Evaluating the Nature of So-Called S*-State Feature in Transient Absorption of Carotenoids in Light-Harvesting Complex 2 (LH2) from Purple Photosynthetic Bacteria

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§Supporting Information

ABSTRACT: Carotenoids are a class of natural pigments present in all phototrophic organisms, mainly in their light-harvesting proteins in which they play roles of accessory light absorbers and photoprotectors. Extensive time-resolved spectroscopic studies of these pigments have revealed unexpectedly complex photophysical properties, particularly for carotenoids in light-harvesting LH2 complexes from purple bacteria. An ambiguous, optically forbidden electronic excited state designated as S* has been postulated to be involved in carotenoid excitation relaxation and in an alternative carotenoid‐to‐bacteriochlorophyll energy transfer pathway, as well as being a precursor of the carotenoid triplet state. However, no definitive and satisfactory origin of the carotenoid S* state in these complexes has been established, despite a wide-ranging series of studies. Here, we resolve the ambiguous origin of the carotenoid S* state in LH2 complex from Rba. sphaeroides by showing that the S* feature can be seen as a combination of ground state absorption bleaching of the carotenoid pool converted to cations and the Stark spectrum of neighbor neutral carotenoids, induced by temporal electric field brought by the carotenoid cation–bacteriochlorophyll anion pair. These findings remove the need to assign an S* state, and thereby significantly simplify the photochemistry of carotenoids in these photosynthetic antenna complexes.

INTRODUCTION

Carotenoids are present in all phototrophic organisms and play important roles in various light-harvesting complexes as accessory light absorbers and photoprotectors of numerous types of (bacterio)chlorophylls.1–4 In light-harvesting (LH) complexes from purple bacteria, carotenoids play a structural role5 and serve as auxiliary light absorbers, harvesting light at wavelengths not covered by bacteriochlorophyll a (BChl a). As the lowest lying excited state S1 is optically forbidden,6 absorption of carotenoids, responsible for a bright yellow or orange coloration of the pigments, is associated with the strongly allowed S0 → S1 electronic transition. In the LH2 complex, this harvested energy is transferred to both the B800 and B850 spectral forms of BChls (Figure 1). Finally, carotenoids are also excellent photoprotectors, efficiently quenching BChl a triplet states, harmful reactive singlet oxygen,7,8 and in some cases also BChl a excited singlet state.9

The standard three-state S0→S1→S2 model satisfactorily accounts for the typical spectroscopic properties of carotenoids in solvents and in protein environments. However, experimental results from femtosecond time-resolved absorption spectroscopy, first applied to photophysical studies of carotenoids in the 1990s10,11 demonstrated that additional optically silent electronic states may lie in the vicinity of the S1 and S2 states.12–15 The most controversial state, S*, was first introduced to explain ambiguous spectral features in transient absorption (TA) spectra of the open-chain carotenoid spirilloxanthin,14 although similar spectral features had been observed previously for long-chain homologues of β-carotene.16 In the case of spirilloxanthin, the S* feature was observed in organic solvents as well as in the LH1 protein complex, and it was natural to use the same term in both cases.14 Two hypotheses were introduced to explain the nature S* state of carotenoids dissolved in solvents. One assumed that the S* is an electronic excited state, resembling the S1 state of a carotenoid with a twisted geometry,17,18 whereas the second argued that it is a highly vibrationally excited (“hot”) ground state, S0 populated via impulsive stimulated Raman scattering19 or intramolecular vibrational redistribution.20 Most recent investigations suggest that the S* feature can be associated with vibronic transition from either S1 or from vibrationally

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excited levels of $S_0$ state, depending on the carotenoid conjugation.21

With respect to protein-bound carotenoids, the $S^*$ feature TA band was initially observed in the LH1 complex from R. rubrum containing spirilloxanthin,14,22 a carotenoid with a long conjugated carbon–carbon double bond system ($N = 13$). Subsequently, TA signals were assigned as an ambiguous $S^*$ state in numerous LH2 antenna complexes with carotenoids such as neurosporene ($N = 9$), spheroidene ($N = 10$), and rhodopin glucoside ($N = 11$).22-24 Some studies on LH2 suggested that $S^*$, although very minor, is an energy donor to BChl $a$ being consistent with $S^*$ as an excited state.23,24 It was also argued that the “$S^*$ state” is a precursor in the ultrafast formation of a carotenoid triplet, further supporting the excited state hypothesis.14,22,25

However, the assumption that the $S^*$ feature in LH2 is associated with a distinct excited state of a carotenoid has a major drawback. Comparison of lifetimes of the $S^*$ feature, obtained for various carotenoids in several LH complexes, did not reveal the expected dependence on the carotenoid conjugation length, $N$, in apparent contradiction of the energy gap law.26 This problem could be disregarded for solvents in which the $S^*$-to-$S_0$ internal conversion depends on the rate of preceding conformational change of molecule; however, this argument does not hold within a protein environment in which the carotenoid geometry is much more rigid due to pigment–protein interactions and steric considerations.

Interestingly, explanations of the origin of the carotenoid $S^*$ feature in LH2 complexes have not taken into account the possibility that it may be somehow associated with the short-lived carotenoid radical cation–B800 anion pair ($Car^*B800^-$) that is formed after direct excitation of a carotenoid, during excitation relaxation from its $S_0$ state. Ultrafast formation of the $Car^*B800^-$ pair in LH2 complexes was not known at the time when $S^*$ was introduced,27 and the potential relation between the $Car^*B800^-$ pair and the $S^*$ feature in LH2 complexes has not been further evaluated.

As formation of a $Car^*B800^-$ pair reduces the initial pool of carotenoids in the excited state, spectral features of $Car^*$, visible as transient absorption band in the NIR spectral range, should also be accompanied by bleaching of a ground state absorption of a carotenoid’s neutral form in the vis range. This fact has not been taken under consideration in fitting models of transient absorption data of carotenoids in LH2 complexes. The TA data show that an $S^*$ feature always comprises negative bands that more or less correspond to carotenoid ground state bleaching. In addition, an $S^*$ feature also consists of an adjacent positive band that has been interpreted as a transient absorption band of the ambiguous $S^*$ state.

However, the $S^*$ feature in the LH2 complexes may have an alternative origin. It seems apparent that the short-lived $Car^*B800^-$ pair will temporarily form quite a strong electric dipole that will provide additional polarization of the nearest environment. It is not difficult to imagine that this temporal polarization brought by the local electrostatic field of a $Car^*B800^-$ pair will lead to an electrochromic response of neighboring pigments (like carotenoid). In such a scenario, TA spectra of the LH2 complex (upon carotenoid excitation) recorded in the range of the carotenoid band should also consist of a spectral feature that will be a mixture of two elements: ground state bleaching (associated with portion of carotenoids converted to $Car^*$) and electrochromic spectrum of a neutral carotenoid nearby to a $Car^*B800^-$ pair. The temporal characteristics of this spectral feature should be completely independent from the conjugation of an involved carotenoid; its decay will be coupled to recombination of $Car^*$ back to a carotenoid neutral form. As shown below, this hypothetical temporal spectral feature fits quite well to the spectral and temporal properties of the $S^*$ feature in LH2 complexes.

To demonstrate that the $S^*$ feature is indeed associated with the $Car^*B800^-$ pair, as suggested above, we have studied two neurosporene-containing LH2 complexes from a Rba. sphaeroides mutant strain,28 either with a native or a severely attenuated B800 BChl $a$ absorption band. These preparations will be referred to as LDAO-Tris-LH2 and DDM-HEP-LH2 where LDAO and DDM are abbreviations for the detergents used during purification processes: N,N-dimethyldodecylamine N-oxide and $\beta$-dodecyl maltoside, respectively.

Time-resolved absorption spectroscopy studies combined with simple modeling of changes in a local electric field brought by the $Car^*B800^-$ pair show that the transient spectral feature previously assigned to an $S^*$ state can be very adequately modeled as combination of a bleaching of ground state absorption of the fraction of the excited carotenoids that had been converted to radical cations and electrochromic response of the neighbor neutral carotenoid. In order to experimentally test this hypothesis, we have also studied LH2 with a depleted B800 BChl $a$ band. It is known that in this type of LH2 a $Car^*B800^-$ pair cannot be formed;29 consequently, an $S^*$ feature should also be absent in TA spectra.

**MATERIALS AND METHODS**

**Bacterial Growth, Isolation, and Purification of LH2 Complexes.** Cells of a Rba. sphaeroides $\Delta rctC$, neurosporene-containing mutant were grown according to the protocol described previously.28 The LH2 complexes were prepared using two different protocols, yielding preparations with a well-developed or severely depleted B800 BChl $a$ absorption band. The LH2 complexes with an intense B800 absorption band were prepared using a protocol published previously.30 The LH2 preparation with a reduced B800 band was obtained according to the protocol published in ref 28. The final sample buffer (pH = 7.5) consisted of 20 mM HEPES and 0.03% $\beta$-dodecyl maltoside ($\beta$-DDM). As shown in ref 30, this protocol
and buffer are perfect for simultaneous preparation of LH2 and LH1 complexes, although prolonged storage of LH2 complex leads to a slow oxidation of B800 BChl a to 3-acetyl-Chl a, probably due to the oxidative properties of HEPES.

Steady-State, Femtosecond, and Nanosecond Time-Resolved Absorption Spectroscopy. Transient absorption experiments were carried out using a Helios femtosecond time-resolved and EOS, nanosecond time-resolved pump–probe absorption spectrometers (Ultrafast Systems LCC, Sarasota, FL) coupled to a Spectra-Physics femtosecond laser system described previously (Spectra-Physics, Santa Clara, CA). The excitation wavelength was preferentially set to excite the B850 (~850 nm) associated with monomeric and excitonically coupled forms of BChl a. The energy of the excitation beam was kept between 200 and 400 nJ, corresponding to an intensity of ~0.5–1 × 10^14 photons/cm^2 per pulse, low enough to prevent singlet–singlet annihilation within the B850 BChl a array. Steady-state absorption measurements were performed using a Shimadzu UV-1800 spectrophotometer.

Data Processing and Fitting. Group velocity dispersion in TA data sets was corrected using Surface Explorer software provided by Ultrafast Systems by building a dispersion correction curve from a set of initial times of transient signals obtained from single wavelength fits of representative kinetics. Target analysis, a directed kinetic modeling of TA results, was performed using CarpetView, data viewing and analysis software for ultrafast spectroscopy measurements (Light Conversion Ltd., Vilnius, Lithuania). The fitting employed the kinetic models with anticipated realistic branches mimicking the true decay pathways following excitation of the carotenoid. If the underlying assumptions are correct, targeted kinetic analysis separates spectral components such as excited state absorption (ESA) of the specific excited state of molecule, etc. The results are commonly abbreviated as SADS or SAS, species associated (decay) spectra. The instrument temporal response function was assumed to have a Gaussian-like shape with the full width at half-maximum (fwhm) of ~200 fs and was used as a fixed parameter in the fitting procedure.

RESULTS

Steady-State Absorption. Steady-state absorption spectra of the LDAO-Tris-LH2 and DDM-HEP-LH2 complexes containing neurosporene are given in Figure 1.

The spectra consist of features associated with electronic transitions of BChl a: Soret bands with maxima at ~370 nm, Qy bands at 590 nm and two Qx bands, B800 (~800 nm) and B850 (~850 nm) associated with monomeric and excitonically coupled forms of BChl a, respectively. The vibronic peaks of the neurosporene S0 → S1 band appear between 400 and 500 nm. The absorption spectrum of the DDM-HEP-LH2 shows a substantial reduction in intensity of the B800 peak and the presence of a new weak band at ~690 nm, indicating that the B800 BChls a are not released from the protein but were oxidized to 3-acetyl-chlorophyll a (ac-Chl a). This structural modification of the pigments in the B800 ring only marginally affects the spectroscopic properties of the carotenoid, which are manifested as a small, ~2 nm, hypochromatic shift of the absorption band. A similar but bathochromic shift of the B850 band in respect to their counterpart from the LDAO-Tris-LH2 is also observed. The overall integrity of the DDM-HEP-LH2 complex is preserved, including carotenoid-B850 BChl a pigment interactions, as shown in previous studies. Because there is a mismatch in the excited state energies between neurosporene and ac-Chl a, the energetic coupling between them will be substantially weakened.

Excited-State Dynamics of LH2-Bound Neurosporene. Transient absorption results taken in the vis and NIR spectral ranges of the LDAO-Tris-LH2 excited at the neurosporene absorption band are given in Figure 2. Figure 2A,B shows 3D-pseudocolor contour maps of TA within 8 ps after excitation in vis and NIR spectral ranges, respectively. Figure 2C,D highlights exemplary TA spectra extracted at various time delays.

The main ESA bands associated with the carotenoid neurosporene are indicated along with spectral features corresponding to bleaching of ground state absorption (GBS) of either carotenoid (Car) or BChls present within 8 ps after excitation. The states from which the transition occurs are indicated. (C, D) Exemplary transient absorption spectra taken at various delay times after excitation. The main transient ESA bands are indicated.

Figure 2. Transient absorption data of the LDAO-Tris-LH2 complex recorded after excitation into the (0–0) vibronic band of carotenoid neurosporene; (A, B) TA contour maps highlighting ESA bands and bleaching of ground state absorption (GBS) of either carotenoid (Car) or BChls present within 8 ps after excitation. The states from which the transition occurs are indicated. (C, D) Exemplary transient absorption spectra taken at various delay times after excitation. The main transient ESA bands are indicated.

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S^+\) suggests that those may have common temporal characteristics.

Transient absorption results taken in vis and NIR spectral ranges of the DDM-HEP-LH2 excited in the neurosporene absorption band are given in Figure 3.

Figure 3. Transient absorption data of the DDM-HEP-LH2 complex recorded after excitation into the \((0-0)\) vibronic band of carotenoid neurosporene. \((A, B)\) TA contour maps highlighting ESA bands and bleaching of ground state absorption (GSB) of either the carotenoid (Car) or BCHls present within 10 ps after excitation. The states from which the transition occurs are indicated. \((C, D)\) Exemplary transient absorption spectra taken at various delay times after excitation. Note that the D0 → D1 absorption band of Neu^+ has disappeared and the S^+ spectral feature is replaced by a noticeably sharper and blue-shifted transient absorption band.

Contour maps of TA shown within the first 10 ps after excitation (Figure 3A,B) demonstrate few significant changes in respect to LH2 with a fully developed B800 absorption band. The neurosporene S1 → S0 ESA band appears at 535 nm and persists for a much longer time indicating weakened energetic coupling with (B)Chls. At the very early delay times, the S1 → S0 ESA band is also red-shifted, representing vibrational equilibration of the S1 state. Most importantly, the broad spectral feature characteristic of “S^+” has substantially changed. It has been replaced by a sharp and slightly blue-shifted positive transient band with a maximum at 507 nm. In the NIR spectral range the band associated with absorption of Neu^+ completely disappeared, demonstrating that neurosporene is not able to form the cation–anion pair with ac-Chl a “B800”.

Further spectroscopic evaluation by measurements in the submicro-second time regime demonstrated that this transient band is identical to triplet–triplet (T1 → Tn) ESA of LH2-bound neurosporene, as demonstrated in Figure 4. It shows the TA spectrum of the LDAO-Tris-LH2 taken at 7 ps, corresponding to so-called S^+ spectral feature overlaid with equivalent TA spectrum of the DDM-HEP-LH2 (because neurosporene decays longer in this LH2 batch it was taken at 30 ps).

The fourth spectrum corresponds to TA of the DDM-HEP-LH2 taken at 50 ns, and due to the long time delay, it can be only associated with the so-called triplet-minus-singlet (T–S) spectrum of neurosporene. It is apparent that both 30 ps and 50 ns TA spectra are essentially identical but are very different from the spectral envelope of the S^+ feature. It is worth noting that, thus, the triplet T1 → Tn band is already clearly resolved in the TA spectrum at 1 ps (Figure 3C).

**DISCUSSION**

Electric Field Established by Neu^+B800^− Pair.

Experimental results shown above clearly suggest a connection between the Neu^+B800^− pair and the S^+ transient spectral feature. As we have hypothesized that the S^+ band can be associated with an electrochromic response of neutral carotenoid to the electric field temporarily induced by the Car^−B800^+ pair, it would be particularly interesting to know what level of electric field the neighboring neutral carotenoid may sense from the cation–anion pair. In order to calculate this, pigment coordinates from the *Rps. acidophila* LH2 crystal structure have been used. As crystal structure of *Rba. sphaeroides* LH2 is not known, the results should be interpreted rather as “qualitative” evidence used to support the concept. Because the pigments are not infinitesimally small, in order to make this simple modeling feasible it was necessary to approximate them by points that lie on the surface that is designated by the plane of the B800 macrocycle. With these assumptions, the arrangement of the pigments of interest can be represented as in Figure 5. The point symbolizing B800 BCHl a has been placed in the center of the pigment's macrocycle. To simplify calculations, the frame of reference was placed in the midpoint between B800^− and Car^+.

The electric field that the nearest neutral carotenoid senses from the electric dipole formed by the Car^−B800^+ pair can be described by following equation:

\[
E_T = \frac{1}{4\pi\varepsilon_0\varepsilon_p} \frac{qd}{R^3} \sqrt{1 + 3 \cos^2 \alpha}
\]

The parameters \(d\), \(R\), and \(\alpha\) are provided in Figure 5; \(q\) is the electron charge, and \(\varepsilon_p\) is the relative dielectric constant of the surrounding protein medium. Its value has been estimated for
Figure 5. Simplified arrangement of the Car*B800$^-$ pair and the nearest neutral carotenoid (Car$_{nn}$) based on pigment coordinates from Rps. acidophila LH2. The axis surface represents the B800 BChl a macrocycle plane. Point symbolizing B800 BChl a is placed in the center of the pigment’s macrocycle.

Various LH2 complexes in the 1.2–1.3 range, and a value of 1.2 has been used in this model. Calculations show that the nearest neutral carotenoid (Car$_{nn}$) senses an electric field of 4.4 MV/cm.

Previous studies of electrochromic responses of carotenoids bound to LH2 suggested that the strong bathochromic shift of the local electric field of 4 MV/cm that is most likely brought by protein residues like Car$_r$ of the carotenoid absorption spectrum (orthogonal to each other) of the B800 BChl a complex of LH2,35 The axis surface represents the B800 BChl a macrocycle plane. Point symbolizing B800 BChl a is placed in the center of the pigment’s macrocycle.

Our model of pigment interactions assumed that a single carotenoid conjugation that crosses the graph surface while bound to LH2 suggested that the strong bathochromic shift of the local electric field of 4 MV/cm that is most likely brought by protein residues like Car$_r$ of the carotenoid absorption spectrum (orthogonal to each other) of the B800 BChl a complex of LH2,35 The axis surface represents the B800 BChl a macrocycle plane. Point symbolizing B800 BChl a is placed in the center of the pigment’s macrocycle.

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Various LH2 complexes in the 1.2–1.3 range, and a value of 1.2 has been used in this model. Calculations show that the nearest neutral carotenoid (Car$_{nn}$) senses an electric field of 4.4 MV/cm.
the protein environment. The TA data from Figure 3 show that $T_1 \to T_n$ and $S_1 \to S_n$ ESA bands appear simultaneously, suggesting that the $S_1$ state cannot be a state in which singlet fission takes place. The second candidate, an optically forbidden $1B_u$ state, has been proposed to lie just below the $S_2$ state for carotenoids with $N \geq 946$ (and that includes neurosporene), and its lifetime could be too short to be temporally resolved from the initially excited $S_2$ state. Therefore, the detailed mechanism of carotenoid singlet fission was not further investigated in this study.

**Simplified Carotenoid Excitation Decay Pathway in LH2.** Target model analysis (Figure 7) can be used to review the study presented here and propose a new, simplified $S^*$-free carotenoid excitation decay pathway in the LH2 complex from purple bacteria.

The model should be seen as data simulation that incorporates numerous known parameters adapted from previous studies of neurosporene in solvents and in LH2. The so-called “$S^*$ state” has been replaced by the Neu"$*"B800"$ pair, evolving from the carotenoid $S_2$ state during its relaxation. It was assumed that the population of BChl $a$ triplets is entirely

![Figure 6](image1)

**Figure 6.** Reconstruction of the $S^*$ transient spectral feature. (A) Spectral components involved in reconstructing the $S^*$ transient feature: cyan line, ground-state absorption spectrum of neurosporene in the LH2; dashed black line, ground-state absorption spectrum of neurosporene affected by the electric field induced by Neu"$*"B800"$ pair; red line, difference spectrum (Stark spectrum) of the previous two spectra; green line, ground-state absorption bleaching of neurosporene involved in formation of Neu"$*"B800"$ pair. (B) The $S^*$ spectral feature cleaned from contribution of transient absorption of BChls by subtracting complementary spectrum of carotenoidless LH2. (C) Pure $S^*$ feature overlaid with its anticipated spectrum (for details refer to the main text): TA, transient absorption, GSB, ground-state bleaching.

![Figure 7](image2)

**Figure 7.** Target analysis of the vis–NIR combined TA data set of the LDAO-Tris-LH2 complex. (A) Kinetic model employed to simulate the TA data set. (B) SADS amplitudes resulting from the kinetic analysis. The yellow compartments in the kinetic models symbolize carotenoid neurosporene (with exception of the Neu"$*"B800"$ pair) while green compartments correspond to BChl $a$. Note that the SADS amplitudes do not correspond to actual contribution of the component in the raw TA data and should be weighted by their time-dependent concentrations; Exc = excitation.
quenched by neurosporene molecules with time constant of ~20 ns, a value obtained from fitting the recovery of BChl a ground state absorption bleaching (Figure S1). This value is similar to triplet lifetime of BChl a in other LH2 complexes.49 Neurosporene triplet lifetime was obtained from fitting of the T1 → Tn band decay (Figure S2). The effective lifetime of the B850 BChl a of ~1.2 ns was obtained from fitting of the kinetic trace recorded at 850 nm (Figure S3) and was decomposed to microscopic decays of 1.4 ns (S1 → S0, radiative and nonradiative) and 7 ns (intersystem crossing, ISC, S1 → Tn) to be in agreement with triplet yield. Due to the temporal resolution limitation of the spectrometer, the effective lifetime of the carotenoid S2 state in LH2 complexes could not be measured and instead was set to ~50 fs as demonstrated in previous studies.24,25 Microscopic parameters of neurosporene S2 state depopulation are described with the following details: the S2 → S1 decay was assumed to have the same characteristics as in solvent; the S1 → BChls decay was set in order to give an overall carotenoid to BChl a energy transfer efficiency of 87% that is known from previous studies of this complex. For simplicity both spectral forms of BChl a, B800 and B850, were combined as identity. It was assumed, in agreement with experimental data, that Neu−* is populated with ~13% quantum efficiency from the S0 state of the neutral form. The SADS (species associated decay spectra) are given in Figure 7B. It should be clarified that the contribution of the SADS component in the raw TA spectra should not be judged on the basis of its relative amplitude but on the basis of the product of SADS amplitude and its concentration at a specific delay time.

## CONCLUSIONS

This study clarifies the origin of the S* feature in LH2 complexes from purple bacterium *Rba. sphaeroides.* We propose that the S* transient spectral feature corresponds to a combination of bleaching of the ground-state absorption of carotenoid involved in the Car−*B800*− pair and the Stark spectrum of the nearest neutral carotenoid. The target analysis model of the TA data set also supports this view. This eliminates the so-called “S* state” from existing kinetic schemes of the carotenoid excitation decay pathway in the LH2 complex, and thereby provides a new framework for investigating the excited states of carotenoids in these photosynthetic antenna complexes.

## ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscb.6b08639.

Recovery of bleaching of the B850 band in the LDAO-Tris-LH2 sample recorded in 7 and 150 ns time windows and decay of neurosporene triplet state (PDF)

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### Notes

The authors declare no competing financial interest.

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