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The electronic structure of $P840^{+\bullet}$

The primary donor of the *Chlorobium limicola* f. sp. *thiosulphatophilum* photosynthetic reaction centre

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Abstract

The radical cation $P840^{+\bullet}$ was studied in frozen suspensions of *Chlorobium limicola* f. sp. *thiosulphatophilum* membranes using ENDOR and Special TRIPLE spectroscopies. The spectra show that $P840^{+\bullet}$ arises from a bacteriochlorophyll *a* 'special' pair with a highly symmetrical distribution of electron spin density between the constituent bacteriochlorophylls. Special TRIPLE spectroscopy has resolved the separate contributions of the two halves of the pair and revealed small deviations from a 1:1 electron spin density distribution. Nevertheless $P840^{+\bullet}$ appears to come the closest yet to the symmetrical 'dimer' originally proposed for the structure of the primary donor radical cation ($P870^{+\bullet}$) in purple non-sulphur photosynthetic bacteria.

Key words: Reaction centre; $P840^{+\bullet}$; ENDOR; Special TRIPLE; Green sulphur photosynthetic bacteria

1. Introduction

The photosynthetic reaction centre of the purple non-sulphur bacterium *Rhodobacter sphaeroides* is a protein heterodimer, employing a bacteriochlorophyll *a* (Bchl *a*) pair as the primary electron donor [1]. The Bchl *a* pair is bound at an apparent C2 symmetry axis of the reaction centre, with each Bchl *a* attached to one peptide of the heterodimer. Spectroscopic analyses using ENDOR [2] and ESEEM [3] have shown that the unpaired electron spin in the oxidised Bchl *a* pair, $P870^{+\bullet}$ is asymmetrically distributed (in a 2:1 ratio) between the constituent Bchl *a* molecules. This asymmetry is attributed to the influence of the surrounding protein environment.

A heterodimer structure is also found in the reaction centres of the two photosystems of oxygenic photosynthesis. Photosystem II (PSII) shows amino acid sequence homology with the purple bacterial photosynthetic reaction centre [4], whereas PSI is homologous to the reaction centres of photosynthetic green sulphur bacteria, the Chlorobiaceae, and the Heliobacteriaceae [5]. Iron-sulphur centres in the *Chlorobium thiosulphatophilum* reaction centre which are similar to iron-sulphur centres X, A and B of PSI, have been identified by EPR spectroscopy [6–8]. Possible analogues of the early PSI acceptors A_0 and A_1 have also been observed [9]. An electron spin polarised signal has been observed in whole cells and membrane fractions of *C. thiosulphatophilum* [8,10]. Such a spectrum can be obtained for PSI, but is only observed in PSII or purple bacteria if the non-heme iron is removed. The observation of an electron spin polarized signal [8,10], and indirect evidence suggesting that the triplet of $P840^{+}$ is affected by conditions that could lead to double reduction of a quinone [7] suggest the presence of an 'A_1' quinone acceptor in *C. thiosulphatophilum*. However, the presence of a quinone acceptor in Heliobacteriaceae is disputed [11]. The primary donor of PSI, P700, is thought to be a chlorophyll *a* (Chl *a*) pair (for a review see [12]), while the primary donor in *C. thiosulphatophilum*, P840, is presumed to be a Bchl *a* pair [7,13,14]. An asymmetric distribution (3:1) of the unpaired electron spin density between the constituent Chl *a* monomers has been determined for the radical cation $P700^{+\bullet}$ [3,15]. EPR spectroscopy of the triplet states of P840 [7], P870 [16] and P700 [17,18] suggests that the electronic structure of P840 (in the triplet state) resembles that of P870 more than P700 [7].

Despite the similarities between PSI and the *C. thiosulphatophilum* reaction centre, recent genetic analysis of *C. limicola* f. sp. *thiosulphatophilum* has found only one gene which shows homology with the large 'core' reaction centre proteins of PSI (the *psaA* and *psaB* gene

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Abbreviations: ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; hfc, hyperfine coupling constant; P700, primary electron donor of photosystem 1 of oxygenic photosynthesis; P840, primary electron donor of the reaction centre of *Chlorobium limicola* f. sp. *thiosulphatophilum*; P870, primary electron donor of the reaction centre of *Rhodobacter sphaeroides*; ST, special TRIPLE resonance; Chl, chlorophyll; BChl, bacteriochlorophyll.

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products) [19]. A similar result was obtained from *Heliobacillus mobilis* [20]. This implies that these two organisms have reaction centres that are protein homodimers [20,21] rather than heterodimers. A protein homodimer reaction centre in *C. thiosulphatophilum* [21] would probably place P840 in a highly symmetrical environment. Such symmetry may be evident in the distribution of the unpaired electron spin of the oxidised species $P840^{+\bullet}$. Here we report the analysis of the unpaired electron spin distribution of $P840^{+\bullet}$ using ENDOR and special TRIPLE spectroscopy.

2. Materials and methods

Chlorobium limicola f. sp. *thiosulphatophilum* (strain Tassajara) was grown as in [6] and harvested by ultrafiltration using a 0.2 μm membrane in a Millipore Pelicon concentrator, followed by centrifugation. Cell pellets were stored in liquid nitrogen. Preparation of a 'cell membrane plus chlorosome' fraction was carried out as in [22] with all steps being carried out under argon as far as possible. The membrane+chlorosome pellets were resuspended in a minimal volume of buffer and stored in liquid nitrogen. The samples for spectroscopy were thawed under argon, placed in 3 mm internal diameter EPR tubes, dark adapted for 30 min and then frozen in liquid nitrogen in the dark.

ENDOR, special TRIPLE (ST) and EPR spectra were obtained at X-band using a Bruker ESP 300 EPR spectrometer in conjunction with a Bruker EN 003 ENDOR/TRIPLE interface, Wavetek 3000–446 radio frequency (r.f.) synthesizer (a Programmable Test Systems, PTS, r.f. synthesizer was used as the second source for ST experiments), EN 370 power amplifier, and EN 801 ENDOR cavity (estimated Q of 800). The Wavetek synthesizer also provided for frequency modulation of the r.f. output. Temperature control was achieved using an Oxford Instruments continuous flow ESR 900 cryostat with an ITC 4 temperature controller. The impedance of the r.f. circuit was 50 Ω. ENDOR spectra were acquired at field values corresponding to the crossing point of the first derivative EPR spectrum, and were corrected for baseline nonlinearity by the subtraction of off-resonance scans which were filtered for noise (standard Bruker software) to avoid reducing the spectrum signal-to-noise ratio. Acquisition conditions for specific spectra are given in the figure captions. Accuracy of hfc determination was ± 0.1 MHz in ENDOR and ± 0.05 MHz in Special TRIPLE.

3. Results and discussion

The radical cation $P840^{+\bullet}$ is formed in *C. thiosulphatophilum* reaction centres by illumination of dark adapted samples at 77 K. The half life of this species is at least 24 h at 77 K and therefore it is stable over the time of the ENDOR/ST experiments (for 3–6 h). Fig. 1 shows the EPR spectrum before and after such illumination. The illuminated spectrum, b, shows a 7.7 G wide isotropic signal that is assigned to $P840^{+\bullet}$. The linewidth of the $P840^{+\bullet}$ EPR spectrum has previously been reported as 9.0 G [13] to 9.3G [14]. These studies, however, employed lower temperatures and higher microwave powers than used here.

The ENDOR spectrum of $P840^{+\bullet}$ (158 kHz r.f. modulation depth) is shown in Fig. 2. ENDOR and Special TRIPLE spectra are presented in first derivative mode. The hyperfine coupling constants are measured from zero crossing points, except for $A_{||}$ features of methyl

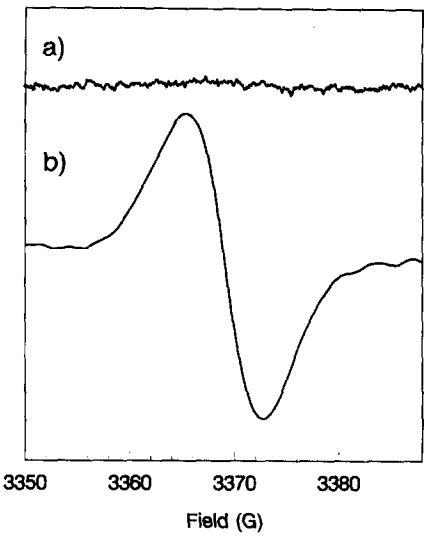

Fig. 1. EPR spectra of *C. thiosulphatophilum* membranes at 80 K, recorded using the ENDOR cavity; (a) dark adapted, (b) after illumination at 77 K showing the $P840^{+\bullet}$ spectrum. Condition: microwave power, 50 μW; microwave frequency 9.46 GHz; modulation amplitude 0.15 mT.

groups where the peak maximum is used. ENDOR resonances in this region arise from hyperfine coupling to protons. The intense features 3 and 4 have lineshapes typical of hfc's to β-methyl protons in frozen solutions [23]. The couplings of each of the three protons of a methyl group are rendered equivalent by its rapid rotation. Bchl *a* has only two methyl groups, β to the π orbital system, at positions 1 and 5 (Fig. 3). Previous ENDOR studies of native and chemically modified Bchl *a* cation radicals in vitro have shown that the hfc to the 5-methyl protons is always larger than that to the 1-methyl group [24]. Therefore we assign spectrum features 3 and 4 to the 1- and 5-methyl groups, respectively. The hfc's of β-methyl groups have high symmetry, showing only weak axial distortion. This arises since the hfc's are dominated by the isotropic component, where electron spin is transferred to the nucleus via hyperconjugation [25]. The perpendicular ($A_{\perp}$) and parallel ($A_{||}$) components of such weakly axial systems become resolved as the coupling increases since the axial distortion is a fixed proportion of the isotropic coupling in all cases ($A_{||}-A_{\perp} \simeq 0.15\ A_{iso}$). The axial distortion is not resolved for the 1-methyl coupling but a shoulder, feature 4a, is observed on the 5-methyl resonance. This we assign as $A_{||}$ of the 5-methyl group, thus feature 4 is more properly the $A_{\perp}$ component.

Only the β-protons at positions 3, 4, 7 and 8 (Fig. 3) are expected to have hfc's larger than those of the 5-methyl group [24]. These protons give rise to weak, broad ENDOR resonances in frozen solutions as observed for $P870^{+\bullet}$ [26], and $P960^{+\bullet}$ of *Rhodopseudomonas viridis* [27]. Features 5 and 6 (Fig. 2) therefore have the properties expected of β-proton resonances. The hfc's of

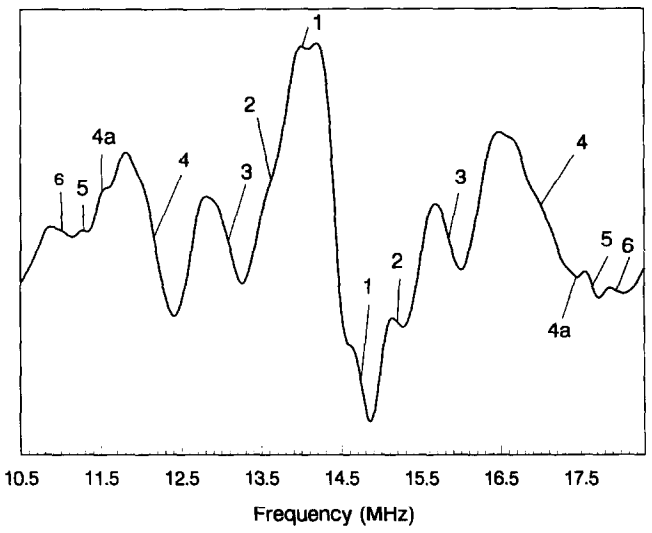

Fig. 2. ENDOR spectrum of $P840^{+\bullet}$ at 60 K. Numbering refers to Table 1 and is discussed in the text. Conditions: microwave power, 7.9 mW; r.f. power 100 W; field 337 mT (EPR crossing point); r.f. modulation depth 158 kHz; scan time 84 s; time constant 655 ms; 80 scans co-added.

these protons depend not only on the electron spin density distribution but also on the geometries of the flexible rings II and IV to which they are attached (Fig. 3) [28]. Therefore it is difficult to assign individual hfc's to particular protons and the possible contribution from the ring geometry to the magnitude of the hfc's must always be considered.

The small hfc's, features 1 and 2 in Fig. 2, could arise from either the meso protons of the methine bridges connecting the pyrrole rings of Bchl *a* (labelled α, β and γ in Fig. 3) or from the proton at position 10 on ring V [24]. Feature 1 could also arise from a protein proton hydrogen bonded to P840. This uncertainty as to the origin of these resonances makes further analysis difficult.

The hfc's of $P840^{+\bullet}$ are collected together in Table 1 together with previously determined hfc's for $P870^{+\bullet}$ [2] and Bchl $a^{+\bullet}$ in vitro[24]. The range of hfc's exhibited by $P840^{+\bullet}$ is smaller than that previously reported for the high spin density (L) half of $P870^{+\bullet}$ or Bchl $a^{+\bullet}$ in vitro. A thorough analysis of the ENDOR spectrum of $P870^{+\bullet}$ in reaction centre single crystals has been published by Lendzian et al. [2]. They used the ratio between the summed methyl group hfc's for each half (L or M) of the Bchl *a* pair to determine the spin density distribution between the constituent Bchl *a* molecules. It is possible to make similar deductions using the ratio of the $P870^{+\bullet}$ hfc's to those of Bchl $a^{+\bullet}$ in vitro.

This method can be extended to the analysis of the hfc's of $P840^{+\bullet}$. Using the data of Table 1, the ratios Σ(methyl hfc's) $P840^{+\bullet}$/Bchl $a^{+\bullet}$, $P870^{+\bullet}$(L)/Bchl $a^{+\bullet}$ and $P870^{+\bullet}$(M)/Bchl $a^{+\bullet}$ are 0.53, 0.66 and 0.33, respectively (the error in such ratios was estimated at $\pm$ 0.02). The ratios calculated for $P870^{+\bullet}$ are the same as those determined by taking the ratio between the two halves of the Bchl *a* pair [2]. The value of 0.53 determined for $P840^{+\bullet}$ suggests that the observed spectrum actually represents the overlap of two spectra with nearly identical hfc's, each arising from one half of a Bchl *a* pair in which the unpaired electron spin density is almost equally shared between the constituent monomers. No published chemical modifications of Bchl *a* would allow for the observed spectrum arising from a monomer in any environment [24].

The broad lines of frozen solution (powder) ENDOR spectra preclude the resolution of the separate spectra of each Bchl *a* monomer in the $P840^{+\bullet}$ pair under the condi-

Table 1
Hyperfine coupling constants (MHz) of Bchl *a* in reaction centres and in vitro

Feature	P840	P840(ST)[a]	P870(L)[b]	P870(M)[b]	Bchl *a*[c]	Assignment
1	0.7	–	–	–	–	? see text
2	1.6	–	–	–	–	? see text
3	2.7	2.50 2.95	3.95	1.45	4.84	1-methyl A_{iso}
4	4.6	4.30 4.80	–	–	–	5-methyl $A_{\perp}$
4a	5.8	5.80 6.40	–	–	–	5-methyl $A_{\parallel}$
	5.0	?[d]	5.66	3.30	9.60	5-methyl A_{iso}
5	6.5	6.6 6.7	8.53	N/A	13.59	3 β-proton
6	6.9	7.15 7.55	9.58	N/A	16.48	4 β-proton

[a] Values from high resolution special TRIPLE.
[b] A_{iso}, average values from [2].
[c] A_{iso} from [24].
[d] See text for possible A_{iso}.
[e] N/A, not available.

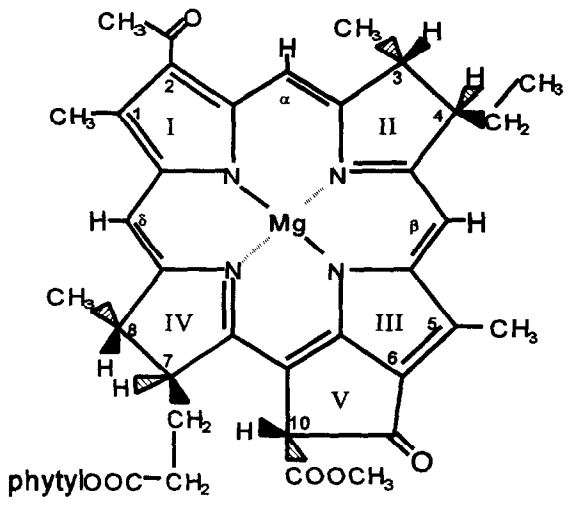

Fig. 3. The structure of bacteriochlorophyll *a* with numbering scheme.

tions employed for Fig. 2. Using a lower modulation depth for the r.f. radiation would increase the resolution, but at the expense of the signal-to-noise ratio. We have overcome this problem using special TRIPLE (ST) spectroscopy [29,30]. This technique is an extension of ENDOR employing a second r.f. It produces a spectrum which appears as half an ENDOR spectrum but with greater resonance intensities than the original ENDOR spectrum. The scale of a ST spectrum is referenced to the centre of the original ENDOR spectrum and thus the line positions can be read off directly as A/2. The increased resonance intensities produced by ST enabled us to use lower r.f. modulation depths without a signal-to-noise penalty (relative to ENDOR). The ST spectrum of $P840^{+\bullet}$, at 50 kHz modulation depth, is shown in Fig. 4. (Note that our spectrometer cannot record ST frequencies below 1 MHz.) The methyl group resonances are expected to be the most intense features of the spectrum, as in ENDOR. Features 3 and 4 are therefore assigned as the 1- and 5-methyl group resonances, respectively. These features can be seen in the ST spectrum (Fig. 4) to consist of two partially resolved lines, showing that the ST technique does enable us to resolve the separate contributions from the two constituent Bchl *a* molecules of $P840^{+\bullet}$. Feature 4 was assigned above, from the ENDOR spectrum of Fig. 2, as the $A_\perp$ feature of the 5-methyl hfc and an $A_{||}$ feature was also observed (feature 4a of Fig. 2). Since each $A_\perp$ should have an $A_{||}$ feature associated with it, we would expect to observe two $A_{||}$ in Fig. 4. The features labelled 4a in Fig. 4 have the exepected characteristics of $A_{||}$ 'turning points'. The separation of these two features is the same as that of the two components of feature 4. This suggests that $A_{||}$-$A_\perp$ is the same for both methyl groups as expected. Four further features (labelled 5 and 6) of the ST spectrum in Fig. 4 are assigned to the β-protons at positions 3 and 4 (Fig. 3). These again show further splitting relative to the ENDOR spectrum of Fig. 2, suggesting that we are also resolving the contributions from the individual constituent Bchl *a* molecules of $P840^{+\bullet}$ for these features. The hfc's determined from the Special TRIPLE spectrum are consistent with those determined from the ENDOR spectrum of Fig. 2 and the assertion that Fig. 2 arises from two overlapping spectra. The hfc's derived from Fig. 4 are tabulated in Table 1. It is not possible to associate particular hfc's with a certain half of the pair. Therefore both the larger 1-methyl and the larger 5-methyl hfc's could arise from the same bacteriochlorophyll, giving Σ(methyl hfc's) $P840^{+\bullet}$/Bchl $a^{+\bullet}$ for this bacteriochlorophyll of 0.57. The smaller methyl group couplings would arise from the other constituent Bchl *a* having Σ(methyl hfc's) $P840^{+\bullet}$/Bchl $a^{+\bullet}$ of 0.51. This would suggest a slightly asymmetric distribution of electron spin density between the components of the P840 Bchl *a* pair. Alternatively the different methyl group couplings could arise from a differing distribution of electron spin density *within* each Bchl *a* component of the pair. Thus the larger 5-methyl hfc could arise from the same Bchl *a* as the smaller 1-methyl hfc and vice versa. This leads to Σ(methyl hfc's) $P840^{+\bullet}$/Bchl $a^{+\bullet}$ of 0.53 for both halves of the P840 pair, i.e. a symmetrical distribution of electron spin between the halves. Note that the ratios discussed above sum to 1.06–1.08 rather than 1. This reflects experimental error and also the influence of the acetyl group at position 2, the orientation of which has been shown to affect the hfc's of methyl groups 1 and 5 slightly (by 1–6%) [24]. It is possible that the orientation of the acetyl group in Bchl $a^{+\bullet}$ in vitro is different from that in $P840^{+\bullet}$.

The ENDOR and ST spectra of $P840^{+\bullet}$ show that the electron distribution is symmetrical, indicating that the environment is symmetrical. Since the electron spin density on the primary donor is thought to be determined

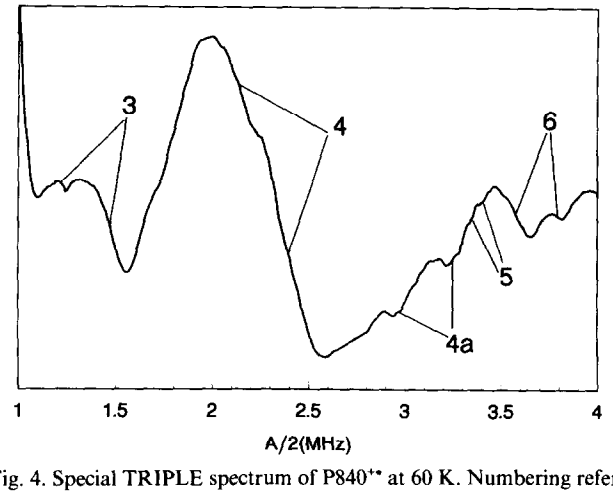

Fig. 4. Special TRIPLE spectrum of $P840^{+\bullet}$ at 60 K. Numbering refers to Table 1 and is discussed in the text. Conditions: microwave power 10 mW; r.f. power (total) 180 W; field 337 mT (EPR crossing point); r.f. modulation depth 50 kHz; scan time 84 s; time constant 655 ms; 160 scans co-added.

by the protein framework, this is consistent with the proposal that the photosynthetic reaction centre of *C. thiosulphatophilum* is a homodimer providing a symmetrical environment for $P840^{+\bullet}$. The function of such pairs may be to stabilize the charge-separated state through delocalisation of the positive charge on the primary donor cation over as large an orbital as possible. This would promote charge separation, while inhibiting back reaction.

The small asymmetry required to account for the non-equivalence of the methyl group hfc's resolved in the ST may reflect deviations from total symmetry in the formation of the homodimer. Alternatively it may be induced by the binding of other reaction centre components [21] to the homodimer. The significance of the symmetry in the selection of electron transfer pathways through the *C. thiosulphatophilum* reaction centre is also unclear at this time. The *R. sphaeroides* photosynthetic reaction centre has been shown to posses two bacteriopheophytin *a* molecules, approximately symmetrically positioned relative to the primary donor, that could act as primary acceptors [1]. However, only one is used under normal circumstances. It is possible that the asymmetric electronic structure of P870 (deduced from the electronic structure of $P870^{+\bullet}$)[2] contributes to this electron transfer pathway selection. The *C. thiosulphatophilum* reaction centre homodimer presumably also contains two primary electron acceptors and therefore two possible routes for electron transfer [5,21]. The more symmetrical electronic structure of $P840^{+\bullet}$ may allow for electron transfer to both putative acceptors with equal efficiency, although recent studies of the *R. sphaeroides* photosynthetic reaction centre suggest that it is the protein structure that determines the route of electron transfer [31] rather than the electronic structure of the primary donor.

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