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THE TRIPLET STATE OF THE PRIMARY DONOR OF THE PHOTOSYNTHETIC BACTERIUM *RHODOPSEUDOMONAS VIRIDIS*

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The absorbance-detected magnetic-resonance technique has been applied to the study of the triplet state of the primary donor in chromatophores of the photosynthetic bacterium *Rps. viridis*. The results confirm the triplet-minus-singlet absorbance-difference spectrum and its interpretation as previously obtained for isolated reaction centers (Den Blanken, H.J. and Hoff, A.J. (1982) Biochim. Biophys. Acta 681, 365–374). Our present results affirm that the primary donor is a bacteriochlorophyll *b* dimer, and that there is no blue exciton band at 850 nm. We show that the reaction centers are not identical, but have a small heterogeneity in their properties. In chromatophores and sometimes in isolated reaction centers a shoulder is observed in the long-wavelength absorbance-difference band of the primary donor. This shoulder is possibly caused by charge transfer interaction of the donor with an adjacent chromophore (Vermeglio, A. and Paillotin. G. (1982) Biochim. Biophys. Acta 681, 32–40; Maslov, V.G., Klevanik, A.V., Ismailov, M.A. and Shuvalov, V.A. (1983) Doklady Akad. Nauk. SSSR 269, 1217–1221) or it reflects a slight heterogeneity in the reaction-center geometry, which cannot be removed with the selection offered by the magnetic resonance technique. The zero-field triplet-ESR spectrum and the sublevel decay rates of the triplet state of the primary donor are presented, as detected in whole cells at the antenna fluorescence, and in chromatophores and isolated reaction centers at the absorbance-difference band at 838 nm. We do not observe the expected reversal of the sign of the ESR transitions monitored with the two techniques. A tentative explanation is given in terms of energy transfer from unrelaxed excited states of the antenna pigments to the reaction center.

Introduction

Understanding the primary photochemistry of bacterial photosynthesis depends on precise knowledge of the structural parameters of the pigments constituting the primary reactants. Optical spectroscopy, including absorbance-difference, linear dichroism and photoselection measurements on various photosynthetic organisms and reaction centers have yielded a wealth of information. Those performed on the BChl *b*-containing purple bacterium *Rps. viridis* are of special interest, because of the wider spectral range and consequently better resolution of its absorbance spectrum compared to that of BChl *a*-containing species. The conversion of spectral data into structural information, however, is severely hampered by the uncertainty regarding the make up of the primary electron donor and the presence of transient acceptors that interact with the donor. Furthermore, there is the problem of the lack of agreement on the interpretation of the absorbance spectrum and the absorbance-difference spectrum (oxidized–reduced) (for recent reviews see Refs. 1 and 2).

For BChl *a*-containing bacteria it is generally

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accepted on the basis of ENDOR data that the primary donor (generically labeled P-870) is a BChl *a* dimer [3–5]. For *Rps. viridis* this is less certain [6]; the ENDOR data of the oxidized donor ($P\text{-}960^+$, note that at cryogenic temperatures the long-wavelength absorption band shifts to 990 nm) support a dimeric model when a twist in the position of the β-protons on ring IV of the BChl *b* molecule is imposed [6], but more definite proof for a dimeric P-960 is clearly desirable. Additional structural information on P-960 may be obtained by the study of its triplet state: the values of the zero-field splitting parameters $|D|$ and $|E|$ and especially the sublevel decay rates k_u ($u = x, y, z$) are sensitive to dimerization [7]. One aim of the present work is a precise determination of those parameters by ESR in zero field, detected optically via the fluorescence (fluorescence-detected magnetic resonance, FDMR) or via the singlet ground-state absorption (ADMR).

We have recently employed the ADMR method to record with high sensitivity and high resolution the triplet – singlet absorbance-difference (T – S) spectrum of isolated reaction centers of *Rps. viridis* at 1.2 K [8]. Another aim of the present work is to extend those measurements to chromatophores, and to achieve even higher resolution by using site-selection methods [9]. This is of importance, because the triplet state is a gentler perturbing probe than the cation and comparison of the triplet – singlet absorbance-difference spectrum (the $P\text{-}960^T$ – P-960 or T – S spectrum) [1,10,11] with the light-induced absorbance-difference spectrum of oxidized minus reduced P-960 (the $P\text{-}960^+$ – P-960 or redox spectrum) aids in the interpretation of the latter. One expects the bleachings of the bands due to P-960 to be identical for both types of absorbance-difference spectrum. Their main difference lies in shifts exhibited by neighboring pigments and in the presence of absorption bands of the triplet or the cation, respectively.

The results of the magnetic resonance experiments are similar to those of P-870 in BChl *a*-containing purple bacteria and are consistent with P-960 having a dimeric structure, but by no means prove this. $|D|$ and $|E|$ are about 30% less than the corresponding values of monomeric BChl *b* in vitro, but the latter are sensitive to ligation effects. Similarly, the small differences between the values of the decay rates for P-960 and those of BChl *b* in vitro could equally well be due to dimerization and/or to environmental effects on a monomer. Therefore, it is not possible to draw firm conclusions regarding the aggregation state of the donor that are based on the values of $|D|$ and $|E|$ and the decay rates alone without knowledge of the ligands of the P-960 complex in the protein matrix and of possible charge transfer contributions.

The sign found for the FDMR resonances in whole cells and the ADMR resonances in chromatophores and isolated reaction centers poses a special problem. The expected sign reversal [12,8] for the FDMR lines compared to the ADMR lines does not occur. A tentative explanation is given based on the fact that the antenna pigments in *Rps. viridis* absorb to longer wavelengths than does the primary donor.

The T – S spectrum of chromatophores is very similar to that found previously for isolated reaction centers [11]. This excludes possible contributions to the latter spectrum of artefacts produced by the isolation procedure. Site-selection reveals a shoulder in the bleached long-wavelength absorption band that corresponds to the shoulder in the 990 nm absorption band found in the 4.2 K absorbance spectrum [13]. Moreover, an overall narrowing of this band is produced by site-selection, which shows that part of the width of the 990 nm absorption band is due to an inhomogeneous distribution of electronic 0–0 transitions that is caused by a heterogeneity in the structure and the optical properties of the reaction center [14,15].

The isolation procedure of the reaction center affects the zero-field splitting parameters and the position of the 990 nm absorption band in the site-selected T – S spectrum, but the 790–890 nm region is identical for isolated reaction centers and for chromatophores. In contrast to Ref. 13 we do not observe a bleaching in this region that can be interpreted as an exciton component of P-960. The bands at about 850 and 870 nm are well described by a shift of the absorption band at about 850 nm of one of the BChl *b* accessory pigments [11]. The band appearing at 838 nm is ascribed to one of the BChl *b* monomers making up P-960 [11].

The T – S spectrum of *Rps. viridis* and its interpretation are analogous to that of P-870 and as such strongly support a dimeric model for the

primary donor of *Rps. viridis*. The splitting in the 990 nm absorption band is possibly caused by a charge transfer interaction of the donor with an adjacent chromophore [13] or the splitting reflects a slight heterogeneity in reaction center geometry that is not removed by the ADMR technique (see appendix Ref. 9).

Materials and Methods

Isolated reaction centers of *Rps. viridis* were prepared as described in Ref. 11. We received from Dr. J.P. Thornber isolated reaction centers in 0.1% nonidet NP-40. The FDMR experiments on whole cells were performed essentially in the same way as described in Ref. 16. We used a Kodak 5233 filter instead of the detection monochromator and in the excitation path a Corning CS-7-59 filter instead of the Corning CS-7-51 filter. The ADMR experiments on chromatophores and isolated reaction centers were performed as described in Ref. 8. The T – S spectra were recorded as in Ref. 11. The kinetics were measured with the pulse method [17,18].

Results and Discussion

Fluorescence- and absorbance-detected triplet-ESR spectra in zero-field and kinetic parameters

Fig. 1. shows the ADMR triplet-ESR spectra in chromatophores monitored at various wavelengths within the 990 nm singlet absorption band of the primary donor. It is seen that the shape of the ADMR spectra is dependent on the wavelength of the monitoring light. Analogous effects were also observed in chromatophores of the BChl *a*-containing purple bacterium *Rps. sphaeroides* R-26 [9]. They are attributed to a heterogeneity in the reaction centers that is (partly) removed by monitoring the changes in absorbance at a particular wavelength, thereby selecting so-called sites [9]. In general, a site is a species with specific physical properties that originate from a specific environment or from a particular configuration of the components making up the species [9]. The inhomogeneous character of the triplet transition lines (i.e., a heterogeneity in the values of $|D|$ and $|E|$) is demonstrated by a hole-burning experiment [19,20]. The width of the hole is 7.0 MHz for the

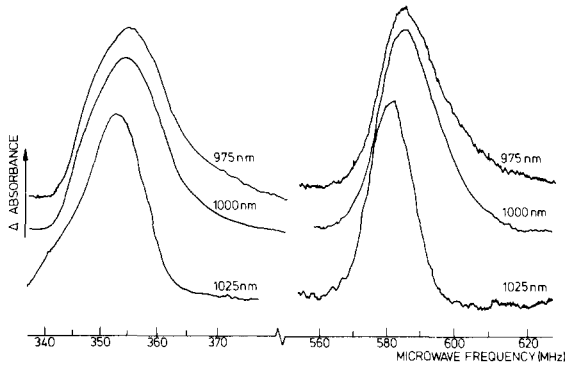

Fig. 1. ADMR transitions of the primary donor in chromatophores at 1.2 K. The detection monochromator was set at various wavelengths as indicated. The spectra are normalized for easier comparison. They are single scans with lock-in detection and amplitude modulation of the microwaves at 313 Hz. Total scan time 100 s, response time 1 s. The optical resolution of the detecton monchromator was 3.2 nm.

$|D|-|E|$ transition and 3.7 MHz for the $|D|+|E|$ transition, which values represent twice the homogeneous linewidth. We believe that the sites correspond to slightly different geometries of the

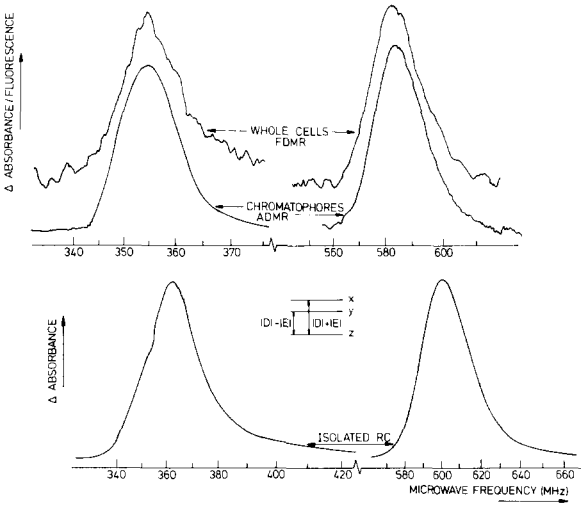

Fig. 2. The zero-field triplet-ESR spectrum of the primary donor in whole cells by FDMR at the total antenna fluorescence, and in chromatophores and isolated reaction centers by ADMR at 838 nm. All spectra are single scans with a scan time of 100 s. The FDMR spectrum of whole cells was recorded with a response time of 3 s, the ADMR spectrum of chromatophores and isolated reaction centers with a response time of 1 s. Other parameters as in Fig. 1. Insert: the triplet sublevels in zero-magnetic field with the two microwave transitions.

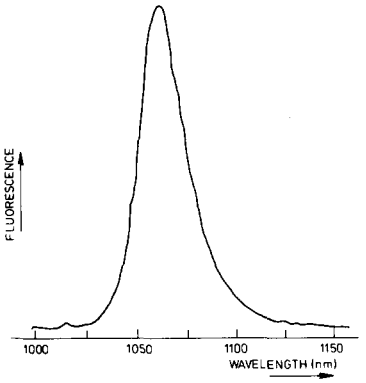

Fig. 3. The fluorescence spectrum of the antenna complex in whole cells at 4.2 K. The optical resolution is 3.2 nm. The fluorescence was detected in a direction perpendicular to that of the excitation light.

BChl molecules making up the primary donor [9]. Small changes in their configuration will lead to small differences in excitonic and/or charge-transfer interaction, which in turn will lead to shifts of the long-wavelength absorbance which is correlated to variations in the values of $|D|$ and $|E|$. (See also the section on the triplet-minus-singlet absorbance-difference spectra.)

The shape of the triplet-ESR spectra monitored at a particular wavelength λ in the 990 nm band results from the product of the distribution of sites coupled to any particular microwave frequency and their oscillator strength at wavelength λ [9]. Only when all sites have the same oscillator strength connected with an absorbance change at a particular wavelength, λ, does the shape of the ADMR line represent the true distribution function of the sites. We have found that all triplet sites are linked to an absorbance change at the sharply defined wavelength of 838 nm (see the section on the T − S spectra). Thus, detection at this wavelength yields an ADMR triplet-ESR spectrum in which all sites are equally weighted [9] (i.e., no optical selection) and whose shape gives the distribution function of the values of $|D|$ and $|E|$ that make up the ensemble of triplet sites. In Fig. 2 is shown the ADMR triplet-ESR spectrum monitored at the 838 nm band in chromatophores

TABLE I

CHARACTERISTICS OF THE TRIPLET STATE OF THE PRIMARY DONOR AND OF MONOMERIC BChl

	$\lvert D\rvert$	$\lvert E\rvert$	k_x	k_y	k_z	Ref.
	(cm^{-1}) ($\times 10^{-4}$)		(s^{-1})			
Rps. viridis						
cells	157	37				[21]
cells	153 ± 2	37 ± 2				[22]
cells	156.2 ± 0.7	37.8 ± 0.7	⩽16000	⩽16000	⩽2600	this work
chromatophores	158	38				[21]
chromatophores	156.2 ± 0.7	37.8				this work
isolated RC	155 ± 1	36 ± 1				[31]
isolated RC	160.3 ± 0.7	39.7 ± 0.7	13700 ± 900	16100 ± 1300	2420 ± 90	this work
BChl *b*						
toluene/10% pyridine	212 ± 6	55 ± 2				[32]
methyltetrahydrofuran	221 ± 2	57 ± 2	12400 ± 900	14900 ± 1300	1300 ± 30	[18]
Rps. sphaeroides R-26						
cells	187.2 ± 0.6	31.2 ± 0.6	9000 ± 1000	8000 ± 1000	1400 ± 200	[11]
isolated RC	188.0 ± 0.4	32.0 ± 0.4				[12]
BChl *a*						
methyltetrahydrofuran	232 ± 4	58 ± 3				[33]
methyltetrahydrofuran	226 ± 5	59 ± 5				[32]
methyltetrahydrofuran	230 ± 2	58 ± 5	11950 ± 700	15900 ± 1300	1635 ± 50	[18]
tetrahydrofuran	238 ± 5	69 ± 3	2887 ± 280	3321 ± 572	661 ± 74	[25]
toluene/10% pyridine	224 ± 7	53 ± 3				[32]
toluene/10% pyridine	227 ± 3	55 ± 3				[33]

and the ADMR triplet-ESR spectrum monitored at the 838 nm band of isolated reaction centers [11]. The notion that Fig. 2 represents the true distribution function of sites can be checked by recording a FDMR triplet-ESR spectrum. Because, according to current models, the antenna pigment complex is coupled to all the reaction centers, monitoring the microwave-induced change in the antenna fluorescence at 1060 nm (see Fig. 3) yields a triplet-ESR spectrum in which all the sites are equally weighted. Hence, this spectrum also gives the true distribution function of the triplet sites. Comparing the FMDR spectrum of whole cells with the ADMR spectrum of chromatophores monitored at 838 nm in Fig. 2, it is seen that the shapes of the two spectra are indeed identical.

The transition frequencies in the optically detected triplet-ESR spectra corresponding to the top of the site-distribution function are identical for whole cells and chromatophores, but differ slightly from those of isolated reaction centers (Fig. 2). We observed similar changes in the ADMR spectrum for isolated reaction centers in 0.1% Nonidet NP-40. For whole cells and chromatophores the values of the corresponding zero-field splitting parameters $|D|$ and $|E|$ agree with those measured with high-field ESR [21,22] but for isolated reaction centers they are slightly different (see Table I). This difference is probably induced by variations in the isolation procedure. A difference for the values of $|D|$ and $|E|$ between cells/chromatophores and reaction centers was also observed for the BChl *a*-containing bacterium *Rps. sphaeroides* R-26. There it was ascribed to a change in the distribution function of sites [9], caused by a slightly altered configuration of the primary donor dimer. The shape of the zero-field triplet-ESR spectra of Fig.2 resembles that of the spectra of the BChl *a*-containing purple bacteria [23,20]. The triplet transition lines are comparatively narrow, and only the $|D| \pm |E|$ transitions are observed. Presumably, the $2|E|$ transition has low intensity because of almost steady-state populations of the two sublevels of the triplet state resonant with the microwave field.

The kinetics of the triplet state of the primary donor were measured as a function of incident light flux after a brief pulse of microwaves with frequency corresponding to the $|D| + |E|$ or the $|D| - |E|$ transition [12,17,18]. In whole cells, the signals were too weak to work in the low-light regime, in which the kinetics are independent of light intensity [17]. With the purified reaction centers, the kinetics at low light intensities were independent of light flux, hence in this region the true molecular decay rates k_x, k_y and k_z were measured (see Table I). The average rate is comparable to the inverse lifetime of the optically detected state that was assigned to the triplet state of P-960 in Ref. 24. Our ratios of $k_x : k_y : k_z = 5.6 : 6.6 : 1.0$ differ somewhat from the ratios $k_x : k_y : k_z = 7.5 : 10.0 : 1.0$ obtained by the inherently less accurate computer simulation of the high-field triplet-ESR spectrum [22]. In agreement we find that k_y is larger than k_x, which contrasts with the $k_{x,y}$ values found in the BChl *a*-containing purple bacteria [12,20].

Attempts have been made [25] to establish the structure (monomer or dimer) of the primary donor of BChl *a*-containing purple bacteria using the values of $|D|$, $|E|$ and the triplet-sublevel decay rates of the primary donor and the corresponding values of monomeric BChl in vitro. The then available values were substituted in the exciton model of Sternlicht and McConnell [7] as applied to a symmetric dimer to evaluate the direction cosines of the spin axes of the two monomers in the dimer relative to the dimer spin axes [25]. Such an exercise with our new data for *Rps. viridis*, however, is only of limited value in our opinion. The influence of ligation on the $|D|$ and $|E|$ values of monomeric BChl *b* in vitro is comparable to the difference between those values and the zero-field splitting parameters of the primary donor [18]. Likewise, the small difference between the values of the triplet-sublevel decay rates of P-960 and those of BChl *b* in vitro [18] could easily be due to ligation or charge transfer effects. Thus, without knowledge of the ligands of the P-960 complex in the protein matrix and of possible charge-transfer contributions to P-960 [26], it is not possible to draw firm conclusions on the structure of P-960 based on the measured values of $|D|$, $|E|$ and the triplet-sublevel decay rates [18].

Energy transfer

The sign of the ADMR transitions monitored at the 990 nm absorption band of the primary donor

corresponds to an increase in the ground-state population (positive sign) and is the same as observed for the BChl *a*-containing purple bacteria [8]. However, the expected reversal [12] of the sign of the FDMR transitions, monitored at the antenna fluorescence, with respect to the sign of the ADMR lines does not occur. We believe that the explanation for this unexpected result lies in the peculiar composition of the antenna system of *Rps. viridis*.

In Ref. 12 the negative sign of the FDMR signal of *Rps. sphaeroides* is explained using a Stern-Volmer type quenching relation for the intensity of the fluorescence as a function of the concentration of 'open' traps. In this model it is assumed that an atenna excitation relaxes vibronically in the S_1 state, after which the antenna excitation is transferred to the reaction center. However, in *Rps. viridis* the longest-wavelength absorption band of the antenna pigments lies about 200 cm^{-1} to the infrared of P-960, in contrast to the situation in *Rps. sphaeroides*, where the antenna pigments absorb about 200 cm^{-1} to the blue of P-870. If excitation transfer were to proceed from a relaxed S_1 state, then at 1.2 K ($kT < 1$ cm^{-1}) the energy-transfer process from the antenna to the reaction center would be virtually blocked. However, we observe large ADMR signals monitoring the 990 nm absorption band of the primary donor, which implicates relatively efficient energy-transfer and charge-separation processes. This suggests that the excitation is transferred from a vibronically excited S_1 state to the reaction center. Energetically, this is quite possible, as in the FDMR and ADMR experiments we excited with broad-banded light of high intensity in the 350–450 nm and 720–1100 nm region, respectively. To explain the positive sign of the FDMR signals of *Rps. viridis* we need, in addition to the above assumptions, to assume that (i) the triplet state of P-960 is a more effective trap for the antenna excitation than the singlet ground state of P-960 and (ii) the energy transfer and trapping processes are faster than other processes that relax the excited singlet states of the antenna such as fluorescence, etc.

The above assumptions can be tested by selective excitation into the antenna absorption band. In Fig. 5 we show that the reaction center has no absorption intensity at 1040 nm, the maximum of the antenna absorption band. Therefore, selective excitation by laser light in the 1040 nm band at liquid helium temperatures, combined with yield measurements of primary-donor triplet formation by monitoring the absorbance changes at 838 nm and by monitoring the antenna fluorescence at 1060 nm, will give direct information on the low-temperature energy transfer and trapping efficiencies.

Triplet-minus-singlet absorbance-difference spectra

In Fig. 4 we show the ADMR-monitored T – S spectra of chromatophores in the 780–890 nm region. It is identical to the T – S spectrum of isolated reaction centers [11], which excludes possible contributions to this spectrum of artefacts produced by the isolation procedure. The bands at about 850 and 870 nm depend in a similar way on site effects and are well described by a shift of the absorption band at about 850 nm of one of the BChl *b* accessory pigments [10,11,27]. In contrast to Ref. 13, we do not observe a bleaching in this region that can be interpreted as an exciton component of P-960. The band appearing at 838 nm is ascribed to one of the BChl *b* monomers making up P-960 [11]. The bands at 822 and 831 nm are ascribed to a shift of the absorption band at about 827–831 nm of another BChl *b* accessory pigment [11].

In Fig. 5 are shown the ADMR-monitored T – S spectra of chromatophores and isolated reaction centers in the 890–1040 nm region. The ADMR-monitored T–S spectra of isolated reaction centers are shifted to the blue with respect to the corre-

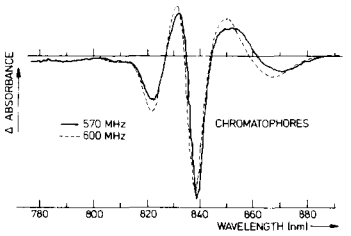

Fig. 4. The ADMR-monitored P-960^T minus P-960 difference spectrum in the 780–890 nm region of chromatophores at 1.2 K. The resonant microwaves were set at 570 and 600 MHz as indicated. The spectra are normalized for easier comparison. The spectra are single scans, other parameters as in Fig. 1.

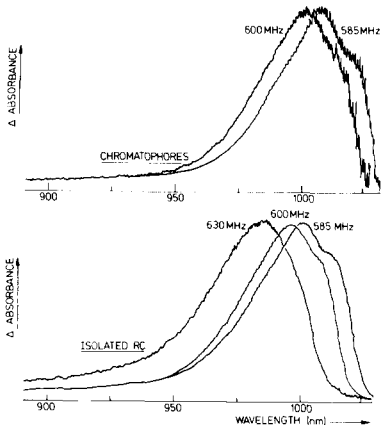

Fig. 5. The ADMR-monitored P-960^T minus P-960 difference spectrum of the long-wavelength absorption band of chromatophores and isolated reaction centers at 1.2 K. The resonant microwaves were set at various frequencies in the $|D|+|E|$ triplet-ESR transition line as indicated. The spectra are single scans and normalized for easier comparison. The small spikes in the spectrum are caused by sudden changes in the intensity of the transmitted light when stepping the detection monochromator over the antenna absorption band at 1040 nm. Their size is proportional to the slope of this absorption band.

sponding T − S spectra of chromatophores. In both preparations the shape of the T − S spectra depends on the frequency of the resonant microwave field. At the lower frequencies a clear shoulder is observed. We have observed a similar shoulder in the long-wavelength bleaching of the T − S spectrum of P-870^T in chromatophores and in isolated reaction centers of *Rps. sphaeroides* R-26 [9]. As discussed in Ref. 9 and in the next section, the variation of the position of the long-wavelength bleaching in the T − S spectrum with microwave frequency demonstrates that the structure of the primary donor as reflected in the optical and triplet properties is not identical for all reaction centers. This heterogeneity is at least partly removed by selecting a particular ADMR frequency. This is direct evidence that there is a correlation between the triplet state parameters and the $S_1 \leftarrow S_0$ spectrum [9]. One possible correlation is that between the redshift of the long-wavelength absorbance of P-960 compared to the in vitro absorption band of BChl *b* (which red shift is generally believed to be at least to an appreciable extent due to exciton interaction within the dimer) and the value of the zero-field splitting parameters. Both are dependent on the characteristics of the exciton interaction, which in turn depend on the detailed geometry of the dimer. The microwave frequency selects a certain slice out of the distribution of geometries, which in turn gives rise to a distribution of $S_1 \leftarrow S_0$ transitions whose shape and peak wavelength differ from that of the envelope of the non-selected long-wavelength absorption band. This interpretation is elaborated in Ref. 9. Another possible cause of the observed correlation is a variation in the degree of admixture of a charge-transfer state caused by variations in the configuration of the reaction center pigments. This possibility will be discussed in the next section.

The shoulder that is observed in some of the ADMR-selected T − S spectra of Fig. 5 indicates that either there is an additional heterogeneity that is not removed by the ADMR selection criterion [9], or that there is a subdistribution of structures (sites) of P-960, in which the long-wavelength absorption band consists of two overlapping transitions [9,13,34]. In support of the first possibility is the observation that in reaction centers of *Rps. viridis*, the intensity of the shoulder is rather variable with slight changes in the isolation procedure, occasionally disappearing altogether. It is noteworthy in this respect that it is possible to isolate reaction centers in which the peak wavelength of the long-wavelength absorption-difference band is shifted up to 100 nm to the blue with respect to that in chromatophores, without apparent detrimental effect on the primary reactions as reflected in the triplet yield. In such reaction-center preparations the ADMR transitions are clearly split and represent two ensembles of reaction centers that significantly differ in their T–S spectrum (data not shown). The second possibility follows a suggestion by Vermeglio and Paillotin [13] and Maslov et al. [34], who observed a shoulder in the low-temperature absorption band and in the oxidized-minus-reduced absorbance-difference spectrum of the primary donor of *Rps. viridis*, and who express the view that the 990 nm band represents the lower-energy exciton component of the primary donor that is split by charge transfer interaction between the donor and a neighboring bacteriochlorophyll. This will be more extensively discussed in the next section.

According to Vermeglio and Paillotin, a bleaching at 850 nm in the oxidized-minus-reduced absorbance-difference spectrum represents the other, high-energy exciton component of P-960 [13]. As is clearly seen in Fig. 4, however, we do not observe a bleaching around 850 nm that could be interpreted to be an exciton component of the primary donor. This is corroborated by the results of polarization spectroscopy applied to the T − S spectrum. Preliminary experiments indicate that the 850 and 870 nm features show about the same polarization, strengthening our earlier interpretation that the positive band at 850 nm does not represent an exciton component of P-960 [11], but a shift of one of the accessory pigments. Our results are in agreement with the linear dichroism experiments of Shuvalov and Asdadov [27]. They suggest that the primary donor is made up of two symmetric parallel BChl *b* molecules aligned head-to-tail. In this configuration only one, red-shifted, exciton band is expected [28]. The 990 nm band with its shoulder does not show polarisation effects, in agreement with Ref. 13.

Charge-transfer states

The suggestion that the long-wavelength singlet absorption band of P-960 is split at temperatures of 4 K and below by the admixture of a charge-transfer state [13,34] merits some further discussion. Maslov et al. [34] argue that the peak of the split band corresponds to a state with predominant ^{1}P*B character and the shoulder to its companion with predominant $^1(P^+B^-)$ character, where P* is the first excited singlet state of the primary donor P and B is the first intermediairy acceptor, a B-830 BChl molecule. Formally, the excited singlet states are then represented by [34,35]:

$$|S^+\rangle = \sqrt{1-\alpha^2}\,{}^1P^*B + \alpha\,{}^1(P^+B^-) \tag{1a}$$

$$|S^-\rangle = \alpha\,{}^1P^*B - \sqrt{1-\alpha^2}\,(P^+B^-) \tag{1b}$$

where α represents the fractional charge-transfer contribution to the $|S^+\rangle$ state, $\alpha \ll 1$. This interpretation of the split band is supported by the correspondence of the energy difference between the main peak and the shoulder (0.02 eV) and the value inferred for the difference between the ^{1}P*B and $^1(P^+B^-)$ levels (0.025 eV [36]). In principle, the reaction-center triplet state can be analogously described as being split by charge-transfer interaction into a combination of two states, each composed of a linear combination of ^{3}PB and $^3(P^+B^-)$ states with the higher lying state having mainly $^3(P^+B^-)$ character [36]. Thus:

$$|T^-\rangle = \beta\,{}^3PB - \sqrt{1-\beta^2}\,{}^3(P^+B^-) \tag{2a}$$

$$|T^+\rangle = \sqrt{1-\beta^2}\,{}^3PB + \beta\,{}^3(P^+B^-) \tag{2b}$$

with β the fractional charge-transfer contribution to the $|T^+\rangle$ state, $\beta \ll 1$. The relative order of the $|T^-\rangle$ and $|T^+\rangle$ levels is inversed with respect to the $|S^\pm\rangle$ levels, since the exchange interaction between the electrons, which gives rise to the energy splitting between the singlet and the triplet state, is much smaller in the $^1(P^+B^-)$ state than in the P*B state. β need not be equal to α, but we assume that they both are a function of some geometrical variable, i.e., they are correlated via the local geometry. At low temperatures, the state $|T^-\rangle$ is not populated as, in accordance with Ref. 36, we do not see a bleaching at 830 nm. This is corroborated by our value for the zero-field splitting parameter D of P-960^T, which is close to the value of D for ^{3}BChl *b* monomers in vitro. Appreciable admixture of the $^3(P^+B^+)$ state would dramatically lower the value of D, because in this state the dipole-dipole interaction between the two unpaired spins is much lower than for a triplet state located on a single BChl molecule. Thus, all microwave transitions that we observe belong to the postulated triplet state with predominant ^{3}PB character. Modulation of the concentration of this state by resonant microwaves causes a modulation of the concentration of the singlet ground state, hence a modulation of the absorbance over the entire long-wavelength singlet absorbance band, peak and shoulder included. It follows that one would expect to observe identical T − S spectra for all microwave frequencies within the ADMR transition band if the observed heterogeneity in the triplet parameters would not be correlated to the singlet absorbance spectra.

From the results displayed in Fig. 5, we see that the above expectation is not borne out. In contrast, there is a manifest dependence of the shape

of the absorbance band on the microwave frequency within one ADMR transition. The most striking variation is the shift of the peak wavelength of the singlet absorbance to shorter wavelength with higher microwave frequency. This demonstrates that the heterogeneity in triplet parameters (reflected in inhomogeneous ADMR transitions) is correlated to a heterogeneity in the singlet energy levels. Such a correlation is not difficult to accomodate in the charge-transfer model if one assumes that the red shift of P-960 with respect to the in vitro long-wavelength absorption band of BChl *b* is partly caused by admixture of charge-transfer states, either within the primary donor itself, or between P and one of the accessory BChl pigments (the state $^1(P^+B^-)$. The degree of admixture of the charge-transfer states depends on the details of the interaction between the relevant BChl pigments, which in turn depend on their precise configuration. The value of D will be sensitive to the degree of admixture of the states $^3(BChl^+BChl^+)$ or $^3(P^+B^-)$, being lower with lesser purity of the ^3P state. The change in E is more difficult to predict. Presumably, more charge-transfer character means higher asymmetry of triplet wave-function and an increase in $|E|$. We observe (Fig. 5), that the red most shifted singlet absorbance bands correspond with the lower frequencies within the $|D|+|E|$ ADMR transition. This would indicate that the variation with charge-transfer admixture of $|D|$ is larger than that of $|E|$.

In addition to the shift of the long-wavelength absorption band in the T − S spectrum, the band exhibits a shoulder when monitored at the lower microwave frequencies within a particular ADMR transition (Fig. 5). This shoulder could be due to additional heterogeneity that is not removed by the ADMR selection criterion, i.e., it represents a heterogeneity without correlation between triplet and excited singlet properties, or it is also caused by charge-transfer effects. Consider, for example, geometries for which there is not or very little admixture of $^1(P^+B^-)$ in the singlet excited state. Then, the singlet absorbance spectrum (and hence the T − S spectrum) would not show a shoulder around 990 nm. For other geometries with appreciable admixture of $^1(P^+B^-)$ the long-wavelength absorption band may show a distinct shoulder, the two components representing the charge-transfer induced splitting of the P-960 band. In agreement with this interpretation, the shoulder is observed only at the lower microwave frequencies, i.e., at the lower values of $|D|$.

The results of Fig. 5 demonstrate that the singlet absorbance spectrum measured at liquid helium temperatures is the envelope of many, slightly different absorbance spectra. The long-wavelength shoulder is not present in all ADMR-selected T − S spectra; hence, its appearance also reflects a heterogeneity, e.g., in charge-transfer mixing. It follows that the low-temperature fluorescence spectrum should then similarly reflect this heterogeneity, and be made up of two components. Note, however, that the ratio of the amplitude of these two components need not be identical to that of the absorbance spectrum, as the radiative properties of the predominantly charge-transfer state may be quite different from those of the predominantly excited singlet state. Interestingly, the low-temperature fluorescence spectra measured by Maslov et al. [34] of open reaction centers of *Rps. viridis* and of reaction centers in which the menaquinone acceptor was chemically reduced, indeed show a bimodal aspect. In the interpretation of Maslov et al. [34], however, the bimodality does not reflect heterogeneity but is due to vibrational structure. In open reaction centers, the 0–0 transition of the fluorescence band appeared to correspond to that of the charge-transfer shoulder rather than to that of the main peak of the absorption band, suggesting that in all open reaction centers at 1.9 K the fluorescence stems almost exclusively from the lower lying, predominantly charge-transfer singlet excited state. At first sight, this interpretation conflicts with our finding that even when one adheres to the charge-transfer model, reaction centers show heterogeneity. Inspection of the charge-transfer model, however, shows that it can accomodate both experimental findings.

Consider Eqns. 1 and 2. The zero-field splitting parameters are a function of the parameter β in Eqn. 2, which is determined by the local geometry and shows a spread $\Delta\beta$. With the spread in β corresponds a spread $\Delta\alpha$. The transition dipole moments of the $|S^+\rangle$ and $|S^-\rangle$ states are connected to those of the pure $^1P^*B$ and $^1(P^+B^-)$ states by equations analogous to Eqn. 1. Assuming that the

transition to the $^1(P^+B^-)$ state is practically forbidden, the transitions of the $|S^-\rangle$ state acquire their absorptive and emissive oscillator strength from the small admixture of the $^1P^*B$ state. If we now admit a small variation in β this will give rise to a small variation in D and E, whereas the concomitant variation in α is reflected in a variation in the position and intensity of the $|S^\pm\rangle$ states. If the ratio of $\Delta\alpha$ to α is small the variation in the properties of the $|S^+\rangle$ state will be small, but for the $|S^-\rangle$ state the oscillator strength will vary almost linearly with α^2. This means that relatively small changes in D and E will correspond to rather drastic changes in the intensity of the absorbance band due to the $|S^-\rangle$ transition. This would explain the disappearance of the shoulder in the spectra of Fig. 5. If we assume that emission from the $|S^+\rangle$ state is quenched by fast radiationless transition to the $|S^-\rangle$ state, then all emission will take place from the $|S^-\rangle$ state, regardless of the precise value of α.

The above explanation reconciles the experimental findings of Maslov et al. [34] and their interpretation with the present work, but of course it does not constitute proof for the factual presence of charge-transfer states. We note that the experimental results of Maslov et al. [34] can in principle be explained assuming reaction-center heterogeneity only with respect to the red shift of P-960, and a temperature-dependent Stokes shift. The narrowing of the fluorescence band compared to the absorption band could then be due to heterogeneity in fluorescence yield, evidence for which was found in reaction centers of *Rps. sphaeroides* R-26 [16]. We are currently extending our ADMR studies of the triplet state to other organisms, and we hope that this work and further studies of the low-temperature fluorescence bands of reaction centers of *Rps. viridis* under various redox conditions will alow us to decide whether the splitting of the long-wavelength absorption band at low temperatures is caused by a heterogeneity (in exciton interaction or in charge-transfer contribution) that is not removed by ADMR selection or that it reflects the presence of well-defined $|S^\pm\rangle$ states.

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References

1 Parson, W.W. (1982) Annu. Rev. Biophys. Bioeng. 11, 57–80
2 Hoff, A.J. (1982) in Light Reaction Path in Photosynthesis (Fong, F.K., ed.), Molecular Biology, Biochemistry and Biophysics, Vol. 35, Ch. 4, Springer Verlag, Berlin
3 Feher, G., Hoff, A.J., McElroy, J.D. and Isaacson, R.A. (1973) Biophys. J. 13, 61a
4 Norris, J.R., Druyan, M.E. and Katz, J.J. (1973) J. Am. Chem. Soc. 95, 1680–1682
5 Lendzian, F., Lubitz, W., Scheer, H., Bubenzer, C. and Moebius, K. (1981) J. Am. Chem. Soc. 103, 4635–4637
6 Davis, M.S., Forman, A., Hanson, L.K., Thornber, J.P. and Fajer, J. (1979) J. Phys. Chem. 83, 3325–3332
7 Sternlicht, H. and McConnell, H.M. (1961), J. Chem. Phys. 35, 1793–1800
8 Den Blanken, H.J., Van der Zwet, G.P. and Hoff, A.J. (1982) Chem. Phys. Lett. 85, 335–338
9 Den Blanken, H.J. and Hoff, A.J. (1983) Chem. Phys. Lett. 98, 235–262
10 Shuvalov, V.A. and Parson, W.W. (1981) Biochim. Biophys. Acta 638, 50–59
11 Den Blanken, H.J. and Hoff, A.J. (1982) Biochim. Biophys. Acta 681, 365–374
12 Hoff, A.J., De Vries, H.G. (1978) Biochim. Biophys. Acta 503, 94–106
13 Vermeglio, A. and Paillotin, G. (1982) Biochim. Biophys. Acta 681, 32–40
14 Litvin, F.F. Personov, R.I. and Korotaev, O.N. (1969) Dokl. Akad. SSSR Biophys. 188, 118
15 Platenkamp, R.J., Den Blanken, H.J. and Hoff, A.J. (1980) Chem. Phys. Lett. 76, 35–41
16 Den Blanken, H.J., Van der Zwet, G.P. and Hoff, A.J. (1982) Biochim. Biophys. Acta 681, 375–382
17 Van Dorp, W.G., Schoemaker, W.H., Soma, M. and Van der Waals, J.H. (1975) Mol. Phys. 30, 1701–1721
18 Den Blanken, H.J. and Hoff, A.J. (1983) Chem. Phys. Lett. 96, 343–347
19 Leung, M. and El-Sayed, M.A. (1972) Chem. Phys. Lett. 16, 454–459

20 Hoff, A.J. (1976) Biochim. Biophys. Acta 440, 765–771
21 Prince, R.C., Leigh, J.S. and Dutton, P.L. (1976) Biochim. Biophys. Acta 440, 662–636
22 Frank, H.A., Friesner, R., Nairn, J.A., Dismukes, G.C. and Sauer, K. (1979) Biochim. Biophys. Acta 547, 484–501
23 Clarke, R.H., Connors, R.E., Norris, J.R. and Thurnauer, M.C. (1975) J. Am. Chem. Soc. 97, 7178–7179
24 Holten, D., Windsor, M.W., Parson, W.W. and Thornber, J.P. (1978) Biochim. Biophys. Acta 501, 112–126
25 Clarke, R.H., Connors, R.E., Frank H.A. and Hoch, J.C. (1977) Chem. Phys. Lett. 45, 523–528
26 Kooyman, R.P.H. and Schaafsma, T.J. (1980) J. Mol. Struct. 60, 373–380
27 Shuvalov, V.A. and Asadov, A.A. (1979) Biochim. Biophys. Acta 545, 296–308
28 Kasha, M., Rawls, H.R. and Ashraf El-Bayoumi, M. (1965) Pure Appl. Chem. 11, 371–392
29 Beens, H. and Weller, A. (1975) in Organic Molecular Photophysics (Birks, J.B., ed.), Vol. 2, Ch. 4, John Wiley Interscience, New York
30 Eccles, J. and Honig, B. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 4959–4962
31 Trosper, T.L., Frank, H.A., Norris, J.R. and Thurnauer, M.C. (1982) Biochim. Biophys. Acta 679, 44–50
32 Norris, J.R., Uphaus, R.A. and Katz, J.J. (1975) Chem. Phys. Lett. 31, 157–161
33 Kleibeuker, J.F. (1977) Dissertation, Agricultural University, Wageningen, The Netherlands
34 Maslov, V.G., Klevanik, A.V., Ismailov, M.A. and Shuvalov, V.A. (1983) Dokladi Akad. Nauk SSSR, 269, 1217–1221
35 Davidov, A.S. Theory of Molecular Excitons (1971) Plenum Press, New York, Ch. 2
36 Shuvalov, V.A. and Parson, W.W. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 957–961