

## PROTON ENDOR STUDY OF THE PHOTOEXCITED TRIPLET STATE P<sup>T</sup> IN *Rps. SPHAEROIDES* R-26 PHOTOSYNTHETIC REACTION CENTRES

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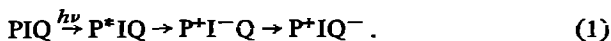
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The photoexcited triplet state P<sup>T</sup> of *Rhodospseudomonas sphaeroides* R-26 has been investigated by ENDOR measurements performed on frozen photosynthetic reaction centre solutions. For the first time hyperfine data could be obtained for P<sup>T</sup>. These data indicate a delocalisation of the triplet state over two bacteriochlorophyll a molecules.

### 1. Introduction

The general sequence of the primary light-induced charge separation in photosynthesis is [1]



Here P denotes the primary electron donor, I the first electron acceptor, Q the secondary acceptor. It has been shown by optical and magnetic resonance spectroscopy that in bacterial photosynthetic reaction centres (RCs) the primary electron donor P is most probably a bacteriochlorophyll a (BChl a) (fig. 1) dimer, I is a bacteriopheophytin a and Q is an ubiquinone molecule. There is evidence from time-resolved optical spectroscopy that a BChl a molecule is involved in the process of electron transfer from P\* to I (for review articles, see refs. [1–6]).

The photochemical function of an RC is strongly related to the structure and relative orientation of the primary reactants in the RC protein complex [4]. The structure of the primary electron donor complex P is of particular interest. For photosynthetic RCs it is extremely difficult to obtain suitable single crystals [7, 8], and a detailed X-ray structure analysis has been performed so far only for the BChl b containing species *Rps. viridis* [9]. However, recent high-resolution

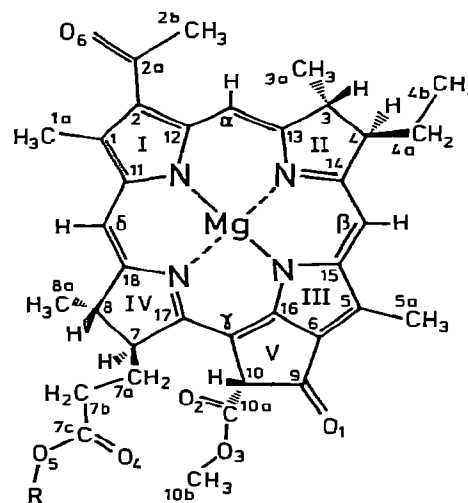


Fig. 1. Molecular structure of bacteriochlorophyll a. R = phytol (C<sub>20</sub>H<sub>39</sub>) for *R. sphaeroides*.

liquid phase ENDOR experiments together with molecular orbital calculations have given structural information about the dimeric cation of the primary donor, P<sup>T</sup>, for two BChl a containing species (*Rps. sphaeroides* and *Rds. rubrum*) [10,11].

When the forward reaction in eq. (1) is blocked either by chemical reduction or by removal of  $Q_+$  recombination of the charges on  $P^+$  and  $I^-$  leads to the triplet state  $P^T$  which, at cryogenic temperatures ( $T < 50$  K), has a lifetime of  $\approx 100 \mu s$  [5].  $P^T$  has been extensively investigated by EPR and by zero-field optically detected magnetic resonance (ODMR) techniques [12–15]. The high-field EPR spectrum shows strong spin polarisation which can be explained by the radical pair recombination reaction [12]. The observed decrease of the zero-field splitting (ZFS) parameters,  $D$  and  $E$ , as compared with the BChl a triplet state has been interpreted as an effect of the sharing of the triplet energy between two BChl a molecules [12–15]. The ZFS values, together with the triplet decay rates, have also been used to derive structural information about  $P^T$  [14,15]. However, because of the unknown amount of charge transfer contributions [12,13] the triplet state data do not give a definitive insight into the structure (refs. [12,13,15] and references therein).

Recently, it has been demonstrated that the ENDOR method can be applied in the study of short-lived triplets randomly oriented in a rigid matrix, provided spin polarisation gives rise to strong EPR signals [16]. Specific orientations of the triplet molecules relative to the magnetic field can be selected by the pumped EPR transition resulting in single-crystal-like ENDOR spectra [17,18]. We have applied this method in the study of the photoexcited triplet state  $P^T$  in frozen reaction centre solutions of *Rps. sphaeroides* R-26.

## 2. Experimental

Chromatophores of *Rps. sphaeroides* R-26 were prepared by standard procedures [19]. Reaction centres were extracted by a modified procedure involving the treatment of chromatophores ( $E_{870} = 50/cm$ ) in tris-HCl buffer (50 mM,  $pH$  8.0) containing NaCl (100 mM) with 0.6% LDAO. The purification over DEAE cellulose [19] and the extraction of quinones [20] followed again standard procedures. The final reaction centre solution was concentrated by membrane filtration to  $\approx 2 \times 10^{-4}$  M.

The RC solutions were treated with sodium dithionite ( $\approx 10^{-2}$  M final concentration) under nitrogen atmosphere prior to illumination to obtain the triplet

state  $P^T$  [21]. The samples were diluted to 60% (v/v) with glycerol to obtain optically clear samples upon freezing. The X-band ENDOR spectrometer has been described previously [22]. For the measurements at low temperatures (10–20 K) the ENDOR cavity has been adapted to an Oxford ESR 10 helium flow cryostat. Samples were illuminated inside the ENDOR cavity with a 500 W xenon arc lamp (Eimac) using a 450 nm UV cut-off filter and a 10 cm water filter for IR absorption.

## 3. Results and discussion

The spin energy level diagram for the three canonical orientations of a triplet state with  $D > 0$  is shown in fig. 2a [13]. From spin-lattice relaxation studies, positive sign of  $D$  has been concluded for  $P^T$  [12]. The heavy lines indicate the predominantly populated levels due to the radical pair recombination reaction which leads to the triplet state  $P^T$  [12]. Fig. 2b shows the EPR spectrum of the randomly oriented triplets  $P^T$  in *Rps. sphaeroides* R-26 RC solutions. The ZFS parameters,  $|D| = 0.0188(1) \text{ cm}^{-1}$  and  $|E| = 0.0032(1) \text{ cm}^{-1}$ , are in good agreement with previously reported values [12,13].

For recording the ENDOR spectra the high-field EPR  $Z_{II}$  peak, which is in emission [12], was saturated. As a result, only molecules having the triplet z-axis parallel to the magnetic field are affected [18].

Fig. 3 shows the triplet spin energy levels for this particular orientation, including first-order hyperfine interaction with one proton. The saturated high-field EPR line corresponds, for  $D > 0$ , to a transition between the  $m_s = 0$  and  $m_s = -1$  levels. There are two ENDOR resonances, at  $\nu_p$  and at  $\nu_p + A_z$ , where  $A_z$  is the component of the hyperfine coupling tensor in the direction of the triplet z-axis [18].

The ENDOR spectrum of  $P^T$  is shown in fig. 4 (see also ref. [23]). The narrow line in the centre of the spectrum stems from the proton Zeeman transition in the  $m_s = 0$  manifold. In addition, there are several hyperfine shifted lines, both towards higher and lower frequencies. If the low-field EPR peak  $Z_I$  (fig. 2a) is saturated, the hyperfine shifted ENDOR resonance for a nucleus,  $i$ , changes to  $\nu_{\text{ENDOR } i} = \nu_p - A_{zi}$  and the lines change side with respect to  $\nu_p$  [18]. Moreover, a phase change of the signals is detected, since the

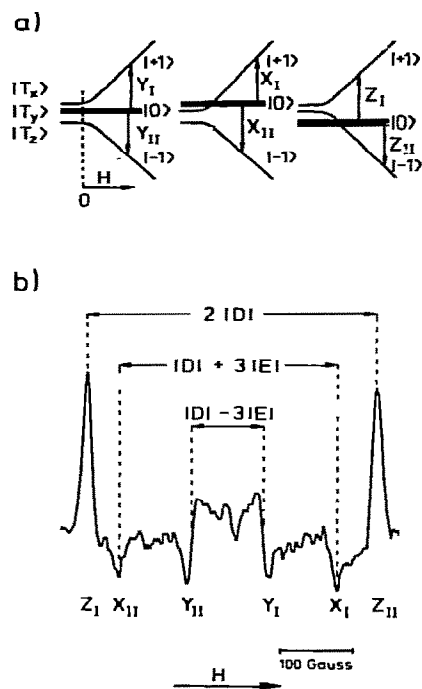


Fig. 2. (a) Field dependence of the spin energy levels of a triplet with  $D > 0$  for the three canonical orientations  $Y$ ,  $X$ , and  $Z$  [13]. The heavy lines indicate the predominantly populated levels due to the radical pair recombination which leads to the triplet state  $P^T$  [12]. The EPR transitions are indicated by arrows. The subscript I denotes transitions between the  $m_s = 0$  and  $m_s = +1$  levels, subscript II between  $m_s = 0$  and  $m_s = -1$  levels [12]. (b) First derivative EPR spectrum of randomly oriented  $P^T$ . RC solutions illuminated at 10–20 K. 1 kHz field modulation (4 G peak–peak) and 130 Hz light modulation. The positions in the spectrum corresponding to the EPR transitions of the canonical orientations in (a) are indicated by  $X_{I,II}$ ,  $Y_{I,II}$  and  $Z_{I,II}$  [12].  $D = 0.0188(1) \text{ cm}^{-1}$ ,  $E = 0.0033(1) \text{ cm}^{-1}$ .

high-field  $Z_{II}$  EPR peak is in emission, whereas the low-field  $Z_I$  EPR peak is in absorption [12]. The hyperfine splitting components  $A_z$  obtained are collected in table 1.  $^{14}\text{N}$  ENDOR signals have also been detected. The interpretation of these signals is complicated by the fact that the resonance positions are affected by quadrupolar, nuclear Zeeman, and hyperfine interactions which are all of the same order of magnitude. A

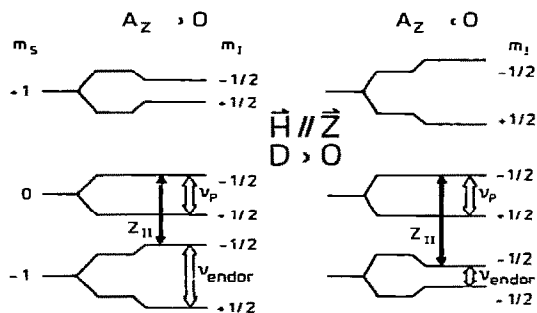


Fig. 3. Spin energy levels for the triplet  $z$ -axis parallel to the magnetic field,  $D > 0$ , showing first-order hyperfine interaction with one proton for  $A_z > 0$  and  $A_z < 0$ , respectively. The pumped EPR transitions ( $Z_{II}$ ) and the ENDOR transitions ( $\nu_p$  and  $\nu_{\text{ENDOR}}$ ) are indicated by arrows.

discussion of these signals will, therefore, be given in a forthcoming paper.

For a preliminary interpretation, the proton couplings may be compared with the hyperfine couplings of the BChl a cation [10,25,27,28] and anion [24,29].

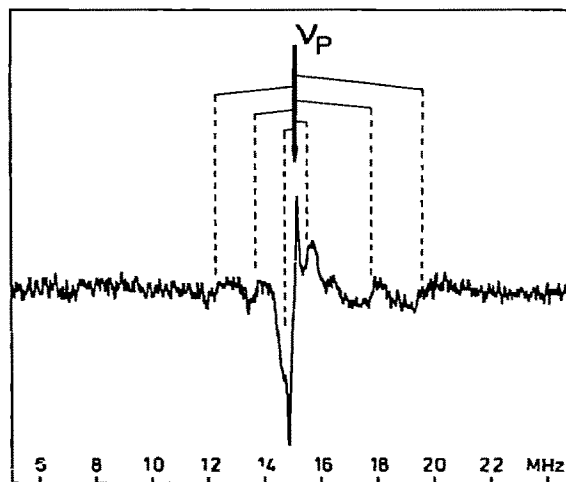


Fig. 4. First derivative proton ENDOR spectrum of  $P^T$ , EPR  $Z_{II}$  peak (fig. 2b) pumped. RC solutions illuminated at  $T = 10\text{--}20 \text{ K}$ . Microwave power: 10 mW, rf power: 100 W (10 G rotating frame), 10 kHz fm of the rf. fm depth:  $\pm 80 \text{ kHz}$ , light modulation: 130 Hz. 20 scans (500 s each), time constant: 400 ms.

Table 1

Proton hyperfine splitting components  $A_z$  (in MHz) of  $P^T$  compared with the isotropic proton hfcs  $a_{iso}$  of  $P^+$  [10], BChl  $a^+$  [10] and BChl  $a^-$  [24]

$P^T$ a) $A_z$ (MHz)	$P^+$ b) $a_{iso}$ (MHz)	BChl $a^+$ c) $a_{iso}$ (MHz)	BChl $a^-$ d) $a_{iso}$ (MHz)	Molecular position e)	Type of proton f)
-0.3		$\pm 0.15$	-6.23	$\left\{ \begin{array}{l} \alpha \\ \beta \\ \sigma \end{array} \right.$	$\alpha$ -protons
-1.4		1.30	-6.91		
-2.8		2.30	-9.65		
0.45	4.00 5.60	4.85 9.50	7.63 9.19	1a 5a	methyl protons
2.7	3.30 4.45	11.61 13.00	0.50 0.95	$\left\{ \begin{array}{l} 3 \\ 4 \\ 7 \\ 8 \end{array} \right.$	$\beta$ -protons
4.5	8.60 9.50	13.59 16.43	-1.60		

- a) Frozen RC solution of *R. sphaeroides* R-26, prereduced with sodium dithionite, illuminated at  $T = 10-20$  K. Hyperfine tensor components (MHz) of  $P^T$  for the magnetic field parallel to the triplet  $z$ -axis, signs for  $D > 0$  [12].  
 b) Liquid RC solutions of *R. sphaeroides* R-26 illuminated at  $T = 298$  K [10].  
 c) Oxidation with iodine in  $CH_2Cl_2/CH_3OH$  (6/1, v/v) at  $T = 255$  K [10].  
 d) Reduction by electrolysis in dimethoxyethane, tetra- $n$ -butylammonium perchlorate as supporting electrolyte.  $T = 255$  K [24].  
 e) See molecular structure, fig. 1. Assignments valid only for  $P^+$ , BChl  $a^+$  and BChl  $a^-$ , see refs. [10,24,25].  
 f) For the characterisation of  $\alpha$ - and  $\beta$ -protons see ref. [26].

In the Huckel MO approximation the normalized triplet  $\pi$ -spin density  $\rho_T(C_1)$  at a carbon position  $C_1$  is given by

$$\rho_T(C_1) = \frac{1}{2} [\rho_C(C_1) + \rho_A(C_1)] \quad (2)$$

where  $\rho_C$  and  $\rho_A$  are the spin densities of the cation and anion radical respectively [26]. Assuming the same  $Q$  factors [26] for the cation, the anion, and the triplet state, eq. (2) should also be valid for the isotropic hyperfine coupling constants (hfcs).

The proton isotropic hfcs of the BChl  $a$  cation and anion together with an assignment to the molecular positions are given in table 1 [10,24]. Using eq. (2) and, to stay in the HMO approximation, neglecting hfcs stemming from negative spin densities, the isotropic hfcs of the triplet state of BChl  $a$  can be estimated. Negative hfcs between  $-3.1$  and  $-4.8$  MHz (corresponding to  $\frac{1}{2} a_{iso}$  for BChl  $a^-$ ) are expected for the  $\alpha$ -protons of the methine bridges (fig. 1). Positive hfcs of  $+6.2$  and  $+9.3$  MHz are expected for methyl protons in positions 1a and 5a. The hfcs of the  $\beta$ -protons in rings II and IV (positions 3, 4 and 7, 8) are expected to be between  $+5.8$  and  $+8.2$  MHz (corresponding to  $\frac{1}{2} a_{iso}$  for BChl  $a^+$ ). The fact that the largest observed hyperfine splitting component in  $P^T$  is positive ( $+4.5$

MHz) is in qualitative agreement with the prediction and can be considered additional evidence for the conclusion that  $D > 0$  in  $P^T$  [12]. However, the observed splittings in  $P^T$  are much smaller than the values expected for a BChl  $a$  triplet.

Since our observed hfcs of  $P^T$  are the components of the hyperfine tensors along the triplet  $z$ -axis they should be compared with the corresponding tensor components in the BChl  $a$  cation and anion. For the BChl  $a$  triplet state it has been shown by magneto-photoselection experiments that the triplet  $z$ -axis is perpendicular to the molecular plane [12]. Therefore the out-of-plane components of the hyperfine tensor should be considered here.

The situation is more complex, however, for  $P^T$ . On the basis of the reduced values of  $D$  and  $E$  as compared with the BChl  $a$  triplet,  $P^T$  is assumed to be a BChl  $a$  dimer [12-15]. Details of its structure and consequently the orientation of the triplet  $z$ -axis relative to the dimer axis system are not known. This implies that the  $\alpha$ -proton hyperfine values are not particularly suited for a comparison with the BChl  $a$  triplet because of their large hyperfine anisotropy [23]. For the methyl protons, another complication arises: It has been shown by ENDOR experiments on BChl  $a$

cations that at temperatures below 40 K (especially below 20 K) hindered rotation of the methyl groups broadens the ENDOR lines. At temperatures between 10 and 20 K, where our ENDOR experiments were performed, the methyl proton lines may be broadened beyond detection [30].

Therefore, the hfcs which should be considered are those of the  $\beta$ -protons in rings II and IV (positions 3, 4 and 7, 8). For these protons the expected isotropic hfcs for the BChl a triplet (+5.8 to +8.2 MHz) are much larger than the observed hyperfine splitting components in  $P^T$  (+2.7, +4.5 MHz). Using a dihedral angle between  $34^\circ$  and  $45^\circ$  for these  $\beta$ -protons, as is assumed in refs. [28,29], it can be calculated that the anisotropic tensor components do not exceed 10% of the isotropic value [31]. The angular dependence of these hyperfine splittings, therefore, cannot explain the small magnitude of the observed values. In the dimer model for  $P^T$ , a reduction of the hyperfine splitting components as compared with the BChl a triplet is expected because of the delocalisation of the unpaired electrons over two BChl a molecules. For the doublet state  $P^+$ , the cation radical of the primary donor, such a delocalisation of the unpaired electron over two BChl a molecules has been postulated on the basis of EPR experiments [32] and has been well established by solid state [30,33] and recent liquid solution ENDOR experiments [10,11,25,34,35].

Since the  $\beta$ -proton hfcs for positions 3, 4, 7, 8 in the BChl a anion are negligibly small (see table 1) the hyperfine splittings for these positions in the triplet state can only arise from the contribution of the "cation orbital" (HOMO). Assuming that these two orbitals in the dimer P have similar behaviour as in monomeric BChl a, it follows from eq. (2) that the expected hyperfine values of these  $\beta$ -protons in  $P^T$  should be one half of the values in  $P^+$ . Indeed, the observed value of the largest positive hyperfine splitting in  $P^T$  is just one half of the mean value of the two largest isotropic hfcs in  $P^+$  (8.6, 9.5 MHz). These hfcs have been unambiguously assigned to the  $\beta$ -protons in ring II and/or ring IV [10,25,30]. This result indicates a delocalisation of the triplet electrons in  $P^T$  over two BChl a molecules.

It is noteworthy that any spectroscopic information is valid only on the time scale inherent in the experiment. In the case of magnetic resonance on  $P^T$ , the time scale is of the order of 1 ns ( $1/D$ ) for EPR and

30 ns ( $1/A_2$ ) for ENDOR. Optical experiments are much faster and there is evidence that on the optical time scale, at low temperature, the triplet excitation sits on one BChl a molecule [36].

#### 4. Conclusion

These proton ENDOR experiments on  $P^T$  give additional support for a delocalisation of the triplet energy over two BChl a molecules on the nanosecond timescale. In order to further corroborate this conclusion one would need the hyperfine couplings of the BChl a triplet state with unambiguous assignment for comparison.

Experiments are in progress with selectively deuterated samples [10,11,37], together with advanced MO calculations [38] for a more quantitative analysis. In addition, ENDOR experiments on  $P^T$  in RC single crystals are planned in order to determine the full hyperfine tensors. The ZFS tensor has been recently determined from EPR experiments on such single crystals [39].

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