Observation of the \( S_1 \) state of spheroidene in LH2 by two-photon fluorescence excitation

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Abstract

The two-photon fluorescence excitation spectrum of LH2, measured by directly exciting the carotenoid \( S_1 \) transition and monitoring fluorescence from the bacteriochlorophyll, provides the first direct experimental verification of carotenoid \( S_1 \) to bacteriochlorophyll energy transfer. It also provides an estimate of the in situ spheroidene \( S_1 \) transition energy of \( 13,900 \pm 150 \text{ cm}^{-1} \), slightly red-shifted from solution estimates. The relative abilities of the carotenoids spheroidene (10 conjugated double bonds) and rhodopin glucoside (11 conjugated double bonds) to transfer energy via their \( S_1 \) states are discussed in terms of spectral overlap factors and competing processes such as \( S_1 \leftrightarrow S_0 \) internal conversion.

1. Introduction

In recent years, carotenoids have been the subject of increasing study as their photophysical properties and vital importance to photosynthetic organisms have become better understood [1,2]. One focus of recent work has been the function of carotenoids in the peripheral light harvesting complex (LH2) of purple bacteria, in which carotenoids harvest light and efficiently transfer excitation energy to nearby bacteriochlorophyll (BChl). In the LH2 of Rhodobacter (\( Rb. \) sphaeroides), the efficiency of this electronic excitation transfer (EET) process approaches unity [3,4]. The mechanisms by which this efficient EET is achieved are poorly understood, as the properties of the pertinent carotenoid excited states are contrary to those of a good energy donor: the \( S_2 \) state has a short lifetime and the \( S_1 \) state is optically forbidden. In this work, we have used two-photon fluorescence excitation to observe the in situ absorption of the \( S_1 \) state of spheroidene, the principal carotenoid in \( Rb. \) sphaeroides. By directly exciting the carotenoid \( S_1 \) state and by observing emission from the BChl, these measurements provide the first direct experimental verification that some carotenoid–BChl energy transfer is mediated by the carotenoid \( S_1 \) state in \( Rb. \) sphaeroides.

In analogy to the closely related linear polyenes, the electronic structure of carotenoids is often described in terms of the idealized \( C_{2n} \) point group. In this description, both the ground \( (S_0) \) and the lowest excited singlet \( (S_1) \) states of carotenoids share \( A_g \) symmetry, so the \( S_1 \leftrightarrow S_0 \) transition is optically for-
hidden. The $S_1$ state has $B_{1u}^g$ symmetry and is responsible for the strong absorption of carotenoids in the 400–550 nm region. Internal conversion from $S_2$ to $S_1$ is highly dependent on solvent but always very rapid, measured as $\sim 200$ fs for spheroidene in methanol solution using fluorescence up-conversion [5]. As mentioned above, the short lifetime of the $S_2$ state, combined with the forbidden nature and short lifetime ($\sim 9$ ps for spheroidene [6–8]) of the $S_1$ state, make carotenoids unlikely donors for EET. Yet, *Rhodopseudomonas* (*Rps.*) *acidophila* has developed an antenna that allows spheroidene to transfer energy to BChl with near unit efficiency, as measured by steady state fluorescence [3].

A decade ago, it was generally accepted that all carotenoid–BChl energy transfer was mediated through the $S_1$ state of the carotenoid because $S_2$–$S_1$ internal conversion was too rapid to permit energy transfer from the $S_2$ state. However, a few authors did propose that $S_2$ may act as a donor state [9,10], and after a thorough transient absorption study of spheroidene dynamics in LH2 and in solution, Shreve et al. [7] suggested that carotenoid–BChl energy transfer proceeds through the $S_2$ state in addition to the $S_1$ state. Several recent studies, including time-resolved measurements of carotenoid–BChl EET in *Chromatium purpuratum* [11] using transient absorption and in *Rhodopseudomonas* (*Rps.*) *acidophila* using fluorescence up-conversion [12], along with two theoretical works [13,14], have confirmed that carotenoid–BChl EET does occur via carotenoid $S_2$ to BChl $Q_y$ transfer. Calculations in a recent work from our group [12] suggest that in antenna with moderate overall carotenoid–BChl EET efficiency, such as 70–75% in the LH2 of *Rps. acidophila* [3,4], the $S_2$ state accounts for nearly all of the carotenoid–BChl energy transfer. Therefore, in *Rps. acidophila*, transfer from carotenoid $S_2$–BChl $Q_y$ (after $S_2$–$S_1$ internal conversion) is only a minor component.

However, in organisms such as *Rh. sphaeroides* where the overall carotenoid–BChl transfer efficiency is very high (>95%) [3,4], it is unlikely that EET from the carotenoid $S_2$ state can account for nearly all of the transfer because EET must compete against the short $S_2$ state lifetime. Ricci et al. measured the dependence of the spheroidene $S_2$–$S_1$ internal conversion time on solvent polarizability, from which they estimated the $S_2$–$S_1$ internal conversion time in LH2 as 150 fs [5]. This rapid, non-radiative process demands that EET times from the carotenoid $S_2$ state to BChl would have to approach unrealistic values of $\sim 10$ fs to achieve overall efficiencies of $\sim 95\%$. Therefore, it seems reasonable to suggest that EET from the carotenoid $S_1$ state (most likely to the BChl $Q_y$ state) accounts for the higher efficiency of carotenoid–BChl transfer in species such as *Rh. sphaeroides* (>95%) compared to species such as *Rps. acidophila* (70–75%).

An important parameter in describing carotenoid $S_1$–BChl $Q_y$ EET is the energy of the carotenoid $S_1$ state. In a series of key experiments, Frank and coworkers [6,10,15,16] used the energy gap law [17] and lifetime measurements on a series of spheroidene analogs to estimate the $S_1$ energy of spheroidene as 14,200 cm$^{-1}$ [15,16]. Recently, Koyama and coworkers have directly observed the $S_1$ state of spheroidene at 14,200 cm$^{-1}$ in absorption [18] using resonance-Raman excitation profiles and in emission [19] using a highly sensitive fluorescence spectrometer. While these measurements may finally begin to settle discussion on the energetic position of the $S_1$ state of spheroidene, they were performed on crystalline spheroidene [18] or on spheroidene in solution [6,10,15,16,19]. The energy of the $S_1$ state likely shifts on incorporation into LH2 relative to solution (the $S_2$ energy shifts 1100 cm$^{-1}$ in LH2 compared to n-hexane) so in situ measurements are needed to determine an $S_1$ energy that is appropriate for discussion of the role of spheroidene as a light harvesting pigment.

Although it is generally accepted that some carotenoid–BChl EET proceeds through the carotenoid $S_1$ state, conclusive experimental evidence does not exist. To our knowledge, all experiments of carotenoid–BChl EET in purple bacteria have involved excitation into the carotenoid $S_2$ state, which necessarily convolves EET from the $S_2$ and $S_1$ states together. We have utilized the presumed carotenoid $S_1$–BChl $Q_y$ EET to perform two-photon fluorescence excitation experiments on the LH2 of *Rh. sphaeroides*. Similar work was performed several years ago by Shreve et al. on the alga *Phaeodactylum tricornutum* in which they monitored chlorophyll $a$ emission while scanning a two-photon excitation source through the estimated $S_1$ energy of
the carotenoid(s) [20]. The two-photon excitation spectrum they observed had a large feature similar to the one-photon absorption and smaller features lower in energy than the onset of one-photon carotenoid absorption. Here, we monitor fluorescence from the B850 BChl of LH2 while scanning a two-photon excitation source through the estimated spheroidened B850 BChl of LH2. We find evidence of a broad absorption with energy and vibronic shape as anticipated for the S1 state of spheroidene. Most of the two-photon absorption assigned to the S1 state occurs in a region where the one-photon spectrum is featureless. Thus, in this work we show that carotenoid S1–BChl Q(EET does occur with significant efficiency and we provide an estimate for the energy of the S1 state of spheroidene in situ in LH2.

2. Experimental methods

Whole cells of *Rh. sphaeroides* strain 2.4.1 were generously provided by Steiger and Sauer. After cells were broken in a French pressure cell at 9000 psi, LH2 was solubilized in a solution of 0.1% LDAO and 20 mM Tris-HCl at pH 8.0 and isolated with a sucrose gradient (0.2–0.8 M sucrose) spun at 150,000 × g overnight according to the method outlined by Cogdell and Hawthornthwaite [21]. One-photon absorption spectra were taken in 1 mm path length cuvettes by a Shimadzu UV-1601 spectro-photometer. Two-photon measurements were performed on ~ 2 ml of sample of O.D. 0.5 per 1 mm path-length at 510 nm. Samples were flowed with a peristaltic pump through a 1 mm path-length quartz cell and maintained at 4–6°C by a recirculating bath that flowed around the sample reservoir.

Excitation light, tunable throughout the region of interest, was provided by a Coherent 9450 optical parametric amplifier (OPA). The OPA was pumped by a Coherent RegA 9000 regenerative amplifier with model 9150 stretcher/compressor and a Mira Seed Ti:Sapphire oscillator. The RegA 9000 was operated at a 250 kHz repetition rate that yielded pulse energies of ~10 nJ from the tunable idler beam of the OPA. A portion of the excitation light was sent to a power meter (Coherent Fieldmaster GS) with wavelength sensitivity correction. The output of this wavelength-corrected one-photon power meter (Ref) was sent to an A/D converter and monitored by computer. A second portion of the excitation light was auto-correlated at each excitation wavelength prior to measuring the fluorescence yield, giving the excitation pulse-width (PW). Typical pulse-widths were ~85 fs and were fairly constant over the entire tuning range.

Excitation light was modulated with an optical chopper (SR-540) and focused into the sample with a f = 5 cm lens. Fluorescence from the sample was collected right angle to the excitation with a f = 2.5 cm lens. A short-wavelength-pass filter (CVI SPF-950) removed any scattered excitation light and a f = 5 cm lens focused the emission through a 870 nm interference filter with 40 nm bandwidth (Corion S40-870) onto a photo-multiplier tube (Hamamatsu R928 red-extended), which was monitored by lock-in amplifier and, in turn, by computer.

At each wavelength, the dependence of sample fluorescence on excitation power was measured. An excitation pulse energy (generally <12 nJ) that yielded a power dependence of 2.0 ±0.1 was used for a fluorescence yield measurement. A measurement consisted of monitoring the fluorescence of the sample \( F_{\text{raw}} \) along with the excitation energy (Ref) once per second for 2 min. The ratio \( F_{\text{raw}} \times PW/\text{Ref}^2 \) was calculated at each point and averaged over the 2 min. With suitable correction for excitation pulse-widths, the square of the excitation energy provides an accurate reference of the effective excitation energy for the two-photon absorption process. The corrected \( F_{\text{raw}} \) is referred to as the two-photon fluorescence excitation (TPE).

Three separate TPE spectra were combined into a single TPE spectrum for use in all of the data analysis. A point from a dataset was discarded if it was considered markedly different (>20% difference in fluorescence signal) from the corresponding points of the other two datasets and if these other two datapoints agreed well (<5% difference) with each other. In addition, points from different datasets were averaged together (both their x and y values) if their x values differed by less than 50 cm^-1 (i.e. they corresponded to the same energy). In the figures, points averaged from all three datasets are designated with filled circles (●), while those averaged from two datasets are designated with open circles (○). The remaining points from each dataset...
3. Results

Fig. 1 shows the one-photon absorption spectrum (dashed line) along with the TPE data (symbols) and fit (solid line) for the LH2 of *Rb. sphaeroides*. The one-photon spectrum shows strong absorptions at 800 and 850 nm from the $Q_y$ bands of the B800 and B850 BCHl, respectively; a single peak at 580 nm from the $Q_z$ bands of the BCHl; and strong vibronic absorption from 430–530 nm due to the $S_1$ band of spheroidene. From 540–570 and from 610–770, the one-photon spectrum is featureless.

The TPE shows a broad absorption and features suggestive of vibronic structure in the 550–700 nm (14 000–18 000 cm$^{-1}$) range, which is shown in more detail in Fig. 2. This absorption extends through the BCHl $Q_z$ region showing significant absorption where there is none in the one-photon spectrum. The lack of coincidence between the one-photon and two-photon spectra strongly indicates that a dipole forbidden transition, which we ascribe as the carotenoid $S_1$ transition, is responsible for the two-photon absorption.

Since BCHl transitions could contribute to the observed TPE spectrum, it is useful to compare the two-photon cross-section of these transitions, $\sigma_{\text{BChl}}$, with the two-photon cross-section of the carotenoids, $\sigma_{\text{Car}}$. To estimate relative values for $\sigma_{\text{BChl}}$ and $\sigma_{\text{Car}}$ we used the two-photon spectrum of *P. tricornutum* measured by Shreve et al., in which the 0–0-transition of Chl $a$ and the tallest peak of the carotenoids clearly can be distinguished [22]. We assumed that the cross-sections of Chl $a$ and the Carotenoids in *P. tricornutum* are of the same order of magnitude as the cross-sections of BCHl and spheroidene. By calculating the ratio of these peaks and correcting it for the stoichiometric amounts of Chl $a$ and energy transferring carotenoids in *P. tricornutum* we obtained $\sigma_{\text{BChl}}/\sigma_{\text{Car}} \sim 0.04$ [23]. Using this value, we can estimate in the same way the BCHl 0–0-transitions in the TPE spectrum of LH2 to be at least 20 times smaller than the spheroidene maximum. The TPE spectrum of isolated Chl $a$ in solution shows, that there are also no unexpected large vibronic peaks [24]. Therefore, we assume that we can neglect contributions of BCHl transitions over the range of 14 000–18 000 cm$^{-1}$. We also have considered the possibility that dipole-forbidden BCHl transitions contribute to the observed TPE spectrum [25]. Recently, such states have been observed in IR absorption experiments of LH2 by Hochstrasser and coworkers [26]. However, none of them matches
exactly the energy which would correspond to our observed TPE. Furthermore, the linewidths of the corresponding transitions in the reaction center of \textit{Rh. sphaeroides}, are completely different from those in our spectrum \cite{27}. In addition, spectra of aggregates such as Chl \textit{a} dimers also show much narrower features than our TPE spectrum \cite{28}.

Finally, both the energy and profile of the TPE spectrum match reasonable parameters for the carotenoid S\textsubscript{1} state (see below). Therefore, although some contributions from BChl cannot be completely excluded, analysis in this work will be conducted assuming that the observed TPE spectrum is entirely due to the carotenoid S\textsubscript{1} transition.

The data alone allow many reasonable lineshapes to be fit through the S\textsubscript{1} region, and, therefore, do not permit quantitative conclusions regarding the shape and position of the S\textsubscript{1} band to be drawn. However, if the fit parameters are restricted to be similar to a mirror image of the \textit{spheroidene} S\textsubscript{1} emission observed in solution by Fujii et al. \cite{19} then the resulting spectral parameters are reasonably robust. The fit parameters were restricted such that the energy of the 0 \leftrightarrow 0 band was allowed to vary within 500 cm\textsuperscript{-1} of the observed by Fujii et al. \cite{19} and the peak spacing was forced to match that observed in solution \cite{19}. The widths of all the peaks were allowed to vary, although they were forced to maintain the same relative widths observed in solution \cite{19}. Finally, the relative amplitudes of the peaks were given complete freedom to vary. Final parameters for the fit of the S\textsubscript{1} lineshape to the TPE fit are given in Table 1 and show that the 0 \leftrightarrow 0 position of the S\textsubscript{1} absorption in LH2 is 13 900 \pm 150 cm\textsuperscript{-1}. (The given uncertainty in the width is based on a fixed value for the center of the 0 \leftrightarrow 0 peak. Shifting the 0 \leftrightarrow 0 peak to a new energy can require large changes in the width, e.g. a 0 \leftrightarrow 0 peak centered at 13 750 cm\textsuperscript{-1} requires a 0 \leftrightarrow 0 FWHM of 800 \pm 50 cm\textsuperscript{-1}, and a 0 \leftrightarrow 0 peak centered at 14 050 cm\textsuperscript{-1} requires a 0 \leftrightarrow 0 FWHM of 1250 \pm 100 cm\textsuperscript{-1}.)

Note that the amplitude of the 0 \leftrightarrow 0 peak is extremely small, 40 times less than the 1 \leftrightarrow 0 band. A satisfactory fit to the TPE spectrum can also be achieved by setting the center of the 0 \leftrightarrow 0 band to 15 100 \pm 150 cm\textsuperscript{-1} and modifying the widths and amplitudes to, effectively, exchange the 0 \leftrightarrow 0 peak for the 1 \leftrightarrow 0, etc. We favor the assignment of the 0 \leftrightarrow 0 band at 13 900 cm\textsuperscript{-1} for three reasons: the quality of the fit is marginally better; the shift of S\textsubscript{1} energy from solution to LH2 should be smaller than, and in the same direction as, the S\textsubscript{2} shift (i.e. to lower energy, as discussed in Section 4.2); and the relative amplitudes of the peaks more closely resemble a mirror image of the Fujii et al. \cite{19} emission spectrum. The last point is also in accord with S\textsubscript{1} spectra of linear polyenes and other carotenoids, which generally show small amplitudes in the 0 \leftrightarrow 0 band, peaking in either the 0 \leftrightarrow 1 or the 0 \leftrightarrow 2 bands. Thus, the Franck–Condon factors associated with the S\textsubscript{1} transitions that have been observed in linear polyenes and carotenoids are generally consistent with a large displacement of the S\textsubscript{1} state relative to the S\textsubscript{0} state, and therefore, a small amplitude for the 0 \leftrightarrow 0 transition. It is possible that the LH2 environment restricts the carotenoid and prevents the large displacement. The 0 \leftrightarrow 0 transition could then have the largest amplitude and would be shifted to

### Table 1

<table>
<thead>
<tr>
<th>Transition</th>
<th>Relative amplitude</th>
<th>FWHM\textsuperscript{a} (cm\textsuperscript{-1})</th>
<th>Center\textsuperscript{a} (cm\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>S\textsubscript{1} 0 \leftrightarrow 0</td>
<td>5\textsuperscript{b}</td>
<td>1200 \pm 100</td>
<td>13 900 \pm 150</td>
</tr>
<tr>
<td>S\textsubscript{1} 1 \leftrightarrow 0</td>
<td>210 \pm 20</td>
<td>1310</td>
<td>15 100</td>
</tr>
<tr>
<td>S\textsubscript{1} 2 \leftrightarrow 0</td>
<td>150 \pm 40</td>
<td>1440</td>
<td>16 300</td>
</tr>
<tr>
<td>S\textsubscript{1} 3 \leftrightarrow 0</td>
<td>60 \pm 40</td>
<td>1590</td>
<td>17 400</td>
</tr>
<tr>
<td>S\textsubscript{1} 4 \leftrightarrow 0</td>
<td>&lt; 1\textsuperscript{b}</td>
<td>1750</td>
<td>18 400</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Relative widths and centers are taken from Fujii et al. \cite{19}, so the widths of all peaks are determined from a single adjustable parameter with one uncertainty, and the centers of all peaks are determined from a second adjustable parameter.

\textsuperscript{b}These parameters are poorly-determined by the data; no uncertainties are given.
higher energy because of the non-equilibrium geometry of the $S_1$ state. However, we feel a 13900 cm$^{-1}$ 0 $\leftrightarrow$ 0 energy is the most reasonable assignment.

4. Discussion

4.1. Spheroidene $S_1$ to BChl energy transfer

As mentioned in Section 1, EET from the carotenoid $S_1$ state is not expected to account for all carotenoid–BChl energy transfer in species that have high overall carotenoid–BChl transfer efficiencies, such as *Rb. sphaeroides*. Recent $S_n \leftrightarrow S_1$ transient absorption measurements by Zhang et al. [8] give the $S_1$ lifetime of spheroidene in n-hexane solution and in LH2 as 9.4 and 2.0 ps, respectively, which indicates a 2.5 ps and 75–80% efficient EET from the carotenoid $S_1$ state to BChl, if the $S_1 \rightarrow S_0$ internal conversion rate is the same in solution and in LH2. These results from Zhang et al. are strong, but indirect, evidence that some carotenoid–BChl EET is mediated by the carotenoid $S_1$ state. The results of the present work show conclusively that, indeed, the spheroidene $S_1$ state does act as an energy donor. Two-photon excitation in the $S_1$ region directly excites the $S_1$ state, making it the only possible energy donor. Subsequent emission from BChl B850 demonstrates that energy transfer did occur.

Why does $S_1 \rightarrow$ BChl energy transfer occur efficiently in *Rb. sphaeroides* but not in *Rps. acidophila*? In general, three factors account for the efficiency of an energy transfer process: the electronic coupling between the donor and acceptor transitions, the spectral overlap of the donor emission and acceptor absorption spectra, and the lifetime of the donor state (in the absence of energy transfer) relative to the energy transfer rate. The first two determine the energy transfer rate while the third accounts for factors that compete against the energy transfer. The three factors are discussed, in turn, below.

Recent calculations have shown that electronic coupling with the dipole-forbidden carotenoid $S_1$ transition is not mediated by the Dexter electron exchange interaction as often presumed [13,14,29]. Other orbital-overlap-dependent mechanisms dominate the Dexter term [30] and, in turn, these are dominated by polarization coupling [14] and high-order Coulombic coupling (e.g., quadrupole–quadrupole) [13,14,29]. While these descriptions of coupling involving the $S_1$ state are still not well understood, it is reasonable that if the structural relationships between carotenoid and BChl are comparable in *Rb. sphaeroides* and *Rps. acidophila*, then the electronic coupling will be as well. Given the similarity of the two known LH2 structures, from *Rps. acidophila* [31] and *Rhodospirillum molischianum* [32], and the similarity of spectral features between the LH2 of *Rb. sphaeroides* and the LH2s of known structure, it seems reasonable that the carotenoid–BChl structural relationships are also similar between the three species. Therefore, for this work, we assume that transfer efficiency differences between the LH2 of *Rb. sphaeroides* and *Rps. acidophila* mainly originate not from differences in electronic coupling, but from differences in spectral overlap and/or donor state lifetime factors.

The spectral overlaps for carotenoid–BChl transfer were estimated by following the procedure outlined in previous work [12,33] and using the emission profile found by Fujii et al. [19]. The 0 $\rightarrow$ 0 band of the emission profile was positioned at energies ranging from 16000 cm$^{-1}$ to 11000 cm$^{-1}$ to examine the dependence of the spectral overlap on the $S_1$ energy (data not shown). Simple Gaussian absorption profiles were assumed for the B800 and B850, as suggested in Ref. [12]. Spectral overlaps peak for $S_1$ energies around 14500–15000 cm$^{-1}$ and are still quite high ($>2 \times 10^{-4}$ cm) in the vicinity of the spheroidene energy at 13900 cm$^{-1}$. However, spectral overlap values decrease quickly as $S_1$ energies fall below 13000 cm$^{-1}$, roughly the energy expected for rhodopin glucoside, the carotenoid in *Rps. acidophila* [15]. Thus, the higher energy $S_1$ state of the 10 conjugated double bond spheroidene yields significantly increased spectral overlap over the $S_1$ state in the 11 conjugated double bond rhodopin glucoside. However, this increase, of roughly a factor of two, accounts for only a portion of the increase in $S_1$ mediated energy transfer efficiency between the two species.

The lifetime of the donor state in the absence of energy transfer is the final factor affecting the efficiency of EET. A longer lived donor state results in higher transfer efficiency simply because there is a
longer period of time available for energy transfer prior to internal conversion. The solution lifetime of the $S_1$ state of spheroidene is roughly 9 ps [4,8], while that of the 11 conjugated double bond spheroidene analog (5',6'-dihydro-7',8'didehydro-spheroidene) is 4 ps [16]. Assuming that these solution lifetimes are representative of the lifetimes in LH2, and that the lifetime of the 11 conjugated double bond rhodopin glucoside is similar to the spheroidene analog, then the $S_1$ transfer efficiency is $\sim 2.25$ times higher in Rh. sphaeroides due to the increased $S_1$ lifetime. In other words, competing effects, primarily $S_1 \rightarrow S_0$ internal conversion, are roughly 2 times faster (more competitive) in rhodopin glucoside than in spheroidene.

The combined increases in spectral overlap and $S_1$ lifetime when going from rhodopin glucoside with 11 conjugated double bonds to spheroidene with 10 conjugated double bonds suggest that energy transfer from the $S_1$ state of spheroidene should be roughly a factor of 4 more efficient than from the $S_1$ state of rhodopin glucoside. In previous work on Rps. acidophila [12] it was estimated that EET from rhodopin glucoside $S$ takes place with a time constant of 15–30 ps, which must compete against $S_1 \rightarrow S_0$ internal conversion of 3.5 ps. Thus, EET from the rhodopin glucoside $S_1$ state to BChl is 10–20% efficient. As mentioned above, the data from Zhang et al. [8] suggest that spheroidene $S_1$–BChl energy transfer is 75–80% efficient in Rh. sphaeroides, an increase of 4–8 times. Our estimates above suggest that spectral overlap and donor lifetime effects each contribute roughly a factor of 2 to the difference in relative energy transfer efficiencies of Rh. sphaeroides and Rps. acidophila, in agreement with the lower end of the factor of 4–8 suggested by experiments.

As pointed out by Frank et al. [16] the total yield of carotenoid–BChl energy transfer is a complex combination of the properties of both the $S_1$ and $S_2$ states of the carotenoid. Carotenoids with longer conjugation lengths (> 10) may generally be poor donors from the $S_1$ state because of shorter $S_1$ lifetimes and reduced spectral overlaps. However, transfer from the $S_2$ state may be better for these carotenoids because $S_2$ lifetimes generally increase with increasing conjugation length, allowing more efficient transfer from this state. Certainly, each individual species has a unique character to its carotenoid–BChl energy transfer pathways.

### 4.2. Energy of the spheroidene $S_1$ state

In addition to demonstrating carotenoid $S_1$–BChl energy transfer, the measured TPE spectrum of the $S_1$ state allows estimation of the in situ $S_1$ energy. The 13900 cm$^{-1}$ estimated here is 300 cm$^{-1}$ lower than estimates based on solution studies [16,19]. This is a relatively small shift compared to the spheroidene $S_2$ state that is lowered 1100 cm$^{-1}$ in LH2 compared to its transition frequency in room temperature n-hexane (19500 cm$^{-1}$ in the present study and 20600 cm$^{-1}$ in Fujii et al. [19]). The change in $S_1$ energy is most likely the result of the protein environment of LH2 having a larger polarizability than the n-hexane solution environment. The $S_2$ state of polyenes, including carotenoids, is known to be highly polarizable and highly sensitive to the polarizability of the surroundings [34,35], while the $S_1$ state is relatively (although not completely) insensitive to its surroundings. Work with linear polyenes [34] demonstrates that changes in solvent polarizability shift the $S_1$ state in the same direction as the $S_2$ state, but with much smaller magnitude to the shift.

The two recent works from Koyama and coworkers found the $S_1 0 \leftrightarrow 0$ band at 14200 cm$^{-1}$ both in emission from n-hexane [19] and in absorption from crystalline spheroidene [18]. Shashima et al. reasoned that because the spheroidene $S_1$ state is highly insensitive to its environment, the energy of the $S_1$ emission should be temperature independent, as observed at 200 and 295 K [19], and the Stokes shift should be small [18]. In fact, because it is the emission in n-hexane that matches the absorption in crystalline spheroidene, the energy difference between absorption and emission in n-hexane (the Stokes shift) equals the energy difference between absorption in n-hexane and absorption in crystalline spheroidene. Both the Stokes shift and absorption shift from n-hexane to crystalline spheroidene are expected to be small, but non-zero [34,35]. Thus, the data of Fuji et al. [19] and Shashima et al. [18] and the behavior of linear polyenes [34] are consistent with our estimate of a small, but non-zero, 300 cm$^{-1}$ red-shift in the $S_1$ energy from solution to protein.
5. Conclusions

We have used two-photon fluorescence excitation to directly excite the S₁ state of spheroidene, the primary carotenoid in the purple bacterium Rb. sphaeroides, while monitoring emission from the B850 BChl. This work provides the first direct experimental verification of carotenoid S₁–BChl energy transfer in purple bacteria. Estimates of spectral overlap factors and S₁ state lifetimes suggest that these two factors contribute roughly equally to ~4 times more efficient carotenoid S₁–BChl EET in Rb. sphaeroides compared to Rps. acidophila. By fitting the absorption profile of the S₁ state to parameters suggested by the emission spectrum found by Fujii et al. [19], we estimate the spheroidene S₁ energy in LH2 to be 13 900 ± 150 cm⁻¹, slightly red-shifted from the 14 200 cm⁻¹ of solution measurements.

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