Integrating the Structural Changes Occurring upon Photoconversion in the Orange Carotenoid Protein from Broadband Two-Dimensional Electronic Spectroscopy

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ABSTRACT: Carotenoids play an essential role in photoprotection, interacting with other pigments to safely dissipate excess absorbed energy as heat. In cyanobacteria, the short timescale photoprotective mechanisms involve the photoactive orange carotenoid protein (OCP), which binds a single carbonyl carotenoid. Blue-green light induces the photoswitching of OCP from its ground state form (OCPO) to a metastable photoprotein (OCPR). OCPR can bind to the phycobilisome antenna and induce fluorescence quenching. The photoswitching is accompanied by structural and functional changes at the level of the pigment and of the bound carotenoid. Here, we use broadband two-dimensional electronic spectroscopy to study the differences in excited state dynamics of the carotenoid in the two forms of OCP. Our results provide insight into the origin of the pronounced vibrational lineshape and oscillatory dynamics observed in linear absorption and 2D electronic spectroscopy of OCPO and the large inhomogeneous broadening in OCPR, with consequences for the chemical function of the two forms.

INTRODUCTION

Light absorption can be dangerous to photosynthetic organisms when it exceeds their capability to convert light energy into chemical energy. To avoid formation of damaging species upon excess light absorption, photosynthetic organisms adopt a series of mechanisms to dissipate light energy safely as heat, collectively referred to as non-photochemical quenching (NPQ). Carotenoids play a critical role in NPQ, as validated by a variety of biochemical, spectroscopic, and theoretical studies.\(^\text{8,9}\) The mechanistic role of carotenoids in NPQ, however, is still not fully understood.\(^\text{10}\) In cyanobacteria, excess light absorption induces a photoprotective mechanism that involves a carotenoid-binding photoactive protein, the orange carotenoid protein (OCP), which both senses light intensity and directly triggers photoprotection.\(^\text{11–13}\)

OCP is a soluble 35 kDa protein found in the inter-thylakoid region, on the same side of the membrane as the phycobilisome antenna, the major light harvester in cyanobacteria.\(^\text{12,14}\) OCP noncovalently binds a single pigment, the carotenoid \(\beta\)-hydroxyechinenone (3-ninecarotene). Absorption of blue-green light by OCP induces conformational changes in both the pigment and the protein, as experimentally demonstrated by Raman, FTIR experiments, and native mass spectrometry.\(^\text{15,16}\) These changes convert the protein from its dark stable photoactive form, the orange OCPO, to a metastable form, the red OCPR. OCPR has been suggested to adopt an “open” conformation with increased accessibility of the carotenoid to solvent.\(^\text{17–20}\)

and a weaker protein–carotenoid binding interaction.\(^\text{16}\) OCPR has been shown to bind to the phycobilisome antenna and induce fluorescence quenching.\(^\text{12,15,21}\) Isolated OCPR spontaneously converts back to the orange form in darkness, and the back-conversion kinetics are independent of illumination but very sensitive to temperature. The conversion takes a few seconds at room temperature \(298 \text{ K}\) and \(\sim 40 \text{ min at } 283 \text{ K}.\(^\text{15}\)

OCPO binds the carotenoid 3-ninecarotene in an all-trans configuration (see Figure 1a).\(^\text{14}\) The carbonyl on the 4-keto-\(\beta\)-ionylidene ring of 3-ninecarotene forms hydrogen bonds at the C-terminal domain of the protein. This carbonyl group is essential for OCP photoactivity, as OCP with zeaxanthin or \(\beta\)-carotene bound (which do not have a carbonyl group) is unable to photoconvert.\(^\text{22}\) The roles of specific pigment–protein interactions responsible for tuning the spectroscopic and photochemical properties of 3-ninecarotene in OCP remain largely uncharacterized. Moreover, the conformation of the carotenoid and its interactions with the surrounding protein in OCPR are not conclusively known, as crystal and NMR structures are unavailable for this form.

In this paper, we investigate the photophysics of 3-ninecarotene in the two forms of OCP using two-dimensional electronic spectroscopy (2DES) to understand how the protein environ-
ment modulates the pigment energetics and dynamics in OCPO and OCPR and relate this to the different biological function of the two forms (photoactive vs quenching). OCPO is an ideal minimal system for the study of protein–pigment interactions, as it binds a single carotenoid. In contrast to other pigment–protein complexes that bind both carotenoids and chlorophyll pigments, the optical response of this minimal system is not complicated by inter-chromophore interactions, allowing direct observation of how the protein environment tunes and alters the properties of the pigment. Carotenoid photophysics are highly dependent both on the structure of the pigment and on the surrounding environment. Therefore, observed differences in the photophysics of the two forms of OCP must arise from changes in the structure of the carotenoid bound to the protein pocket and in the pigment–protein binding interaction.

Previous time-resolved fluorescence and transient absorption experiments have investigated the mechanism played by OCP in energy dissipation. Transient absorption experiments in the visible region suggested that OCPR could accept excitation energy from the phycobilisome bilin $S_1$ state to the carotenoid intramolecular charge transfer (ICT) state. The ICT state can subsequently relax to the ground state allowing direct observation of how the protein environment for the carotenoid 3'-hECN and then decay. Many open questions remain about the molecular origin of the different spectroscopic behavior observed for OCPO and OCPR. For example, the origin of the broadness and red shifting of the OCPR linear absorption has not been fully elucidated, and the origin of the ICT character enhancement in OCPR is incompletely described.

2DES presents numerous advantages over other nonlinear spectroscopic techniques such as transient absorption (for recent reviews, see refs 27–30). 2DES affords time and spectral resolution over both excitation and emission processes and has been very successful in unraveling the complicated structure–function relationship of natural photosynthetic systems. Broadband 2DES probing the visible region allows us to investigate and disentangle a spectral region usually congested in carotenoid photophysics and simultaneously provides the time resolution to investigate the ultrafast processes associated with the excited-state relaxation of carotenoids.

Our 2DES results for the two forms of OCP are remarkably different. OCPO shows pronounced vibrational dynamics, while OCPR gives a highly inhomogeneously broadened signal. We discuss how the differences in protein environment for the carotenoid in the two forms might relate to their specific biological roles (photoactivity and quenching).

### EXPERIMENTAL METHODS

**Sample Preparation.** OCPO was purified from a frozen paste of *Arthrospira platensis* cells (a generous gift of Dr. Gerald Cysewski, Cyanotech Corporation) using a procedure similar to that described by Holt and Krogmann, with isoelectric focusing omitted. Following anion exchange and gel filtration chromatography, the purified OCP had an $A_{496}/A_{280}$ ratio of 1.8:1. To prepare OCP for spectroscopic measurements, purified OCP in 50 mM Tris-HCl, 100 mM NaCl, pH 8 (24 °C) was concentrated to OD 30/cm in a centrifugal spin concentrator. For the OCPR sample, a 30 μL volume of concentrated OCP was initially converted to OCPR by 15 min of illumination with a 505 nm LED (Luxeon Rebel LXML-PE01-0070, Philips Lumileds, 40 nm FWHM) at 273 K. The illuminated sample was thoroughly mixed with 70 μL of chilled glycerol and illuminated for an additional 5 min. The sample was then transferred to a 200 μm path length quartz cuvette and cooled to 77 K in an optical cryostat (Oxford Instruments). The OCPR sample was continuously illuminated with the LED during the cooling to cryogenic temperature. The OCPO sample was prepared similarly but in complete darkness. The maximum OD was 0.28 at 502 nm for the OCPO sample and 0.25 at 518 nm for the OCPR sample. Linear absorption traces were collected before and after every 2DES measurement to check for sample stability at 77 K, and no measurable difference was observed in either sample. We cannot exclude a minor presence of RCP (red carotenoid protein) in our OCP preparations based on the linear absorption alone. However, its

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**Figure 1.** (a) Structure of the carotenoid 3'-hECN in the protein pocket in OCPO. (b) Normalized linear absorption spectra of OCPO (orange line) and OCPR (red line) at 283 K (dashed line) and at 77 K (solid line). Pictures of the cuvettes containing the samples are shown next to the corresponding absorption spectra. (c) Energy level scheme of the key transitions within the main molecular pathways for the carotenoid 3'-hECN. The numbered transitions are: (1) $S_0 \rightarrow S_2$ ground-state bleach; (2) $S_2 \rightarrow S_m$ excited-state absorption; (3) $S_2$ emission; (4) $S_1$ excited-state absorption. The experimentally observable transitions are described for the two forms of OCP in the text.
contribution would be the same in both OCPO and OCPR spectra, and we do not expect it to contribute to any observed differences in the dynamics of 3'-hECN in the two forms.

**Two-Dimensional Electronic Spectroscopy.** The 2DES experimental apparatus has been described in detail previously.36,39,40 A home-built Ti:sapphire regenerative amplifier laser system pumped a home-built noncollinear optical parametric amplifier, producing laser pulses centered at 540 nm (550 nm for OCPR measurements) with 60 nm FWHM. The combination of a prism compression line followed by a diffraction-based SLM (spatial light modulator) pulse shaper compressed the pulses to a duration of 12 fs, as characterized by TG-FROG (transient grating frequency-resolved optical gating).41 The beam was split into two replicas by a beam splitter, delayed with respect to each other via a retroreflector delay stage; this allowed the control of the waiting time \( T \) between pulses 2 and 3. The two beam pairs were further split into a total of four beams by a diffractive optic optimized for first-order diffraction, allowing for passive phase stabilization.39 The delay between pulses 1 and 2, which corresponds to the coherence time \( \tau \), was implemented by means of movable glass wedges, allowing for interferometric precision in the control of \( \tau \).39

The beams were focused to the sample position in a box geometry and had an energy of \( \sim 10 \) nJ per pulse. The interaction with pulses 1, 2, and 3 generates a third-order signal in the phase-matched direction, \( \vec{k}_1 = -\vec{k}_2 + \vec{k}_3 \) collinear with beam 4, the local oscillator (attenuated by 4 orders of magnitude so as to prevent any strong interaction with the sample). This signal was spectrally dispersed and heterodyne-detected on a CCD camera.

The coherence time \( \tau \) was scanned from \(-360\) to \(+360\) fs in 0.8 fs time steps for fixed values of waiting time \( T \). The resulting array of interferograms collected for each value of \( T \) was Fourier-transformed to produce the final 2DES spectrum. Negative values of the coherence time generated the non-rephasing signal (free induction decay), while positive coherence times generated the rephasing signal (photon echo signal). The two signals were obtained experimentally by inverting the ordering of pulses 1 and 2. The total 2DES spectrum, the relaxation spectrum, corresponds to the combined rephasing and non-rephasing components.

Dynamics were monitored by varying the waiting time \( T \); spectra were collected every 10 fs from 0 to 100 fs, then at 120, 150, 300, 450, 1000, 2000, 5000, 7000, 10000, 15000, and 20000 fs. In order to phase the 2DES data, spectrally resolved pump–probe experiments were collected separately for the same values of \( T \) under the same experimental conditions.40,42

### RESULTS

**Linear Absorption.** Figure 1b shows the linear absorption spectrum of the two forms of OCP at 283 and 77 K. OCPO, represented by an orange line, has resolvable features that correspond to the vibronic structure of the optically bright \( S_2 \rightarrow S_1 \) transition. The 0–0 transition is at 499 nm at 283 K and is red-shifted to 502 nm at 77 K. The OCPO sample appears orange, as shown in the inset on the left in Figure 1b. The structure of 3'-hECN in OCP is shown in Figure 1a.35 The linear absorption spectrum at 283 K is asymmetrically broadened toward longer wavelengths. This broadening is reduced in the 77 K spectrum.

Upon blue-green light illumination, the absorption spectrum broadens and red-shifts. The main peak for the red form OCPR is at 512 nm at 283 K and red shifts to 522 nm at 77 K. The vibrational structure that was visible in OCPO is lost, and the spectrum appears nearly Gaussian, as seen in Figure 1b. No apparent band narrowing or increased resolution of vibronic structure is observed in the OCPR spectrum at 77 K.

**Two-Dimensional Electronic Spectroscopy of OCPO.** Figure 2a shows the laser spectrum used in the experiment, superimposed on the absorption spectrum of OCPO at 77 K. The laser pulse is resonant with the \( S_1 \rightarrow S_2 \) electronic transition of the carotenoid, covering the 0–0 vibrational transition and the red edge of the 0–1 transition.

Figure 2b–d displays representative 2DES real-valued relaxation spectra of OCPO. The corresponding absolute-valued spectra are shown in the Supporting Information. Spectra acquired at long waiting times \( T \) (see \( T = 7 \) ps in Figure 2d) have two main features, A and B, with opposite signs. Short time spectra \( (T < 1 \) ps) are more complex (see Figure 2b,c), with multiple overlapping positive and negative features.

The laser pulse excites the system into the \( S_1 \) state; the positive feature centered around 515 nm (boxed region in Figure 2b) is assigned to a ground-state bleach (GSB)/stimulated emission (SE) signal from the \( S_2 \rightarrow S_3 \) transition (see Figure 1c for the energy level scheme). At early waiting times \( T \), we observe the presence of a negative signal centered around 502 nm excitation and 527 nm emission (see Figure 2b, circled region in \( T = 50 \) fs spectrum). At this waiting time, the negative signal has mostly decayed, so it appears blue-shifted along the excitation axis, as the bleach signal becomes stronger. Given the spectral position of this negative band and the time scale of decay (the signal disappears after \( \sim 50 \) fs), we assign it to an \( S_2 \rightarrow S_m \) excited-state absorption (ESA). ESA from \( S_2 \) has previously been observed in the visible region in 2DES experiments on \( \beta \)-carotene in solution.36,44 As the decay of the \( S_2 \) ESA signal proceeds, we see the appearance of two separate regions of intensity in the GSB/SE band, labeled \( A_1 \) and \( A_2 \) in the \( T = 50 \) fs spectrum in Figure 2b. \( A_1 \) and \( A_2 \) have an energy separation of \( \sim 1000 \) cm\(^{-1}\), and they oscillate in
frequency is close to the frequencies of \( \text{C}=\text{C} \) and \( \text{C}−\text{C} \) stretching of the ground state of the carotenoid \( \text{3'-hECN} \) (21 and 28 fs, respectively\(^{15} \)). We might also expect the intense 1008 cm\(^{-1}\) methyl rocking and 980 cm\(^{-1}\) hydrogen-out-of-plane (HOOP) wagging modes observed in prior Raman studies\(^{19,25} \) to contribute to this oscillatory signal, the latter being unique to \( \text{3'-hECN} \) in OCPO. The two peaks \( A_1 \) and \( A_2 \) are part of the same band and decay with the \( S_1 \) lifetime; therefore, they are most likely due to stimulated emission rather than ground-state bleach processes. They likely oscillate in phase due to the creation of ground-state wavepackets.

Even at early waiting times \( T \), the \( A \) band (see Figure 2) is well rounded, meaning it does not exhibit any diagonal elongation. This can be explained as a very fast randomization on the \( S_2 \) potential energy surface of molecules that unsuccessfully pass through the conical intersection\(^ {45−47} \) with the \( S_1 \) state and are scattered in a range of trajectories.

At later times, the system undergoes ultrafast internal conversion to the \( S_1 \) state. A negative feature centered at \( \lambda = 550 \) nm (feature \( B \), \( T = 7 \) ps spectrum in Figure 2d) appears, corresponding to \( S_1 \rightarrow S_0 \) ESA. The spectra at waiting times \( T \) larger than \( \sim 2 \) ps show little evolution of the two main GSB/SE and ESA features. By 20 ps, the signals have entirely decayed, corresponding to the complete relaxation of the population from \( S_1 \) to the ground state (spectra not shown).

At early times \( T \), in the region of emission wavelengths between the GSB/SE and the ESA signals (emission wavelength between 535 and 565 nm), we observe the presence of additional positive and negative features, overlapping spectrally and evolving in time. This region is highlighted by a rectangular box in the \( T = 100 \) fs spectrum in Figure 2c. This spectral region would be difficult to resolve or interpret in a 1D transient absorption experiment (which integrates over the excitation axis), as there are multiple overlapping signals of opposite sign arising from \( S_2 \), \( S_1 \), and possible contributions from other states such as \( S^* \).\(^{23,48−50} \) 2DES, however, can resolve the evolution of all these features and track them as a function of waiting time, \( T \).

To understand the origin of these peaks, we refer to the rephasing and non-rephasing components of the early time spectra. Figure 4 shows the rephasing and non-rephasing components of the \( T = 100 \) fs spectrum, with a rectangular box highlighting the spectral region of interest. The rephasing spectrum shows a similar pattern to the relaxation spectrum in the boxed region, with alternating positive and negative peaks, while in the non-rephasing component, a single positive peak appears. The peaks are observable in the spectra for waiting times shorter than \( T = 1 \) ps (data not shown), longer than the \( S_1 \) lifetime.\(^{19} \) A dispersive lineshape for cross-peaks in the rephasing component of 2DES spectra has been previously observed in both theoretical and experimental work.\(^ {36,44,51} \) This particular lineshape has been attributed to coupling of an electronic transition to a high frequency vibration. In 2DES experiments on \( \beta \)-carotene in solution, an analogous signal has been assigned to the population of a hot ground state via Impulsive Stimulated Raman Scattering (ISRS).\(^ {56} \) Given the similarity in dynamics, lineshape and spectral position of the cross-peaks in our spectra, we propose that they arise from the formation of a wavepacket on the hot ground state.

**Two-Dimensional Electronic Spectroscopy of OCPR.**

Figure 5 shows absolute-valued 2DES spectra for selected waiting times \( T \) for OCPR at 77 K. Figure 6 shows real-valued spectra for \( T = 30 \) fs and \( 7 \) ps.\(^ {52} \) The laser pulse excites the \( S_0 \) \( \rightarrow \) \( S_1 \) transition, and a positive signal appears in the 2D spectra, corresponding to GSB/SE. After excitation, the \( S_1 \) population undergoes ultrafast internal conversion to the \( S_0 \) state, but the \( S_1 \) lifetime cannot be determined with precision. Due to the spectral position of our laser pulse, we are not able to observe the negative peak corresponding to the \( S_1 \rightarrow S_0 \) excited-state absorption signal in our 2D spectra. This ESA signal is in fact red-shifted in OCPR and falls outside the bandwidth of our laser pulse.\(^ {19,25} \) No additional features appear: the GSB/SE signal decays as the molecule relaxes back to \( S_0 \) and this relaxation is complete by 20 ps. The OCPR 2D spectra are
broadened in this form even at 77 K. Interestingly, previous studies have shown that even upon absence of the C-terminal domain the shape and width of the spectrum are unchanged.20,53 This suggests that the origin of this particular lineshape is not highly dependent on the interactions between the C-terminal domain and the carbonyl group of 3′-hECN.

Further investigation of the molecular origin of the static broadening will be provided in the discussion of the 2D results.

Two-Dimensional Electronic Spectroscopy. 2DES allows us to compare 3′-hECN photophysics in OCPO versus OCPR, decongesting vibrational and electronic dynamics occurring on femtosecond to picosecond time scales over ~100 nm bandwidth. Our ultrashort (<15 fs) pulses have allowed us to directly investigate with high time resolution the early time dynamics of 3′-hECN in the protein environment of OCP.

Vibrational activity is visible in both real and absolute-valued 2DES spectra of OCPO. Multiple vibrational modes are initially photoexcited on the S2 state, and the action of ground-state wavepackets persists for up to ~1 ps. The 2DES results for OCPO resemble those obtained at cryogenic temperatures on β-carotene in a 2-methyl tetrahydrofuran glass.56 OCPR, on the other hand, has no observable vibrational features and appears largely broadened, as expected from the linear absorption spectrum.

The precise molecular origin of the observed large inhomogeneous broadening requires further investigation. Conformational heterogeneity could play an important role if 3′-hECN is exposed to bulk solvent due to a loss of pigment—protein interactions attributed to the C-terminal domain of OCPO.20 Solvent-induced broadening of the electronic absorption spectra of carbonyl carotenoids has previously been attributed to mixtures of conformational isomers preferentially formed in polar versus nonpolar solvents.54,55 Exposure of the carbonyl group of 3′-hECN to bulk solvent may create a distribution of conformers at the carbonyl-containing end ring (from s-trans, as in OCPO, to s-cis, as in solution).56 Additionally, fluctuations in the electrostatic environment of the chromophore produced by an ensemble of protein conformations or differences in H-bonding interactions with amino acids or water molecules could create a distribution of 0−0 energies that could further broaden the absorption spectrum of this form. Further experiments on OCP reconstituted with non-carbonyl carotenoids (such as β-carotene or zeaxanthin) and lacking the C-terminal domain could help disentangle these different possible contributions to the inhomogeneous broadening.

Our 2DES data on isolated OCP do not provide evidence for a direct role of 3′-hECN in OCPR-based quenching. We do not, for example, observe any significant shortening of the S1 lifetime in OCPR versus OCPO, consistent with the results in ref 19, and while it is generally accepted that 3′-hECN plays a direct role in the quenching process,24,56 the precise mechanism by which excess energy is dissipated remains under debate. 3′-hECN could play a direct role via energy or charge transfer,24,56 the precise mechanism by which excess energy is dissipated remains under debate. 3′-hECN could play a direct role via energy or charge transfer,24,56 or the OCPR complex could act allosterically to initiate quenching within the phycobilisome in analogy to the mechanism proposed for zeaxanthin in plant non-photochemical quenching by Horton et al.57 Polivka et al.26 recently determined only a small energetic advantage for the S1 state of 3′-hECN in OCPR versus OCPO relative to the phycobilisome bilin S1 state. We are not able to observe signatures of the ICT state in our data, due to the bandwidth of the laser pulse used,
and thus we cannot make conclusions on the enhancement of the ICT character for 3′-hECN in OCP nor draw conclusions regarding its potential involvement in a quenching mechanism. Our data do, however, provide insight into the perturbed local environment provided by the protein pocket in OCPO and OCPR.

Interestingly, it has not been conclusively demonstrated that the 4-keto-β-ionyliden ring, nor the ICT state resulting from the presence of the conjugated carbonyl group, is necessary for quenching activity in an OCP—phycobilisome complex. Could the presence of the 4-keto group be more strictly required for photoactivity rather than quenching activity? OCP is a photoswitch, and the unique photochemistry of 3′-hECN in OCPO must be responsible for driving structural changes in the protein that ultimately lead to formation of the OCPR form, binding of OCP to the phycobilisome, and induction of the quenching mechanism. It is known that interactions between the 4-keto group and absolutely conserved residues in the C-terminal domain (Y203 and W290) are strictly required for OCP photoactivity.22,58 While a relevant modulation of 3′-hECNs conformation(s) and/or photophysical properties in the OCPR form certainly may occur in the context of quenching activity, we must also consider the possibility that the photophysical properties of OCPR’s 3′-hECN chromophore in vitro could simply arise as a secondary consequence of other mechanistically critical changes in protein structure and chromophore solvent accessibility that occur during the photochemical mechanism and allow OCP to bind to the phycobilisome. Further studies of the quenching complex formed by OCP bound to the phycobilisome antenna21 are clearly needed to elucidate the specific nature of the quenching mechanism.

**CONCLUSIONS**

Here we have presented 2DES results comparing the excited-state dynamics of 3′-hECN in OCPO and OCPR. The study investigates the photophysics of a carbonyl carotenoid in two different electrostatic environments following photoconversion of the OCP holoprotein. Our results show resolvable and rich vibrational dynamics in OCPO, consistent with the carotenoid being held in a tightly locked conformation by the protein environment, with consequences for the photoactivity of this form. OCPR, on the other hand, shows a highly inhomogeneously broadened behavior. The origin of this large inhomogeneous broadening can be attributed to conformational heterogeneity due to exposure of the pigment to free solvent or to variations in the electrostatic environment experienced by the carotenoid. Further studies targeted to the investigation of the specific pigment—protein interactions in the C-terminal domain of OCP should help elucidate the role of the carbonyl group and the protein environment in tuning OCP photochemistry.

**ASSOCIATED CONTENT**

*Supporting Information*

Absolute-valued 2DES spectra for OCPO at selected waiting times. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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(52) It was only possible to retrieve the phase factor from pump–probe data for 2DES spectra at select waiting times \( T \), as shown in Figure 6. For comparison, absolute-valued OCPO spectra are shown in the Supporting Information.


