.8 ± 1.1       |
| OCPWW           | 2.3 ± 0.4        | 34.3 ± 1.0       |
| OCPYA           | 2.6 ± 0.3        | 38.2 ± 0.6       |

Average power of actinic LED light was set to 200 mW in all experiments. Experiments were conducted in a range of temperatures from 25 to 40 °C. Values are given as means ± S.D. and resulted from three measurements.
Thus, application of global and target analysis to transient absorption spectra of WT OCP and OCPWW, in which substitution of Tyr-201 by tryptophan increased the yield of the S* state at least fivefold compared to WT OCP and OCPWW, allowed us to disentangle the sequence of ultrafast photoinduced reactions, and to propose a comprehensive kinetic model, which places the S* state (Fig. 3E) in a critical position for proceeding towards the physiologically active red state. The model also indicates that the appearance of the S* state is associated with an intermediate state with ICT state features. In accordance, using ultrafast pump-probe fluorescence techniques applied to an engineered single-tryptophan OCP variant, we have recently found that breakage of the hydrogen bond between the keto oxygen of ECN and Trp-288 occurs with a time constant of 22.9 ± 2.0 ps. This is in line with our suggestion that H-bond disruption must be initiated by transition of the carotenoid into a long-lived S* state. Further, we discuss the possible nature of these states and their role in the photoactivation of OCP.

Possible mechanism of hydrogen bond dissociation upon OCP photoactivation. It is generally accepted that hydrogen bonding of ECN by Tyr-201 and Trp-288 prevents OCP from spontaneous transition into the active state. However, it is well known that OCP activation can occur without photoexcitation of the carotenoid, and some OCP variants balance between the orange and active red state due to the reduced stability of their compact form. Therefore, one cannot exclude processes of spontaneous hydrogen bond breaking under the influence of certain forces arising in the protein environment. But, since OCP is a photoreceptor, answering the question how the excitation energy absorbed by the carotenoid can force the breakage of hydrogen bonds is crucial. To address this question, we performed a series of quantum chemistry (QC) calculations to estimate the energetics of this process.

The energy of hydrogen bonds in the equilibrated ternary ECN/Tyr-201/Trp-288 complex (see Fig. 4A and Supplementary
distortion decreases the hydrogen bond energy in OCP down to 8 kcal/mol (see Supplementary Table 3), which is in good agreement with our experimentally determined values for the apparent activation energy of the OCP$^{\text{OCPR}}$ transition (see Table 2). Such an amount of energy is required to break hydrogen bonds and release ECN, Tyr-201, and Trp-288 as products (Fig. 4A, Reaction Pathway A). According to our calculations, the formation of hydrogen bond(s) between ECN and Tyr-201/Trp-288 causes a bathochromic shift of carotenoid absorption. Thus, dissociation of the hydrogen bond via Reaction Pathway A (Fig. 4A) would inevitably lead to a hypsochromic shift of the absorption spectrum of ECN (see Supplementary Table 5 for excitation energies for ECN and the ECN/Tyr-201/Trp-288 complex). However, this has never been reported in any femtosecond transient absorption experiment. The calculated energies of ECN in the excited states show that hydrogen bonds become about 3.5 kcal/mol stronger in the S$_2$ state, while staying almost the same in S$_1$ as in the ground state (Supplementary Table 5). Since hydrogen bond energy reduction in the excited states is not observed, it can be inferred that Reaction Pathway A is highly unlikely (see Fig. 4A, B) and alternative mechanisms should be considered.

Several hypotheses on the mechanism of photoinduced hydrogen bond breakage have been considered previously. Due to a different configuration of the carotenoid’s β-ring in crystals of full-length WT OCP and its NTD (the so-called red carotenoid protein, RCP) it was proposed$^{15}$ that the C6 − C7 trans−cis isomerization happens during the initial stages of OCP phot activation, however, no evidence for such a process was found on a ps timescale$^9$. Thus, it is reasonable to assume that isomerization of the carotenoid occurs due to the difference in potential energy of trans (C6 − C7 dihedral angle ~ 130°) and cis conformation (~45°)$^{57}$ in the absence of hydrogen bonds as a consequence of hydrogen bond breakage, but not as a prime cause for the breakage of the latter. Alternatively, keto-enol tautomerization of ECN in the excited state was proposed in$^{47}$; however, such a mechanism is highly unlikely due to the huge energy difference between the hypothetical tautomers.

Thus, we consider an alternative mechanism for the disruption of the hydrogen bond in the ECN/Tyr-201/Trp-288 complex involving charge separation (Fig. 4A, Coordinate B). Assuming a protonated ECN(H$^+$) oxocarbenium cation and a deprotonated amino acid (a Tyr or Trp anion) as products, the energy of such a reaction could be estimated as the difference in proton affinity (PA) between the donor and the acceptor of the proton. Calculations show that the proton affinity of ECN in vacuo (see Supplementary Table 6) is lowest compared to the corresponding PA values of Trp or Tyr or a Tyr−Trp complex. The energy difference between the corresponding compounds, which is necessary to conduct the proton transfer, ranges from 82 to 106 kcal/mol (considering a Tyr−Trp complex or Trp as a hydrogen donor, respectively). This energy is extremely high even compared to the excitation by a blue photon (about 65 kcal/mol), suggesting that in vacuum such a reaction is impossible.

However, the proton affinity dramatically depends on the strength of the local electric field. For instance, a local environment with a negative charge will enhance Coulomb interaction with a proton, thereby causing an increase of the proton affinity for a compound. A peculiar feature of conjugated molecules is their high polarizability due to the vast delocalization of the π-electrons$^{59,60}$. The distribution of the charged protein groups gives rise to an electrostatic potential gradient on the surface of the carotenoid-binding cavity of OCP$^{61}$. Noteworthy, the charge distribution within the carotenoid-binding cavity of OCP is highly asymmetric, with mostly positively charged residues concentrated in the CTD, and negatively charged residues dominating in the NTD (Fig. 4C).
and Supplementary Fig. 5). We assume that these external charges located near the polyene chain have a pronounced impact on the PA, even at a substantial distance to the actual protonation site due to polarization of the conjugated system. To test this hypothesis, we performed PA calculations for ECN in several model potentials (see Supplementary information) based on the OCP structure. Notably, the consideration of the local electric field within the protein in our calculations increased the relative PA of ECN and diminished the required proton transfer energy (Supplementary Table 6). According to our estimation, the chloride ion, which can be present in the OCP WW structure (Fig. 2), provides an additional reduction of the PA difference, which might promote proton transfer. Interestingly, similar to the first WT OCP structure, we observe a chloride ion in the interdomain cavity in two of the three structures we obtained for OCP WW (Fig. 2A, B). In spite of the recurrent presence of the chloride ion in OCP crystals and its proximity to the carotenoid molecule, its functional role has not been elucidated yet. The chloride ion is located approximately 14 Å away from both Tyr-201 and Trp-288, but only 4 Å away from the C12 atom of ECN, reducing the electrostatic potential along the ECN conjugated system, especially in the CTD (see Supplementary Fig. 4). Additionally, we tested whether pH affects OCP photoactivity. We found that the pH value affects the charge distribution in the protein by changing the protonation state of charged amino acids; however, the relative PA remains unchanged (see Supplementary Table 7 and Supplementary Fig. 6). Theoretical results suggest that a change in solution pH would not change the efficiency of the photoinduced proton transfer in OCP. In an additional experiment, we show that WT OCP is photoactive even at extremely low (3) and very high (11) pH (see Supplementary Fig. 7), although the protein stability is limited to a pH 4–10 range.

We assume that the disruption of the hydrogen bond via Reaction Pathway B is facilitated by the asymmetric protein environment due to its impact on the local electric field. Apparently, such a local charge distribution must also interfere with the carotenoid’s excited charge transfer states. The rise of an ICT state requires extensive mixing of the lowest-lying $1B_u$-like ionic and $2A_1$-like covalent states (see Mixed State Figs. 3 and 4), thus the ICT state is a charge transfer (ionic-like) state with extensive (covalent-like) bond order reversal and a very large (~25 Debye) dipole moment. Thus, photoexcitation of ECN in OCP, leading to the accumulation of the ICT state, results in a shift of electron density towards the keto oxygen, thereby increasing its negative charge. We suggest that, promoted by the electric field of the protein environment, this effect causes ionization of one of the hydrogen bond donors and favors excited state proton transfer (ESPT) towards the ECN, with transient formation of a highly unstable oxocarbenium ion.

To directly assess the optical properties of protonated ECN, we performed steady-state absorption and Raman spectra of protonated ECN and CAN, pure carotenoids were dissolved in chloroform with a strong organic acid (1 M trifluoroacetic acid, TFAA) as described in. In polar solvents, both carotenoids are characterized by the $S_0$--$S_1$ absorption maximum at ~480 nm; however, protonation by TFAA dramatically decreases the energy of this transition as seen from the shifts of the absorption spectra by 350 and 440 nm, respectively, into the near-infrared region, as well as a large reduction of the ratio of $v_2/v_1$ Raman bands (C=C and C=C stretching modes, Fig. 5). These effects are explained by a redistribution of electron density, which in turn affects the bond length alternation (BLA) pattern. We would like to note that all these effects in our model experiments, including the relative reduction of C=C stretching intensity and the red shift of $S_0$--$S_1$ absorption (Fig. 5A–C), were completely predicted by our QC calculations with excellent accuracy.

Thus, the accumulation of protonated ECN (the oxocarbenium ion) upon photoexcitation of OCP should be accompanied by an increase of absorption in the IR region (800–1000 nm, see Figs. 3 and 5), considering that formation of the ground state occurs faster than ECN deprotonation. We assume that such signatures of a protonated carotenoid could be identified in IR transient absorption or fs-Raman experiments. However, it is possible that rapid deprotonation occurs within the excited state of ECN. Due to the high proton affinity of Trp and Tyr (see Supplementary Table 6), protonation of ECN must be readily reversible, which might explain the overall low yield of the primary photoproduct (1.5% according to ref. 9) upon photoactivation of WT OCP. However, we note that the equilibrium geometry of a protonated carotenoid is different compared to that of ECN in the ground state (Fig. 5D). If deprotonation occurs after conformational relaxation of the oxocarbenium ion, the resulting configuration of ECN could be distorted (and likely not suitable for the formation of hydrogen bonds), which would promote carotenoid isomerization and the following stages of OCP activation.

Conclusions

The existence of hydrogen bonds between the keto oxygen of the carotenoid and Tyr-201/Trp-288 was revealed by structural studies long before photoactivity and the functional role of OCP were established. Since then, multiple works have proposed the significance of Tyr-201/Trp-288 as hydrogen bond donors for the functional activity of OCP, or rather for its low quantum yield of photoconversion, since these residues are necessary to keep the protein in a compact and physiologically inactive state. Despite the high evolutionary conservation of these residues, we found that both of them do not need to be in place simultaneously to keep the protein photoactive. Our work shows that the presence of two hydrogen bonds in WT OCP causes spectral heterogeneity. However, the complications entailed by this heterogeneity could be overcome by protein modifications which reduce the effective number of hydrogen bonds, but still, keep the protein stable in its dark-adapted state. By this artifice, we obtained an OCP variant (OCP WW) with the lowest contribution of the red state reported so far, and, consequently, a beneficially reduced spectral heterogeneity in the dark-adapted state (Fig. 1). Concomitantly, the OCP WW variant proved to have an increased quantum yield of specific carotenoid states which appear on a picosecond timescale upon OCP photoexcitation (Fig. 3). In particular, the yield of $S^*$ features in OCP WW was at least 5 times larger compared to WT OCP, which permitted quantitative analysis of the excited state evolution (Fig. 3) and revealed an interplay between the charge transfer (ICT) states and long-lived products (such as $S^*$) of the photochemical reaction. Considering various possible mechanisms for hydrogen bond breaking, we put forward the hypothesis that electronic excitation of the ketocarotenoid in OCP induces accumulation of an ICT state, the features of which—together with the local electric field provided by the specific protein environment within the carotenoid tunnel—promote redistribution of the electron density in a way that it induces proton transfer from Tyr-201 or Trp-288 to the keto oxygen of the carotenoid leading to the formation of a metastable oxocarbenium ion (see Figs. 4 and 5). We assume that the chloride ion, observed in the interdomain cavity of OCP in several crystal structures, might promote transient protonation of the carotenoid as it contributes to the local electrostatic potential. The features of protonated carotenoids, as characterized here by absorption and Raman spectroscopy and supported by quantum chemical calculations, reveal structural determinants forcing the
Cloning, protein expression, and purification. Production of wild-type *Synechocystis* OCP and variants thereof in ECN/CAN-producing strains of *Escherichia coli* followed previously published protocols. All OCP variants were produced by site-directed mutagenesis on the basis of the pQE81-L plasmid for the wild-type OCP using Q5 High-Fidelity DNA polymerase (New England Biolabs, USA) and primers indicated in Supplementary Table 8. The integrity and correctness of the resulting constructs were verified by DNA sequencing. The proteins carried an N-terminal His-tag (MRGSHHHHHHTDPATM) and were purified using a combination of immobilized metal-affinity and size-exclusion chromatography as described before. Protein concentrations were determined by spectrophotometry at 280 nm using the sequence-specific extinction coefficients calculated by the ProtParam tool in ExPasy.

Crystal structure solution and refinement. Diffraction data were processed using XDS. The structures were solved using molecular replacement in MOLREP and the PDB ID 4XB5 structure as a search model, yielding one OCPWW molecule per asymmetric unit. Before refinement, the canthaxanthin molecule present in the 4XB5 structure was removed to avoid bias in structure modeling. ECN was then manually built in the omit density in Coot and used for refinement using REFMAC. The refinement strategy included rigid-body refinement and then restrained refinement using TLS and individual anisotropic B-factors. Occupancies of alternative conformations of some residues including the engineered Trp-201 residue were also addressed to better describe the electron density map. Atomic coordinates and structure factors are deposited with the Protein Data Bank (PDB) under accession numbers indicated in Table 1 and Supplementary Table 1.

Molecular dynamics simulations. The initial conformations of Trp-201 ("IN" and "OUT" rotamers) of OCPWW were taken from our crystallographic models. The model of the apo-protein and carotenoid molecules were composed using the
OPLS-AA forcefield exactly in the same way as it was made earlier for similar simulations\textsuperscript{26}. The molecular model of the carotenoid molecule was created using standard OPLS atom types with the diene types for the conjugated chain. The model globules were dissolved in 100 mM NaCl solution (TIP3P water model). The molecular scene was orthorhombic with periodic boundary conditions and an inter-planar distance of 10 nm. The reference temperature was 310 K (Nose-Hoover thermostat) and the reference isotropic pressure was 1 Bar (Parrinello-Rahman thermostat). The integration step was 2 fs and the hydrogen bonds were constrained using LINKS algorithm. At the first stage of the simulation, the backbone atoms and heavy atoms of the carotenoid molecule were restrained in the space and the surrounding solution and amino acids' side chains were equilibrated for 10 ns. Then, the restrains were removed and the structure of the initially restrained and "OUT" OCP\textsuperscript{WW} globules was equilibrated in 100 ns simulation (10 ns tails of the trajectories were used for conformation analysis). Two independent simulations were made for each rotamer.

Size-exclusion spectrophotometry. OCP WT and OCP\textsuperscript{WW} were analyzed by size-exclusion chromatography with full absorbance spectrum detection. Purified protein samples (50 μM) were loaded on a Superdex 200 Increase 5/150 column (GE Healthcare) equilibrated with a filtered and degassed 20 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl and operated at a 0.45 ml/min flow rate using a Varian 335/Varian 363 HPLC system (Varian Inc., Melbourne, Australia). During the run, absorbance in the 240–900 nm range with 1-nm steps (4 nm slit width) was recorded with a frequency of 2.5 Hz. The profiles contained the symmetrical peaks whose absorbance spectra corresponding to the peak maximum and to the apparent MW of 35 kDa are presented as extracted from diode-array detector data using a custom Python-based script. Apparent MW for the peaks were determined using column calibration with BSA dimer (132 kDa), BSA monomer (66 kDa), ovalbumin (45 kDa), and α-lactalbumin monomer (15 kDa). The calculated MW for a OCP WT monomer is 34.6 kDa.

Steady-state absorption measurements. Steady-state absorption spectra and the time-courses of absorbance changes at 550 nm were recorded as described earlier\textsuperscript{33}. A blue light-emitting diode (M455L3, Thorlabs, USA), with a maximum emission at 455 nm was used for the photococonversion of the samples (actinic light for OCP\textsuperscript{W}→OCP\textsuperscript{P} photococonversion). The temperature of the sample was stabilized by a Peltier-controlled cuvette holder Qpod 2e (Quantum Northwest, USA) equipped with a magnetic stirrer. Amplitudes of photococonversion and OCP\textsuperscript{P}→OCP\textsuperscript{W} (R-O for simplicity) relaxation rates were determined according to procedures described earlier\textsuperscript{34} after a 10 s exposure to actinic light at temperatures from 5 to 35 °C. Each experiment was repeated at least three times. Rate constants (k) of a single temperature-dependent process yield a straight line within an Arrhenius plot (lnk versus 1/T), from which both the activation energy (E_a) and the pre-exponential factor were determined.

Transient absorption spectroscopy. Transient absorption spectra were measured using a femtosecond pump-supercontinuum probe setup. The output of Ti:Sap phire oscillator (800 nm, 80 MHz, 80 fs, "Tsunami", Spectra-Physics, USA) was amplified by a regenerative amplifier ("Spphire", Spectra-Physics, USA). The repetition rate of the amplified laser pulses was set at 100 Hz. The amplified pulses (800 nm, 100 Hz, 1.2 mJ, 80 fs) were split into two beams. One of the beams was attenuated to 0.4 mJ and directed to a non-collinear optical parametric amplifier (Clark-MXR), the radiation of which was used as a pump pulse. The pump pulse had a Gaussian pulse shape centered at a wavelength of 520 nm, 26 fs FWHM, and attenuated to 50 nJ pulse energy. The second beam was attenuated to 1 μJ and focused into a 3 mm quartz cell with pure H_2O to produce a supercontinuum probe pulse. The supercontinuum probe pulse had a smooth spectrum in the wavelength range of 400–900 nm.

The pump and probe pulses were delayed relative to each other by a computer-controlled delay line in the range of 0–500 ps, with a resolution of 3.3 fs to 1 ps. The pulses were then attenuated, recombined, and focused in a sample flow cell with an optical path of 0.5 mm. The pump and probe light spots had diameters of 200 and 80 μm, respectively. The relative polarizations of pump and probe beams were adjusted to 54.7° (the so-called "magic angle").

The experiments were carried out at 293 K. The circulation rate in the flow cell was 8 ml/min. The supercontinuum probe signal out of the sample was dispersed by a holographic grating with a spectral resolution of 0.023 nm/pixel. The signal was detected by a 1024-channel MWIR (800–1700 nm) detector (PIL.backend, USA). Transient absorption spectra were acquired at excitation wavelengths of 520 nm.

Quantum chemistry. In the present work, the following DFT functionals were used for geometry optimization: B3LYP\textsuperscript{72}, CAM-B3LYP\textsuperscript{73}, PBE\textsuperscript{74}. Dispersion correction D3\textsuperscript{75} was used in all cases. Basis sets used were: 6–311++G**(8s6p1f) for SCF, 6–311++G(2d2p) for optimization. To calculate the interaction energy in H-bonded complexes, the BSSE approach was used\textsuperscript{76}. For proton affinities, a direct A approach via the energy difference between two calculations of the initial and final states was used\textsuperscript{77}. To simulate the electrostatic surrounding, point charges were introduced to the quantum chemical system. For S1 calculations, TDDFT/TDA/ B3LYP, CAM-B3LYP\textsuperscript{78}, wB97X\textsuperscript{74},80, RI-wB2PLYP\textsuperscript{81} functionals were used. These calculations were done using ORCA 4.2 software package.
Kerfeld, C. A. Structure and function of the water-soluble carotenoid-binding protein.

Bulat, F. A., Toro-Labbé, A., Champagne, B., Kirtman, B. & Yang, W. Density-functional theory (hyper)polarizabilities of push-pull π-conjugated systems: trends and an accurate exchange and role of correlation. J. Chem. Phys. 123, 014319 (2005).

Otsuka, M., Mori, Y. & Takano, K. Theoretical study on photophysical properties of 3′-hydroxychinenone and the effects of interactions with orange carotenoid protein. Chem. Phys. Lett. 647, 95–102 (2016).

Kerfeld, C. A. Structure and function of the water-soluble carotenoid-binding protein of cyanobacteria. Photosynth. Res. 81, 215–127 (2004).

Enriquez, M. M. et al. The intramolecular charge transfer state in carboxyl-containing polynenes and carotenoids. J. Phys. Chem. B 114, 12416–12426 (2010).

Femtosecond transient absorption with chirped pump and supercontinuum probe: Perturbative calculation of transient spectra with general lineshape functions, and simplifications. Chem. Phys. 347, 127–138 (2008).

Moldenhauer, M. et al. Assembly of photoactive orange carotenoid protein from its domains unravels a carotenoid shuffle mechanism. Photosynthesis Res. 133, 327–341 (2017).

Dobryakov, A. L., Pérez Lustres, I. L., Kovalenko, S. A. & Ernsting, N. P. Femtosecond transient absorption with chirped pump and supercontinuum probe: Perturbative calculation of transient spectra with general lineshape functions, and simplifications. Chem. Phys. 347, 127–138 (2008).

Shelaev, C. V. et al. Femtosecond primary charge separation in Synechocystis sp. PCC 6803 photosystem I. Phys. Rev. A 38, 3098–3100 (1988).

Yanai, T., Tew, D. P. & Handy, N. C. A new hybrid exchange–correlation functional using the Coulomb-attenuating method (CAM-B3LYP). Chem. Phys. Lett. 393, 51–57 (2004).

Adamo, C. & Barone, V. Toward reliable density functional methods without adjustable parameters: The PBE0 model. J. Chem. Phys. 110, 6158–6170 (1999).

Ditchfield, R., Hehre, W. J. & Pople, J. A. Self-consistent molecular-orbital methods. IX. An extended Gaussian-type basis for molecular-orbital studies of organic molecules. J. Chem. Phys. 54, 724–728 (1971).

Hehre, W. J., Ditchfield, R. & Pople, J. A. Self-consistent molecular orbital methods. XII. Further extensions of Gaussian-type basis sets for use in molecular orbital studies of organic molecules. J. Chem. Phys. 56, 2257–2261 (1972).

Kendall, R. A. Jr., Dunning, T. H. & Harrison, R. J. Electron affinities of the first-row atoms revisited. Systematic basis sets and wave functions. J. Chem. Phys. 96, 6796–6806 (1992).

Gutowski, M. & Chalasinski, G. Critical evaluation of some computational approaches to the problem of basis set superposition error. J. Chem. Phys. 98, 5540–5554 (1993).

Su, N. Q. & Xu, X. Insights into direct methods for predictions of ionization potential and electron affinity in density functional theory. J. Phys. Chem. Lett. 10, 2692–2699 (2019).

Lin, Y.-S., Li, G.-D., Mao, S.-P. & Chai, J.-D. Long-range corrected hybrid density functionals with improved dispersion corrections. J. Chem. Theory Comput. 9, 263–272 (2013).

Casanova-Pérez, M., Dardis, M. B. & Geerigk, L. wB2PLYP and wB2GPPLYP: the first two double-hybrid density functionals with long-range correction optimized for excitation energies. J. Chem. Theory Comput. 15, 4735–4744 (2019).

Schmidt, M. W. et al. General atomic and molecular electronic structure system. J. Computational Chem. 14, 1347–1363 (1993).

Acknowledgements

E.G.M. thanks professor John T.M. Kennis for discussion and valuable suggestions. We acknowledge the support of the Russian Science Foundation (Grant no. 18-44-04002), and the German Research Foundation (DFG grant no. FR1276/5-1). Transient absorption experiments were performed using the facilities of Semenov FRC RAS CCE (no. 506694). Work was partially supported by the Semenov FRC RAS State task AAAA-A19-11901289006-7. Protein crystallization (I.G. and A.R.) was supported by the Ministry of Science and Higher Education of the Russian Federation (agreement #075-00337-20-03, project FSMG-2020-0003). Size-exclusion chromatography with full-spectrum detection was supported by the Ministry of Science and Higher Education of the Russian Federation (N.N.S. and Y.B.S.: AAAA-A19-11901050010-3). T.P. thanks the Czech Science Foundation grant 18-21631 S for financial support. This research has been supported by the Interdisciplinary Scientific and Educational School of Moscow University - Molecular Technologies of the Living Systems and Synthetic Biology.

Author contributions

I.A.Y. proposed an idea and performed quantum chemical calculations; E.G.M. designed and performed experiments, analyzed the data, and wrote an article with contributions from all co-authors; N.N.S. designed and performed experiments, analyzed the data, solved, refined, and analyzed the crystal structures; D.V.Z. conducted experiments on molecular dynamics; A.Y.S. designed, expressed, and purified proteins; E.A.S. expressed and purified proteins; Y.B.S. expressed and purified proteins; Y.S.B. expressed and purified proteins; A.R. crystallized the protein; K.K. collected the diffraction data; V.I.G. supervised the diffraction data collection; I.G. solved, refined, and analyzed the crystal structures; I.V.S. measured femtosecond transient absorption; P.E.G. measured femtosecond transient absorption; V.V.P. performed quantum chemical calculations; D.A.C. analyzed femtosecond transient absorption; T.P. analyzed femtosecond transient absorption; M.K. analyzed femtosecond transient absorption; T.S.G. analyzed femtosecond transient absorption; V.A.N. analyzed femtosecond transient absorption; D.K. analyzed the data; T.F. analyzed the data, V.Z.P. analyzed the data; A.B.R. analyzed the data; M.P.K. analyzed the data.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42003-021-02022-3.

Correspondence and requests for materials should be addressed to E.G.M.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2021