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TRIPLET-MINUS-SINGLET ABSORBANCE DIFFERENCE SPECTRA OF REACTION CENTERS AND ANTENNA PIGMENTS OF THE GREEN PHOTOSYNTHETIC BACTERIUM *PROSTHECOCHLORIS AESTUARII*

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We have applied absorbance-detected electron spin resonance in zero magnetic field to several pigment-protein complexes that belong to the membrane-bound photosystem of the green sulfur bacterium *Prosthecochloris aestuarii*. It was found that three triplet states can be discerned, that are formed in the light-harvesting bacteriochlorophyll *a* protein, the core complex and in the primary donor P-840, respectively. Triplet-minus-singlet absorbance difference spectra of the latter two states are presented. The spectrum of the core complex shows a bleaching at 837 nm and an absorbance increase at 808 nm. This suggests a strong electronic interaction between at least two of the constituent BChl *a* molecules of the complex. The triplet-minus-singlet spectrum of P-840 shows two negative bands at 826 and 837 nm, that, according to their linear dichroism, have almost parallel polarization. It is shown that no spectral evidence exists for the presence of two resolved dimer exciton bands of P-840. We conclude that P-840 either consists of two weakly coupled BChl *a* molecules or of a strongly coupled pair with one allowed exciton band at 837 nm, the other blue-shifted exciton component being very weak. Decay rates of P^T-840 of 6790 (±500) s^{-1}, 3920 (±300) s^{-1} and 1275 (±100) s^{-1} were observed for the *x*, *y* and *z* triplet sublevels, respectively.

Introduction

The photosystem of the green sulfur bacterium *Prosthecochloris aestuarii* consists of a large BChl *c* containing antenna structure, the chlorosome, connected to the cytoplasmic membrane by a baseplate that contains the well-characterized light-harvesting BChl *a* protein (LH-BChl *a* protein) [1,2]. Excitation energy that is captured by the chlorosome is funnelled through this baseplate to the membrane-bound antenna complexes, and is finally transferred to the primary electron donor P-840.

So far isolated, photochemically active, reaction centers have not been obtained from the *Chlorobiaceae*. Information about the nature of the primary donor P-840 has been derived from absorbance difference spectroscopy and ESR measurements on relatively large pigment-protein complexes [3–6], and is still rather limited as compared to the extensive body of data on the reaction center of purple bacteria [7]. The oxidized-minus-reduced absorbance difference spectrum of P-840 [4,5] clearly deviates from that observed for the primary electron donor of purple bacteria and *Chloroflexaceae* [7–9]. The main features are a bleaching at 830–840 nm, a blue absorbance shift around 800 and a red shift around 670 nm. From ESR

Abbreviations: ADMR, absorbance-detected electron spin resonance in zero magnetic field; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; LD-ADMR, linear dichroism of ADMR; LH-BChl *a* protein, light-harvesting BChl *a* protein; P-840, primary electron donor; T – S spectrum, triplet-minus-singlet absorbance difference spectrum.

measurements, it was concluded that the odd electron is shared by two BChl *a* molecules when P-840 is oxidized [6]. The redox potential of P-840 is 200 mV lower than usually found for other photosynthetic bacteria [3] and the green sulfur bacteria are capable of generating directly a reductant that is strong enough to reduce NADP$^+$ [10].

We have applied absorbance-detected electron spin resonance in zero magnetic field (ADMR) to photochemically active pigment-protein complexes of *P. aestuarii.* These complexes, designated photosystem-pigment complex and reaction center pigment-protein complex, respectively, are capable to form a stable charge separation [4,5]. They consist of the so-called core complex, that is associated with the reaction center [11,12] and in addition they contain different amounts of the LH-BChl *a* protein.

In the present paper, we report a study of the properties of the triplet states that are formed in the core complex and in the reaction center and present their triplet-minus-singlet (T − S) absorbance difference spectra. We have also measured the linear dichroism of the T − S spectrum of P-840, and implications for the possible structure of the primary electron donor will be considered. The triplet state of the LH-BChl *a* protein will be the subject of a subsequent publication.

Materials and Methods

Prosthecochloris aestuarii, strain 2K, was grown anaerobically in a mixed culture originally known as *Chloropseudomonas ethylica* [13], as described by Holt et al. [14]. Photosystem-pigment and reaction center pigment-protein complexes were prepared according to Ref. 4, the core complex as described in Ref. 11, and the LH-BChl *a* protein according to Ref. 15.

The ADMR experiments were performed at 1.2 K as described in Refs. 16 and 17. The techniques used to obtain the polarized ADMR spectra (LD-ADMR) have been described in Ref. 18. Kinetics of the absorbance changes were measured with the pulse method [19,20].

Samples were suspended in 10 mM phosphate-ascorbate buffer (pH 7.4) containing 0.05% (v/v) Triton X-100 and diluted to about 65% (v/v) with ethylene glycol; the absorbance was adjusted to approx. 0.5 at 1.2 K at 810 nm; just before the experiment, sodium dithionite to a final concentration of 10 mM was added to keep the secondary electron acceptors (FeS_I and FeS_{II}) in the reduced form.

Results

Absorbance-detected ESR spectra and kinetic parameters

Fig. 1A and B show the absorbance-detected ESR spectra at 1.2 K of the reaction center pigment-protein complex, monitored at 836.5 nm within the singlet absorbance band of P-840. Two strong transitions were observed at 515 and 735 MHz which gave rise to identical T − S spectra (see below). The same transitions were observed in the photosystem-pigment complex. The zero field splitting parameters $|D|$ and $|E|$ of this triplet state are 208.3 $(\pm 0.7) \cdot 10^{-4}$ and 36.7 $(\pm 0.7) \cdot 10^{-4}$ cm^{-1}, respectively, which is in excellent agreement with those previously reported by Swarthoff et al. [6] for the spin-polarized light-induced ESR spectrum of the photosystem-pigment complex (207 $(\pm 2) \cdot 10^{-4}$ and 37 $(\pm 1) \cdot 10^{-4}$ cm^{-1}, respectively).

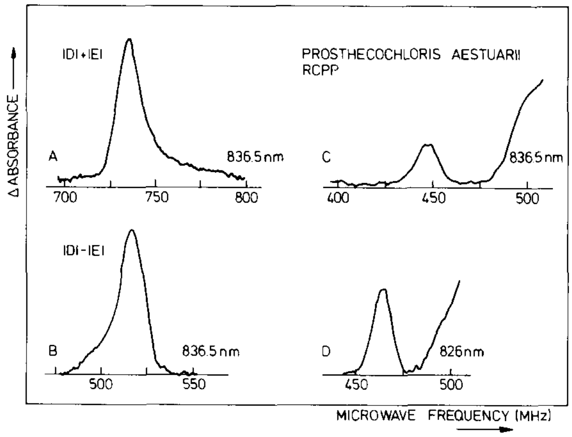

Fig. 1. Zero field triplet ESR spectrum at 1.2 K in the reaction center pigment-protein (RCPP) complex from *P. aestuarii.* The detection monochromator (optical resolution 3.2 nm) was set at various wavelengths as indicated. The spectra are single scans of 100 s duration obtained with lock-in detection and amplitude modulation of the microwave radiation at 313 Hz; time constant 1 s. (Note that in C and D, the reaction center microwave transition at 515 MHz is also detected.)

We therefore assume that these microwave transitions belong to the reaction center triplet which results from radical pair recombination. The high quantum yield of this triplet state [5] is in accordance with the high intensity of its microwave transitions relative to the other microwave transitions detected (see below).

Sublevel decay rates of the reaction center triplet state were measured as a function of the flux of incident exciting light after a brief irradiation with microwaves swept rapidly (1 MHz/μs) through the $|D|-|E|$ or $|D|+|E|$ transition. The true molecular decay rates k_x, k_y and k_z were measured at low light intensity and are given in Table I. The k_x and k_y values differ appreciably from those determined by conventional ESR techniques. Conventional ESR necessitates extrapolation of the decay rates to low microwave power [21], whereas with the ADMR technique, the only requirement for the correct determination of sublevel decay rates is that they be independent of the incident light flux. The microwaves are only used to perturb the system from equilibrium and not to monitor population differences. Hence, we assume that the decay rates presented here are more reliable. The fact that optically a quasi-monophasic decay rate at 5 K was reported for the triplet state [6] is explained by the use of a time resolution that was too low to resolve the fastest component (k_x).

Another distinct triplet state with a microwave transition centered at 450 MHz was observed upon detection at 836.5 nm (Fig. 1C). Its low intensity is indicative of an internal conversion mechanism for the formation of this triplet. The corresponding T − S spectrum differed from that of P-840 and in the following section we will present evidence that this triplet state originates in the core complex. We attribute the 450 MHz ESR line to the $|D|-|E|$ transition. The $|D|+|E|$ transition was located at 785 MHz in isolated core complex, from which $|D| = 205.8 \cdot 10^{-4}$ and $|E| = 55.8 \cdot 10^{-4}\,\mathrm{cm}^{-1}$ were calculated (see Table II).

Upon detection at 826 nm, a third triplet state appeared, with a microwave transition centered at 464 MHz (Fig. 1D). The same microwave transition, with a similar T − S spectrum was observed in isolated LH-BChl *a* protein, where also a second transition at 795 MHz was detected. Thus, this triplet state is formed in the LH-BChl *a* protein. Its microwave transitions and the associated T − S spectra will be discussed in a subsequent publication.

Table II summarises the microwave transition frequencies and the zero field splitting parameters obtained for the reaction center pigment-protein complex of *P. aestuarii* for the three triplet species that were detected. Note that the $|D|+|E|$ transitions of the core complex and of the LH-BChl *a* protein were not resolved in the reaction center pigment-protein complex.

Triplet-minus-singlet absorbance difference spectra

Reaction center triplet states. The ADMR-monitored T − S spectra of the reaction center triplet obtained when the $|D|+|E|$ transition was irradiated at 735 MHz is shown in Fig. 2 for the reaction center pigment-protein complex. The sign of the microwave transition monitored at 836.5 nm corresponds to an increase in the ground state population under resonant conditions, as was also found for the reaction center triplets of BChl *a* and BChl *b* containing purple bacteria [17]. The spectrum shows sharp negative bands at 837 and 826 nm, an absorbance increase at 814 nm and weak bands in the Q_x and Soret region of BChl *a*. Especially in the region 360–650 nm, the shape of the spectrum

TABLE I

TRIPLET SUBLEVEL DECAY RATES

Species	k_x (s^{-1})	k_y (s^{-1})	k_z (s^{-1})	Average (s^{-1})	Method
P^T-840, *P. aestuarii*	6790 ± 500	3920 ± 300	1275 ± 100	3995 ± 300	ADMR (this work)
	3000 ± 400	3000 ± 400	1300 ± 150	2430 ± 300	ESR [6]
	–	–	–	2475 ± 300	absorbance [6]
Monomeric BChl *a* in methyltetrahydrofuran	11950 ± 700	15900 ± 1300	1635 ± 50	9830 ± 700	ADMR [20]

TABLE II

MICROWAVE TRANSITION FREQUENCIES AND ZERO FIELD SPLITTING PARAMETERS

Triplet state	ν_1 (MHz)	ν_2 (MHz)	$\|D\|$ ($\times 10^{-4}$ cm^{-1})	$\|E\|$ ($\times 10^{-4}$ cm^{-1})	Ref.
Reaction center	515	735	208.3 ± 0.7	36.7 ± 0.7	this work
Reaction center	–	–	207.0 ± 2.0	37.0 ± 2.0	6
Core complex	450	785	205.8 ± 0.7	55.8 ± 0.7	this work
LH-BChl *a* protein	464	795	209.8 ± 0.7	55.2 ± 0.7	this work
Monomeric BChl *a* in methyltetrahydrofuran	516	864	230 ± 2	58 ± 2	20

is similar to the absorbance difference spectra obtained by Swarthoff et al. [5], which supports the assignment of these spectra to a BChl *a* triplet state. The spectrum in the region 600–700 nm is shown in 10-fold enlargement in the insert of Fig. 2. In addition to a bleaching of the Q_x band of BChl *a*, three distinct bands are present around 670 nm with minima and maxima at 665, 670 and 672 nm, respectively. These bands may be ascribed to BPh *c* or related pigments (Braumann, Th. and Vasmel, H., unpublished data) that exhibit absorbance bands at 4 K at 665, 670 and 675 nm, respectively [22]. Recent evidence from nanosecond-resolved spectroscopy suggests that a pigment absorbing at 670 nm (probably BPh *c*) acts as an intermediary electron acceptor in the electron-transport chain [23]. The absorbance changes in the T – S spectrum in this region agree with the notion that this pigment is situated in close vicinity to the primary donor.

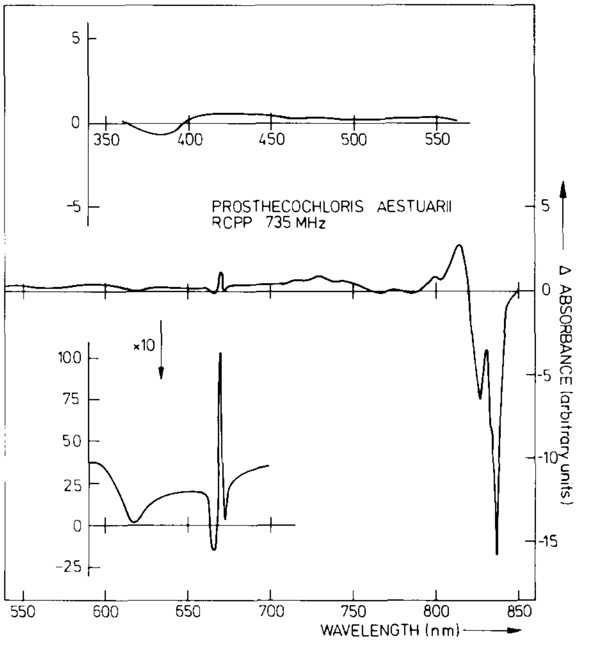

Fig. 2. The ADMR-monitored reaction center T – S spectrum at 1.2 K in the reaction center pigment-protein (RCPP) complex, recorded with the resonant microwaves set at 735 MHz (|*D*| + |*E*| transition). Spectral resolution is 3.2 nm for the whole range, except for the insert 600–700 nm, where it is 1.6 nm; the spectrum was constructed from single scans at different sensitivities for the various wavelength regions; time constant 1 s.

The bleaching in the Soret region and in the Q_x region of the spectrum is remarkably small compared to the absorbance decrease in the Q_y region of BChl *a*, as was also found for the reaction center triplets of all purple bacteria studied so far [17,27,34]. This is probably due to a strong BChl a^T absorbance, as the triplet-triplet absorbance spectrum of BChl *a* in vitro shows an oscillator strength in these regions that is comparable to that of the singlet-singlet absorbance [24].

The T – S spectrum of the reaction center pigment-protein and photosystem-pigment complexes in the region 790–850 nm is shown in more detail and at higher resolution in Fig. 3. The two spectra are similar, though not identical, and when they are normalized at their maxima at 836.8 nm, it becomes clear that the shoulder at 833.7 nm and the maximum at 830.8 nm can be attributed to a band shift which is more prominent in the photosystem-pigment than in the reaction center pigment-protein complex (Fig. 3C). A band at 834 nm is indeed resolved in the 4 K absorbance spectrum of both preparations [22] and we suggest that this band shifts to slightly shorter wavelength upon triplet formation of P-840.

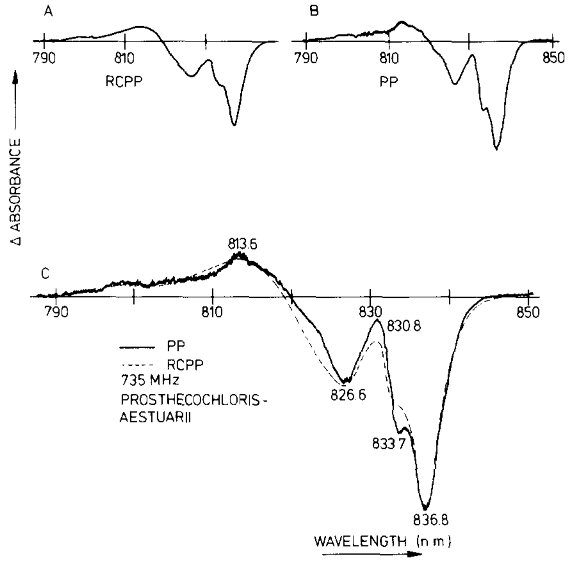

Fig. 3. Comparison of the ADMR-monitored reaction center $T-S$ spectra of reaction center pigment-protein (RCPP) and photosystem-pigment (PP) complexes between 790 and 850 nm. Spectral resolution 1.6 nm; other conditions as for Fig. 2. The spectra are single scans of 3 s per 0.2 nm step, time constant 1 s. (A) reaction center pigment-protein complex; (B) photosystem-pigment complex; (C) the two spectra normalized at their maxima at 836.8 nm.

The two most prominent features in the near-infrared region of the $T-S$ spectrum are negative bands at 826.6 and 836.8 nm. If these are due to simultaneous bleachings of two absorbance bands of the primary donor, they would be expected to be exciton-coupled. Alternatively, the 836.8 nm band could be the only band that signifies a bleaching of the primary donor, the other bands, including the positive band at 813.6 nm, being due to absorbance shifts and absorbance changes that occur in neighboring pigments. To discriminate between these possibilities, we have measured the polarization of the triplet-minus-singlet absorbance changes with the recently developed method of LD-ADMR spectroscopy [18]. The method is analogous to that used for photoselection, except for the fact that the selection of a particular distribution of molecules in the isotropic sample is achieved by excitation with polarized microwaves instead of polarized light. The transition moment of a microwave transition is polarized normal to the plane formed by the dipolar axes of the two triplet levels between which the transition takes place, which usually coincide with molecular symmetry axes [25]. The difference of the $T-S$ spectra for light polarized parallel and perpendicular to the direction of microwave polarization is detected.

By excitation in the $|D|-|E|$ and $|D|+|E|$ transitions, orientations corresponding to two mutually perpendicular axes of the reaction center are selected and the orientation of the optical transitions in the unpolarized $T-S$ spectrum relative to

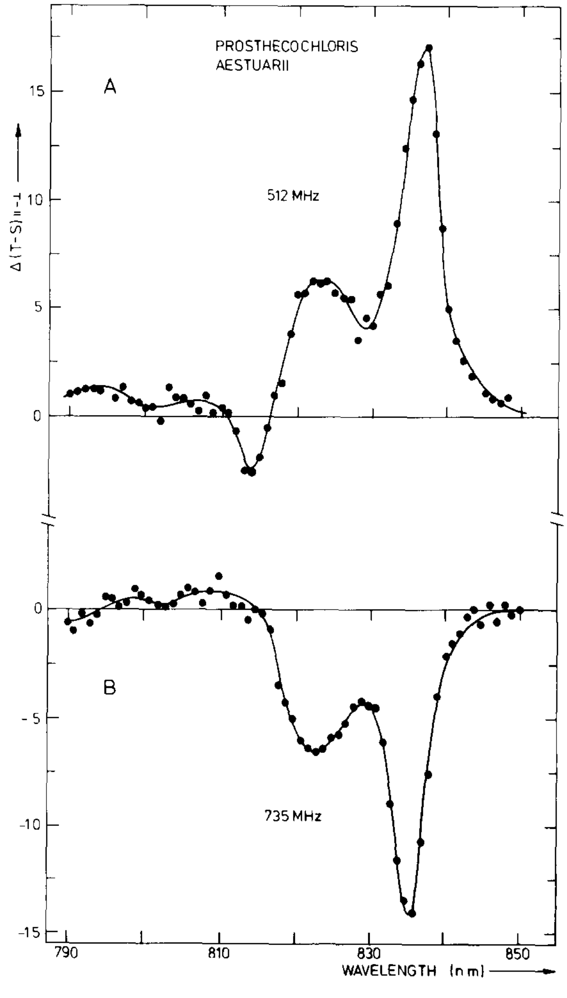

Fig. 4. Linear dichroic $T-S$ spectra of P-840 recorded in the reaction center pigment-protein complex. The spectra are the hand-average of two scans, with a time constant of 3 s and spectral resolution of 3.2 nm. The microwave frequency corresponds to the $|D|-|E|$ transition (A) and the $|D|+|E|$ transition (B), respectively.

these axes can be derived. The two LD-ADMR spectra of the reaction center pigment-protein complex thus obtained are shown in Fig. 4. In both spectra, the two principal long-wavelength bands (826.6 and 836.8 nm) are seen to have the same sign of polarization with respect to the selected axes. Moreover, the ratio of their intensity is practically the same as in the unpolarized T − S spectrum. This is strong evidence that the two bands are approximately parallel. This means that they cannot be due to an exciton pair, because the transition moments of the members of an exciton pair are mutually perpendicular.

Comparison of the two LD-ADMR spectra also suggests that the band at 814 nm does not form part of a band shift but represents a real absorbance increase, because its polarization relative to the two triplet axes is clearly different from that of the band(s) in the region 820–825 nm. The shoulder at 833.7 nm was not resolved in these spectra.

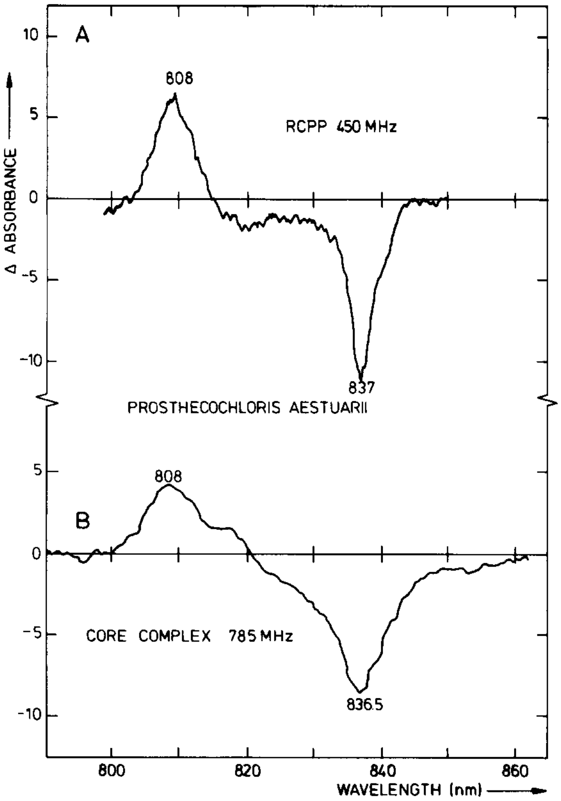

Fig. 5. ADMR-monitored difference spectra of the reaction center pigment-protein (RCPP) complex (A) and the isolated core complex (B). Microwave frequencies as indicated. The spectra are single scans of 600 s, time constant 3 s. Other conditions as for Fig. 2.

We will return to the interpretation of the reaction center triplet absorbance difference spectrum in the Discussion and Conclusions.

Core complex triplet state. A triplet was also detected in the core complex. This complex lacks the LH-BChl *a* protein and is closely associated with the reaction center [11,12].

Fig. 5 shows that the 450 MHz microwave transition observed in the reaction center pigment-protein complex (Fig. 1) can be ascribed to the core complex. Its T − S spectrum is shown in Fig. 5A. In the isolated core complex, a similar spectrum was obtained at 450 MHz ($|D|-|E|$ transition). The corresponding $|D|+|E|$ transition was located at 785 MHz in the isolated core complex (Table II) and yielded the spectrum shown in Fig. 5B. Except for small differences in the region 815–830 nm, possibly due to additional pigment shifts in the spectrum of the isolated core complex, the two spectra agree quite well. This supports the conclusion [11] that the structure of the core complex is left virtually unchanged upon isolation.

Discussion and Conclusions

Three different complexes derived from the membrane-bound photosystem of *P. aestuarii* have been investigated by ADMR: the photosystem-pigment, reaction center pigment-protein and core complex. The reaction center pigment-protein complex contains only the membrane-bound complexes of the photosystem (subunits of LH-BChl *a*, core complex and reaction center), while the photosystem-pigment complex comprises in addition two more loosely bound molecules of the LH-BChl *a* protein [26]. In agreement with this proposed organization, we detect by ADMR three well-defined triplet states in the reaction center pigment-protein and photosystem-pigment complexes. Two of these triplet states will be considered below; the triplet state of the LH-BChl *a* will be discussed in a subsequent paper. As expected, this triplet state was present in both photosystem-pigment and reaction center pigment-protein complexes but not in the isolated core complex.

The triplet state with microwave transitions at 450 and 785 MHz is formed in all three complexes. This indicates that it originates from the core complex. The main features of its T − S spectrum (Fig. 5) are a negative band at 837 and a positive band at 808 nm. These can be interpreted by the assumption that a strong electronic interaction, at least on the level of a dimer, is responsible for the band at 836 nm in the absorbance spectrum of the core complex. Apparently, the triplet state is localized on one of the constituent BChl *a* molecules of the dimer. Because of the ensuing strong decrease in the electronic interaction between the members of the dimer upon triplet formation, the absorbance band of the dimer (837 nm) is bleached and a monomer BChl *a* absorbance band appears around 808 nm. A similar interpretation has been applied to the T − S spectra of isolated reaction centers of purple bacteria [17]. Although recently a fraction similar to the core complex has been isolated that retained photochemical activity [12], our preparation showed none [11], and no triplet state of the primary donor was detected in the isolated complex.

The triplet state with microwave transitions at 515 and 735 MHz can be identified as being the triplet state of the reaction center. It was only formed in photosystem-pigment and reaction center pigment-protein complexes and its $|D|$ and $|E|$ values are identical to those of the spin-polarized triplet found earlier in the photosystem-pigment complex by Swarthoff et al. [6]. The T − S spectrum of this state should therefore contain the bleaching of the absorbance band(s) of P-840. In addition, triplet-triplet absorbance of P-840 and singlet-singlet absorbance bands of the new electronic configuration (including band-shifts of neighboring pigment molecules) can be expected to contribute to the difference spectrum.

The unpolarized T − S spectrum shown in Fig. 2 and in more detail in Fig. 3 contains two negative bands, located at 826.6 and 836.8 nm. If the corresponding bands in the singlet absorbance spectrum form an exciton pair, then the two bleachings should possess a perpendicular polarization. The polarized T − S spectra measured for two mutually perpendicular orientation axes (Fig. 4) show that for the 826 and 837 nm bands:
(1) the ratio and sign of the intensities in the polarized spectra is practically identical to that in the unpolarized T − S spectrum (Figs. 2 and 3);
(2) in the region of these bands, the two polarized spectra have opposite sign.
These requirements can be separately satisfied by two perpendicular transitions, but not simultaneously. The only configuration that is compatible with both observations is that in which the two transitions are approximately parallel (Hoff, A.J., Den Blanken, H.J., Vasmel, H. and Meiburg, R.F., unpublished data).

The linewidth of the ESR signal of P^+-840 (9.0 ± 0.2 G compared to 13.5 G for monomeric BChl a^+ in vitro; Ref. 6) argues against a monomeric primary donor (but note that an alternative interpretation has been proposed recently [27]). The narrowing of the ESR line has been ascribed to delocalization of the positive charge over the two BChl molecules, but the interaction between the two molecules that is responsible for this delocalization may be weak, as a hopping rate larger than about 20 MHz would suffice, corresponding to about $1 \cdot 10^{-3}$ cm^{-1}, i.e., a splitting of only $1 \cdot 10^{-4}$ nm. Thus, the 826 and 837 nm bands might be weakly coupled bands of a dimeric primary donor. In that case, the different locations of the absorbance bands of the BChl *a* molecules could be explained by different red shifts with respect to the monomer transitions due to electrostatic effects that result from pigment-protein interactions [28]. One would then expect the triplet to be localized on the pigment with the lowest lying excited state. The bleaching at 837 nm then would conceivably be caused by triplet formation on one of the component BChl *a* molecules, whereas a redistribution of oscillator strengths between a pigment absorbing at 814 nm and the other component of P-840 may explain the absorbance changes at shorter wavelengths. The former pigment may be the BChl *a* molecule that can be photoreduced at low redox potential [29]. The alternative explanation is that only one of the bands (presumably the 837 nm band) is due to the primary donor; it might then be the allowed exciton component of a strongly coupled dimer or be composed of two nonresolved absorbance bands of weakly coupled BChl *a* molecules. In the latter case, the exciton coupling can only be weak, since the bleaching at 837 nm has a uniform polarization across the band (Fig. 4). Also, if the 826 nm is not due to P-840, a redistribution of oscillator strength between the pigments absorbing at 826 and 814 nm must be assumed to occur. Then, both bands must be ascribed to pigments in close proximity to the primary donor that are not resolved in the absorbance spectrum.

The presence of another porphyrin molecule close to the reaction center is indicated by the absorbance changes around 670 nm (Fig. 2). These absorbance difference bands are much weaker than those observed in the same wavelength region when P-840 is photooxidized [5]. They may be due to a slightly altered exciton interaction between a pigment absorbing in this region (probably BPh *c*) and the primary electron donor upon triplet formation. By means of nanosecond flash spectroscopy, BPh *c* has been tentatively identified as the primary electron acceptor [23].

In conclusion, we remark that the application of ADMR to the photosystem of the green sulfur bacterium *P. aestuarii* has allowed us to gain insight in the spectroscopy of its primary donor P-840. In the course of the last years, several explanations have been presented to account for the spectral properties of the primary donor of bacterial photosynthesis. These include the charge transfer model [30,31], the implication of local pigment-protein interactions [28] and exciton theory (discussed in Ref. 32). We conclude from our data, that there is no spectral evidence for the existence of two resolved exciton bands due to the primary donor of *P. aestuarii.* The same conclusion has been drawn for the primary donor of the gliding green bacterium *Chloroflexus aurantiacus* [33] and of several species of purple bacteria [17]. Whether the primary donor of *P. aestuarii* consists of two relatively weakly coupled BChl *a* molecules (absorbing either both at 837 nm or at 826 and 837 nm) or of a strongly coupled BChl *a* pair with one allowed exciton band at 837 nm has to be decided from future experiments.

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