Fluorescence and Absorption Detected Magnetic Resonance of Membranes from the Green Sulfur Bacterium *Chlorobium limicola*. Full Assignment of Detected Triplet States

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Optically detected magnetic resonance of chlorosomes-containing membranes from the green sulfur bacterium *Chlorobium limicola* has been performed both by fluorescence and absorption detection. Triplet states localized in the chlorosomes and in the FMO complex have been characterized. After chemical reduction with dithionite followed by illumination at 200 K, a recombination triplet state localized in the primary donor P840 becomes populated under illumination at low temperature. A reaction center triplet state characterized by slightly different ZFS parameters, grows up irreversibly after prolonged illumination at low temperature in the presence of reductant. We were able to obtain the T−S spectra of the FMO complex and of the primary donor P840 in their native environment and to compare them to the spectra obtained in isolated complexes previously published, revealing differences in the spectra. Fluorescence detected magnetic resonance measurements demonstrate that the BChl *c* antenna pigments are connected via energy transfer to the BChl *a* molecules at the low temperature of the measurements (1.8 K) and that all the pigments carrying the triplet states are sensitive to the redox treatment. Dithionite reduction, in fact, induces an enhancement of the BChl *c* and, at a major extent, of the BChl *a* fluorescence yield accompanied by an increase of the yield of all the triplet states of the pigments. Evidence for the presence of BChl *c* excited states quenchers in the core chlorosome and for their selective effect at low temperature is provided, and the location of the quenchers close to BChl *c* molecules absorbing at longer wavelengths discussed.

Introduction

The photosynthetic bacterium *Chlorobium limicola* belongs to the family of green sulfur bacteria which share with other green (non-sulfur) bacteria the characteristic of possessing a peripheral light-harvesting system consisting of large bacteriochlorophyll containing organelles, the so-called chlorosomes, connected to the cytoplasmic membrane by a "baseplate". Excitation energy is funneled from the chlorosomes, through the baseplate and a crystalline array of water-soluble BChl *a* proteins, known as FMO, to the membrane bound core complexes containing the reaction centers.[1] Chlorosomes contain preponderantly BChl *c* (*d* or *e* in other species) assembled as molecular aggregates into rodlike elements. Pigment−pigment interactions are important in determining the supramolecular organization in the rods and the optical spectroscopic characteristics of the BChl *c* molecules are dominated by exciton states.[1,2] The role played by the proteins, which are present in the organelle's envelope, formed by a monolayer of galactolipids, is still a matter of debate.

A small number of chlorosomal BChl *a* molecules (up to 1% in *Cb. limicola*, with respect to the total amount of BChl *c*) is associated to proteins and thought to be located in the baseplate assembly.[3] As indicated above, BChl *a* is also found in the FMO proteins which are assumed to mechanically couple the chlorosome of green sulfur bacteria to the cytoplasmic membrane,[1] and in the photosynthetic reaction center.

Carotenoids contribute to the whole pigment composition (10% in *Cb. limicola*), chlorobactene being the prevailing species. Quinones are also found in chlorosomes and seem to play an important role in regulating the energy transfer under oxic conditions in green sulfur bacteria.[4]

Under continuous illumination triplet states are formed in the chlorosomes of green bacteria due to excitation of bacteriochlorophylls and carotenoids, both at room temperature and at low temperature.[5−7] These triplet states have been used as probes of the chlorosomes topology and function. In this paper we use the powerful technique of zero-field optically detected magnetic resonance, in the fluorescence (ZF-FDMR) and absorption (ZF-ADMR) detection modes to investigate the triplet states formed in intact membranes of *Cb. limicola* under illumination at low temperature. FDMR of whole cells and isolated chlorosomes of *Cb. phaeobacteroides* and *Cb. limicola* has been already reported by Psencik et al.[5], and several triplet species have been detected and assigned to BChl *a*, carotenoids and BChl *e* (or *c*) molecules of the chlorosomes. In our recent comparative study of the isolated chlorosomes of *Cb. tepidum* and *Cf. aurantiacus*,[6] we found that triplet states can easily be detected in both species

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under steady-state illumination at 1.8 K. The observed triplet signals have been assigned to BChl *c*, BChl *a*, and carotenoid molecules on the basis of their ZFS parameters and their T−S spectra. From the data it was concluded that some carotenoids are localized in the baseplate because they are able to quench BChl *a* triplet states. On the other hand, no evidence of quenching of BChl *c* triplet states in the core of chlorosomes has been found. In *Cb. tepidum*, a green sulfur bacterium belonging to the same family as *Cb. limicola*, different BChl *c* oligomers, connected via energy transfer to BChl *a*, were distinguished by detection of slightly different triplet state parameters.[6]

In this work we extend the previous FDMR study of Psencik et al.,[5] on prereduced whole cells, to untreated samples using also the absorption detection (ADMR). In this way we are able to fully assign the observed triplet states to chlorosome pigments, to FMO pigments and to the primary electron donor in the RC. We will discuss the effect of quenchers on the triplet populations of the different pigments, which yields information on the energy transfer pathways and on the photoprotective processes. Last, but not least, we will compare the T−S spectra of the triplet species detected in intact membranes with the results previously published for isolated FMO−protein[8,9] and for core complexes and RC preparations from *Prosthecochloris aestuarii*.[10] We have shown previously that the T−S spectra of pigments, when measured in isolated small particles, are often affected by the biochemical procedure and important features of pigment interactions may be lost.[11,12] It is therefore of great relevance to obtain reliable spectra in large particles maintaining native photosystems.

Materials and Methods

Cells of *Cb. limicola* (strain Tassajara) were grown as described in Jennings and Evans.[13] Subsequent preparation of membrane fractions[14] was performed under argon in 20mM Tris-Tricine buffer at pH 8.0 with an oxygen trap utilizing Tricine and flavin mononucleotide (FMN) as described by Hager-Braun et al.[15] The absorption spectrum of isolated membranes was the same as that reported in the literature for whole cells.[16] Fluorescence spectra of prereduced samples taken at 1.8 K show two bands centered at 783 nm and at 827 nm due to the emission of BChl *c* and BChl *a* respectively in a ratio of about 4:1. The intensity of the BChl *c* fluorescence in untreated samples is reduced by a factor of about 2 while the BChl *a* fluorescence by a factor of about 3.

ODMR Spectroscopy. The samples were diluted to 200 μg BChl/ mL and in some cases preincubated with 30 μM methyl viologen and 20 mM dithionite, after addition of few microliters of a 1 M dithionite solution freshly prepared by dissolving sodium dithionite into 20mM tris-tricine/pH 8.0 buffer. This chemical reduction procedure improves significantly the signal-to-noise ratio of the triplet spectra as expected on the basis of the redox potential control of fluorescence and energy transfer yields in the chlorosomes.[4] Illumination of the prereduced samples at 200 K for 30 min was also performed directly in the cryostat to achieve the reduction of secondary electron acceptors in the reaction centers and allow the formation of recombination triplet state localized in the primary donor, under continuous illumination at 1.8 K. The procedure has been optimized carefully to obtain a high triplet yield without damaging the photosystem.

Glycerol 66% vol/vol was added to the samples just before freezing to avoid matrix cracking. After flushing the solution with argon the residual oxygen was removed using a glucose/ glucose oxidase system.

The home-built FDMR apparatus was described previously.[17] The detection wavelength was selected by cutoff and band-pass filters in the range 770−900 nm (bandwidths 10 nm). The ADMR apparatus, together with the description of the experimental setup for the detection of triplet minus singlet (T−S) spectra, was given in ref 12. Different modulation frequencies for the amplitude-modulated microwaves have been used, depending on the triplet lifetime: 325 Hz for carotenoid and BChl *a* triplet states and 33 Hz for BChl *c* triplet states.

Double resonance FDMR experiments have been performed using a RF oscillator kept at the fixed frequency chosen depending on the experiment, and amplitude modulated, while sweeping a second RF field in the resonance range of the FDMR detected triplet states. These experiments make possible to distinguish among different triplet states having one overlapping transition.

Fluorescence spectra at 1.8 K were detected using the same setup as for FDMR except for a monochromator (Spex232) which has been used before the photodiode instead of band-pass filters.

Results

Triplet States Formation under Reducing Conditions. The absorption spectra of the membranes are dominated by the bands of BChl *c* in oligomeric form. At room temperature the Q_y band is found at 753 nm, largely red-shifted with respect to the monomer absorption (672 nm). The BChl *a* molecules, which are present in the chlorosomes, FMO proteins, and reaction centers, absorb in the long wavelength tail of the Q_y band of BChl *c*, mainly in the range 795−840 nm. A shoulder at 510 nm is indicative of the presence of carotenoids (not shown).

Figure 1 (inset) shows the emission spectrum of the membranes at 1.8 K, after chemical reduction by dithionite. The spectrum shows two bands. The first, centered at 783 nm, is due to the emission from BChl *c*, the other, centered at 827 nm, is due to BChl *a* molecules from the baseplate and the FMO proteins. The intensity of the BChl *c* emission is reduced by a factor of ∼2 at 1.8 K in the absence of reductant, while the intensity of the BChl *a* emission is reduced by a factor of ∼3 (data not shown).

In Figure 1 the FDMR spectra of prereduced samples, detected at different wavelengths in the emission spectrum, are shown. Many transitions are observed, the number and the intensity of which depend on the detection wavelength. The changes in the BChl *c* emission (780−820 nm) due to resonant microwave fields reveal the presence of triplet states, formed under illumination at 1.8 K. Starting from 780 nm three main transitions centered at 210, 638, and 856 MHz are found, corresponding to the 2|E|, |D|−|E|, and |D|+|E| transitions of a triplet state. Two shoulders are also clearly distinguishable on the two sides of the 638 MHz peak**.** On the basis of the ZFS parameters (see discussion and Table 1), the signals detected are certainly due to BChl *c* triplet states in oligomeric form.[5,6] Moreover the microwave induced T−S spectrum taken at 636 MHz (Figure 2a) shows a bleaching at 768 nm, corresponding to the singlet ground-state absorption of the species carrying the triplet state, which is in the red-tail of the absorption band of the chlorosome BChl *c* oligomers. Similar results were also obtained in isolated chlorosomes from *Cb. tepidum*.[6]

Interestingly, together with those mentioned above, other transitions are clearly found when detecting the emission at 790 nm, a wavelength close to the maximum of the BChl *c* fluorescence. The weak and broad transition centered at about 1100 MHz is due to carotenoid triplet state |D|+|E| transition,

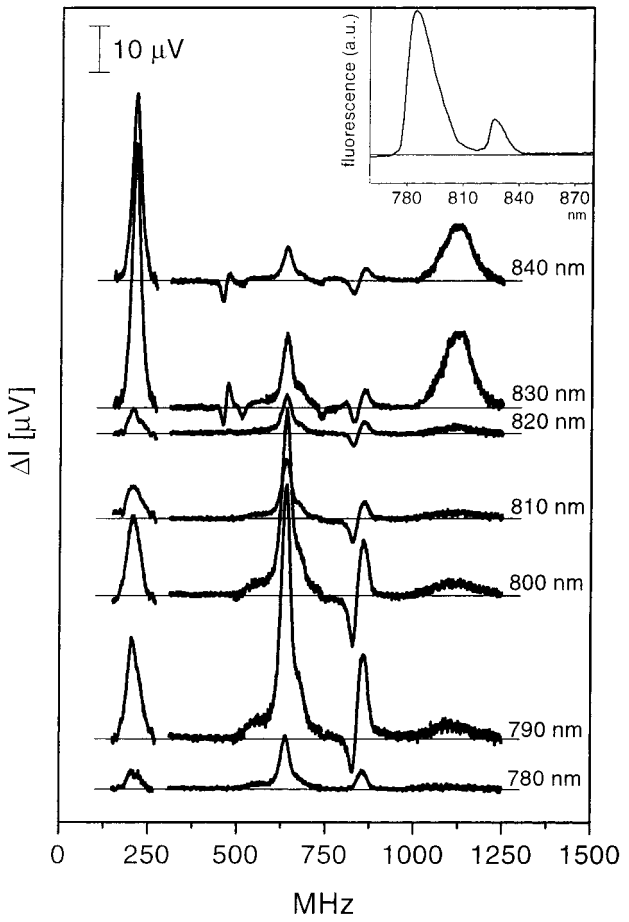

Figure 1. FDMR spectra of prereduced (20 mM dithionite) and preilluminated membranes from *Chlorobium limicola* detected at different wavelengths as indicated in the figure. Experimental conditions: $T =$ 1.8 K; modulation frequency, 320 Hz for carotenoids triplet states; 33 Hz for BChl(s) triplet states; microwave power, 20 mW; scan rate, 0.83 MHz/s. Inset: fluorescence spectrum at 1.8 K of prereduced and preilluminated membranes; excitation obtained by white light filtered by water (7 cm) and 700 nm cutoff filter; optical resolution, 5 nm.

detected by an induced change of the BChl *c* emission. The 2|E| transition, at about 200 MHz, which is usually the most intense in the characteristic polarization pattern of carotenoid triplet states in zero magnetic field, overlaps with the 2|E| transition of BChl *c* triplet states observed also at 780 nm. At 828 MHz a negative band is present. We assign this band to a second BChl *c* triplet state (BChl *c* II), distinct to that observed also at 780 nm (BChl *c* I). In fact, as shown in Figure 3, double resonance experiments obtained pumping at 829 MHz, while sweeping the microwave field in the range 600−700 MHz and 150−250 MHz, show that the |D|−|E| transition of this triplet state is centered at 633 MHz and the 2|E| transition is centered at 192 MHz. In the same figure, trace b, the result obtained by pumping the |D|+|E| transition at 860 MHz, assigned to BChl *c* I triplet state, is also shown: a double resonance transition is observed correspondingly at 639 MHz. The intensity of the FDMR signal due to BChl *c* II triplet state, reaches the maximum at 800 nm, red shifted with respect to the maximum of BChl *c* I triplet state (Figure 1). The results are shown, after decomposition of the spectra by Gaussians in Figure 4 (see Discussion). The T−S spectrum detected at 832 MHz, in the spectral region where Q_y absorption bands of BChl *c* are expected, confirms the assignment to a BChl *c* triplet state and shows a red-shifted bleaching when compared to the T−S spectrum detected at 860 MHz, that is in correspondence to the |D|+|E| transition of BChl *c* I triplet state (Figure 2a). All the above-mentioned signals can be followed in the fluorescence detection mode, up to 820 nm, even though the intensity decreases following the BChl *c* fluorescence profile (see insert of Figure 1). On the other hand all the signals due to BChl *c* triplet states increase again when monitoring at longer wavelengths (BChl *a* emission) and are easily detected up to 840 nm. These data show that energy transfer processes occur between the BChl *c* molecules carrying the triplet states and the emitting BChl *a* molecules. As expected, the sign of BChl *c* FDMR signals does not change when the detection moves from the emission band of BChl *c* itself to the lower energy emission band of BChl *a*.

Since the signals due to BChl *c* I and BChl *c* II show a different dependence on the frequency of microwave amplitude and on the lock-in detection phase (not shown), it is clear that the spectroscopic characteristics of the two triplet states are different. However the relative sign and the absolute polarization pattern cannot be determined in the present experiments, due to the low intensity of the signals which precludes the direct detection. The two triplets states belong to oligomeric BChl c pools which have likely a different molecular environment. These BChl *c* molecules, carrying the triplet state, are all connected via energy transfer to BChl *a* emitting molecules. Similar results have been obtained also in isolated chlorosomes from *Cb. tepidum*;[6] however, we obtained a better signal-to-noise ratio in these membranes preparations from *Cb. limicola.*

By monitoring the microwave induced fluorescence changes in the emission band of BChl *a*, starting from 830 nm, FDMR signals in a different microwave range have also been detected. These transitions can be assigned to BChl *a* triplet states by comparing the ZFS parameters of the observed triplet states with the in vivo and in vitro values.[18] The transitions found at 472 and 806 MHz can easily be assigned to triplet states belonging to BChl *a* located in the FMO antenna complexes, by comparing the resonance frequencies with those of the ADMR transitions reported for isolated FMO protein.[8] It is worth noting that these transitions were absent in the isolated chlorosomes from *Cb. tepidum*.[6] The T−S spectrum (Figure 2b) detected while irradiating the sample at 470 MHz shows the characteristic features of the spectrum obtained by ODMR, in isolated FMO-protein from *P. aestuarii*.[8] Some details of the spectrum however are different. The three negative bands are found at 806, 816, and 825 nm instead of at 805, 814, and 827 nm, and the positive band is found at 820 nm instead of 817 nm. The intensities of the two bands associated with the band shift (the central bands) are here the most intense while they are the weakest in ref 8.

By monitoring the BChl *a* fluorescence, two weak signals of comparable intensity are also found at 512 and 736 MHz which can be assigned to the |D|−|E| and |D|+|E| transitions of the primary donor (P840) recombination triplet state.[10] The calculated ZFS parameters are $|D| = 0.0208$ cm^{-1} and $|E| = 0.0037$ cm^{-1}, close to the values measured by EPR in membranes from *Cb. limicola f. thiosulfatophilum*[19] and to those measured by ADMR in the reaction center pigment protein-complex from *P. aestuarii*.[10] The T−S spectrum recorded at 733 MHz is shown in Figure 2c. The spectrum in the region 760−850 nm shows two strong negative bands at 837 and 826 nm and a weaker, positive, band at 815 nm. The spectrum is similar to the one already reported for the reaction center pigment−protein complex from *P. aestuarii*,[10] but no shoulder at 833 nm has been detected. The transitions, which have been observed in the FDMR spectra, are found also in the ADMR spectra obtained when monitoring the absorption at 837 nm (not shown). In

TABLE 1: Gaussian Components, $c_i = a_i e^{-2(x - xc_i)^2/w_i^2}$, Used in the Decomposition of the Spectra of Figure 1[a]

c_i	xc_i MHz	w_i MHz	780 nm	790 nm	800 nm	810 nm	820 nm	830 nm	840 nm	assignment
c_1	638 ± 1	24 ± 0.5	8.90	41.5	28.00	9.60	5.85	10.50	5.50	|D|−|E| BChl *c* I
c_2	856 ± 1	26 ± 0.5	4.00	18.5	13.00	3.95	2.65	4.30	2.50	|D|+|E| BChl *c* I
c_3	210 ± 1	35 ± 0.5	2.25	10.65	7.35	2.00	1.15	2.75	1.50	2|E| BChl *c* I
c_4	562 ± 1	55 ± 0.5	0.85	4.00	3.25	0.75	0.65	1.50	0.75	n.a.
c_5	651 ± 1	60 ± 0.5	2.65	15.00	13.75	3.35	2.35	5.35	2.00	n.a.
c_6	828 ± 1	18 ± 0.5		−8.75	−10.5	−5.20	−2.80	−4.00	−2.95	|D|+|E| BChl *c* II
c_7	198 ± 1	24 ± 0.5		5.00	6.00	2.35	1.10	2.25	2.00	2|E| BChl *c* II
c_8	472 ± 1	12 ± 0.5						5.00	1.80	|D|−|E| FMO
c_9	806 ± 1	12 ± 0.5						2.50	0.90	|D|+|E| FMO
c_{10}	460 ± 1	9 ± 0.5						−3.75	−4.50	|D|−|E| BChl *a*
c_{11}	831 ± 1	15 ± 0.5						−1.25	−1.75	|D|+|E| BChl *a*
c_{12}	512 ± 1	12 ± 0.5						−2.15	−0.85	|D|−|E| P840
c_{13}	736 ± 1	15 ± 0.5						−2.40	−0.85	|D|+|E| P840
c_{14}	210 ± 1	24 ± 0.5		7.00	6.30	2.55	3.50	52.50	37.0	2|E| car
c_{15}	1120 ± 1	80 ± 0.5	0.50	2.5	2.25	1.00	1.40	15.85	11.9	|D|+|E| car

[a] The values represent the amplitudes (a_i) in μV (errors ± 0.05 μV) at different detection wavelengths. (n.a. = not assigned.)

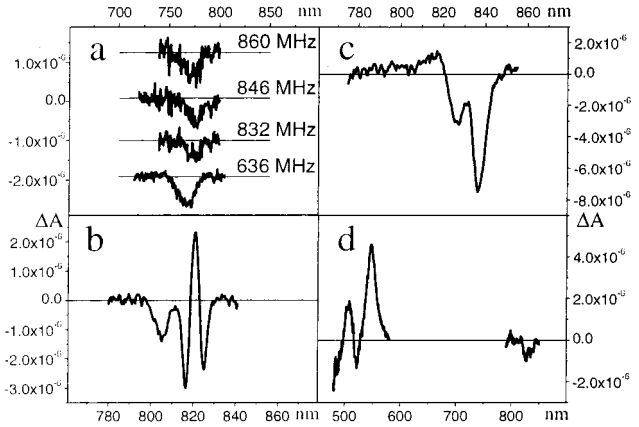

Figure 2. T−S spectra detected in prereduced and preilluminated membranes from *Chlorobium limicola*. (a) T−S spectra of the BChl *c* triplet states detected at different microwaves frequencies, as indicated in the figure; modulation frequency, 33 Hz; microwave power, 20 mW; resolution, 1.5 nm; scan rate, 0.1 nm/s. (b) T−S spectrum of the BChl *a* triplet state assigned to the FMO complex detected at 470 MHz; modulation frequency, 320 Hz; microwave power, 800 mW; resolution, 2.1 nm; scan rate, 0.1 nm/s. (c) T−S spectrum of the P840 recombination triplet state detected at 733 MHz (|D|+|E| transition); modulation frequency, 320 Hz; microwave power, 20 mW; resolution, 2.4 nm; scan rate, 0.1 nm/s. (d) T−S spectrum of the carotenoid triplet state detected at 207 MHz (2|E| transition); modulation frequency, 320 Hz; microwave power, 1 W; resolution, 1.8 nm; scan rate, 0.1 nm/s.

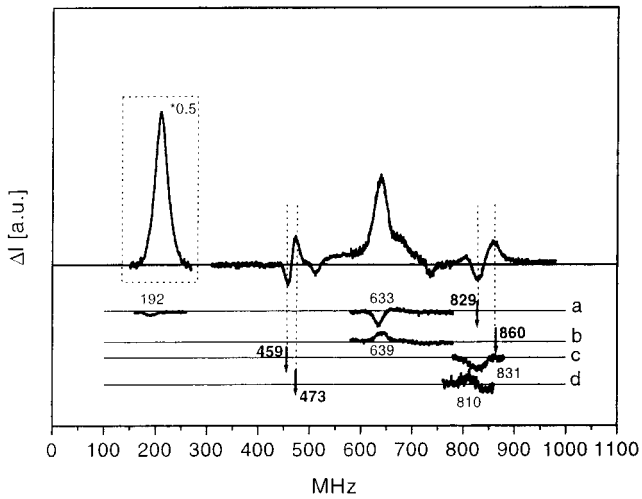

Figure 3. Double resonance spectra of prereduced and preilluminated membranes; the arrows indicate the pumping microwave frequency (microwave power, 800 mW). On the top the FDMR spectrum of Figure 1, detected at 830 nm, is reported (a) pumping frequency, 829 MHz (BChl *c* II |D|+|E| transition); emission detected at 810 nm; modulation frequency, 33 Hz; microwave power, 20 mW; scan rate, 0.83 MHz/s. (b) Pumping frequency, 860 MHz (BChl *c* I |D|+|E| transition); emission detected at 810 nm; modulation frequency, 33 Hz; microwave power, 20 mW; scan rate, 0.83 MHz/s. (c) Pumping frequency, 459 MHz (BChl *a* |D|−|E| transition); emission detected at 850 nm; modulation frequency, 320 Hz; microwave power, 20 mW; scan rate, 0.83 MHz/s. (d) Pumping frequency, 473 MHz (FMO BChl *a* |D|−|E| transition); emission detected at 830 nm; modulation frequency, 320 Hz; microwave power, 20 mW; scan rate, 0.83 MHz/s.

contrast to the results previously reported[10] we did not detect any transition in the 400−450 MHz region. Moreover we found that the |D|+|E| transition splits into two components centered at 704 and 733 MHz. The second component (704 MHz) can be shown to grow after prolonged illumination of the samples at 1.8 K and may be detected both by fluorescence and absorption changes. The T−S spectrum detected at 704 MHz is identical to the one detected at 733 MHz (data not shown). This suggests that the resonance observed at 704 MHz is due to a slightly different population of primary donor triplet state.

When detecting the BChl *a* emission, another transition is found at 460 MHz that we assign to the |D|−|E| transition of BChl *a* triplet states in the chlorosomes from a comparison with the results obtained in isolated chlorosomes of *Cb. tepidum*.[6] The |D|+|E| transition overlaps with the 830 MHz transition of the BChl *c* mentioned above, as proved by double resonance experiments (Figure 3). We therefore assign this triplet state to

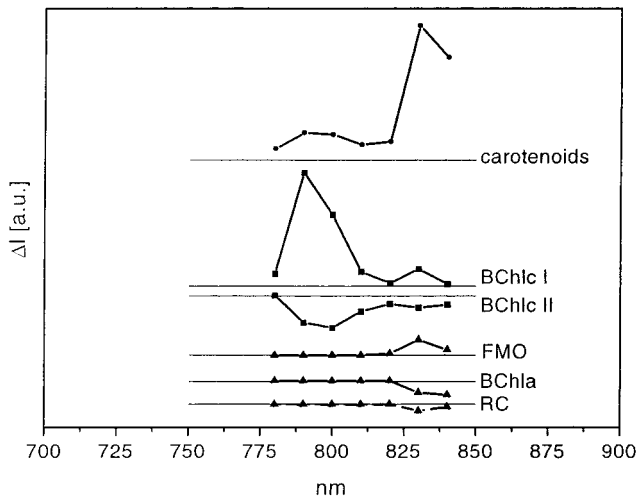

Figure 4. Reconstructed MIF (microwave induced fluorescence spectrum) of all the pigments present in prereduced (dithionite) and preilluminated membranes from *Chlorobium limicola* after decomposition and assignment according to the data reported in Table 3. As representative of each triplet state, one transition has been chosen: from the top: 1. transition at 1120 MHz (|D|+|E| carotenoids), 2. transition at 638 MHz (|D|−|E| BChl *c* I), 3. transition at 828 MHz (|D|+|E| BChl *c* II), 4. transition at 472.5 MHz (|D|−|E| BChl *a* FMO), 5. transition at 460 MHz (|D|−|E| BChl *a* chlorosomes), 6. transition at 736 MHz (|D|+|E| ^{T}P840).

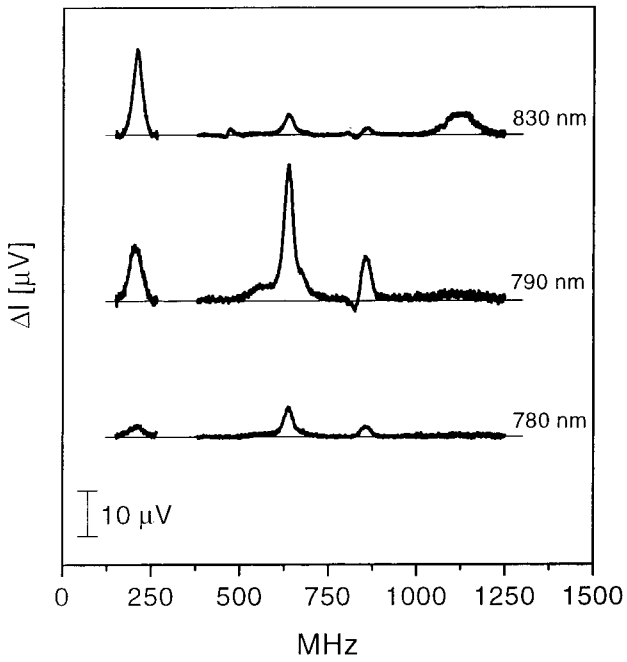

Figure 5. FDMR spectra of untreated membranes from *Chlorobium limicola* detected at different wavelengths as indicated in the figure. Experimental conditions: $T = 1.8$ K; modulation frequency, 320 Hz for carotenoid triplet states and 33 Hz for BChl(s) triplet states; microwave power, 20 mW; scan rate, 0.83 MHz/s.

the BChl *a* in the chlorosomes. All the triplet states assigned to BChl *a* molecules have been detected up to 850 nm; however, the intensity of the FDMR signals due to the FMO and to P840 reaches a maximum at 830 nm, while the FDMR signal assigned to chlorosomal BChl *a* increases up to 840 nm (Figure 4). The BChl *a* FDMR transitions are not observed when detecting the BChl *c* emission.

The T−S spectrum of the chlorosomal BChl *a* pool has not been detected, probably due to the low population of this triplet state.

Together with the signals due to BChl *a* triplet states, strong FDMR transitions due to carotenoids are found when monitoring the fluorescence changes in the BChl *a* emission band. The 2|E| and |D|+|E| transitions of carotenoid triplet states give ZFS parameters which correspond roughly to a number of 9−10 conjugated double bonds[20] ($|D| = 0.0339$ cm^{-1}; $|E| = 0.0035$ cm^{-1}). The |D|−|E| transition is expected at about 900 MHz. In this microwave range the transitions due to BChl *c* triplet states are also found. However we can exclude the presence of an overlapping |D|−|E| transition due to carotenoids in those transitions assigned to BChl *c* because changing the frequency modulations of the microwave amplitude from 33 to 325 Hz causes the signal in that frequency region to disappear completely. In contrast the |D|+|E| and the 2|E| transitions of the carotenoids show, as usually observed for carotenoids triplet states, a maximum of intensity at the latter modulation frequency, due to lifetime of carotenoids triplet state which is close to 10 microseconds. The T−S spectrum, recorded at 207 MHz (Figure 2d), shows positive bands at 508 and 550 nm and negative bands at about 485 and 525 nm, assigned respectively to triplet−triplet and singlet−singlet absorption of carotenoids. The T−S pattern is similar to those detected in photosynthetic antenna complexes from higher plants[21,22] and from purple bacteria.[23] The intensity of the T−S signal is low indicating a small carotenoid triplet population. For this reason the T−S spectrum was not detected within the broader |D|+|E| transitions. As already found for all the carotenoid triplet states reported in photosynthetic systems, special features are seen in the BChl *a* Q_y absorption region of the T−S spectra. These features are due to the change in the electronic state of the nearby carotenoid molecules,[21−23] while no effect is found in the corresponding BChl *c* absorption range. The same results have been already discussed in our previous work on isolated chlorosomes.[6]

The dependence of the intensity of FDMR signals due to carotenoid triplet states on the detection wavelength is presented in Figure 4 (see Discussion). When monitoring the BChl *a* fluorescence, the carotenoid triplet state FDMR signals are about seven times stronger with respect to those detected by monitoring the BChl *c* fluorescence at 790 nm. FDMR spectra detected at 780 nm are likely free of carotenoid contributions and the transition observed at about 200 MHz must be assigned to the 2|E| BChl *c* triplet transition. The line shape and the frequency corresponding to the maximum of the signals due to carotenoids change somewhat, depending on the detection wavelength, suggesting some heterogeneity of the carotenoid population.

Triplet States Formation under Nonreducing Conditions. In the absence of the incubation with dithionite the intensity of the FDMR signals is strongly decreased, in parallel with the decrease of the emission intensity. The effect has been ascribed to the action of endogenous quenchers (possibly quinone molecules[4]), which contribute, in their oxidized form, to the deactivation of the BChl *c* excited states. The FDMR experiments show that the sensitivity to the quenchers, at least at 1.8 K, is specific for different pigments in the chlorosomes and in the membranes. In Figure 5 the FDMR signals at three representative wavelengths of detection are shown. All the triplet states, which are detected under reducing conditions, are still present except for the P840 triplet state. However, a closer inspection of the spectra reveals that some triplet state transitions decrease relatively more than others. The quantitative analysis (see Discussion) shows that triplet state populations from the BChl *c* II pool and from the baseplate BChl *a*, decrease to the largest extent, followed by the carotenoid and FMO BChl *a*

TABLE 2: Transitions and ZFS Parameters of the Assigned Triplet States

	2|E| (MHz)	|D|−|E| (MHz)	|D|+|E| (MHz)	|D| (cm^{-1})	|E| (cm^{-1})
BChl c I	210	638	856	0.0249 ± 0.0002	0.0036 ± 0.0002
BChl c II	198	n.o.	828	0.0243 ± 0.0002	0.0033 ± 0.0002
FMO	n.o.[a]	472.5	806	0.0213 ± 0.0002	0.0056 ± 0.0002
BChl a	n.o.	460	831	0.0212 ± 0.0002	0.0058 ± 0.0002
P840	n.o.	512	736	0.0208 ± 0.0002	0.0037 ± 0.0002
carotenoids	210.5	n.o.	1120	0.0339 ± 0.0002	0.0035 ± 0.0002

[a] n.o. = not observed.

triplet states. The smallest effect is observed for the BChl *c* I pool.

Discussion

The energy transfer processes, which occur in the green sulfur photosynthetic apparatus, are aimed to funnel the energy from the chlorosomes toward the reaction center, located in the cytoplasmic membrane. Energy can be transferred in a few tens of picoseconds from BChl *c* to BChl *a* pigments, which are probably coordinated to proteins in the baseplate.[3,24] The efficiency of this process ranges from 30 to 90% depending on the system and on the experimental conditions.[25−29] The excitation which reaches the BChl *a* bound to the FMO proteins is finally transferred to the RC; however, quite unexpectedly, the efficiency of this process is low (20−30%).[1,2] A redox-dependent quenching of BChl *c's* excited states takes place in the chlorosomes,[24] and a decrease of energy transfer to BChl *a* is observed under nonreducing conditions.[4] The quenchers, thought to be quinones,[4] provide a rapid nonradiative decay pathway, which competes with energy transfer. A similar effect has also been reported in the FMO protein[30] but the quenching mechanism is, in this case, thought to involve tyrosine radicals. The mechanism of quenching should act as a protection from oxidative damage preventing charge separation in the reaction center during occasional exposure of the system to oxygen.

Carotenoids are assumed to transfer excitation to BChl *c* in the core chlorosomes and probably most of the singlet energy transfer from the carotenoids to BChl *a* proceeds via BChl *c*.[29] Psencik et al.[5] have shown that carotenoid triplet states can be formed under illumination of cells and of isolated chlorosomes suggesting a possible photoprotective role for these pigments, similar to that they play in purple bacteria and higher plants.[29,32] In our recent work we have found by ODMR that, in isolated chlorosomes from *Cb. tepidum* and *Cf. aurantiacus*, carotenoid triplet states, which become populated under illumination at 1.8 K, are closely related to BChl *a* rather than to BChl *c* molecules, and carotenoids carrying the triplet states are therefore likely to be located close to BChl *a* in the chlorosome baseplate.[6] Recently, time-resolved absorption measurements on isolated chlorosomes have also been reported,[7] supporting the conclusion that at room temperature carotenoids are able to quench both BChl *c* and BChl *a* triplet states with nearly 100% efficiency, suggesting a temperature effect in the populating mechanisms. Moreover, in our previous work,[6] we identified several other triplet states which become populated under continuous illumination at 1.8 K and which have been assigned to BChl *a* in the baseplate and to three different BChl *c* populations in the chlorosomes. The present extension of the work to intact membranes under different redox conditions allows to give full significance to the results obtained in isolated chlorosomes and to study the energy transfer processes in the pathway to the RCs. In fact, the location of triplet states can be used as a probe of the antenna structure and of the energy transfer processes, especially when optical detection of magnetic resonance is used so that absorption and emission spectra of the whole system can be correlated to the presence of a specific triplet population.

Since in the FDMR spectra, shown in the previous section, several transitions belonging to different triplet states overlap in the same microwave and optical spectral regions, to fully assign the transitions and calculate the ZFS parameters, decompositions of the spectra with Gaussian curves have been performed. In the global analysis the fact that transitions belonging to the same triplet state must behave in the same way with respect to the detection wavelengths, to the modulation frequencies and to the microwave power, has been taken into account. Moreover double resonance experiments were used to find the center of the "missing" components in the fittings. The results and the assignments are reported in Table 1, while the ZFS parameters are summarized in Table 2.

BChl *c* Triplet States. Under our experimental conditions, BChl *c* triplet states become populated in membranes of *Cb. limicola*. Different BChl *c* pools are observed as triplet state traps (see Table 2, BChl *c* I and BChl *c* II). In fact, only BChl *c* I can be detected at 780 nm, and it shows different optical and magnetic parameters with respect to the triplet state of BChl *c* II, indicating some heterogeneity of BChl *c* oligomers in green sulfur bacteria. As seen in the T−S spectra taken in the range 825−860 MHz, the BChl *c* oligomers, which undergo the change in the electronic state, absorb at 767−775 nm. These values are very close to the absorption wavelengths of the BChl *c* molecules found by optical experiments.[33−35] The intensity of the T−S spectra reveals that the triplet populations are very low.

Very recently van Rossum et al.[36] have shown that the absorption band of chlorosomal BChl *c* is fully described by the exciton splitting produced by short-range interactions involving five monomers in a stack and by the interactions among different stacks in the rods. However, an inhomogeneous broadening of the single bands is evaluated to be of the order of 350 cm^{-1}.[37] What is probably seen by ODMR at 1.8 K is a number of low energy oligomers separated from the bulk population at this temperature, where the ISC probability becomes higher owing to the reduced back energy transfer. Both the observed BChl *c* pools are however directly and/or indirectly connected to BChl *a* molecules via energy transfer, as proven by the observation that FDMR of BChl *c* triplet states may be performed, not only by detection of BChl *c* emission, but also by detection of BChl *a* fluorescence. Some selection in the BChl *c* emission band is also observed for the two different triplets (780 vs 810 nm). The effect of the sodium dithionite on the intensity of the FDMR signals of the BChl *c* triplet states allows us to infer that the molecules carrying the triplet states belong to the antenna population which "feels" the effect of the quenchers. As a matter of fact, the FDMR signals (ΔI) slightly increase in the case of BChl *c* I triplet and greatly increase (5 times) in the case of BChl *c* II triplet, when the reductant is added to the samples before the measurement (Table 3). The stronger effect found for the BChl *c* II triplet state seems to

TABLE 3: Dithionite Effect on the FDMR Signal Intensity Measured as the Ratio: ΔI(Treated Sample)/ΔI(Untreated Sample)

	BChl *c* I	BChl *c* II	FMO	BChl *a*	P840	car
790 nm	1.8 ± 0.2	3.0 ± 0.3				
830 nm	2.7 ± 0.3	5 ± 0.3	3.5 ± 0.3	5.8 ± 0.3	>10	3.5 ± 0.3

indicate that the quenchers are mainly located close to long-wavelength-absorbing (776 nm) and emitting (800−810 nm) BChl *c* molecules. This "local effect" of the quenchers may not be observed at physiological temperature since the excitation is spread in the whole chlorosome and the quenching of the excited states is about 20 times more efficient with respect to the low temperature,[4] due to the mobility of the excitations.

Compared to the isolated chlorosomes of *Cb. tepidum*,[6] the intensity of BChl *c* FDMR signals measured in membranes are not reduced, meaning that the probability of populating triplet states is not greatly affected by the presence of the energy-transfer processes to the FMO, at least at 1.8 K.

BChl *a* Triplet States. The organization of BChl *a* molecules in the chlorosomes is still uncertain, and their location in the baseplate has been proposed on the basis of several pieces of experimental evidence.[3] The role of the proteins present in the envelope is also unclear. Energy transfer is thought to occur between the BChl *a* molecules in the baseplate and the BChl *a* molecules in the FMO proteins before reaching the reaction centers. As already reported for isolated chlorosomes,[6] BChl *a* triplet states are found in membranes under illumination at 1.8 K, with the same spectroscopic characteristics as those reported for isolated chlorosomes. The ZFS parameters are reported in Table 2. Under nonreducing conditions, the FDMR signals of this BChl *a* triplet state decrease by a factor of 5 suggesting that it belongs to BChl *a* molecules which are part of the fully connected BChl *c*/ BChl *a* antenna system of the chlorosomes (Table 3). The trapping of such triplet states, even in the presence of FMO and open RCs, may be due to a loss of efficiency of the energy transfer at low temperature. However, their relative FDMR intensity with respect to the BChl *c* signals is reduced about 2 times when compared to the signals detected in isolated chlorosomes from *Cb. tepidum*[6] indicating that the energy transfer process toward the RC has affected the triplet yield of the BChl *a* triplet states. A second BChl *a* triplet state population has been detected in the membranes, which is not present in isolated chlorosomes and which can be assigned to BChl *a* molecules located in the FMO protein. The signal is dithionite sensitive (Table 3), but at a smaller extent compared to the BChl *a* triplet state located in the chlorosomes. It is observed even when the recombination triplet state is detected at the same time (see below), meaning that the energy transfer from the FMO complexes to the reaction centers in our experimental conditions is not very efficient. Zhou et al.[30] have proven the presence of quenchers of BChl *a* excited states in nonreduced, isolated, FMO proteins at room temperature, while at low temperature the fluorescence yield was not affected by the presence of dithionite. Therefore the effect observed in our experiments is likely due to the reduced energy transfer from the chlorosomes rather than to the intrinsic quenchers.

The microwave induced T−S spectrum of FMO shows some band shifts and a changed relative intensity of the bands, when compared to the microwave induced T−S spectrum of isolated FMO complexes from *P. aestuarii*.[8] Moreover, the comparison of the T−S spectra of isolated FMO proteins from *Cb. tepidum* and *P. aestuarii*, detected by optical spectroscopy at 6 K, reported by Vulto et al.[9], indicates that the differences between the two species concern mainly the frequencies of the peaks while the relative band intensities are very similar. In particular the most intense negative band is, in both cases, the low energy band at about 826 nm. In view of the 97% homology in amino acids sequences and of the similarity of the absorption and CD spectra between the FMO complexes from *Cb. tepidum* and *Cb. limicola*[38] we would have expected a T−S spectrum from *Cb. limicola* much more similar to those reported for the other two species. On the contrary, the T−S spectrum reported here differs especially with respect to the relative intensity of the low energy band where the triplet state is expected to be localized. Interestingly it has recently been reported[39] that FMO complexes copurified with the RC show an extra BChl, possibly involved in the energy transfer to the RC. In this context the observed differences in the T−S spectra could be due to the conserved environment of the FMO complexes in our samples with respect to the isolated FMO complexes, rather than to different species. This means that the FMO exciton properties are modulated "in situ" by the interactions with components which are lost in the isolation of the complexes.

When the membranes are prereduced and preilluminated at 200 K for 30 min, a new triplet state is detected both by FDMR and ADMR, which we have assigned to the reaction center recombination triplet state. We followed the procedure previously described by Nitschke et al.[19] who found that the recombination triplet state becomes populated in green sulfur bacteria membranes only after a prereduction step. The reduction induces the full double reduction and protonation of a quinone acceptor (A_1) and, after prolonged illumination at 200 K, to reduce the iron−sulfur centers. Recent reports in the literature[40,41] have shown that a semiquinone electron acceptor can be reduced by these treatments, although the presence and function of quinones in the electron transfer chain of the reaction centers of green sulfur bacteria is still a controversial topic. The procedure allows in any case to produce a detectable yield of recombination triplet state and to precisely measure its ZFS parameters: $|D| = 0.0208$ cm^{-1} and $|E| = 0.0037$ cm^{-1}. The reduction of the ZFS compared to monomeric BChl *a* in vitro, (the |D| value is reduced by 6%), suggests a dimeric structure for the donor, which is paralleled by the finding that P_{840}^+ is quite symmetric with respect to the charge distribution in the two halves.[42] On the other hand the T−S spectrum, which is quite similar to the previously detected one of *P. aestuarii*,[10] does not show the characteristic oscillations found in the T−S spectra of the primary donor of purple bacteria[43−45] but resembles closely the T−S spectrum of P700[11] which is localized over only one-half of the dimer, suggesting similar pigment arrangements and interactions among the primary donor and the nearby pigments. In summary, the evidence for a dimeric structure of the triplet state in the primary donor P840 in *Cb. limicola* is not conclusive. Concerning the analogies between PSI and green sulfur bacteria RC, it is also interesting to note that we observed a similar phenomenon for P700 and P840 triplet states, after prolonged illumination at very low temperature. A second form of the primary donor triplet state, having slightly different ZFS parameters, but the same T−S spectrum, becomes populated in both systems. The effect was reversible for P700 after raising the temperature up to 50 K keeping the sample in the dark,[11] but it is not so for P840, at least up to 100 K. The reversibility of the effect suggested for PSI a possible conformational change upon illumination at low temperature or, alternatively, a photoinduced reduction of partially reduced RCs. The different behavior observed in the recovery of the initial state in green sulfur bacteria might be due to the difference in the redox potential of the iron−sulfur centers. In principle it

is even possible that the two different triplet states correspond to localization of the triplet in the two halves of the dimer, particularly since a recent report[46] suggests that electron transfer can occur on both branches in the PSI reaction center. Unfortunately we did not obtain a triplet yield sufficiently high to allow an accurate comparison of the two T−S spectra. For this reason we are now trying to obtain membranes with a reduced antenna size with the aim of increasing the RC/antenna ratio and consequently the recombination triplet yield.

Carotenoids. By ODMR we found that both BChl *c* and BChl *a* triplet states become populated in the membranes, at least at low temperature. However the main triplet species, populated among the antenna pigments under illumination, are carotenoid triplet states, as evidenced also by the relatively high intensity of their T−S spectra.

In photosynthetic systems carotenoids act as efficient antenna pigments and transfer excitation to (B)Chl molecules in the pigment−protein light-harvesting complexes. Second, they function as quenchers of the (B)Chl triplet states to avoid singlet oxygen formation.[31,32] Green sulfur bacteria are anaerobes, however they may encounter oxic conditions in their environment due to vertical mixing of zones in the chemocline in stratified lakes. In fact it is known that *Cb. tepidum*[4] under oxidizing conditions activates a mechanism in the chlorosomes which quenches excited BChl *c* singlet states decreasing the energy transfer to the BChl *a* in the baseplate. This uncoupling mechanism is reversible and prevents the formation of toxic reactive oxygen species via photoproduced reactants in the reaction centers.

As already found in isolated chlorosomes, FDMR signals of carotenoids triplet states in the membranes are mainly detected by induced changes in the BChl *a* fluorescence.

We found that the carotenoid triplet states are only weakly detected by BChl *c* fluorescence at 790 nm, even though, as is the case of *Cb. tepidum*, the fluorescence yield is higher at 780 nm with respect to 830 nm. The results are summarized in Figure 4. By ODMR spectroscopy one selects only the carotenoids which are able to quench BChl triplet states and therefore we cannot exclude the possibility that the main carotenoid population is able to transfer singlet excitation also to BChl *c* molecules in the core of the chlorosomes. It is interesting to note that under non reducing conditions the signals of carotenoid triplet states almost disappear, as is also observed for BChl *c* II and BChl *a* triplet states (Table 3). This suggests that the quinone mediated quenching mechanism, which acts to prevent new charge separations, is quite efficient at the level of blocking the excitation before it reaches the baseplate and represents the main photoprotective mechanism in the antenna. The presence of detectable triplet states belonging to BChl *c* and BChl *a* molecules indicates that the carotenoids present in the chlorosomes are not able to completely quench the BChl triplets in the antenna complexes, at least at very low temperature. In several antenna complexes of higher plants and purple bacteria, T−S spectra, taken at the resonance frequencies of carotenoid triplet states, show characteristic features in the Q_y absorption region of (B)Chls.[21−23] These spectral features have been interpreted as due to electronic interactions between the carotenoid molecule carrying the triplet state and Chl molecules close to it and therefore the most likely to be involved in the triplet−triplet transfer process which populates the carotenoid triplet state. At wavelengths corresponding to the Q_y absorption region of BChl *a* small negative bands seem indeed to be present in the T−S spectrum at about 830 nm, recorded at the resonant frequency of the carotenoid triplet state. It has been noted[23] that the intensity of these interaction bands is directly correlated to the efficiency of the triplet−triplet energy transfer, which, in these systems, is not very high at low temperature. Following these experimental results we may conclude that the carotenoids carrying the triplet state are mainly interacting with BChl *a* molecules and are therefore located close to them.

Conclusions

The high resolution and the selectivity of the ODMR technique allows to distinguish triplet states belonging to different BChl *c* pools localized in the chlorosomes. These pools are different in terms of optical and magnetic properties and in terms of sensitivity to the action of endogenous quenchers, meaning that they are likely located in different points with respect to the baseplate. In proximity to the baseplate the quenching effect is the highest as suggested by the strong decrease of the signal of BChl *a* triplet states under oxidizing conditions. The FDMR experiments prove that the BChl *c* antenna pigments are connected via energy transfer to the BChl *a* at the low temperature of the measurements (1.8 K) and that all the pigments carrying the triplet states are sensitive to the redox treatment even though to different extents. Carotenoid triplet states are populated in the chlorosomes by quenching of BChl triplet states especially chlorosomal BChl *a* molecules. The quenching, which is not very efficient at low temperature, may however be improved at high temperature by triplet−triplet transfer among oligomers, as already reported for chlorosomes from *Cb. tepidum* and *Cf. aurantiacus*.[7]

After chemical reduction with dithionite followed by illumination at 200 K, a recombination triplet state localized in the primary electron donor of the reaction center P840 becomes populated under illumination at low temperature. A reaction center triplet state characterized by slightly different ZFS parameters grows up irreversibly after prolonged illumination at low temperature. If this second form of recombination triplet state is due either to a different conformation of the protein or to a different localization of the triplet state remains unclear. The T−S spectrum detected for the first time from membranes of the green sulfur bacterium *Cb. limicola* is quite similar to the previously detected one of *P. aestuarii*,[10] indicating a similar arrangement of the pigments surrounding the primary donor. Moreover, the T−S spectrum of the FMO complex in its native environment is here obtained for the first time. The comparison with the spectrum obtained in isolated complexes from *P. aestuarii*,[8] previously published, shows some interesting new features. Further investigation will reveal whether these features are due either to the different species or to the "intact" environment and organization of the FMO complexes in the membranes. In any case the BChl *a* involved in triplet localization is the same as observed in isolated complex, a long-wavelength-absorbing (826 nm) molecule.

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Abbreviations

ODMR, optically detected magnetic resonance

ZF-FDMR, zero field-fluorescence detected magnetic resonance

ZF-ADMR, zero field-absorption detected magnetic resonance

MIA, microwave induced absorption

T−S, triplet-minus-singlet

ZFS, zero field splitting
BChl, bacteriochlorophyll
Car, carotenoid
Cb., *Chlorobium*
RC, reaction center

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