Excited States of the Inactive and Active Forms of the Orange Carotenoid Protein

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Supporting Information

ABSTRACT: The orange carotenoid protein (OCP) is a crucial player in the process of nonphotochemical quenching in a large number of cyanobacteria. This water-soluble protein binds one pigment only, the keto carotenoid 3'-hydroxyechinenone, and needs to be photoactivated by strong (blue-green) light in order to induce energy dissipation within or from the phycobilisome, the main light harvesting system of these



organisms. We performed transient-absorption spectroscopy on OCP samples frozen in the inactive and active forms at 77 K. By making use of target analysis we determined the excited state properties of the active form. Our results show that OCP photoactivation modifies the carotenoid excited state energy landscape. More specifically the photoactivated OCP is characterized by one state with predominantly ICT character (ICT/S₁) and a lifetime of 2.3 ps, and another state with mainly S₁ character (S₁/ICT) with a lifetime of 7.6 ps. We also show that the kinetic model is fully consistent with the RT data obtained earlier (Berera et al., *J. Phys. Chem. B* **2012**, *116*, 2568–2574). We propose that this ICT/S₁ state acts as the quencher in the OCP mediated nonphotochemical quenching.

INTRODUCTION

Carotenoids are a class of pigments widespread in nature where they play a number of functions. Their common structural feature is a series of alternating carbon-carbon single and double bonds, while their specificity is attained by the composition of side groups and structures integrated in the carbon-carbon backbone.1 In photosynthetic organisms, carotenoids are active in energy transfer to neighboring (bacterio)chlorophylls, thereby increasing the absorption cross section for photosynthesis, in structure stabilization and in photoprotection.^{1,2} The simplest representation of their singlet excited state manifold consists of a three state model based on the C_{2H} point symmetry group for linear polyenes: the ground state is denoted as $1A_g^+$, the first singlet excited state $2A_{g^{+}}(S_{1})$, and the second singlet excited state, $2B_{u^{-}}(S_{2})$. Because of symmetry reasons, the transition from the ground to the first excited state is forbidden,³ while the transition to the second excited state is strongly allowed and responsible for the color of the molecule. Besides these states, evidence for other carotenoid excited states, such as S^* , $S^\#$, and Sx has have been reported in the literature.^{4–8} Carbonyl carotenoids, characterized by a keto carbonyl group partly conjugated with the carbon-carbon backbone, are among the most abundant carotenoids in nature, being the main carotenoid group in photosynthetic marine organisms.⁹ The presence of the keto carbonyl group, confers to the molecule a number of interesting and important features. Carbonyl carotenoids possess an intramolecular charge transfer (ICT) state which is usually strongly coupled to the S₁ state.¹⁰ This state makes the excited state properties of the molecule sensitive to the polarity of the environment; polar solvents stabilize the ICT state, and this generally leads to a shortening of the S₁/ICT lifetime. Carbonyl carotenoids also show a smaller S₁/ICT-S₂ energy gap, and this feature is particularly useful in marine organisms where the S₂ state is sufficiently red, i.e., sufficiently low in energy, to allow efficient green light absorption and yet the S₁/ICT state energy is high enough to guarantee very efficient energy transfer to neighboring bacteriochlorophyll molecules.¹¹

Cyanobacteria regulate the energy flow within the phycobilisome, their main light harvesting antenna, according to environmental (light) conditions.^{12–14} Under strong light, cyanobacteria decrease the energy arriving to the reaction center by increasing thermal energy dissipation at the level of the phycobilisome. A crucial role in the process is played by the orange carotenoid protein (OCP).¹⁵ This water-soluble protein binds one pigment only, the keto carotenoid 3'-hydroxyechinenone. When the organism is exposed to strong (blue-green) light illumination, OCP is photoactivated and both carotenoid and protein conformational changes are induced¹⁶ converting the inactive orange form (OCP°) to a red metastable active

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Received:
July 26, 2012

Revised:
June 14, 2013

Published:
June 25, 2013
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form (OCP^r). Only OCP^r is able to bind to the phycobilisome and to induce photoprotective energy dissipation.¹⁷ In our previous study on OCP we reported on the excited state properties of the orange and red OCP at room temperature. In that study, however the samples were composed of mixtures of inactive and active OCP, which prevented us from extracting the detailed pathways of excited state decay. As a consequence we could not draw a clear conclusion on the effect of OCP photoactivation on the potential energy landscape of its S_1/ICT states. In this paper we report results from transient absorption spectroscopy at 77 K on OCP in its inactive and active forms. The aim of the experiments was to determine the spectroscopic properties of OCP in its orange (inactive) and red (active) forms on samples containing either only orange or only red OCP. By performing these experiments at low temperature (77 K) we obtained a fully converted sample where 100% of OCP is in the red form. At this temperature the effect of the excitation light on photoconversion is negligible, thus allowing us to measure on a sample of purely inactive OCP. By making use of global and target analysis techniques, we disentangled the excited state manifold of the active form. We also show that the kinetic model we applied for the 77 K data can successfully be applied to the RT data¹⁸ where the sample contains a mixture of orange and red OCP. Our results show that OCP photoactivation modifies the potential energy surfaces of the S₁/ICT states by altering their excited state energy landscape.

MATERIALS AND METHODS

Sample Preparation. Purification of OCP was performed as previously described¹⁶ form the overexpressing C-terminal His-tagged OCP Δ CrtR strain of *Synechocystis* PCC 6803.¹⁹ To convert OCP from the orange to the red form, OCP was illuminated for 15 min with white light of ~800 μ mol of photons m⁻² s⁻¹ at 5 °C. Then, the samples were mixed with glycerol [~50% vol/vol] and immediately placed in a cryostat at 77 K.

Time-Resolved Measurements. Low temperature (77 K) transient absorption spectroscopy²⁰ was performed on a Coherent MIRA seed and RegA amplifier system. The initial 800 nm (\sim 60 fs) pulse is split into two beams. One is used to pump an optical parametric amplifier to generate the pump beam at 550 nm with \sim 100 fs bandwidth. The pulse energy was ~24–25 nJ. The second beam is focused on a CaF₂ plate to generate a white light continuum, the probe beam. The repetition rate was set to 40 kHz. The polarization between the pump and the probe beam was set at magic angle (54.7°) . The detection system consists of a 75 diode array coupled to a shot to shot detector. This new detection system allows faster measuring times and very high S/N ratio. The room temperature measurements were carried out on a kHz system described in ref 20. The data were analyzed by making use of global and target analysis techniques.²¹

RESULTS

Figure 1 shows the steady state absorption spectrum of the orange and red OCP at 77 K as well as the spectrum obtained after the red form was allowed to reconvert back to the orange form at room temperature in the dark. The spectra corresponding to the orange and red after samples show peaks at 473 and 496 nm while the spectrum of the activated OCP displays a peak at 506 nm. These spectra are fully consistent with those previously published;^{16,18} in particular,



Figure 1. Steady state absorption spectra of the photoconverted OCP sample (red), of the same sample after reconversion to the inactive form at RT (black) and of the orange OCP at 77 K (orange). The orange spectrum has been normalized to the amplitude of the black spectrum at 498 nm.

the red spectrum shows that the OCP sample was fully (100%) converted into its red form. When the sample is brought back to RT it fully converts back to the orange form (cf. black and orange lines). Note that the orange spectrum was measured at 77 K while the black one was measured at RT.

In the following we will describe the results of transient absorption experiments on orange and red OCP at 77 K and the modeling of those results by global and target analysis. We also show results from target analysis of the RT data in.¹⁸

Inactive (Orange) OCP: Global Analysis. In order to avoid photoactivation of the orange OCP, the sample was prepared and frozen in the dark. Figure 2 shows the results of a global analysis of the 77 K time-resolved transient absorption data for the orange OCP. An overview of 112 data traces and their fits is shown in Figure S1. The decay associated difference spectra (DADS) are obtained using a parallel model, i.e., a model consisting of a number of compartments which decay independently and in parallel in a monoexponential fashion.²¹ In this way one can extract the number of components and lifetimes necessary for a good fit of the data. The DADS, however, do not necessarily correspond to spectra of pure molecular species.²¹ Three components are required for a good fit. The first spectrum (black dotted line) with a lifetime of \sim 0.34 ps corresponds to the fastest decaying component and shows decay of ground state bleach (GSB) and stimulated emission (SE) below ~585 nm as well as a broad decay of excited state absorption (ESA) from ~585 nm up to ~830 nm. This spectrum is rather noisy in particular in the ESA region due to cross-phase modulation and coherent artifacts occurring around time zero. The red DADS, with a lifetime of ~ 2 ps, shows negative signal below ~540 nm mainly due to a loss of GSB and positive signal above this wavelength corresponding to the decay of ESA. Note that its isosbestic point (zero crossing) is blue-shifted compared to the black DADS due to the presence of SE from the S₂ state in the latter. The blue spectrum has a lifetime of ~ 7.5 ps and displays a similar spectral shape compared to the red DADS up to 675 nm. Above this wavelength it shows smaller amplitude suggesting a less pronounced decay in this spectral region.

The upper panel of Figure 2 shows selected kinetic traces for the orange form of OCP. The top trace at 501 nm is taken as representative of the GSB region. The second trace at 538 nm shows the very fast decay of the S_2 state mainly due to SE. The



Figure 2. (top) Representative traces and fits (dashed) of the 77 K time-resolved transient absorption data for the orange form of OCP. The ordinate label indicates wavelength and the scale is in mOD. Note that the time axis is linear up to 3 ps, and logarithmic thereafter. (bottom) DADS (mOD) estimated from a global analysis.

635 and 748 nm traces correspond to different ESA regions; while the 635 nm trace reflects both S_1 and ICT ESA contribution, in the 748 nm trace the ICT ESA dominates.

Activated (Red) OCP: Global Analysis. The 77 K decay DADS for the activated (red) OCP are shown in the lower panel of Figure 3 while selected kinetic traces are displayed in the upper panel. An overview of 112 data traces and their fits is shown in Figure S2. The black dotted spectrum with a lifetime of ~0.26 ps features the decay of SE in the 530 to 630 nm region as well as decay of ESA above ~650 nm. Its spectrum, in particular in the red region above ~625 nm, is quite noisy and its spectral shape not fully reliable. The red DADS, with a lifetime of ~2.3 ps, shows decay of GSB and SE below 620 nm as well as ESA decay above 620 nm. Its spectral shape is very different compared to the conventional carotenoid S₁ spectrum.²² The blue DADS with a lifetime of \sim 7.6 ps shows negative signal in the ~470-580 nm region corresponding to decay of GSB as well as loss of ESA above ~590 nm. Its spectral shape is similar to that of a carotenoid S₁ state with a broad tail above 700 nm. The most interesting result from this analysis is in the spectral shape of the red and blue spectra. In fact, while the blue spectrum reflects an S₁ state with a partial ICT character, the red spectrum mainly shows ICT state like features.

The upper panel of Figure 3 shows selected kinetic traces. The first trace at 538 nm is taken as representative of the carotenoid GSB, while the 580 nm trace corresponds to the SE region where the fast decay component due to S_2 SE is clearly visible. The 654 and 748 nm traces are taken as representative of the S_1 and ICT ESA region. The 748 nm trace clearly shows faster decay components when compared to the 654 nm trace.



Figure 3. (top) Representative traces and fits (dashed) of the 77 K time-resolved transient absorption data for the red form of OCP. Note that the time axis is linear up to 3 ps, and logarithmic thereafter. (bottom) DADS (mOD) estimated from a global analysis.

Note that the 748 nm trace also contains decay from S_2 ESA. Overall, the traces show clear differences for the orange and red forms. It is interesting to note that, after the initial, fast decay corresponding to the S_2 state, the decay is more homogeneous for the orange form over the whole spectral range (*vide infra*). Besides showing a somewhat different time-evolution, the red form shows a much more pronounced ICT character; this can be seen by comparing the ratio of GSB and ESA signal for the 748 and 536 nm traces. Overall, note the higher S/N ratio obtained at 77 K on the 40 kHz system coupled to the multidiode shot to shot detection system when compared to the kHz RT data.¹⁸

Target Analysis. In order to better understand the excited state energy landscape of the orange and red forms, a target analysis was applied to the data. The kinetic models are shown in Figure 4(a) and (b) for the orange and red forms, respectively. The model consists of three compartments: the S₂ state, created upon excitation, decays in parallel to a state with predominantly S_1 properties denoted S_1/ICT (33% for OCP^o vs 43% for OCP^r) and to a state with predominantly ICT properties (ICT/S₁) (33% vs 35%). In addition, part of the S₂ state decays directly to the ground state (34% vs 22%). This channel is necessary to account for the loss of GSB at early times (~300 fs at 77 K). The loss of GSB together with the absence of a spectroscopic signature of photoproducts suggests that no intermediate states are involved in this decay. The lifetimes we obtained from the target analysis are identical to those from the global analysis. The relative fractions for the branching are estimated by comparing amplitudes in the GSB region. The estimated species associated difference spectra (SADS) are shown in Figure 4(c) and (d) for the orange and red forms, respectively. Note that in the kinetic models there is

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Figure 4. Kinetic model employed for the target analysis for the orange (A) and red (B) OCP, respectively. SADS estimated from the target analysis for the orange (C) and red (D) forms of OCP at 77 K. Lifetimes: orange OCP 0.34 ps (black dotted), 2.0 ps (red), and 7.5 ps (blue); red OCP 0.26 ps (black dotted), 2.3 ps (red), and 7.6 ps (blue). SADS estimated from the target analysis for the orange (E) and red (F) forms of OCP at RT. Lifetimes: orange OCP 0.06 ps (black dotted), 0.8 ps (red), and 4.3 ps (blue); red OCP 0.06 ps (black dotted), 0.9 ps (red), and 3.3 ps (blue). Note that the RT SADS are more uncertain around the 550 nm excitation wavelength.

no interconversion between the ICT/S₁ and S₁/ICT states, and consequently they decay independently. Figure 4(c) shows the SADS for the orange form. The black SADS corresponds to the time zero spectrum, populated upon excitation and features GSB and SE features below ~560 nm and a region of very broad ESA above this wavelength. The blue and red SADS are very similar and their main difference is above ~700 nm in the ICT ESA region where the red SADS shows larger amplitude most likely due to a stronger ICT character. Figure 4(d) displays the SADS corresponding to the red form of OCP. The black dotted SADS, which corresponds to the S₂ state shows GSB and SE below ~580 nm and a region of ESA above this wavelength. The blue SADS (S₁/ICT) displays negative signal below ~585 nm (GSB) and a broad region of ESA above this

wavelength. The positive signal shows a peak at ~655 nm and a broad tail. Thus this spectrum contains mainly S₁ character and some ICT character. The red SADS corresponding to the ICT/S₁ state again shows GSB and SE below 620 nm and a region of ESA between ~620 and ~830 nm. Unlike the blue SADS, the red SADS shows a maximum at ~750 nm and only a tail in the S₁→S_n ESA region. Thus the red spectrum displays mainly ICT character. The difference between the red and blue spectra is rather striking. In Figure 4(c) the spectral shape of the black dotted SADS (S₂) is very similar to that of the red SADS (ICT/S₁) suggesting that it may contain contributions from the ICT/S₁ and S₁/ICT states; it is possible that its actual lifetime is shorter than the one we estimate from the analysis (~0.34 ps). The same may be true for the red form where the black SADS

already contains some contribution from the red SADS (Figure 4(d)). If we compare the red SADS for the orange and red forms we see that the spectrum corresponding to the activated OCP shows a more pronounced ESA in the red region of the spectrum, above ~675 nm, an indication of an increased CT character of the associated state. The blue SADS also show some clear differences upon photoactivation; more specifically, unlike the blue SADS for the inactive OCP, the blue SADS for the photoactivated OCP shows a spectral shape strongly reminiscent of that of a pure carotenoid S₁ state. Thus photoactivation leads to a loss of ICT character for this state.

Target Analysis of the Room Temperature Data Using the 77 K Target Model. In ref 18, we demonstrated that at room temperature in the dark adapted state a small fraction of OCP is present in the red form, whereas in the light adapted state under our experimental conditions, approximately equal fractions of OCP are present in the orange and red forms. In Figure 4(c) and (d), we presented a target analysis at 77 K of samples were OCP is exclusively present in either the orange or the red form. With the kinetic model in Figure 4 at hand, we can now apply it to the room temperature data which consist of three measurements at 550 nm excitation, namely, dark, light, and dark after.¹⁸ In the analysis we assumed a loss channel of 30% for the S_2 state, and fixed the S_1/ICT and ICT/S_1 fractions at 35%. More specifically, since at room temperature both inactive and active OCP coexist in the sample mixture in both the dark and light measurements, the kinetic models in Figure 4(a) and (b) are applied simultaneously to the RT data. The estimated SADS and lifetimes with corresponding fractions are shown in Figure 4(e) and (f) for the orange and red OCP and Table 1, respectively. Qualitatively, the SADS show consistent

Table 1. Estimated Lifetimes and Fractions of the Red and Orange Forms of OCP at 77 K and RT^a

	red S ₂	$\operatorname{red}_{\operatorname{S_1}}^{\operatorname{ICT}/}$	red S ₁ /ICT	orange S ₂	orange ICT/S ₁	orange S ₁ /ICT
77 K	0.26 ps	2.3 ps (35%)	7.6 ps (43%)	0.34 ps	2.0 ps (33%)	7.5 ps (33%)
RT	0.06 ps	0.9 ps (35%)	3.3 ps (35%)	0.06 ps	0.8 ps (35%)	4.3 ps (35%)
^a Relative	e error on	the lifetim	nes is estim	nated to be	10%. exce	ept for the

Relative error on the inferimes is estimated to be 10%, except for the S_2 state where it is 30%.

differences between the red and orange forms at both temperatures. The S₁/ICT SADS of the orange form displays GSB below 550 nm and a relatively flat ESA up to ~700 nm. The S₁/ICT SADS of the red form shows GSB below 575 nm and ESA up to \sim 750 nm. The ICT/S₁ SADS of the orange form displays GSB below 550 nm (77 K) and 575 nm (RT) and a relatively flat ESA extending up to \sim 800 nm. The ICT/S₁ SADS of the red form shows GSB below 600 nm and a large (ESA that increases up to \sim 775 nm. Note that compared with the evolution associated difference spectra (EADS) in ref 18 the ICT/S_1 SADS displays less S_1 character, while the S_1/ICT SADS shows increased S1 character suggesting that the separation between the S₁/ICT and ICT/S₁ states is successful. Table 1 collates the 77 K and RT results, showing that the fractions are consistent and that the lifetimes show similar trends.

DISCUSSION

Carotenoid ICT states carry great biological significance. In the peridinin chlorophyll complex they are responsible for the very efficient light harvesting of peridinin which transfers energy to chlorophyll with ~63% efficiency via its S_1/ICT state.^{23–26} We have shown that the carotenoid ICT state can act as a mediator in the process of energy dissipation (fluorescence quenching) in a model system.²⁷ A similar mechanism may be responsible for nonphotochemical quenching in plants²⁸ and cyanobacteria.²⁹ In our previous study on OCP at room temperature we have shown that a very pronounced ICT state is created upon photoactivation.¹⁸ Even though we could separate the spectra of the inactive and active forms of OCP, we could not draw specific conclusions about the effect of photoactivation on the S₁ and ICT states' excited state energy landscape. The reason for that is that the samples we used contained a mixture of inactive and active OCP forms. Thus the many parameters and degrees of freedom in the system did not allow us to apply a specific, physical kinetic scheme to the data. In the present study performed at 77 K the amount of orange and red OCP in the corresponding samples is $\sim 100\%$ as shown in Figure 1. This greatly simplifies the analysis of the data and the interpretation of the kinetics.

The S₁ and ICT states have originally been proposed to be 2 distinct excited states.^{30,31} This hypothesis was supported by time dependent density functional theory calculations.³ Subsequent work has however challenged this interpretation. Several studies on different carotenoids and organisms have shown that the S1 and ICT states follow the same decay kinetics, suggesting that they belong to the same potential energy surface.^{10,33,34} Studies on peridinin, on the other hand, have shown that the S_1 and ICT states can be distinguished by multi pulse experiments.^{35,36} Recent one- and two-photon excitation studies have provided evidence that the S1 and ICT states behave as 2 separate excited states in fucoxanthin.^{37,38} If we look at the DADS in Figure 2 for the inactive orange OCP, we see that the red and blue spectra are rather similar in shape and display features of both S1 and ICT states. In Figure 3 on the other hand, the blue and red DADS are more reminiscent of those of pure S₁ and ICT states. The different kinetics in the S_1 and ICT spectral regions can be seen in Figure 3(upper panel) where the kinetic traces at 654 and 748 nm, taken as representative of the S₁ and ICT decay, show different time evolution. Thus upon photoactivation the ICT/S_1 state gains ICT character, while the S_1/ICT state loses ICT character and gains S_1 character, thereby resembling a pure S_1 state. These results show that the protein environment modulates the ICT and S1 properties of echinenone excited states upon photoactivation.

The biexponential excited state decay of OCP in both the inactive and active forms was suggested to originate from sample heterogeneity. More specifically the OCP sample may be intrinsically inhomogeneous, by containing at least two OCP subpopulations.³⁹ This conclusion was based on the fact that, at room temperature, different excitation wavelengths, namely, 495 and 530 nm, lead to different OCP excited state spectral shapes.³⁹ More recently it was suggested that the biexponentiality could be due to the coexistence of the inactive and active OCP in the sample; the faster decay was associated to the activated OCP while the slower decay to the inactive one.⁴⁰ If this is the case, excitation at different wavelength where different ratios of orange and red OCP are excited and photoactivation are expected to lead to the same excited states but with different relative amplitude. To the contrary, the spectra we obtained at 77 K are clearly different in shape in the inactive and active samples (Figure 4c,d) showing that

photoactivation leads to new excited states. Our RT results also argue against the above interpretation. We excited the darkadapted OCP sample at four different excitation wavelengths, 480, 495, 540, and 550 nm at RT. Figure S.3 of the Supporting Information shows the EADS we obtained upon 495 and 550 nm excitation. As one can readily see the spectra do not show the pronounced differences reported in ref 39 upon 495 and 530 nm excitation. The EADS in Figure S.3 do show some small differences and we have shown that such differences can be accounted for by taking into consideration a small excitation wavelength-dependent fraction of activated OCP present in the sample¹⁸ (this fraction is induced by the pump beam and the redder the excitation, the higher the relative fraction of activated OCP). If the red and blue EADS were associated with the inactive and active OCP, one would expect a large increase in amplitude for the red with respect to the blue spectrum upon going from 495 to 550 nm excitation (the relative increase in absorption for the active vs inactive OCP forms is \sim 2.5 times from 495 to 550 nm, cf. Figure 1). This is not the case and the ratio between the amplitudes is virtually identical for the two excitation wavelengths. Thus, our results argue against the interpretation that the origin of the biexponential decay is due to a mixture of orange and red OCP in the sample preparation. Our results also agree with those from low temperature Raman spectroscopy, a technique very sensitive to small changes in carotenoid conformation and configuration,¹⁶ and independently refined OCP structures obtained from X-ray data;⁴¹ neither technique showed any evidence of sample heterogeneity. We also note that our samples are obtained from the cyanobacterium Synechocystis PCC 6803 while those in refs 39,40 were obtained from another organism, namely, Arthrospira maxima, and the two OCPs, though similar, show some functional differences which may be related to different protein stability (Kirilovsky, unpublished results). We note that the isolation protocol is also different for the two OCPs.⁴¹ The discrepancy between our results and interpretation and those in refs 39,40 could also be due to different experimental conditions, such as different sample refreshment rate at RT.

It is interesting to note that, upon photoactivation, the extinction coefficient of both the S_1/ICT and ICT/S_1 state increase as they lose S₁ and ICT character, respectively, as one can see by looking at the ratio between ESA and GSB signals for the red and blue spectra in Figure 4(c,d). Thus both the $S_1/$ ICT and ICT/S₁ state gain dipole strength upon photoactivation. This suggests that the two states may be associated with the same Hamiltonian; the effect of photoactivation would be to stabilize the ICT character of the ICT/S_1 state and to decreased the ICT character (and increase the S1 character) of the S_1/ICT state. This would imply the splitting of the carotenoid S₁/ICT state, as found in solution,³⁹ into two ICT/ S_1 and S_1 /ICT states, when echinenone is bound to the protein. To this end it is worth noting that for fucoxanthin in solution two distinct S1 and ICT states have been proposed to be present in the carotenoid excited state manifold.^{37,38} On the other hand the steady state absorption spectrum of echinenone in hexane and methanol, a polar and nonpolar solvent, respectively, only shows small differences, more specifically a loss of vibrational structure and a small red shift in methanol vs hexane; yet, the ICT character of the S₁/ICT state shows clear spectroscopic changes and is much more pronounced in methanol.³⁹ In this respect it is possible that the ICT/S_1 and S₁/ICT states we detected in this study originate from two

OCP subpopulations with similar steady state absorption spectra.

Taken together our results suggest the following interpretation for the origin the ICT/S_1 and S_1/ICT states:

If the two states originate from two OCP subpopulations, the two subpopulations show similar (steady state) spectroscopic features.

The two states could originate from the same OCP population.

Further work on OCP mutants with altered carotenoid– protein interaction is necessary to better understand the origin of the two states.

If we compare the SADS we obtained for both the orange and red forms, with the EADS we obtained at RT,¹⁸ apart from the S₂ state whose spectral shape is sometimes notoriously difficult to accurately estimate, the SADS corresponding to the S_1/ICT and ICT/S_1 states show increased and decreased S_1 character, respectively. The fluctuation of hydrogen bond between the keto carbonyl group of echinenone and the protein at RT has been suggested to be a possible cause of sample heterogeneity and thus to be at the origin of the biexponential decay of the OCP excited states. Our low temperature results argue against this interpretation and against sample heterogeneity, since again, upon 550 nm excitation and with a purely orange sample at 77 K we obtained spectra very similar to those in the literature upon 480 nm¹⁶ and 495 nm³⁹ excitation wavelength. The lifetimes we obtained at 77 K are somewhat longer than those at room temperature;¹⁸ a similar trend has been reported for other carotenoids.²²

It is very likely that the spectroscopic changes of the S₁/ICT and ICT/S₁ states upon photoactivation are due to the opening of OCP,42 which leads to an increased exposition of the carotenoid to the solvent, and to the modulation of the hydrogen bond between the keto group and the protein. The carotenoid in the orange form of OCP is almost entirely buried in the protein and 4% only is solvent-exposed.⁴¹ Upon absorption of a photon, the carotenoid remains in the alltrans conformation, but its structure becomes more planar and as a consequence its effective conjugation length increases. This causes a conformational change in the protein leading to the formation of the red form of OCP.¹⁶ The red OCP has no longer a hydrogen bond between its N- and C-terminal domains⁴² and the cavity between the domains remains open potentially facilitating the access of solvent to the carotenoid. The pronounced separation of the ICT/S_1 state, which is sensitive to the polarity of the environment, could be mediated by the presence of an increased number of solvent molecules near the carotenoid and by the modulation of the hydrogen bond between the keto group of hydroxyechinenone and the protein.

Future work on OCP mutants with altered carotenoid– protein interaction will possibly allow us to better understand how the protein environment can tune the carotenoid excited state energy landscape.

CONCLUSIONS

In this study we have shown that OCP photoactivation modifies the carotenoid excited state potential energy landscape. When compared to the inactive form, the activated OCP shows a state ICT/S_1 with increased ICT character and a state S_1/ICT with increased S_1 character. The kinetic model we applied at 77 K and RT shows consistent spectra of both states.

A keto carotenoid ICT state has been shown to be responsible for energy dissipation in a biomimetic light-harvesting antenna where the carotenoid moiety accepts energy from a linked phthalocyanine (a chlorophyll mimic) in a process mediated by the carotenoid ICT state. We suggest that, upon OCP photoactivation, the echinenone ICT/S_1 state may be the key player in the quenching of phycobilisome fluorescence. The ICT/S_1 state can potentially act as an energy acceptor as we proposed in ref 18 and would effectively dissipate excited state energy since its lifetime is \sim 3 orders of magnitude shorter than that of the excited bilin. Alternatively the ICT/S_1 state may act as a mediator in a charge transfer quenching mechanism.^{43,44} The recent successful reconstitution of the OCP mediated nonphotochemical quenching in vitro¹⁷ has opened up the possibility to investigate the biophysical mechanism underlying the OCP mediated NPQ by making use of transient absorption techniques.

ASSOCIATED CONTENT

S Supporting Information

Kinetic traces at 77 K; global analysis of the RT data upon 495 and 550 nm excitation; estimated lifetimes from a RT measurement of the dark adapted OCP sample upon 495 and 550 nm excitation. 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.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Jos Thieme for excellent technical support. R.B. was supported by The Netherlands Organization for Scientific Research (NWO) through a Veni grant and by Laserlab Europe through a LCVU-1626 grant. R.v.G. acknowledges financial support from a TOP grant (700.58.305) from the Foundation of Chemical Sciences and from an Advanced Investigator Grant (267333, PHOTPROT) from the European Research Council. This work was partially supported by the European community via the HARVEST project. M.G. has a fellowship of the European project HARVEST.

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