Functional characteristics of spirilloxanthin and keto-bearing Analogues in light-harvesting LH2 complexes from Rhodobacter sphaeroides with a genetically modified carotenoid synthesis pathway

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Light-harvesting 2 (LH2) complexes from a genetically modified strain of the purple photosynthetic bacterium Rhodobacter (Rba.) sphaeroides were studied using static and ultrafast optical methods and resonance Raman spectroscopy. Carotenoid synthesis in the Rba. sphaeroides strain was engineered to redirect carotenoid production away from spheroidene into the spirilloxanthin synthesis pathway. The strain assembles LH2 antennas with substantial amounts of spirilloxanthin (total double-bond conjugation length $N \geq 13$) if grown anaerobically and of keto-bearing long-chain analogs [2-keto-2,2'-diketospirilloxanthin ($N = 13$), 2-ketospirilloxanthin ($N = 14$) and 2,2'-diketospirilloxanthin ($N = 15$)] if grown semi-aerobically (with ratios that depend on growth conditions). We present the photophysical, electronic, and vibrational properties of these carotenoids, both isolated in organic media and assembled within LH2 complexes. Measurements of excited-state energy transfer to the array of excitonically coupled bacteriochlorophyll $\alpha$ molecules ($B850$) show that the mean lifetime of the first singlet excited state ($S_1$) of the long-chain ($N \geq 13$) carotenoids does not change appreciably between organic media and the protein environment. In each case, the $S_1$ state appears to lie lower in energy than that of $B850$. The energy-transfer yield is $<0.4$ in LH2 (from the strain grown aerobically or semi-aerobically), which is less than half that achieved for LH2 that contains short-chain ($N \leq 11$) analogues. Collectively, the results suggest that the $S_1$ excited state of the long-chain ($N \geq 13$) carotenoids participates little if at all in carotenoid-to-BChl $\alpha$ energy transfer, which occurs predominantly via the carotenoid $S_2$ excited state in these antennas.

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1. Introduction

The antennas of many purple photosynthetic bacteria consist of two types of light-harvesting (LH) complex, LH1 and LH2. LH2 is a peripheral pigment-protein that absorbs light and transfers excitation energy to LH1, which is closely associated with the reaction center, the ultimate trap for the harvested excitation energy. High resolution structures of LH2 complexes from two bacterial species, Rhodopseudomonas (Rps.) acidophila strain 10050 and Phaeospirillum (Phs.) molischianum, have been determined using X-ray crystallography [1–4]. The antennas have a circular structure consisting of either eight or nine identical protein subunits, each of which is a heterodimer of $\alpha$ and $\beta$ polypeptides that span the photosynthetic membrane. Each $\alpha$-$\beta$-protein subunit accommodates three bacteriochlorophyll $\alpha$ (BChl $\alpha$) and one carotenoid. The macrocycles of two BChl $\alpha$ of each subunit are perpendicular to the membrane plane and are electronically (excitonically) coupled to one another and to the pairs from adjacent subunits. These interactions produce a ring-like array of closely-spaced BChl $\alpha$ (B850) that has its lowest energy absorption band (the Qy band) at $\sim 850$ nm. The remaining monomeric BChl $\alpha$ molecules (B800), one from each subunit, lie parallel to the membrane plane and between polyketides of adjacent subunits, and have their Qy band at $\sim 800$ nm [1–3].

Abbreviations: ACN, acetonitrile; BChl $\alpha$, bacteriochlorophyll $\alpha$; EADS, evolution-associated difference spectra; ESA, excited-state absorption; FWHM, full width at half maximum; GSB, bleaching of a ground-state-absorption profile; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; IRF, instrument response function; LDAO, lauryldimethylamine-oxide; LH1, light-harvesting 1 complex; LH2, light-harvesting 2 complex; n-hex, n-hexane; 2-MTHF, 2-methyltetrahydrofuran; VIS, visible; NIR, near infrared; Rba, Rhodobacter; Rps, Rhodopseudomonas; Phs, Phaeospirillum; P, Pantoec, TA, transient absorption; RT, room temperature; THF, tetrahydrofuran; Tris, tris(hydroxymethyl)aminomethane; RR, resonance Raman

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Carotenoids play a dual role in purple bacterial antennas. They assist in light harvesting by absorbing photons in a spectral range (450–550 nm) that is mainly inaccessible to BChl a. The excited carotenoid then transfers energy to the BChl a species (B800 or B850 in LH2), hereafter denoted Car → BChl energy transfer, with efficiency \( \Phi_{\text{Car} \to \text{BChl}} \). Carotenoids also play a photoprotective role in efficiently quenching the triplet excited state of the BChl a, which otherwise may produce deleterious reactive oxygen species [5]. It has been reported that the propensity for quenching of BChl a triplet states in LH1 or LH2 appears to be independent of the type of carotenoid present [6], although a recent study shows that spheroidenone has some unique attributes in this respect, when bound within the LH1 antenna of \( \Phi_b \) *Rba. sphaeroides* [7].

\( \Phi_{\text{Car} \to \text{BChl}} \) varies between different purple bacterial species, but the reasons for this variability are not fully delineated. Among the many contributing factors are the excited-state properties of the carotenoid (e.g., energy and lifetime), the orientation and distance of the carotenoid with respect to the neighboring BChl a, and the double-bond conjugation length, and therefore more extended \( \pi \)-electron system, of the carotenoid. For simplicity, the total number of double bonds in the carotenoid conjugation pathway will be designated \( \text{carotenoid} \). For the LH2 complexes from purple sulfur bacteria such as \( \Phi_b \) *Rba. sphaeroides* [12,13], similarly, LH2 from \( \Phi_{\text{N}} \) *Allochromatium vinosum* [14,15], the latter two LH2 complexes combine up to 8 different carotenoids (and shorter analogues) with conjugation lengths in the range 11–13, while each type of complex has \( \Phi_{\text{Car} \to \text{BChl}} \approx 80\% \). The ratios of keto-bearing carotenoids (and shorter analogues) depend on the growth light conditions [15].

Previous studies on LH2 have shown that \( \Phi_{\text{Car} \to \text{BChl}} \) is greater for carotenoids with lower \( N \) values. LH2 from the \( \Phi_b \) *Rba. sphaeroides* G1C mutant that incorporates neurosporene \( (N = 9) \) has \( \Phi_{\text{Car} \to \text{BChl}} \approx 90\% \) [8,10,11]. LH2 from wild-type \( \Phi_b \) *Rba. sphaeroides* (2.4.1, which contains spheroidene \( (N = 10) \) when grown photosynthetically (anaerobically) and spheroidene \( (N = 11, N_{\text{car}} = 10, N_{\text{c-O}} = 1) \) when grown semi-anaerobically has \( \Phi_{\text{Car} \to \text{BChl}} \approx 95\% \) [8]. However, a previous study of a \( \Phi_b \) *Rba. sphaeroides* strain genetically engineered to produce the \( N = 11 \) carotenoid lycopene found \( \Phi_{\text{Car} \to \text{BChl}} < 54\% \) for lycopene–LH2 complexes purified from this strain [12,13]. Similarly, LH2 from \( \Phi_b \) *Rps. acidophila* 10050 that contains rhodopin glucoside \((N = 11)\) has \( \Phi_{\text{Car} \to \text{BChl}} < 55\% \) [8,10,11]. An even lower \( \Phi_{\text{Car} \to \text{BChl}} \) of 30–40% has been obtained for the LH2 complexes from purple sulfur bacteria such as \( \Phi_{\text{N}} \) *Thermochromatium tepidum* and \( \Phi_{\text{N}} \) *Allochromatium vinosum* [14,15]. The latter two LH2 complexes combine up to 5 different carotenoids with conjugation lengths in the range 11–13, with ratios that depend on the growth light conditions [15].

Attempts to isolate the impact of individual factors on \( \Phi_{\text{Car} \to \text{BChl}} \) have been hindered by the limited number of different carotenoids that can be incorporated into LH2 of a given bacterial species. Carotenoid function could vary between species due to differences such as precise LH2 structures and local/global electrostatics derived from non-identical amino-acid sequences. Genetic engineering of the carotenoid synthesis pathway of \( \Phi_b \) *Rba. sphaeroides* [16] offers some advantages by providing a set of LH2 and RC–LH1–PuX complexes that incorporate diverse carotenoids with \( N = 9–15 \), while each type of complex has the same polypeptides and associated architectural and electrostatic characteristics [17]. The series of LH2 complexes analyzed in the present study, and the analogous RC–LH1–PuX set, provide new vehicles for investigating \( \Phi_{\text{Car} \to \text{BChl}} \) and associated energy-transfer dynamics in bacterial light-harvesting systems. Initial static spectrosopic studies on this engineered set of \( \Phi_b \) *Rba. sphaeroides* of LH2 antennas revealed the general trend of decreasing energy-transfer efficiency with increasing double-bond number for the primary carotenoid produced \( (\text{Car} \to \text{BChl} : N) \): neurosporene \((94\%, 9)\), spheroidene \((96\%, 10)\), spheroidene \((95\%, 11)\), lycopene \((64\%, 11)\), rhodopin \((62\%, 11)\), spirilloxanthin \((39\%, 13)\), and diketospirilloxanthin \((35\%, 15)\) [17].

The present work greatly expands the scope of characterization to include static and ultrafast optical measurements and resonance Raman (RR) studies. The investigations focus on LH2 complexes from the \( \Phi_b \) *Rba. sphaeroides* \( \text{crt}:\text{crt}^\text{Ps} \) strain, which produces large quantities of the longest–chain carotenoids \( (N = 13 \text{ to } 15) \) found thus far in antennas from the purple photosynthetic bacteria (Fig. 1). This strain contains the four-step phytoene desaturase from *Pantoea (P.) agglomerans*, which feeds carotenoid production into the spirilloxanthin pathway, rather than the native three-step phytoene desaturase that directs carotenoid production along the spheroidene synthesis pathway [17].

Carotenoid production in \( \text{crt}:\text{crt}^\text{Ps} \) affords LH2 complexes with carotenoids with conjugation lengths up to spirilloxanthin \((N = 13, N_{\text{car}} = 13, N_{\text{c-O}} = 0) \) when grown under photosynthetic (anaerobic) conditions; smaller amounts of other carotenoids including anhydrorhodovibrin \((N = 12, N_{\text{car}} = 12, N_{\text{c-O}} = 0) \) are also present. When the same strain is grown semi-anaerobically, terminal keto groups are added to the carotenoid carbon–carbon bond backbone, yielding 2-ketoanhydrorhodovibrin \((N = 13, N_{\text{car}} = 12, N_{\text{c-O}} = 1) \), 2-ketospirilloxanthion \((N = 14, N_{\text{car}} = 13, N_{\text{c-O}} = 1) \), and 2,2’-diketospirilloxanthin \((N = 15, N_{\text{car}} = 13, N_{\text{c-O}} = 2) \) [17]. Hereafter, the long-chain keto-bearing carotenoids will be referred to simply as ketoanhydrorhodovibrin, ketospirilloxanthin and diketospirilloxanthin. The ratios of keto-bearing carotenoids (and shorter analogues) depend on growth conditions, specifically the extent of oxygenation of the culture.

LH2 antennas from aerobically or anaerobically grown \( \Phi_b \) *Rba. sphaeroides* \( \text{crt}:\text{crt}^\text{Ps} \) are of particular interest among the entire set of engineered strains [17] because, aside from LH2 complexes with purple sulfur bacteria that have small quantities of carotenoids with \( N = 12 \) or \( 15 \) [15,18,19], no other LH2 complexes are known to preferentially bind \( (\text{keto-}) \) carotenoids with such long conjugation lengths \((N \geq 13) \). To assess the impact of high \( N \) value carotenoids on the functional properties of these LH2 complexes, the carotenoid electronic, vibrational, and excited-state properties as well as Car → BChl energy transfer were examined using static and ultrafast optical techniques (at room temperature and 77 K) and RR spectroscopy. In addition, the spectral and excited-state properties (energies, lifetimes) of ketoanhydrorhodovibrin, ketospirilloxanthin and diketospirilloxanthin were studied in representative organic solvents and compared with the properties of the carotenoids within LH2.

2. Materials and methods

2.1. \( \Phi_b \) *Rba. sphaeroides* strains and growth conditions

The \( \text{crt}:\text{crt}^\text{Ps} \) strain of \( \Phi_b \) *Rba. sphaeroides* was constructed and grown either anaerobically or semi-anaerobically as described by Chi et al. [17]. After reaching stationary stage the cultures were harvested and pelleted by centrifugation.

2.2. Isolation and purification of LH2

Pelleted bacterial cells were resuspended in 20 mM tris(hydroxymethyl)-aminoethane (Tris) buffer \((pH = 8.0)\) and the membranes were released by ultrasonication and then pelleted by centrifugation. Subsequently, the pellet was resuspended in 20 mM Tris buffer \((pH = 8.0)\) to \( D_{\text{B500}} \approx 20 \text{ (cm path)} \) and mixed with lauryldimethylamine-oxide (LDAO) to a final concentration of \(-0.5\% \) for 20 min at room temperature (RT). The insoluble material was pelleted by centrifugation. Final purification of the complexes was carried out using an anion exchange chromatographic column (Q Sepharose High Performance, GE Healthcare) equilibrated with 20 mM Tris buffer \((pH = 8.0)\) with 0.06% LDAO by applying 50 mM gradient steps of NaCl from 150 and 500 mM. The protein-containing fraction was eluted with 300–400 mM NaCl. Samples for 77 K studies were re-suspended in a 50:50 (v/v) glycerol:buffer mixture.
2.3. Purification of long-chain keto-bearing carotenoids and analysis of LH2 carotenoid content

Carotenoids ketoanhydrorhodovibrin, ketospirilloxanthin and diketospirilloxanthin were extracted from pelleted bacterial cells using a technical grade methanol/acetone (1/1, v/v) mixture. The extract was injected into an Agilent 1100 high-performance liquid chromatography (HPLC) system employing a Zorbax Eclipse XDB-C18 reverse phase column (250 mm × 4.6 mm) and eluted using HPLC grade acetonitrile/tetrahydrofuran (ACN/THF) (95/5, v/v) mixture as a mobile phase with a 1.5 ml min$^{-1}$ flow rate. For analysis of LH2 carotenoid content, the pigment extract from the LH2 was injected into the same HPLC setup programmed for a gradient mobile phase of 100% ACN to 60/40 ACN/THF (v/v) within 30 min. Carotenoid identities are based on the HPLC and mass spectroscopy analysis of the pigment content of these LH2 complexes as described in Chi et al.[17]. The identification of purified carotenoids was similarly made.

2.4. Steady-state and femtosecond time-resolved absorption spectroscopy

Time-resolved pump-probe absorption experiments were carried out using a Helios femtosecond transient absorption (TA) spectrometer (Ultrafast Systems LCC, Sarasota, FL) coupled to a Spectra-Physics femtosecond laser system described previously[15]. Before TA measurements of the LH2 complexes the regular buffer was replaced with D$_2$O-based buffer to enhance transparency in the near-infrared (NIR) region. For preferential excitation of the LH2-bound spirilloxanthin, the excitation wavelength was set to 500 nJ in a spot size of 1 mm diameter, corresponding to an intensity of –2 × 10$^{14}$ photons/cm$^2$. For carotenoids in the LH2 complexes, the energy of the pump was lowered to 200 nJ (to minimize the possibility of excited-state annihilation in the BChl$\alpha$ array, B850) corresponding to an intensity of ~7 × 10$^{13}$ photons/cm$^2$. For diketospirilloxanthin and ketoanhydrorhodovibrin (in n-hex at RT), the excitation wavelengths were 550 nm and 535 nm, respectively. For the carotenoids in organic solvents, the energy of the pump beam was set to 500 nJ in a spot size of 1 mm diameter, corresponding to an intensity of ~2 × 10$^{14}$ photons/cm$^2$. For carotenoids in the LH2 complexes, the energy of the pump was lowered to 200 nJ (to minimize the possibility of excited-state annihilation in the BChl$\alpha$ array, B850) corresponding to an intensity of ~7 × 10$^{13}$ photons/cm$^2$. All steady-state absorption measurements were performed using Shimadzu UV-1800 spectrophotometer. For low temperature measurements of diketospirilloxanthin, the purified pigment was dissolved in spectroscopic grade 2-MTHF, transferred to a 1 cm square cryogenic quartz cuvette (NSG Precision Cells, Inc) and frozen in the SVP100 liquid nitrogen cryostat from Janis (Woburn, MA). The optical density (OD) of the sample was set to ~0.5 at the maximum of carotenoid absorption band. For low temperature measurements of the LH2 proteins the buffer was mixed with glycerol in 1:1 (v/v) ratio, transferred to a 1 cm square plastic cuvette and frozen in the above-mentioned cryostat. Fluorescence and fluorescence excitation spectra were acquired at RT using a Horiba-Spekman Nanolab fluorometer with 2–4 nm excitation and detection bandwidths and were corrected for the instrument spectral response. Samples had an OD ≤ 0.1 at the excitation and emission wavelengths to avoid front-face and inner-filter effects.

2.5. Data processing and fitting

Group velocity dispersion in the TA datasets was corrected using Surface Explorer Pro software (v.2.3) (Ultrafast Systems LCC) by building a dispersion correction curve from a set of initial times of transient signals obtained from single wavelength fits of representative kinetics. Global analysis of the TA datasets was performed using a modified version of ASUfit 3.0, program provided by Dr. Evaldas Katilius at Arizona State University.
State University. The full width at half maximum (FWHM) of a temporal response function was assumed to be ~150 fs for all the measurements in the visible (VIS) spectral range and ~200 fs in the NIR spectral range and was used as a fixed parameter in the fitting procedures. In an idealized scenario the TA data should be fitted with anticipated, realistic kinetic model mimicking the true decay pathways following excitation. However, for structurally complicated systems like LH2 the precise decay scheme is complex and uncertain. Alternatively the datasets may be fitted with a non-branching, sequential, irreversible scheme \( A \rightarrow B \rightarrow C \rightarrow D \), where arrows represent increasingly slower monoeponential decays, with time constants corresponding to lifetimes of the transient species \( A, B, C, D \). The spectral profiles of these species are termed Evolution-Associated Difference Spectra (EADS) [20]. EADS do not necessarily correspond to a particular process, excited state, or individual species (carotenoid or the BCHl \( a \) entities B800 or B850); however, the EADS help visualize the time evolution of the spectral features of the whole system (e.g., LH2). This type of fitting was applied to all TA data in this work.

2.6. RR spectroscopy

The RR spectra of the isolated carotenoids were obtained at 4 °C in THF solutions with the samples contained in 1 mm diameter capillary tubes. The spectra of the carotenoids in the LH2 complexes were obtained at 4 °C in buffer solutions (either 20 mM Tris at pH 8.0 with 0.06% LDAO and 400 mM NaCl or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.5 with 0.03% n-dodecyl-beta-D-maltoside) with the samples contained in 1 mm diameter capillary tubes.

The RR spectra were acquired with a triple spectrograph (SpeX 1877) equipped with holographically etched 1800 groove/mm gratings in the third stage. A UV-enhanced charge-coupled device was used as the detector (Princeton Instruments LN/CCD equipped with an EEV1152-UV chip). The excitation wavelength of 532 nm was provided by the discrete output of a diode-pumped solid-state laser (Coherent Verdi-V6). The laser power was typically 5 mW and the scattered light was collected at 90° using a 50 mm f/1.4 Cannon camera lens. The spectra were acquired with 1–2 h of signal averaging (20 × 180 s to 40 × 180 s scans). The spectral resolution was ~2 cm\(^{-1}\) at a Raman shift of 200 cm\(^{-1}\). The spectra data were calibrated using the known frequencies of indene.

3. Results

3.1. Analysis of carotenoid content in the LH2 complexes

Fig. 1A shows the native carotenoid biosynthesis pathway for Rba. sphaeroides and the altered pathway leading to spirilloxanthin and diketospirilloxanthin (Fig. 1B), resulting from a gene replacement that swaps the 3-step phytoene desaturase for a 4-step enzyme. The final step in both pathways (spheroidene/spheroidenone; spirilloxanthin/diketospirilloxanthin) is catalyzed by a monooxygenase, so the oxygen level of the culture determines the extent of this reaction [21,22]. Representative chromatograms of the LH2 from Rba. sphaeroides crt:crtP recorded at 530 nm (anaerobically grown strain) and at 540 nm (semi-anaerobically grown strain) are shown in Fig. 1C. The chromatograms were analyzed using the simplifying assumption that all carotenoids present have very similar molar extinction coefficients \( (\varepsilon_{\text{molar}}) \) at their absorption maximum in the solvent mixture used for the HPLC runs. This is reasonable because most open-chain carotenoids from purple photosynthetic bacteria have \( \varepsilon_{\text{molar}} \) in range of 145,000–160,000 M\(^{-1}\) cm\(^{-1}\) at their (1,0) vibronic band [23]. The carotenoid content of the samples is summarized in Table 1. The major carotenoids in the LH2 complex from anaerobically grown bacteria are spirilloxanthin \( (N = 13, 60\%) \) and anhydrohodovibrin \( (N = 12, 15\%) \). The major carotenoids in the LH2 from semi-anaerobically grown bacterium are keto-bearing carotenoids with the increased double-bond length and are ketoanhydrohodovibrin \( (N = 13, 28\%) \), ketospirilloxanthin \( (N = 14, 16\%) \), and diketospirilloxanthin \( (N = 15, 21\%) \).

The chromatograms in Fig. 1 also indicate that the carotenoids all primarily have the all-\( \text{trans} \) configuration, in agreement with the RR data on the isolated carotenoids from the same extracts (vide infra). The cis isomers of the carotenoids present elute at the edge of the main chromatographic peaks, as can be seen for spirilloxanthin. The amounts of cis isomers present are typical for minor spontaneous, thermal isomerization after extraction from LH2. Thus, there appears to be no or only trace amounts of cis isomers of these carotenoids when bound to LH2.

3.2. Absorption spectra of LH2 complexes at RT and at 77 K

Fig. 2A and B show RT and 77 K absorption spectra of LH2 from the Rba. sphaeroides c_\text{rt}::crtP strain grown photosynthetically (anaerobically) or semi-aerobically, respectively. The B800 band (BChl \( a \) monomers) and B850 band (BChl \( b \) oligomer) appear at 798 and 849 nm, respectively, in both complexes. Upon lowering the temperature, the B850 band bathochromically shifts 3 nm and the B800/B850 peak-intensity ratio increases from 0.75 to 0.91. These spectral characteristics demonstrate good overall assembly of the BChl \( a \) molecules into polypeptide matrix [8,24].

The carotenoid absorption contours are more complex and differ with carotenoid content, which for Rba. sphaeroides c_\text{rt}::crtP depends on growth conditions (anaerobic or semi-aerobic) (Table 1). In the case of LH2 that contains primarily spirilloxanthin, the vibronic bands of the carotenoid absorption spectrum (420–580 nm) are clearly resolved even at RT (Fig. 2A). However, for LH2 with keto-bearing carotenoids lowering the temperature to 77 K does not substantially improve the vibronic resolution (Fig. 2B), indicating substantial overlap of the absorption features of the bound pigments. Nonetheless the carotenoid absorption contours can be reconstructed from the spectra of the contributing carotenoids because the positions of the respective (0,0) bands are predictable. Based on previous studies of reconstitution of the LH1 complex from R. rubrum with various types of carotenoids and on the LH2 complexes containing only single carotenoid [8,25], the positions of (0,0) absorption bands of the non-ketolated carotenoids are expected to be as follows: spirilloxanthin (~550 nm), anhydrohodovibrin and didehydrohodopin (~536 nm), lycopene (~516 nm), and neurosporene (~491 nm). The absorption spectra of keto-bearing counterparts (obtained from HPLC) show that introduction of one terminal C=O group onto a C=C backbone shifts the absorption spectrum by 500 cm\(^{-1}\) to lower energy. Therefore, the spectral positions of keto-bearing carotenoids in LH2 from the semi-aerobically grown bacterium are predicted to be as follows: diketospirilloxanthin (~582 nm), ketospirilloxanthin (~565 nm), and ketoanhydrohodovibrin (~550 nm).

The absorption spectra of the individual carotenoids can be used along with the percent contributions from HPLC (Table 1) to accurately reconstruct (simulate) the observed spectra of both LH2 complexes containing only single carotenoid [8,25], the positions of (0,0) absorption bands of the non-ketolated carotenoids are expected to be as follows: spirilloxanthin (~550 nm), anhydrohodovibrin and didehydrohodopin (~536 nm), lycopene (~516 nm), and neurosporene (~491 nm). The absorption spectra of keto-bearing counterparts (obtained from HPLC) show that introduction of one terminal C=O group onto a C=C backbone shifts the absorption spectrum by 500 cm\(^{-1}\) to lower energy. Therefore, the spectral positions of keto-bearing carotenoids in LH2 from the semi-aerobically grown bacterium are predicted to be as follows: diketospirilloxanthin (~582 nm), ketospirilloxanthin (~565 nm), and ketoanhydrohodovibrin (~550 nm).

The absorption spectra of the individual carotenoids can be used along with the percent contributions from HPLC (Table 1) to accurately reconstruct (simulate) the observed spectra of both LH2 complexes (Fig. 2C and D). The fidelity of the fits was judged by the difference between the raw LH2 absorption spectrum and the fitted trace, which should mimic the absorption spectrum of the BChl \( a \) content, which can be seen independently from LH2 that naturally lacks carotenoids [26]. Using the same assumption that in LH2 the peak molar absorptivity \( \varepsilon_{\text{molar}} \) is the same for each carotenoid, the percent contribution of each pigment to the total composition based on the spectral reconstruction can be calculated. These values along with the wavelength positions of the (0,0) vibronic bands used are listed in the last four columns of Table 1. The agreement with the carotenoid compositions obtained from the HPLC analysis shown in columns 5 and 6 of the table are excellent. These comparisons demonstrate consistency in the analysis of the carotenoid content of the LH2 complexes under study.
3.3. RR spectra of carotenoids in LH2 and organic media

RR spectra of the isolated carotenoids in THF solutions were obtained for comparison with results for the LH2 complexes (Fig. 3). The \( \nu_1 \) vibrational energies of the carotenoids in solution are generally consistent with an all-trans conformation, in keeping with the HPLC data. The \( \nu_1 \) energies generally track the conjugation length [27–29]. This point is illustrated by the RR spectra of anhydrorhodovibrin (\( N = 12 \)), spirilloxanthin (\( N = 13 \)), ketoanhydrorhodovibrin (\( N = 13 \)), ketospirilloxanthin, (\( N = 14 \)) and diketospirilloxanthin (\( N = 15 \)) for which \( \nu_1 \) is found at 1509, 1507, 1507, 1505 and 1501 cm\(^{-1}\), respectively.

Fig. 3 also shows RR spectra of the LH2 complexes from the \( \textit{Rba. sphaeroides} \) strain grown anaerobically (blue) and semi-aerobically (magenta). The breadth of the spectra is consistent with contributions of mixtures of carotenoids. Visually, the general nature of the required mixtures of individual spectra (based on the solution RR data) are consistent with the pigment content (Table 1) and Fig. 2.

![Fig. 2](image.png)

**Table 1**

Carotenoid content in LH2 from \( \textit{Rba. sphaeroides} \) strain isolated and purified as described in the Materials and methods section. See Figure 51.

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>( N )</th>
<th>( N_{C_C} )</th>
<th>( N_{C_O} )</th>
<th>HPLC analysis (^a)</th>
<th>Spectral reconstruction analysis (^a)</th>
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<td>Neurosporene</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td>4%</td>
<td>5%</td>
</tr>
<tr>
<td>Lycopene</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td>13%</td>
<td>15%</td>
</tr>
<tr>
<td>Didehydroxanthin</td>
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<td>12</td>
<td>0</td>
<td>8%</td>
<td>6%</td>
</tr>
<tr>
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<tr>
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<td>60%</td>
<td>4%</td>
</tr>
<tr>
<td>Ketoanhydrorhodovibrin</td>
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<td>13</td>
<td>1</td>
<td>28%</td>
<td>16%</td>
</tr>
<tr>
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<td>12%</td>
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<td>13</td>
<td>2</td>
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</tr>
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</table>

\(^a\) Strains grown photosynthetically (PS) or semi-aerobically (SA) and LH2 isolated and purified as described in the Materials and methods section.

\(^b\) See Figure S1.

\(^c\) Performed using the simplifying assumption that the carotenoids all have the same peak extinction of the (0,0) band.

\(^d\) The total number of double bonds (\( C\_C \) and \( C\_O \)) in the conjugated \( \pi \)-electron system.

\(^e\) Wavelength for the (0,0) band returned from the spectral reconstruction for LH2 from the photosynthetically grown strain.

\(^f\) Wavelength for the (0,0) band returned from the spectral reconstruction for LH2 from the semi-aerobically grown strain.

\(^g\) Combined value for the two carotenoids.

Fig. 2. (A, B) Steady-state absorption spectra of the LH2 complexes containing spirilloxanthin and diketospirilloxanthin (along with other carotenoids) recorded in buffer at RT and in the glycerol/buffer glass at 77 K. The spectra are normalized at the B850 band. (C, D) Spectral reconstruction of the carotenoid absorption contour of LH2 at RT. The actual positions of the absorption spectra of carotenoids show minimal deviation from those expected (see Table 1 for details). The dotted line represents the raw absorption of the LH2. The solid black line is obtained from subtraction of the sum of carotenoid features from the raw LH2 absorption and resembles the anticipated absorption spectrum of BChl a.
expected relative absorption at the 532 nm excitation wavelength seen from the optical spectral reconstructions (Fig. 2C and D). For example, for LH2 from the strain grown anaerobically, the RR spectrum can be understood as deriving from contributions from anhydrorhodovibrin and spirilloxanthin (with a small shift to lower cm$^{-1}$ from the position in THF). In turn, the RR spectrum of LH2 from the strain grown semi-aerobically can be understood in terms of a mixture of ketoanhydrorhodovibrin, ketospirilloxanthin, and diketospirilloxanthin, with perhaps a small contribution from other carotenoids present (Table 1). Spectral fits were not performed because the inclusion of resonance energy denominators (including RR and absorption positions of the individual carotenoids in LH2) entails such a large number of parameters.

3.4. Steady-state and TA studies of long-chain keto-bearing carotenoids

3.4.1. Absorption spectra of diketospirilloxanthin in organic media at RT and 77 K

Steady-state absorption spectra of diketospirilloxanthin are given in Fig. 4. In n-hex the (0,0) vibronic band of the $S_0(1^1A_g^-) \rightarrow S_2(1^1B_u^+)$ transition is at 554 nm (18,050 cm$^{-1}$) and the absorption spectrum peaks at the 518 nm (1,0) vibronic band. The ratio of (0,0) and (1,0) bands is 0.8. A similar spectrum but with small bathochromic shifts is observed upon changing the solvent to the more polarizable and polar 2-MTHF. Upon freezing to 77 K in 2-MTHF, the spectral features are further bathochromically shifted such that the (0–0) vibronic band is at 602 nm (16,600 cm$^{-1}$). The vibronic resolution is also enhanced at 77 K.

The temperature induced spectral shift is caused by the large polarizability of 2-MTHF 77 K glass (versus at RT for 2-MTHF and n-hex) that energetically stabilizes the $S_2(1^1B_u^+)$ state. The improved vibronic resolution allows a more precise estimate of the spacing of the absorption bands. In general, the vibronic structure of the $S_0(1^1A_g^-) \rightarrow S_2(1^1B_u^+)$ absorption contour of carotenoids can be modeled as combinations of the progressions of two vibrational modes with energies of ~1200 cm$^{-1}$ (totally symmetric C–C stretch) and ~1600 cm$^{-1}$ (totally symmetric C=C stretch) [30]. The spacing between successive pairs of bands in the 77 K absorption spectrum of diketospirilloxanthin (Fig. 1) is ~1280 cm$^{-1}$, which suggests that the progression is dominated by the totally symmetric C–C stretching vibration.

3.4.2. TA studies of long-chain keto-bearing carotenoids in organic solvents

Fig. 5 panels A–F show TA data for diketospirilloxanthin in n-hex at RT. The left stack of panels (A–C) show data in the visible (VIS) region and the right stack of panels (D–F) in the NIR region. The top row of panels (A, D) displays contour plots of the TA data. The middle row of panels (B, E) shows spectra obtained from global analysis (EADS), which employed four components (exponential) in the VIS and one or two in the NIR because some states/processes contribute significantly to the former and not the latter. The bottom row of panels (C, F) shows representative kinetic profiles and fits using the time constants derived from global analysis, normalized to the amplitude at t = 0. Analogous data for diketospirilloxanthin in 2-MeTHF at 77 K are shown in Figure S1. Similar data were acquired in n-hex at RT for the two keto-bearing carotenoids with shorter conjugation lengths. The results for ketospirilloxanthin are shown in Fig. 5 panels G–L and those for ketoanhydrorhodovibrin in Fig. 5 panels M–R.

In the following, the data for diketospirilloxanthin ($N = 15, N_{C=C} = 13, N_{C=O} = 2$) at the two temperatures are discussed first, followed by the main differences for ketospirilloxanthin ($N = 14, N_{C=C} = 13, N_{C=O} = 1$) and ketoanhydrorhodovibrin ($N = 13, N_{C=C} = 12, N_{C=O} = 1$). The key issues are the lifetime and spectral positions (energies) of the transient features associated with the $S_0(1^1A_g^-)$ and $S_1(1^1A_g^-)$ excited states, because these two states are donors for energy transfer...
Fig. 5. Transient absorption results for keto-bearing carotenoids diketospirilloxanthin (A–F), ketospirilloxanthin (G–L) and ketoanhydrorhodovibrin (M–R) in n-hex at RT in the VIS region (A–C, G–I, M–O) and NIR region (B–E, J–P, R) upon excitation at 560 nm (A–F), 550 nm (G–I), or 540 nm (M–R). The top panels show 2D contours, middle panels results of global analysis (EADS) of the data sets, and bottom panels representative kinetic profiles (symbols) and multi-exponential fits using the time constants obtained from global analysis (solid lines), normalized to the same amplitude at t = 0. The dynamics in (C, I, O) shows decay of the S1 (21Ag+) state probed at the S1 (21Ag+) → S0 ESA peak on the short- or long-wavelength-wavelength edge. The profiles in (F, L, R) are for decay of the S1 (11Ag+) state (1180, 1210, 1190 nm) and the S1 (21Ag+) state (1515, 1400, 1450 nm). To enhance visibility of the NIR contours, the ΔA magnitude is shown in logarithmic scale in figures D, J and P.

The spectral changes associated with the S2 (11Bu+) excited state for diketospirilloxanthin and its decay at RT are given by the first EADS in Fig. 5B (VIS) and Fig. 5E (NIR); the data at 77 K are given in Figure S1. The S2 (11Bu+) lifetime is at or below the width of the instrument response function (IRF; 150 fs for VIS and 200 fs for NIR). The spectral signatures of this state are as expected: The spectrum in the VIS includes combined bleaching of the S0 (11Ag+) → S2 (11Bu+) ground-state absorption profile (GSB) and stimulated S2 (11Bu+) → S0 (11Ag+) emission (with shape somewhat obscured by solvent-response features during the ~100 fs excitation flash). The spectrum in the NIR includes a distinct band at ~1200 nm due to S2 (11Bu+) → S0 absorption, in which n stands for a higher excited state with Ag-like symmetry [31].

The spectral changes associated with the S1 (11Ag+) excited state and its decay for diketospirilloxanthin are given by the second EADS in Fig. 5B and E (RT) and Figure S1-B and S1-E (77 K). The S1 (11Ag+) lifetime is 800 fs at RT [average of 790 fs (VIS) and 810 fs (NIR)] and lengthens to 1.1 ps at 77 K [average of 1.2 ps (VIS) and 1.0 ps (NIR)]. The spectral signatures (EADS) associated with this state are as expected: The spectrum in the VIS includes a negative feature due to GSB of the S1 (21Ag+) → S0 (11Ag+) → S1 (21Ag+) ESA band at ~1200 nm due to S2 (21Ag+) → S0 (11Ag+) → S1 (21Ag+) ESA band [34–36]. The ESA band has a maximum at ~1400 nm at RT and shifts bathochromically so much at 77 K that part of the profile is past the NIR probe limit of 1600 nm (Figures S1-D and S1-E).

The impact of cryogenic temperature on the ground- and excited-state absorption features is noteworthy because the spectral positions provide an estimate of the energy of the S1 (21Ag+) excited state (vide infra). The bathochromic shift of the S1 (21Ag+) → S0 ESA band from 650 nm at RT to 700 nm at 77 K (i.e., a reduced energy gap between the states) derives from the large polarizability of 2-MTHF glass that energetically stabilizes the S0 state(s). A similar stabilization of the S2 (11Bu+) state underlies the bathochromic shift (554 to 602 nm) of the S0 (11Ag+) → S2 (11Bu+) profile in the ground-state absorption spectrum (Fig. 1) and the main S1 (21Ag+) → S0 (11Ag+) → S1 (21Ag+) feature (~1400 nm to >1600 nm) in the ESA spectrum (Figs. 5E and S1-E).

The origin of the two additional EADS components (1.7 and 6.2 ps at RT; 1.6 and 5.6 ps at 77 K) in the VIS data sets for diketospirilloxanthin (Figs. 5B and S1-B) is not clear. Previous TA studies of carotenoids with long conjugation lengths showed similar spectral/kinetic features that were assigned to undefined excited state(s) denoted S*, which were first observed in long-chain analogues of β-carotene [37]. It has been hypothesized that S* is a vibrationally non-equilibrated ground state [37,38], a distinct electronic state in the energy vicinity of S1 (11Ag+) [39], or the S1 (11Ag+) itself but with a twisted configuration [35,40].

The TA for ketospirilloxanthin (Fig. 5 panels G–L) and ketoanhydrorhodovibrin (Fig. 5 panels M–R) in n-hex at RT can be similarly analyzed. The key differences from diketospirilloxanthin are two-fold. One is a lengthening of the S1 lifetime in n-hex at RT with a decrease in the number of double bonds in the carotenoid conjugation length: from 0.8 ps for diketospirilloxanthin (N = 15,
Figs. 4, 5 and S1 provide the means to extend analysis of the S1 ener-
path (Fig. 2E, K, Q). This trend translates to a shift in the energy of the
feature in the NIR TA data to a longer wavelength (lower energy) with
S1(21Ag)
carotenoids is a trend of the position of the S1(21Ag
at RT[39,41]. The second major difference for the three keto-bearing
excited-state absorption [S1(21Ag
N
enoids from purple bacteria from studies at 77 K[15,42]. Such energies
determined previously for numerous non-carbonyl open-chain carot-
spirospirilloxanthin and diketospirilloxanthin because transition
ges to both longer conjugation lengths and additional keto-bearing ca-
For diketospirilloxanthin, following conversion of wavelengths to

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<th>N_Od</th>
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<th>S2 (cm⁻¹)</th>
<th>τ_S1 (ps)</th>
<th>System</th>
<th>Φ_Car → BChl (%)</th>
<th>Temp</th>
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|                        | a n.d. — not determined, EPA — (diethyl ether/isopentane/ethanol, 5/5/2, v/v/v), v.s. — various solvents.
b Carotenoid-to-BChl energy-transfer yield obtained from comparison of 1−T and fluorescence excitation profiles. The values for the new set of engineered Rba. sphaeroides mutants obtained in the initial study[17] are as follows: (Φ_Car → BChl): neurosporene (94%, 9), spheroidene (96%, 10), spheroidenone (95%, 11), lycopene (64%, 11), rhodopin (62%, 11), spirilloxanthin (39%, 13), and diketospirilloxanthin (35%, 15).

3.4.3. The S2(21Ag) state energy of long-chain keto-bearing carotenoids in organic media

The energies of the S1(21Ag) and S2(11Bu⁺) states have been
determined previously for numerous non-carbonyl open-chain carot-
}
strain grown semi-aerobically. The fluorescence spectra (dashed black) were obtained using excitation of the BChls at 590 nm; spectra with the same shapes (but different amplitudes) were obtained at other excitation wavelengths, including exciting the carotenoid(s) in the 450–580 nm range. Fluorescence-excitation spectra (red) were obtained by monitoring the emission from the B850 BChls at 850 nm and scanning the excitation wavelengths between 350 and 1000 nm. The fluorescence-excitation spectra and absorptance (1 − T, where T is transmittance) spectra (blue) were normalized at 850 nm.

Fig. 7 shows the value of $\Phi_{\text{Car} \rightarrow \text{BChl}}$ obtained by measuring the amplitude ratio of the fluorescence-excitation versus absorptance spectra ratio as a function of wavelength across the carotenoid absorption region. The figure also shows the (0,0) positions of the carotenoids that contribute substantially to the content of one or both of the LH2 complexes (from strains grown anaerobically or semi-aerobically). As shown in Fig. 2C and D for each carotenoid, relative to the (0,0) band the absorption-contour maximum at the (1,0) band is tens of nanometers to shorter wavelengths and the entire contour spans over 100 nm to shorter wavelengths. Thus the various carotenoids present (Table 1) contribute broadly across the region shown in Fig. 7C.

Examination of the spectral reconstructions for the two LH2 samples (Fig. 2C and D) indicates that 540 nm is a reasonable compromise wavelength to obtain a yield value that has the least contributions from (1) shorter carotenoids (N < 13) that may be present (Table 1) and absorb at shorter wavelengths, and (2) direct excitation of B800 and B850 BChls in their Q$_o$ band that peaks to longer wavelengths (~590 nm). A
contribution of either such species may shift the average $\Phi_{\text{Car} \rightarrow \text{BChl}}$ measured by excitation of a pigment mixture at a given wavelength to values larger than may be correct for individual or mixtures of carotenoids with $N = 13-15$. For example, the small increase in apparent $\Phi_{\text{Car} \rightarrow \text{BChl}}$ in progressing from 540 to 550 to 560 nm in Fig. 7C may arise from a contribution from (direct excitation in) the short-wavelength tail of the BChl $Q_b$ band. Nonetheless, the data in Fig. 7C suggests that the $\Phi_{\text{Car} \rightarrow \text{BChl}}$ at 540 nm will give a good average for the key contributing carotenoids.

In this way, $\Phi_{\text{Car} \rightarrow \text{BChl}} = 0.41$ was determined for the LH2 from the *Rba. sphaeroides* crtI:*crtIPa* strain grown photosynthetically and basically the same value (0.42) was found for LH2 from the same strain semi-aerobically. Assessing these values in terms of the contributing carotenoids can be placed in a larger context by drawing up the analogous data shown in Fig. 7D. Those data were obtained from the earlier study of LH2 complexes derived from the same *Rba. sphaeroides* strain but using different growth batches (and purification protocols) and thus possessing different proportions of carotenoids.

The curves for $\Phi_{\text{Car} \rightarrow \text{BChl}}$ versus excitation wavelength for LH2 from the anaerobically strain studied here (Fig. 7C) are quite similar to those from the prior study (Fig. 7D), although perhaps shifted to slightly higher average values (e.g., 0.41 versus 0.40 at 540 nm and 0.40 versus 0.39 on the average at shorter wavelengths). Such a difference (if real) could be accounted for by the slightly lower (60%) spirilloxanthin $(N = 13)$ in the sample studied here (Table 1) [along with 15% anhydrorhodovibrin $(N = 12)$ and 8% lycopene $(N = 11)$] compared to (70%) spirilloxanthin in the sample studied previously [along with 13% anhydrorhodovibrin and 9% lycopene] [17].

The $\Phi_{\text{Car} \rightarrow \text{BChl}}$ levels are somewhat more different for LH2 obtained from the *Rba. sphaeroides* crtI:*crtIPa* strain grown semi-aerobically and studied here compared to that studied previously. In particular the $\Phi_{\text{Car} \rightarrow \text{BChl}}$ Value using 540 nm excitation obtained here (0.42, Fig. 7C) is greater than the value from the prior study [17] (0.33, Fig. 7D). The average values encompassing shorter-wavelength excitation are 0.44 versus 0.35. Such modest differences are consistent with the altered ratios of the three key keto-bearing carotenoids $(N = 13-15)$ and shorter $(N < 13)$ carotenoids. In particular, the LH2 complexes isolated previously from semi-aerobically grown cells had 62% diketospirilloxanthin $(N = 13)$, 16% ketospirilloxanthin $(N = 14)$ and 14% ketoanhydvorhodovibrin $(N = 13)$ and only very small amounts of shorter carotenoids such as anhydvorhodovibrin $(N = 12)$ or lycopene $(N = 11)$ [17].

The LH2 used for the present work had respectively 28%, 16% and 21% of the three keto-bearing carotenoids along with somewhat larger amounts of anhydvorhodovibrin (7%), dihydroanhydvorhodovibrin (6%) and lycopene (15%). The small amounts of these $N = 11-12$ carotenoids (and ratios of keto-bearing carotenoids) can easily account for the small increase in mean energy-transfer yield (0.44 versus 0.35) between the two samples when integrating over the entire absorption contour. Such a difference is truly modest when placed in the overall landscape of $\Phi_{\text{Car} \rightarrow \text{BChl}}$ values in LH2 complexes, including >90% typical for $N \leq 11$ carotenoids (Table 2) as noted in the Introduction and considered further below. The collective results speak to the low inherent $\Phi_{\text{Car} \rightarrow \text{BChl}}$ values for individual $N \geq 13$ carotenoids.

3.5.2. TA studies of energy flow in spirilloxanthin-containing LH2

Fig. 8 shows TA results for LH2 complexes from the photosynthetically grown *Rba. sphaeroides* crtI:*crtIPa* strain acquired at RT, and Figure S3 shows analogous data obtained at 77 K. This LH2 contains spirilloxanthin (60%) as the primary carotenoid, along with a lesser amount of anhydvorhodovibrin (15%) and still smaller amounts of shorter carotenoids (Table 1). The excitation wavelength (550 or 555 nm) was chosen to selectively excite spirilloxanthin, and no signals that can be easily associated with anhydvorhodovibrin are observed. In Fig. 8, the left panels are for the VIS region and the right panels for the NIR. The top panels give TA spectra at representative times, the middle panels give the results of global analysis (EADS) and the bottom panels representative kinetic profiles and fits.

The spectra at the earliest times and the associated first EADS, which evolves within the instrument response function (~150 fs VIS, ~200 fs NIR), has characteristics expected for the $S_2(2^1A_g)$ state of spirilloxanthin and its decay and include GSB for the carotenoid absorption profile in the VIS and $S_1(1^3B_{b'})$ state of spirilloxanthin probed at the maximum of the $S_1(2^1A_g) \rightarrow S_0$ ESA band (620 nm) and the initial rise of the bleaching and slow recovery of the B850 excited state. The EADS also shows simultaneous bleaching of both the B800 and B850 bands and a contribution of ESA in the VIS and NIR from the excited states of these BChl $a$ species (B800* and B850*). The finding of BChl-associated features at these earliest times reflects ultrafast (~150 fs) energy transfer from the $S_0(1^3B_{b'})$ state of spirilloxanthin to both BChl $a$ classes. The spirilloxanthin–to-BChl energy-transfer process may be less efficient at 77 K than at RT: the bleachings of the B800 and B850 bands are substantially smaller in relation to GSB of spirilloxanthin observed at RT. This effect is most likely associated with reduced energy transfer from the $S_0(1^3B_{b'})$ state due to reduced spectral overlap of fluorescence from the carotenoid $S_0(1^3B_{b'})$ state and the BChl Q absorption as the bands narrow at low temperature. Once energy arrives at B800, the 800-nm bleaching persists for a couple of picoseconds and then...
disappears as B800$^\rightarrow$ B850 energy transfer occurs, in concert with direct transfer from the carotenoid S$_1$(1$^1$Bu$^+$) to B850.

The spirilloxanthin S$_1$(2$^1$Ag$^+$) excited state and its decay apparently primarily underpin the second EADS (red spectra in Fig. 8C and D). This assessment based on the similarity of the spectral shape to that obtained for the isolated carotenoid in various solvents and at various temperatures [35,39,41–45]. This includes a sharp positive S$_1$(2$^1$Ag$^+$) → S$_0$ ESA band at 620 nm at RT at 624 nm at 77 K. The state decays with a time constant at RT of 1.4 ps (squares and blue curve for decay of GSB and circles and red curve for decay of S$_1$(2$^1$Ag$^+$) → S$_0$ ESA in Fig. 8E). The lifetime of 1.4 ps is practically identical to values obtained from prior TA studies of spirilloxanthin in solution. This finding shows that the S$_1$(2$^1$Ag$^+$) state is not involved in energy transfer to the B800 or B850 BChl α [35,39,41–45]. The S$_1$(2$^1$Ag$^+$) lifetime lengthens slightly to 1.6 ps at 77 K (Figure S3), as observed previously for spirilloxanthin in solution [35,42].

The 8.3 ps EADS seen in the VIS (Fig. 8B) has some characteristic similarities of those of the so-called carotenoid S$^*$ state (vide supra). It has been suggested that the S$^*$ state has properties that differ in LH2 versus organic media and is a precursor of the carotenoid triplet state [8,39,46,74]. The 28 ps EADS in the NIR data set that does not contribute significantly in the VIS may reflect decay of the spirilloxanthin radical cation formed in low yield via carotenoid–B800(BChl) interactions, as reported for numerous LH2 complexes [8,48,49].

The spectral shape of the EADS with an ~800 ps time evolution at RT [740 ps in VIS (Fig. 8C) and 860 ps in NIR (Fig. 8D)] is related to decay of B850$^*$, the lowest singlet excited state of the excitonically coupled BChls. The B850$^*$ lifetime at 77 K lengthens to ~1100 ps (1140 ps in VIS and 1050 ps in NIR; Figure S3). In addition, the profile for B850 ground-state bleaching plus B850$^*$ stimulated emission is broader at 77 K than at RT. This effect may arise from a larger absorption–emission Stokes shift at low temperature, giving less overlap of the two contributions than at RT.

The longest “infinite” EADS (Fig. 8D) has a very small amplitude but has ESA characteristics expected for the spirilloxanthin triplet excited state. This state in LH2 is thought to form via direct quenching of BChl α species. The same species, along with lower N-value carotenoids including anhydrorhodovibrin (Table 1), shows with the LH2 that contains spirilloxanthin (Fig. 2D). A lifetime 4.4 ps corresponds well to the B850$^*$ state(s) of the contributing long-chain keto-bearing carotenoids (circles and blue-line fit at 520 nm) along with lower N-value carotenoids including anhydrorhodovibrin (Table 1). The 542 nm excitation wavelength will excite all of these carotenoids to some extent, as indicated from the absorption spectral reconstruction (Fig. 2D). Thus, it might be expected that this LH2 shows more complex kinetic behavior than for LH2 containing a single or predominant carotenoid. However, apparently the spectral and temporal overlap of the features associated with the S$_1$(2$^1$Ag$^+$) state of the three keto-bearing carotenoids blend into average characteristics.

The raw spectra and EADS in Figs. 9 and S4 show features analogous to those described above for the LH2 from the same strain grown anaerobically and containing primarily spirilloxanthin (Figs. 8 and S3). The features are associated with the GSB and ESA of carotenoids and with bleaching of the B800 and B850 bands. The evolution of the carotenoid S$_2$(1$^1$Bu$^+$) dominates the spectral and time evolution during the IRF, including rapid energy flow to the B800 and B850 BChl α species. The EADS with a 1.1–1.2 ps time evolution shows a broad carotenoid GSB profile that does not match the ground-state profile of any individual carotenoid. The spectrum also contains a somewhat broad ESA feature near 650 nm (Fig. 9C, red) that remains broad at 77 K although the time constant increases to 1.8 ps (Figure S4). This EADS likely reflects the combined S$_1$(2$^1$Ag$^+$) excited states two or all three of keto-bearing diketospirilloxanthin, ketospirilloxanthin, ketoanhydrorhodovibrin and the observed time constant is an average S$_1$(2$^1$Ag$^+$) lifetime (1.2 ps at RT, 1.8 ps at 77 K).

The next EADS observed at RT with a 4.4 ps lifetime has a much smaller amplitude than the 1.1 ps EADS (green versus red spectra in Fig. 9C), and is absent at 77 K when the excitation wavelength is shifted from 542 to 560 nm. The shape of this minor EADS resembles the vibronic bands at 535 and 500 nm expected for anhydrorhodovibrin/didehydrorhodopin (Fig. 2D). A lifetime 4.4 ps corresponds well to the S$_1$(2$^1$Ag$^+$) lifetime of anhydrorhodovibrin measured in organic media [35].

The kinetic traces for decay of bleaching of carotenoid, B800 and B850 ground-state absorption bands (normalized at the t = 0 amplitude) are given in Fig. 9E. It is clear that the decay of the S$_2$(1$^1$Bu$^+$) state(s) of the contributing long-chain keto-bearing carotenoids (circles and blue-line fit at 520 nm) does not induce additional bleaching of the B850 band (triangles and red-line fit at 854 nm). The rise of the B850 bleaching is coupled to decay of the B800 band (triangles and black-line fit) as energy flows rapidly from B800$^*$ to B850. Thus, by analogy with the LH2 that contains spirilloxanthin (N = 13) the S$_1$(2$^1$Ag$^+$) state of keto-bearing carotenoids (ketoanhydrorhodovibrin (N = 13),
ketospirilloxanthin \((N = 14)\) and diketospirilloxanthin \((N = 15)\) does not participate in energy transfer to the BChl a molecules.

Once formed via energy transfer from carotenoid \(S_2(1^1B_u)\) and B800\(^*\), the B850\(^*\) state of the excitonically coupled BChls in LH2 from semi-aerobically grown \(Rba.\ sphaeroides\) \(crt::crt\) decays with a time constant at RT of \(-720\) ps [740 ps VIS (Fig. 9C) and 690 ps NIR (Fig. 9D)], lengthening to 1.3 ps at 77 K (Figure S4). The decay time at both temperatures is comparable to that found above for the LH2 from semi-aerobically grown \(Rba.\ sphaeroides\) \(crt::crt\) that contains primarily spirilloxanthin (Figs. 8 and S3).

### 3.5.4. The \(S_1(2^1A_g)\) energy of spirilloxanthin and long-chain keto-bearing carotenoids in LH2

The TA data presented herein for the LH2 complexes from the \(Rba.\ sphaeroides\) \(crt::crt\) strain grown anaerobically, which contains primarily spirilloxanthin \((N = 13)\) and grown semi-aerobically, which contains a mixture of keto-bearing long-chain analogues [ketoanhydrorhodovibrin \((N = 13)\), ketospirilloxanthin \((N = 14)\) and diketospirilloxanthin \((N = 15)\)], affords a means to estimate the energy of the \(S_1(2^1A_g)\) states of these carotenoids and compare with the results for the isolated carotenoids in organic media. A prior such comparison showed that the \(S_1(2^1A_g)\) energy for spirilloxanthin in LH1 is the same as in solution [41], and thus that the protein environment does not alter the structural/electronic characteristics of this carotenoid. However, such a comparison has not been made for spirilloxanthin in LH2, or for the long-chain keto-bearing carotenoids.

The energy of the \(S_1(2^1A_g)\) state for LH2-bound carotenoids for \(Rba.\ sphaeroides\) \(crt::crt\) strain grown anaerobically and semi-aerobically was calculated from spectroscopic data using the same methodology described above for isolated diketospirilloxanthin, ketospirilloxanthin and ketoanhydrorhodovibrin in \(n\)-hex at RT (Fig. 6) and diketospirilloxanthin at 77 K (Figure S2). The results are presented in Fig. 10.

Before spectral matching, the NIR TA spectrum with the \(S_1(2^1A_g)\) \(\rightarrow S_2(1^1B_u)\) ESA band present was corrected for overlap with the ESA band of the long-lived B850\(^*\) excited state. The latter was taken from the TA spectrum recorded at later delay times, after complete decay of the carotenoid \(S_1(2^1A_g)\) state. The resulting corrected \(S_1(2^1A_g) \rightarrow S_2(1^1B_u)\) ESA profiles are the red traces in Fig. 10. The solid black traces are the observed \(S_1(2^1A_g) \rightarrow S_0(1^1B_u)\) steady-state absorption profiles of the LH2, which includes contributions from all carotenoids present as well as the Qo absorption band of the B800 and B850 BChls \((-17,000 \text{ cm}^{-1})\). The dashed black spectrum in Fig. 10A is the steady-state absorption of spirilloxanthin obtained from spectral reconstruction of the carotenoid contour in that LH2 (anaerobic growth) (Fig. 2C). The dashed spectrum in Fig. 10B reflects a mixture of the steady-state absorption spectra diketospirilloxanthin, ketospirilloxanthin and ketoanhydrorhodovibrin (Fig. 2D) with contributions given by their percent contribution (Table 1) to the total carotenoid content of that LH2 (semi-anaerobic growth). In both cases, the carotenoid contour chosen should reflect the major carotenoids elevated to the excited state by the excitation flash in the TA experiments that afford the \(S_1(2^1A_g) \rightarrow S_2(1^1B_u)\) ESA profile. Because of the mixture in the semi-aerobically grown strain, there is necessarily some uncertainty in matching the \(S_1(2^1A_g) \rightarrow S_2(1^1B_u)\) ESA profile with the \(S_0(1^1B_u)\) vibronic contours to obtain the \(S_0(1^1B_u)\) energy. Additionally, the higher-energy side of the ESA profile may be enhanced by absorption of carotenoid cation present on the same time scale as the \(S_2(1^1B_u)\) state, resulting in poor alignment on the higher energy portion of the contours. Although these factors may affect the \(S_0(1^1B_u)\) energies derived, they do not compromise the overall conclusions.

The derived \(S_1(2^1A_g)\) state energy of 11,300 \text{ cm}^{-1} for spirilloxanthin in LH2 (Fig. 10A) is practically the same as in organic solvents (Table 2); thus the effective conjugation length is not affected by protein binding pocket. The derived \(S_1(2^1A_g)\) state energy of 11,300 \text{ cm}^{-1} for the mixture of diketospirilloxanthin, ketospirilloxanthin and ketoanhydrorhodovibrin in LH2 (Fig. 10B) is comparable to the average value for the three keto-bearing carotenoids in \(n\)-hex (Fig. 6). The implications of these findings for the functional characteristics of these carotenoids in the LH2 antennas are given in the Discussion section.

### 4. Discussion

The rates, efficiencies and mechanisms of Car \(\rightarrow\) BChl energy transfer in LH2 complexes from a variety of bacterial species have been studied extensively by static and time-resolved absorption spectroscopy, as noted in the Introduction. The carotenoid content of LH2 was varied by choice of bacterial strain, growth conditions and reconstitution into carotenoid-free LH2 complexes. Such studies have primarily involved LH2 containing carotenoids with conjugation lengths up to \(N = 11\), for which spheroidenone \(\left(N_{C=C} = 10, N_{C=O} = 1\right)\) is the prime keto-bearing example. The photophysical properties of such carotenoids

![Fig. 10. Analysis of the \(S_1(2^1A_g)\) energy from data acquired at RT for LH2 complexes from \(Rba.\ sphaeroides\) \(crt::crt\) grown anaerobically (A) and containing primarily spirilloxanthin, and grown semi-aerobically and containing a mixture of diketospirilloxanthin, ketospirilloxanthin and ketoanhydrorhodovibrin (B). The solid black spectrum in each panel is the observed steady-state \(S_1(2^1A_g) \rightarrow S_2(1^1B_u)\) absorption spectrum. The \(S_1(2^1A_g) \rightarrow S_2(1^1B_u)\) TA spectra (red) were extracted from the NIR TA datasets at delay times at which contribution from the \(S_1(2^1A_g) \rightarrow S_0(1^1B_u)\) ESA is negligible and shifted in energy to give best coincidence with steady-state absorption of the contributing carotenoid(s) in that LH2 (dashed lines). The latter utilized the spectra obtained from the spectral reconstructions in Fig. 2C and D. For panel A, the dashed spectrum is that for spirilloxanthin, the predominant carotenoid. For panel B, the dashed spectrum reflects a mixture of diketospirilloxanthin, ketospirilloxanthin and ketoanhydrorhodovibrin in proportion to percentage of the LH2 content (Table 1). The magnitude of the energy shift of the TA spectra determines the energy of the \(S_1(2^1A_g)\) state of the carotenoids.](image-url)
(including spirilloxanthin, \( N = 13 \)) also have been studied in organic solvents with a range of polarities and polarizabilities at RT and 77 K [8,35,50–52]. Table 2 collects the excited-state lifetimes and energies from some of the prior work, along with the data obtained herein. The present results on spirilloxanthin (\( N = 13 \)) and long-chain keto-bearing carotenoids ketoanhydrorhodovibrin, ketospirilloxanthin and diketospirilloxanthin (\( N = 12–15 \)) in LH2 and organic media add to the large body of prior work by many groups. Key findings and comparisons are highlighted in the following.

The recent study by Chi et al. [17] introduced a series of genetically engineered \( \textit{Rba. sphaeroides} \) strains that produce LH2 (and LH1) housing carotenoids from \( N = 9 \) to 15. The LH2 from some strains contain primarily one carotenoid while others afford mixtures with ratios that depend on growth conditions. This is not surprising for purple sulfur bacteria that employ the spirilloxanthin biosynthesis pathway. The LH2 sample studied here from \( \textit{Rba. sphaeroides} \text{crtI::crtIPa} \) grown anaerobically contains 60% spirilloxanthin (Table 1), which is similar to the 70% obtained for a different growth batch from the initial study [12]. In both studies, \( \Phi_{\text{car} \rightarrow \text{Bchl}} \) is found when the excitation light is absorbed predominantly by spirilloxanthin or in part by other carotenoids present (Fig. 6).

The LH2 sample studied here from \( \textit{Rba. sphaeroides} \text{crtI::crtIPa} \) grown semi-aerobically contains a mixture of ketoanhydrorhodovibrin, ketospirilloxanthin and diketospirilloxanthin (Table 1) whereas LH2 from the prior study had 60% diketospirilloxanthin. Nonetheless, \( \Phi_{\text{car} \rightarrow \text{Bchl}} \) in the range -0.35 to -0.45 is found with differences consistent with the measured carotenoid ratios.

These results show that \( \Phi_{\text{car} \rightarrow \text{Bchl}} \) is -0.4 (or slightly lower) for \( N = 13 \) carotenoids anhydrorhodovibrin (\( N_{\text{car} \rightarrow \text{Bchl}} = 12, N_{\text{deg} \rightarrow \text{Bchl}} = 1 \)) and spirilloxanthin (\( N_{\text{car} \rightarrow \text{Bchl}} = 13, N_{\text{deg} \rightarrow \text{Bchl}} = 0 \)) and upon extension via incorporation of the terminal keto groups in ketospirilloxanthin (\( N_{\text{car} \rightarrow \text{Bchl}} = 13, N_{\text{deg} \rightarrow \text{Bchl}} = 1 \)) and diketospirilloxanthin (\( N_{\text{car} \rightarrow \text{Bchl}} = 13, N_{\text{deg} \rightarrow \text{Bchl}} = 2 \)). It is also found that the lifetime of the \( S_1(2^{1}A_g) \) excited state of spirilloxanthin in the anaerobically grown \( \textit{Rba. sphaeroides} \text{crtI::crtIPa} \) and the average \( S_1(2^{1}A_g) \) lifetime of the trio of keto-bearing long chain analogs in the semi-aerobically grown strain are similar to the values for the isolated carotenoids in organic media. The combined results suggest that the \( S_1(2^{1}A_g) \) excited state of the \( N \geq 13 \) carotenoids participates little if at all in energy transfer to the B800 or B850 BCHls. This conclusion supports the hypothesis from studies of carotenoids up to \( N = 11 \) that only carotenoids with \( N \leq 10 \) transfer energy via their \( S_1(2^{1}A_g) \) states to BChl a in LH2 to any significant degree [53].

The finding of modestly greater \( \Phi_{\text{car} \rightarrow \text{Bchl}} \) of -0.5 to -0.65 for \( N = 11 \) carotenoids (e.g., llycopene, rhodopin, rhodopin glucoside) [8,12,13,54] would then be consistent with the \( S_1(2^{1}A_g) \) pathway remaining partially active or that the yield (and rate constant) of the \( S_2(1^{1}B_u) \) pathway increases modestly as \( N \) decreases, and by extension contributing modestly more than the \( S_1(2^{1}A_g) \) pathway for the \( N = 9–11 \) carotenoids in LH2 antennas. The present results also bear on the finding that \( N = 11 \) spheroidene (\( N_{\text{deg} \rightarrow \text{Bchl}} = 10, N_{\text{car} \rightarrow \text{Bchl}} = 0 \)) has the high \( \Phi_{\text{car} \rightarrow \text{Bchl}} \) (0.90–0.95) of the shorter carotenoids (\( N = N_{\text{deg} \rightarrow \text{Bchl}} = 9–10 \)). In this molecule the normal \( S_1(2^{1}A_g) \) and \( S_2(1^{1}B_u) \) pathways may be augmented by participation of keto-associated excited states such as intramolecular charge-transfer (ICT) state [55] which is not formed in longer-chain (\( N \geq 11 \)) keto-bearing carotenoids as studied here: ketoanhydrorhodovibrin, ketospirilloxanthin and diketospirilloxanthin. It has also been suggested that change in symmetry engendered by addition of a keto group enhances the efficiency of energy transfer via the \( S_1(2^{1}A_g) \) pathway [56]. Regardless, such mechanisms do not provide such enhancements for the longer-chain keto-bearing carotenoids.

The simplest, unifying explanation for all the above-noted observations is if the \( S_1(2^{1}A_g) \) state for the \( N \geq 13 \) carotenoids (with or without keto groups) has dropped energetically near or below the B850° excited state of the excitonically coupled BChl a array, and thus energy transfer is ineffective on energetic grounds. This question was addressed here via TA studies to determine the energies of \( S_1(2^{1}A_g) \) and \( S_1(1^{1}B_u) \) excited states of these carotenoids in LH2 complexes from \( \textit{Rba. sphaeroides} \text{crtI::crtIPa} \) grown anaerobically or semi-aerobically and isolated in organic media. As noted above, prior studies have provided such information for numerous carotenoids without keto groups and for the keto-bearing spheroidene (\( N = 11 \)). The results are listed in Table 2 and shown graphically in Fig. 11. Notable findings are as follows.

(1) The energy of the \( S_1(2^{1}A_g) \) state for \( N \geq 13 \) carotenoids (e.g., spirilloxanthin, ketospirilloxanthin, diketospirilloxanthin) measured in \( n \)-hex at RT is close to or below that for B850° (11,780 cm\(^{-1}\), black dotted line in Fig. 11A). The same holds for these carotenoids when bound in LH2. As noted above, the \( S_1(2^{1}A_g) \) energy for spirilloxanthin in LH2 is the same in LH2 (anaerobically grown \( \textit{Rba. sphaeroides} \text{crtI::crtIPa} \)) as in organic

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**Fig. 11.** (A) The \( S_1(2^{1}A_g) \) and \( S_1(1^{1}B_u) \) state energies of two classes of open chain carotenoids found in LH2 complexes from either sulfur on non-sulfur purple bacteria: non-keto carotenoids (blue circles) ([spheroidenone (Spho), rhodopin glucoside (RdGlu), anhydrorhodovibrin (Anh), spirilloxanthin (Spex)]), and keto-bearing carotenoids (red triangles) ([spheroidenone (Spho), ketospirilloxanthin (kSpex), ketoanhydrorhodovibrin (kAnh), diketospirilloxanthin (dikSpex)]) obtained at RT (closed symbols and solid lines) and at 77 K (open symbols and dashed lines) as a function of the total number of conjugated double bonds (\( N \)). The energies of the \( S_1(1^{1}B_u) \) state originate from steady-state absorption spectra taken in \( n \)-hex (except for rhodopin glucoside that was measured in methanol) while the energies of the \( S_1(1^{1}B_u) \) state are obtained from measuring the \( S_1 \rightarrow S_2 \) energy gap by transient absorption in various solvents (see Table 2 for details). The dotted black horizontal line in (A) is the energy of the B850° excited state of the BChl a array in LH2 at RT. (B) The \( S_1(2^{1}A_g) \) state energy as a function of the \( S_1(1^{1}B_u) \) state energy. The data were fit with linear functions having equations given in the graph.
media, and the average value for ketoanhydrorhodovibrin, ketospirilloxanthin, diketospirilloxanthin (semi-aerobically grown Rba. sphaeroides crtI::crtIPa) is comparable to the average for the three individual carotenoids in n-heptane at RT.

(2) Freezing the medium at cryogenic temperature has relatively small impact on the $S_1(2^1A_g)$ state energy of the non-keto open-chain carotenoids.

(3) As expected based on prior work, the large polarizability of the 2-MTHF glass at cryogenic temperature downshifts the energy of $S_2(1^1B_u)$ for the non-keto carotenoids. However, the trend lines for the state energy versus 1/N at RT (n-heptane) and 77 K (2-MTHF) are not parallel and temperature-induced energetic shift becomes larger as N increases, as exemplified by the points for neurosporene and spirilloxanthin in Fig. 11A. Perhaps in addition to the increased medium polarizability in 2-MTHF at 77 K, torsional motions involving the terminal regions of the longer chains are reduced in the frozen medium, thereby slightly increasing the effective conjugation with the carbon–carbon backbone.

(4) The trend-line for the $S_2(1^1B_u)$ energy versus 1/N or (N) for the keto-bearing carotenoids (solid red line) lies vertically below that for analogs that do not have a keto group (solid blue line), whereas the reverse is true for $S_1(2^1A_g)$. To obtain overlap, the trend-line for $S_2(1^1B_u)$ of keto-bearing carotenoids (red) must be shifted (left) along the 1/N (or N) by an amount corresponding to one additional C=C bond (blue), whereas the reverse is true for $S_1(2^1A_g)$. In other words, addition of one C=C bond decreases the $S_2(1^1B_u)$ energy by an amount that would be expected upon increasing N with two C=C bonds, while addition of a C=C bond has a relatively small effect on the $S_1(2^1A_g)$ energy (about one less than addition of a C=C bond). Thus, depending on what is being probed, a C=C bond addition may appear to have anywhere between zero and twice the effect of a C=C bond. One view of these findings is that the carotenoids differ structurally in the two states, perhaps with different torsion angles between terminal C=C group(s) and the carbon–carbon backbone, such that in $S_2(1^1B_u)$ effective conjugation is longer than for $S_1(2^1A_g)$. Another view is that the electronic structures and excited-state manifolds of keto-bearing carotenoids differ sufficiently to warrant treatment as a distinct carotenoid class; thus, there is a risk of oversimplifying the relative effects of adding C=C or C=C bonds.

Finally, the analysis given above has been mainly focused on the effects of addition of C=C and especially C=C bonds to achieve nominal conjugation lengths $N$ ranging from 13 to 15. It has been shown that the $S_2(1^1B_u)$ state energies for such carotenoids (ketoanhydrorhodovibrin, spirilloxanthin, ketospirilloxanthin and diketospirilloxanthin) lie energetically near or below the excited B850 ring of coupled BChls. Thus, direct quenching of B850 by the carotenoid is energetically favorable, especially in diketospirilloxanthin-containing LH2 (i.e., Rba. sphaeroides crtI::crtIPa grown semi-aerobically). Direct quenching of BChls by carotenoid via energy transfer has been reported previously in TA studies of isolated LH2 complexes from higher plants [57] and in IsiA complex [58], and HIID protein from cyanobacteria [59]. However, to our knowledge this effect has not been observed for an LH2 complex, likely due to the lack of LH2 antennas that contain substantial amounts of carotenoids with very long conjugation lengths.

A hint that carotenoid-mediated BChl $a$ quenching in these LH2 complexes may occur is an apparent shortening of the B850* lifetime. In Rba. sphaeroides crtI::crtIPa grown anaerobically (containing primarily spirilloxanthin) and semi-aerobically (containing a mixture of keto-carotenoids including diketospirilloxanthin), the B850* lifetime at RT is ~0.8 ns. This value is modestly shorter than the value of ~1 ns or slightly longer than found previously for most LH2 complexes (with generally containing shorter carotenoids) [60–62]. However, assessing whether this difference reflects carotenoid quenching of B850* or arises from differences in the time-resolved measurements (e.g., excited-state annihilation) and/or LH2 samples (e.g., different extent of interactions between LH2 rings) must await studies of a unified set of LH2 complexes differing only in carotenoid conjugation length. The availability of Rba. sphaeroides mutants [17] that can produce LH2 (and LH1) complexes with such a diversity of carotenoids in a single organism will facilitate this endeavor and other comparisons of carotenoid functional properties in photosynthetic light-harvesting systems.

**Transparency document**

The Transparency document associated with this article can be found in the online version.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2015.04.001.

**References**


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