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

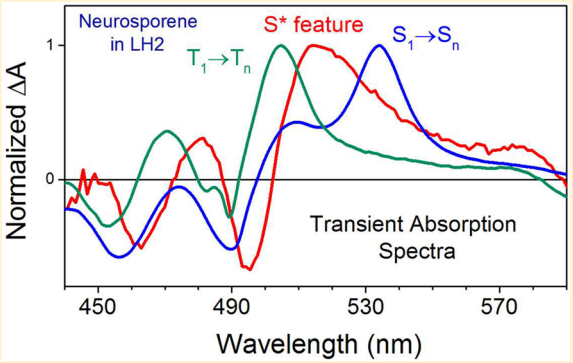
Dariusz M. Niedzwiedzki,*[,†] C. Neil Hunter,[‡] and Robert E. Blankenship[†,§]

[†]Photosynthetic Antenna Research Center and [§]Departments of Biology and Chemistry, Washington University, St. Louis, Missouri 63130, United States

[‡]Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, United Kingdom

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 role[5] and serve as auxiliary light absorbers, harvesting light at wavelengths not covered by bacteriochlorophyll *a* (BChl *a*). As the lowest lying excited state $S_1$ is optically forbidden,[6] absorption of carotenoids, responsible for a bright yellow or orange coloration of the pigments, is associated with the strongly allowed $S_0 \rightarrow S_2$ 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 $S_0-S_1-S_2$ 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 1990s,[10,11] demonstrated that additional optically silent electronic states may lie in the vicinity of the $S_1$ and $S_2$ 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 $S_1$ state of a carotenoid with a twisted geometry,[17,18] whereas the second argued that it is a highly vibrationally excited ("hot") ground state, $S_0$, populated via impulsive stimulated Raman scattering[19] or intramolecular vibrational redistribution.[20] Most recent investigations suggest that the S* feature can be associated with vibronic transition from either $S_1$ or from vibrationally

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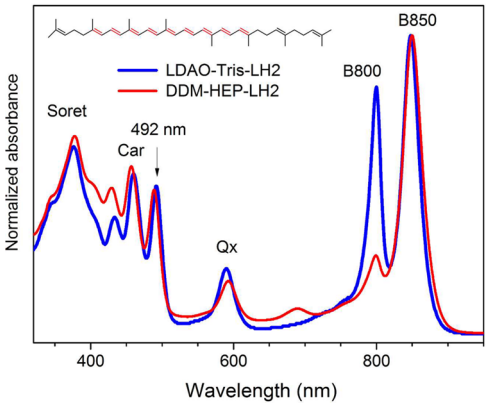


**Figure 1.** Steady-state absorption spectra of two batches of LH2 complex from the Δ*crtC* mutant of *Rba. sphaeroides* along with the chemical structure of carotenoid neurosporene. The major absorption bands are marked. The excitation wavelength used for time-resolved studies is indicated by an arrow.

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, $N$,[14,22−24] 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 ($Car^{+\bullet}B800^{-}$) that is formed after direct excitation of a carotenoid, during excitation relaxation from its $S_2$ state. Ultrafast formation of the $Car^{+\bullet}B800^{-}$ pair in LH2 complexes was not known at the time when S* was introduced,[27] and the potential relation between the $Car^{+\bullet}B800^{-}$ pair and the S* feature in LH2 complexes has not been further evaluated.

As formation of a $Car^{+\bullet}B800^{-}$ pair reduces the initial pool of carotenoids in the excited state, spectral features of $Car^{+\bullet}$, 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 $Car^{+\bullet}B800^{-}$ 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 $Car^{+\bullet}B800^{-}$ 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 $Car^{+\bullet}$) and electrochromic spectrum of a neutral carotenoid nearby to a $Car^{+\bullet}B800^{-}$ 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 $Car^{+\bullet}$ 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 $Car^{+\bullet}B800^{-}$ 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 *β*-dodecyl maltoside, respectively.

Time-resolved absorption spectroscopy studies combined with simple modeling of changes in a local electric field brought by the $Car^{+\bullet}B800^{-}$ 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 $Car^{+\bullet}B800^{-}$ 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* Δ*crtC*, 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% *β*-dodecyl maltoside (*β*-DDM). As shown in ref 30, this protocol


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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).[31] The excitation wavelength was preferentially set to excite the first vibronic band of the $S_0 \rightarrow S_2$ carotenoid absorption at 490 nm. 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 Xplorer 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.[32] 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, $Q_x$ bands at 590 nm and two $Q_y$ 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 $S_0 \rightarrow S_2$ 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, hypsochromic 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.[28] 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-

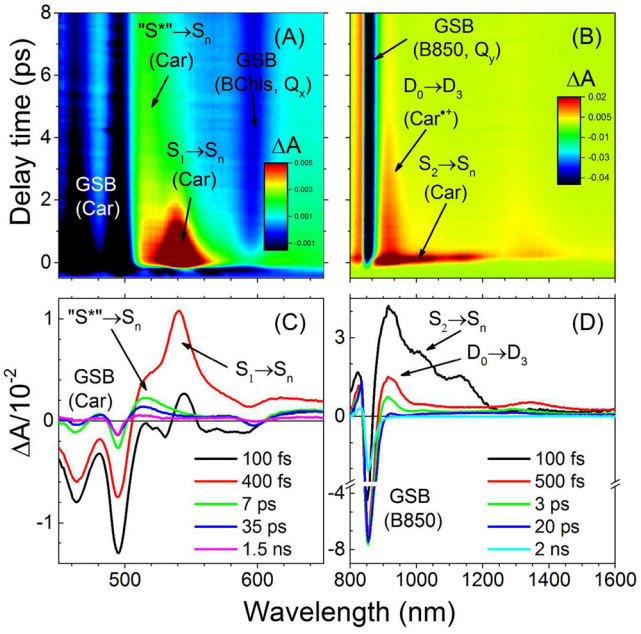


**Figure 2.** Transient absorption data of the LDAO-Tris-LH2 complex recorded after excitation into the (0−0) vibronic band of carotenoid neurosporene; (A, B) TA contour maps highlighting ESA bands and bleaching of ground state absorption (GSB) of either carotenoid (Car) or BChls present within 8 ps 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.

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 (GSB) of either carotenoid or BChls. In the LDAO-Tris-LH2 sample, the $S_1 \rightarrow S_n$ ESA band with maximum at 540 nm is instantaneously populated and then fades away within 3 ps. The remaining spectrally broad signal with a maximum at ~520 nm represents the ESA associated with an ambiguous "S* state". Another noticeable band present in the NIR range appearing only within a few hundred femtoseconds after excitation (Figure 2B, deep-red color; Figure 2D, black line) is associated with the short-lived neurosporene $S_2$ state. Previous experimental and theoretical evaluations of that band demonstrated that it is associated with the $S_2 \rightarrow S_n$ transition.[33] Simultaneously with the S* feature, another spectral band is clearly present in the NIR range between 920 and 1000 nm. This band is associated with $D_0 \rightarrow D_3$ absorption of carotenoid radical cation[23,27,29,34] that is formed shortly after excitation via interaction with B800 BChl *a* that leads to ultrafast formation of the temporarily existing $Neu^{\bullet+}B800^{-}$ pair. Simultaneous appearance and decay of both transient bands ($D_0 \rightarrow D_3$ and


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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.

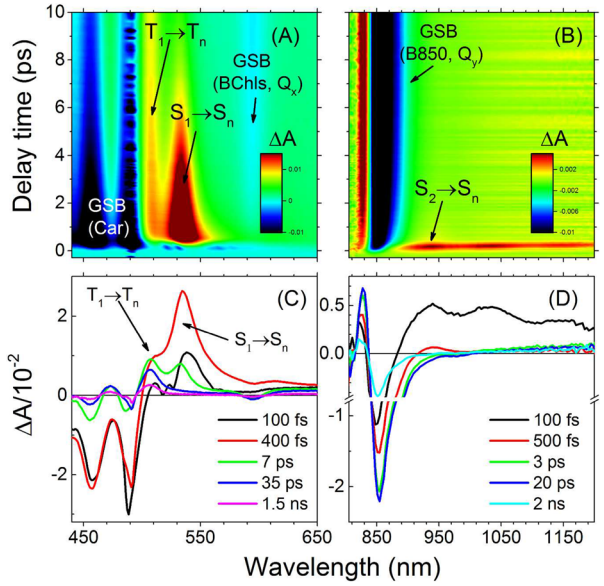


**Figure 3.** Transient absorption data of the DDM-HEP-LH2 complex recorded after excitation into the (0−0) vibronic band of carotenoid neurosporene. (A, B) TA contour maps highlighting ESA bands and bleaching of ground state absorption (GSB) of either the carotenoid (Car) or BChls present within 10 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 $D_0 \rightarrow D_3$ absorption band of $Neu^{\bullet+}$ 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 $S_1 \rightarrow S_n$ 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 $S_1 \rightarrow S_n$ ESA band is also red-shifted, representing vibrational equilibration of the $S_1$ 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 $Neu^{\bullet+}$ completely disappeared, demonstrating that neurosporene is not able to form the cation−anion pair with ac-Chl *a* "B800".[34] Further spectroscopic evaluation by measurements in the submicrosecond time regime demonstrated that this transient band is identical to triplet−triplet ($T_1 \rightarrow T_n$) 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 $T_1 \rightarrow T_n$ band is already clearly resolved in the TA spectrum at 1 ps (Figure 3C).

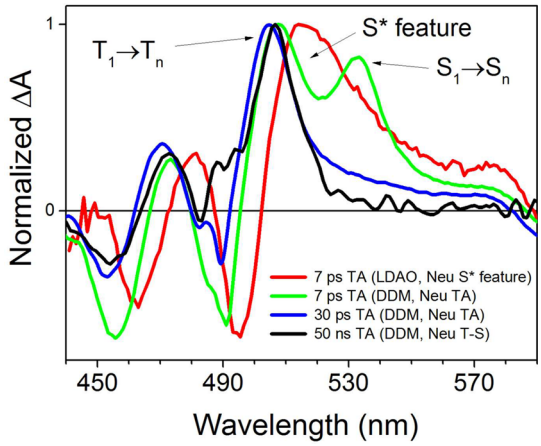


**Figure 4.** Comparison of transient absorption spectra taken for both LH2 batches (LDAO and DDM preps) at time delays at which the main $S_1 \rightarrow S_n$ ESA of neurosporene decayed. For the LDAO-Tris-LH2, the TA spectrum taken at 7 ps corresponds to S* spectral feature. For comparison, also TA spectrum of the DDM-HEP-LH2 at the same delay time is shown. The 30 ps spectrum (free of $S_1 \rightarrow S_n$ ESA band) is identical to the triplet-minus-singlet (T − S) spectrum of neurosporene taken at 50 ns after excitation. All spectra were normalized to unity at their maxima.

## DISCUSSION

**Electric Field Established by $Neu^{\bullet+}B800^-$ Pair.** Experimental results shown above clearly suggest a connection between the $Neu^{\bullet+}B800^-$ 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 $Car^{\bullet+}B800^-$ 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.[35] 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 $Car^{\bullet+}B800^-$ pair can be described by following equation:

$$E_T = \frac{1}{4\pi\varepsilon_0\varepsilon_p}\frac{qd}{R^3}\sqrt{1 + 3\cos^2\alpha} \quad (1)$$

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


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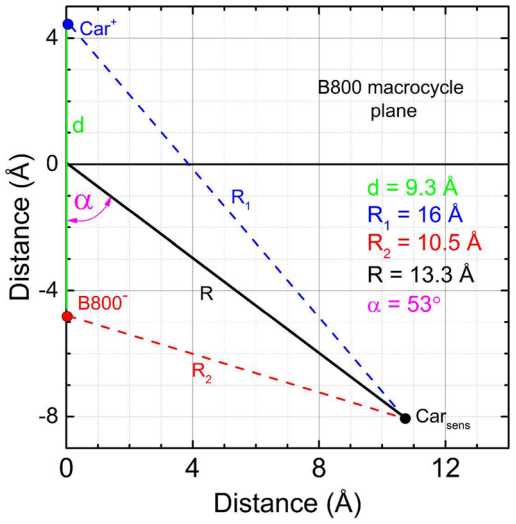


**Figure 5.** Simplified arrangement of the $Car^{\bullet+}B800^{-}$ pair and the nearest neutral carotenoid ($Car_{sens}$) based on pigment coordinates from *Rps. acidophila* LH2.[35] The axis surface represents the B800 BChl *a* macrocycle plane. Point symbolizing B800 BChl *a* is placed in the center of the pigment's macrocycle.

various LH2 complexes in the 1.2−1.3 range,[36] and a value of 1.2 has been used in this model. Calculations show that the nearest neutral carotenoid ($Car_{sens}$) 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 carotenoid absorption observed in the LH2 protein, with respect to low polarizable solvents like *n*-hexane, is caused by a local electric field of 4 MV/cm that is most likely brought by protein residues like *β*Arg-10.[37,38] If those two above-mentioned factors, a static local electric field from protein residue ($E_{loc}$) and a temporal electric field brought by $Car^{\bullet+}B800^{-}$ pair ($E_T$), will simultaneously perturb the carotenoid $S_2$ state, the overall temporal electrochromic shift of the carotenoid absorption spectrum ($\Delta\tilde{\nu}$, in wavenumbers) can be calculated on the basis of the following equation for a Stark shift:[39]

$$\Delta\tilde{\nu} = -\frac{1}{hc}\left(\Delta\mu\mathbf{E}_T + \Delta\alpha\mathbf{E}_{loc}\mathbf{E}_T + \frac{1}{2}\Delta\alpha\mathbf{E}_T^{\,2}\right) \quad (2)$$

Here, $\mathbf{E}_{loc}$ and $\mathbf{E}_T$ are electric field vectors; $\boldsymbol{\Delta\mu}$ and $\Delta\alpha$ are changes in the vector of dipole moment and polarizability tensor, respectively, of the carotenoid between its $S_0$ and $S_2$ states. For simplicity, $\Delta\alpha$ is assumed to be a scalar with value of $\Delta\alpha$ = 1350 Å$^3$ that was previously calculated for structurally similar open-chain carotenoid lycopene[40] (1 Å$^3$ = 1.113 × 10$^{-40}$ C m$^2$ V$^{-1}$). The values of electric vectors are $E_{loc}$ = 4 MV/cm,[38] $E_T$ = 4.4 MV/cm, and $1/hc$ = 0.5 × 10$^{25}$ C J$^{-1}$ m$^{-1}$. It was also assumed that the first term in eq 2 is negligible. According to the simplistic model in Figure 5, $\mathbf{E}_T$ and $\boldsymbol{\Delta\mu}$ vectors would be orthogonal to each other ($\boldsymbol{\Delta\mu}$ will be essentially in line with carotenoid conjugation that crosses the graph surface while $\mathbf{E}_T$ will lie on the graph surface). Then, if $\mathbf{E}_T$ and $\mathbf{E}_{loc}$ are mutually oriented such that the dot product is maximized and positive (similar orientation, parallel), the second term will give an electrochromic shift of 1330 cm$^{-1}$ while the third one will be 730 cm$^{-1}$ and an overall red-shift of 2060 cm$^{-1}$ in respect to absorption in a low polarizable medium like *n*-hexane. As in *n*-hexane, the (0−0) vibronic band of neurosporene appears at 467 nm (21 410 cm$^{-1}$),[41] so that the shifted spectrum should appear at 19 350 cm$^{-1}$, which corresponds to 517 nm.

**Reconstructing the S* Transient Spectral Feature.** We have shown that this carotenoid may temporarily experience additional a 26 nm red-shift (~1000 cm$^{-1}$) of its steady-state absorption in LH2 (normally the (0−0) band is at 491 nm). As demonstrated in Figure 6, this ultrafast Stark shift is strong enough to very adequately reconstruct the S* transient spectral feature. Figure 6A shows the expected transient Stark spectrum of the neurosporene, induced by the electric field of the $Neu^{\bullet+}B800^{-}$ pair (red line). The spectrum is a spectral difference of shifted (dashed black line) and nonshifted (blue solid line) absorption spectra of neurosporene with spectral shift of 26 nm. The pure transient Stark spectrum is similar to the S* transient feature; however, it does not mimic it correctly, as demonstrated in Figure 6B. This suggests that other factor(s) could influence its spectral shape. In order to be sure that raw S* feature spectrum is not significantly distorted by coexisting transient bands of BChls, those bands have been removed. It was done by subtracting a corresponding transient absorption spectrum of the carotenoidless LH2 from *Rba. sphaeroides* R.26.1 (Figure 6B, red line). The "pure" S* feature spectrum (blue line) still substantially deviates from the Stark spectrum, mainly in the range of bleaching of the carotenoid absorption band. However, it should be emphasized that, in addition to the transient Stark spectrum, an additional transient band should be also observed. This band will correspond to bleaching of a ground-state absorption (GSB) of the carotenoid pool fraction that is involved in ultrafast formation of the $Neu^{\bullet+}B800^{-}$ pair. Our model of pigment interactions assumed that a single molecule of $Neu^{\bullet+}$ affects a single molecule of the neutral carotenoid, and such stoichiometry should be also reflected in the spectroscopic measurements. Therefore, the amplitude of GSB should be equal with the amplitudes of shifted and nonshifted absorption spectra as shown in Figure 6A (green line). Because both spectral features, GSB and Stark spectrum, have identical temporal characteristics, both should appear as one spectro-temporal feature that spectrally will resemble the sum of both features. This spectral-temporal feature is given in Figure 6C (red line), along with the "pure" transient S* spectrum (blue line). Importantly, now both spectral profiles match each other very well.

**Formation of Carotenoid Triplet State.** Another interesting aspect revealed by this study is the ultrafast formation of the carotenoid triplet state in situations when the $Car^{\bullet+}B800^{-}$ pair cannot be formed due to an absence of B800 BChl *a*. Triplet formation within 1 ps after excitation strongly suggests that the carotenoid triplet pool is not populated in the typical way via sensitization by BChls triplets, and therefore, carotenoid singlet fission has to be involved. This process in carotenoids was first suggested after observation of a high quantum yield of carotenoid triplet formation in whole cells of the purple bacterium *R. rubrum*.[42] It has been suggested that $S_1$ and $1^1B_u^-$, a state without a precise $S_x$ designation (as it may lie below or above $S_2$ depending on length of carotenoid conjugation), could be viewed as a singlet-coupled combination of two triplets localized in the different parts of a carotenoid chromophore: $1^1B_u^- = T_2 \otimes T_1$ and $S_1 = T_1 \otimes T_1$.[43] Singlet fission, in which T⊗T decouples into physically independent triplet states, was reported to have very low yields in solvents,[44−46] but it is high (up to 30%) in proteins,[25,43,47] suggesting that the decoupling process could be facilitated by a conformational distortion of a carotenoid structure provided by


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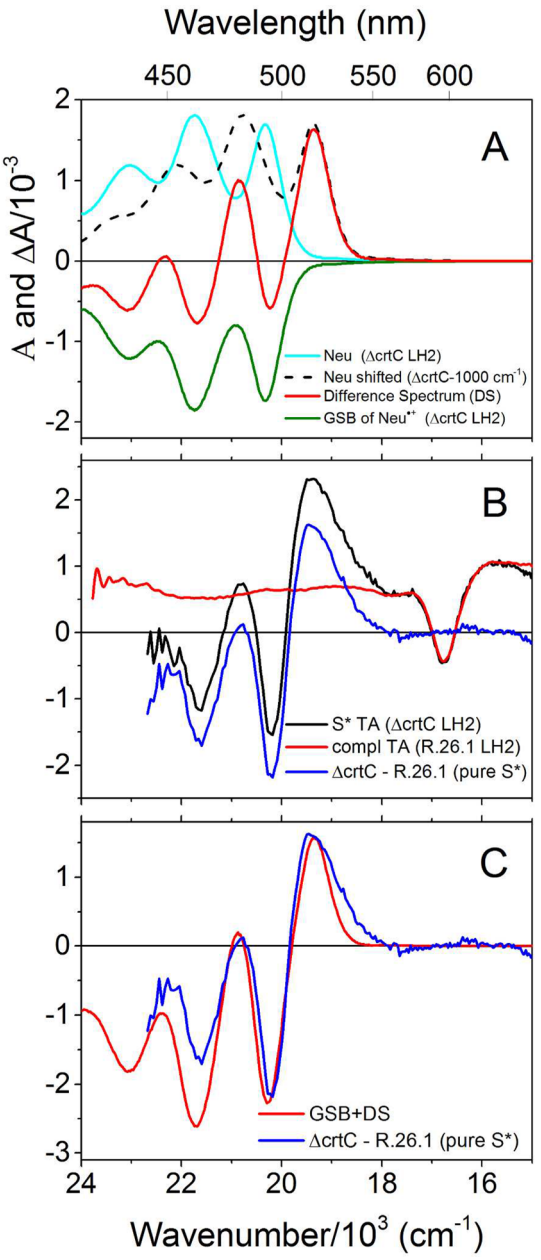


**Figure 6.** Reconstruction of the S* transient spectral feature. (A) Spectral components involved in reconstructing the S* transient feature: cyan line, ground-state absorption spectrum of neurosporene in the LH2; dashed black line, ground-state absorption spectrum of neurosporene affected by the electric field induced by $Neu^{\bullet+}B800^{-}$ pair; red line, difference spectrum (Stark spectrum) of the previous two spectra; green line, ground-state absorption bleaching of neurosporene involved in formation of $Neu^{\bullet+}B800^{-}$ 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 absorption bleaching.

the protein environment. The TA data from Figure 3 show that $T_1 \rightarrow T_n$ and $S_1 \rightarrow S_n$ 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 $1^1B_u^-$ state, has been proposed to lie just below the $S_2$ state for carotenoids with $N \geq 9$[46] (and that includes neurosporene), and its lifetime could be too short to be temporally resolved from the initially excited $S_2$ state.[48] 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 $Neu^{\bullet+}B800^{-}$ pair, evolving from the carotenoid $S_2$ state during its relaxation. It was assumed that the population of BChl *a* triplets is entirely

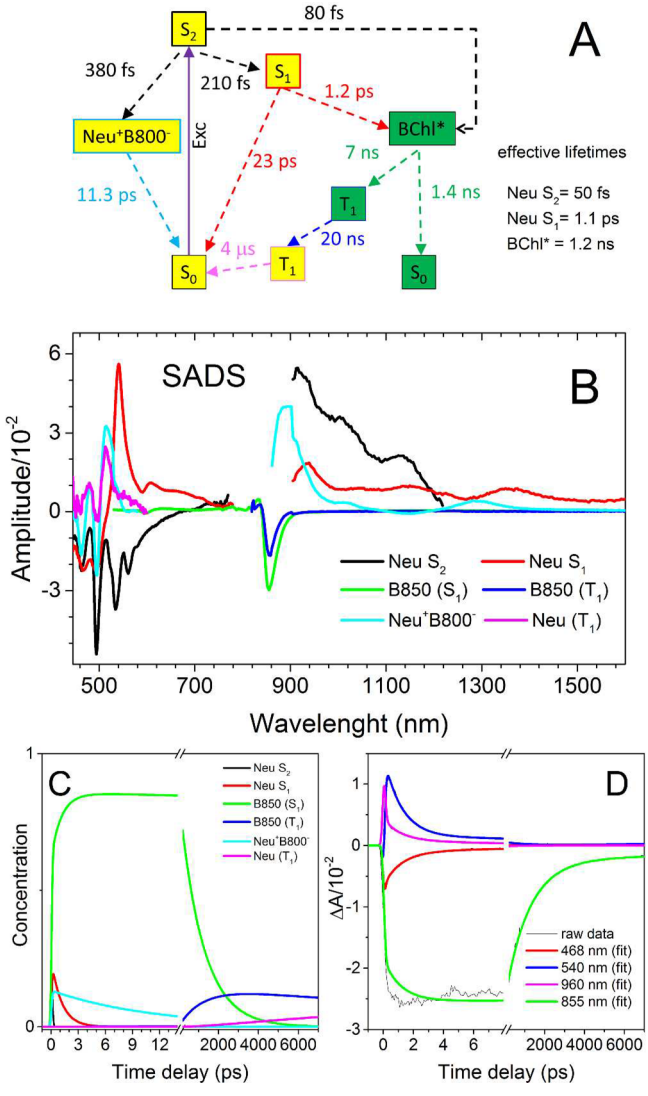


**Figure 7.** Target analysis of the vis–NIR combined TA data set of the LDAO-Tris-LH2 complex. (A) Kinetic model employed to simulate the TA data set. (B) SADS amplitudes resulting from the kinetic analysis. The yellow compartments in the kinetic models symbolize carotenoid neurosporene (with exception of the $Neu^{\bullet+}B800^{-}$ 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.



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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 $T_1 \rightarrow T_n$ 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 ($S_1 \rightarrow S_0$, radiative and nonradiative) and 7 ns (intersystem crossing, ISC, $S_1 \rightarrow T_1$, to be in agreement with triplet yield). Due to the temporal resolution limitation of the spectrometer, the effective lifetime of the carotenoid $S_2$ 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 $S_2$ state depopulation are described with the following details: the $S_2 \rightarrow S_1$ decay was assumed to have the same characteristics as in solvent; the $S_2 \rightarrow$ 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.[9] For simplicity both spectral forms of BChl *a*, B800 and B850, were combined as unity. It was assumed, in agreement with experimental data, that $Neu^{\bullet+}$ is populated with ~13% quantum efficiency from the $S_2$ 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 $Car^{\bullet+}B800^{-}$ 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/acs.jpcb.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)

## AUTHOR INFORMATION

### Corresponding Author

*Phone: 314-935-8483. E-mail: niedzwiedzki@wustl.edu.

### Notes

The authors declare no competing financial interest.

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