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Volume 2 Contents

CHAPTER 8 PHOTOBIOLOGY—OPTICAL TECHNIQUES

CHAPTER 11 PHOTOCHEMISTRY—LINEAR AND NONLINEAR

Electron–Nuclear Multiple Resonance Studies on Primary Products of Bacterial Photosynthesis

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ENDOR and TRIPLE resonance techniques are applied to bacterial photosynthetic pigment anion and cation radicals in solution, yielding an almost complete set of isotropic H- and ^{14}N-hyperfine couplings including signs. Assignments to molecular positions are attempted on the basis of the experiments including specific D-labeling. The results from the bacteriochlorophyll *a* cation radical are compared with those of the *in situ* light-induced primary donor cation P 870$^+$ (protonated and partially deuterated) in the bacterium *Rhodopseudomonas sphaeroides* R-26. The latter is also studied by liquid state ENDOR/TRIPLE under physiological conditions (25°C, water). This comparison shows that P 870$^+$ is a bacteriochlorophyll *a* dimer. The different shifts of the individual hyperfine couplings contain information about the structure of this dimer. On the basis of the experiments and MO calculations a specific dimer model is proposed.

INTRODUCTION

In recent years the mechanism of light-induced charge separation in photosynthesis has roused the interest of scientists from different fields.[1] In such investigations photosynthetic bacteria[2] are easier to study than plants since they have only *one* photosystem, and the reaction centers (RC's)—in which the primary events take place—have been isolated and well characterized.[3] In the non-sulfur purple bacteria *Rhodopseudomonas* (*Rp.*) *sphaeroides* R-26 and *Rhodospirillum* (*R.*) *rubrum* G-9, which are studied in this paper, the RC's

contain three protein subunits, four bacteriochlorophyll (BChl) *a* and two bacteriopheophytin (BPh) *a* molecules, one or two ubiquinones (UQ), and one non-heme iron (3+).[3] The structure of BChl *a* is shown in Figure 1.

In the RC the light energy is trapped by singlet excitation of a pigment absorbing at 870 nm (P 870), which donates an electron to an electron transport chain in a few picoseconds. P 870 is currently believed to be a "special pair" of BChl *a* molecules; the series of electron acceptors involves probably monomeric BChl *a*, BPh *a*, and iron ubiquinone complexes.[4] During the charge separation process cation (P 870) and anion (BChl *a*, BPh *a*, UQ) radicals of the various pigments are formed. Much of our present knowledge about these species and their interactions in the RC has therefore evolved from the application of paramagnetic resonance methods[5]—used in conjunction with optical techniques. The dimeric nature of $P\,870^{+}$ was originally proposed by Norris *et al.*[6] on the basis of EPR data. Further support for this model came from electron–nuclear double resonance (ENDOR) studies.[7-11] Similar investigations were performed in order to identify and characterize the anion radicals of the primary electron acceptors.[9,12-17]

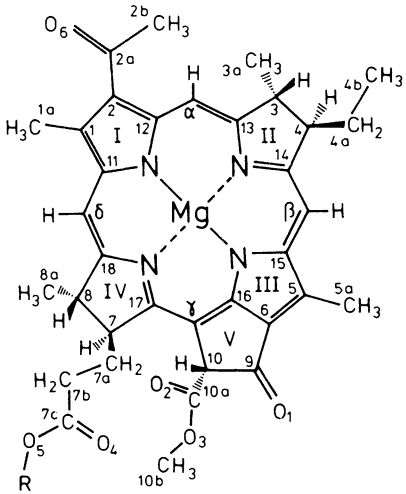

FIGURE 1 Molecular structure of BChl *a* and numbering scheme; R = phytyl, p (in *Rp. sphaeroides*), R = geranylgeranyl, gg (in *R. rubrum*). In BPh *a* the central Mg is replaced by two hydrogens bound to N_I and N_{III}.

In all these studies ENDOR proved to be superior to EPR for the determination of the electron nuclear hyperfine coupling constants (hfc's) due to its much higher spectral resolution.[18] Most of the *in vivo* ENDOR work has been carried out in frozen matrices,[7-11] where dipolar broadening masks all interactions except those belonging to nuclei with relatively small hfs anisotropy, e.g., rotating methyl groups.[19] It was only recently that the first detection of liquid state ENDOR on a bacterial RC (H_2O, 25°C) was reported.[20] In these experiments the additional application of electron–nuclear–nuclear TRIPLE resonance[18] proved to be very helpful for increasing the ENDOR sensitivity and resolution ("Special TRIPLE") and for determining the relative signs of the hfc's ("General TRIPLE").

In the present paper it is demonstrated that an almost complete set of isotropic ^{14}N- and H-hfc's can be obtained by ENDOR/TRIPLE-in-solution for the *in vitro* prepared monomeric pigment radical cations (see also Refs. 21, 22) and anions (see also Refs. 16, 23). These systems are important as model compounds for the *in vivo* occurring radical ions. The data for BChl a^+ are compared to those of P 870^+ in bacterial RC's, allowing conclusions about the electronic and geometric structure of the primary donor cation which is of importance for the understanding of the first events in photosynthesis.

EXPERIMENTAL

Rp. sphaeroides R-26 and *R. rubrum* G-9 were grown in Hutners medium.[24] *Rp. sphaeroides* R-26 RC's were isolated by the method of Clayton and Wang[25] and further purified on DEAE cellulose. Lauryldimethylaminoxid (LDAO) was exchanged against Triton X-100 by chemical reduction on a DEAE cellulose column. Ubiquinone was removed by the method of Okamura *et al.*[26] RC's of *R. rubrum* G-9 were prepared according to Snozzi and Bachofen.[27] The RC samples were concentrated by membrane filtration. They were filled under Argon in the sample tubes, which were then sealed. The RC concentration was *c.* $2\text{–}3 \times 10^{-4}$ M.

The bacteriochlorophylls were isolated by the procedure of Strain and Svec.[28] BPh *a* was obtained from BChl *a* by treatment with hydrochloric acid.[23] The cation radical of BChl *a* was prepared by

iodine oxidation in CH_2Cl_2/CH_3OH (*c.* 6:1, by vol.) under high vacuum conditions.[21,22] The anion radicals of BChl *a* and BPh *a* were generated by potentiostatically controlled electrolysis in 1,2-dimethoxyethane (DME), using tetra-*n*-butylammonium perchlorate (TBAP) as supporting electrolyte.[23] Optimum ENDOR signals[29] were obtained at *c.* 3×10^{-4} M for all three radicals.

The measurements were performed with a self-built computer-controlled spectrometer which allows the registration of EPR, ENDOR and General/Special TRIPLE resonance spectra. The experimental details have already been described elsewhere.[18,30]

RESULTS AND DISCUSSION

ENDOR and TRIPLE resonance on isolated pigment radicals

As a representative example the BChl a_p^+ is chosen. The EPR spectrum ($g = 2.0026$) is completely unresolved. The ENDOR-in-solution spectrum, however, reveals eleven H-hfc's (Figure 2, bottom); additionally, all four ^{14}N-hfc's could be observed at lower frequencies. For BChl a_{gg}^+ the same H- and ^{14}N-hfc's are obtained within experimental error ($\lesssim$10 kHz), clearly showing that the hydrophobic side chain (see Figure 1) has no influence on the spin density distribution of the isolated cation radicals. The hfc's including their signs (from General TRIPLE) are collected in Table I.

Following the method of Katz *et al.*[31] partially deuterated BChl *a* was extracted from biosynthetically labeled bacteria. In such preparations large abundances of protons are only found at positions 1a, 5a, 2b and more than two bonds away from the π-system; the methine and β-proton (rings II, IV) positions are highly deuterated. Such D-labeling and further D-exchange experiments were used for assigning the H-hfc's to specific groups of protons in the radical (see Table I).

Our results are in agreement with those of Borg *et al.*[21] who could resolve seven proton couplings in solution. Earlier frozen solution ENDOR experiments on BChl a^+ gave less detailed results.[7-11]

The H- and ^{14}N-ENDOR and TRIPLE resonance spectra of the anion radicals of BChl *a* and BPh *a* in solution have been analyzed in a similar way.[23] The isotropic hfc's including their signs and the assignments to molecular positions are also given in Table I. Although

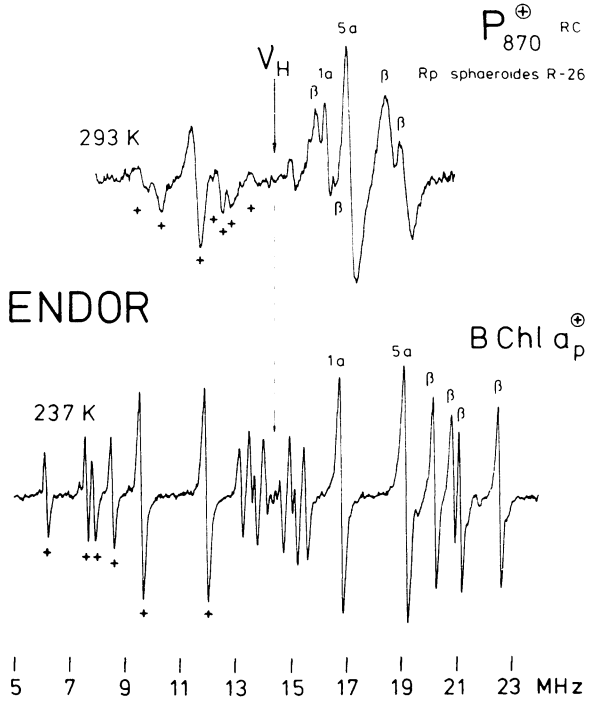

FIGURE 2 H-ENDOR-in-solution spectra of P 870$^+$ in RC's and BChl a_p^+ (both from *Rp. sphaeroides* R-26) at the given temperatures, the signs and assignments of the six largest hfc's (see Table I and II) are indicated. Experimental conditions, BChl a^+: mw power 20 mW, r.f. power 100 W (11 G, rot. frame), 10 kHz fm of r.f. field, ±50 kHz deviation, no field modulation, time constant 1 s, 4 scans; P 870$^+$: *in situ* illumination (450–900 nm, 500 W Xe high pressure lamp), mw power 80 mW, ±70 kHz fm deviation, 9 scans, rest as above.

the EPR spectra of BChl a^- and BPh a^- are very similar, a distinction of these species is clearly possible by their ENDOR and TRIPLE spectra.[18] For all three radical ions discussed in this paper, Pariser–Parr–Pople[16,32] and molecular fragment *ab initio*[33] calculations are available which support the above assignments (see Ref. 30).

A comparison between the cation and anion radicals (Table I) on the basis of known relationships between hfc's and π-spin densities[23,30] yields the following result: In the *cation* there is little—or even negative—spin density at the four nitrogens and the methine carbons. High values are found for all carbons in the pyrrole rings I

TABLE I
Comparison of isotropic hfc's a (MHz) of BChl a^+, BChl a^-, and BPh a^- [a]

Position[b]	Type	BChl a^+	BChl a^-	BPh a^-
1a	methyl protons	+4.85	+7.63	+7.10
5a		+9.50	+9.19	+8.32
α, β, δ	methine protons	±0.15	−6.23	−6.94
		+1.30	−6.91	−8.02
		+2.30	−9.65	−8.50
3, 4, 7, 8	β-protons	+11.61		−1.50
		+13.00	+0.50	−1.84
		+13.59	+0.95	−2.63
		+16.43	−1.60	
	other protons	−0.50		+0.48
		−1.65		+0.98
Rings II, IV, I, III	nitrogens	−3.17	+6.52	+7.19
		−3.05	+5.86	+6.16
		−2.35	(−)1.18	(−)1.20
		±0.45	(−)0.52	(−)0.60

[a] BChl a^+: (I_2, CH_2Cl_2/CH_3OH), H-hfc's: 255 K, ^{14}N-hfc's 270 K; BChl a^-, BPh a^-: (electrolysis, DME, TBAP), H-hfc's: 255 K: BChl a^-, 270 K: BPh a^-, ^{14}N-hfc's: 280 K. Hfs data from ENDOR and Special TRIPLE, signs from General TRIPLE; for details see Refs. 23 and 30.

[b] For numbering of positions see Figure 1. Except for methyl protons (see Ref. 9, 10, 41), individual assignments in the various types of nuclei cannot be given.

to IV (from β-proton and methyl hfc's). In both *anions* of BChl *a* and BPh *a* the nitrogens in rings II and IV have large and the other two have small spin densities. At the methine carbons and at carbons 1 and 5 the spin density is large. It has been argued by Fajer *et al.*[34] that the similarity of the primary chlorophyll donor and the intermediate chlorophyll and/or pheophytin acceptors favour electron transfer on a picosecond timescale. The striking differences between the generated cation and anion radicals, however, minimize orbital overlap and thereby inhibit wasteful back reactions.

ENDOR and TRIPLE resonance on pigment radicals in RC's

Illumination of RC's from *Rp. sphaeroides* R-26 results in a reversible bleaching of the optical band at 870 nm. Concomitantly a narrow Gaussian EPR signal at $g = 2.0025$ appears which is attributed to the

cation radical of P 870. *In situ* illumination in the ENDOR cavity allows the detection of the ENDOR spectrum (25°C, water) depicted in Figure 2 (top).

It has been shown[20] that in P 870$^+$ all proton nuclei are still in the fast tumbling limit in spite of the long rotational correlation time τ_R of 30 ns (molecular weight of RC *c.* 100 000 daltons, water, 25°C). Therefore, a liquid solution ENDOR spectrum is obtained. However, the τ_R value is far from optimum ENDOR detection conditions[29] (τ_R^{opt} *c.* 1 ns), implying lower intensities and fairly large linewidths as compared to the BChl a^+ spectrum (Figure 2).

Seven H-hfc's can be deduced in P 870$^+$ which are all positive and belong to *one* species according to a General TRIPLE experiment. Highest resolution is achieved by use of the Special TRIPLE technique (Figure 3, top). The same spectra are observed when the RC's are chemically oxidized by $K_3[Fe(CN)_6]$. On thermal denaturation of the protein, EPR and ENDOR signals disappear.

In order to assign the ENDOR lines to specific groups of protons in the radical we have also studied deuterated RC's, *vide supra*. In Figure 3 the Special TRIPLE spectra of P 870$^+$ in protonated and partially deuterated RC's are compared. Since in the latter case only lines belonging to the methyl groups in positions 1a and 5a show up (the acetyl and γ-proton hfc's are very small) an assignment as given in Figure 3 is straightforward. The additional line in the P 870$^+$ spectra (smallest hfc) might be assigned to the acetyl group (2b) or to γ-protons (3a, 4a, 7a, 8a) or to protons from the protein. It is remarkable that there are only two hfc's for the methyl groups in positions 1a and 5a (Figures 2 and 3). Assuming a bacteriochlorophyll dimer for P 870$^+$, this finding already indicates a symmetrical spin distribution over both halves.

Since an assignment of the measured hfc's to molecular positions was achieved in both BChl a^+ and P 870$^+$, the data can be directly compared (Table II). It is obvious that in P 870$^+$ all hfc's are reduced in magnitude but not by a constant factor of 2 as proposed by the simple "special pair" model.[6-11]

It is interesting that an average reduction factor defined as the ratio of average hfc's in each species is very close to two (1.95). The procedure of comparing hfc's is, however, questionable (unknown changes in the dihedral angles of the β-protons).[32] A better approach to confirm the dimeric nature of P 870$^+$ is to simulate the EPR

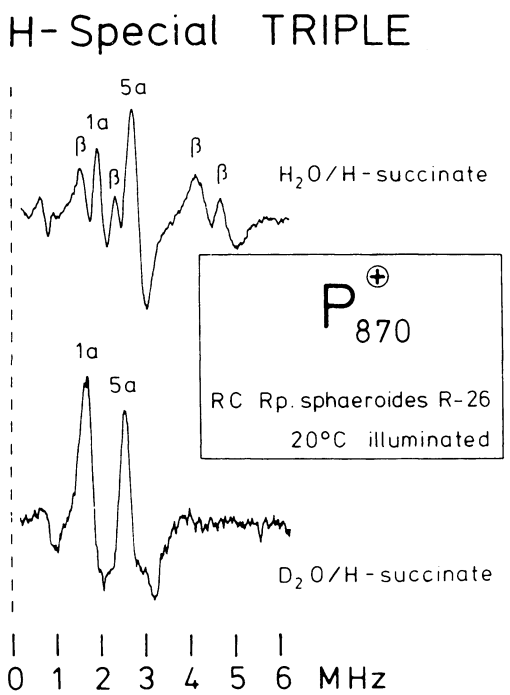

FIGURE 3 H-Special TRIPLE spectra of light-induced P 870$^+$ in solution; bacteria grown with protonated succinate in H_2O (top), and in 99.9% D_2O (bottom). Experimental conditions: total r.f. power 200 W, 4 scans, ±50 kHz fm deviation, rest as for ENDOR (Figure 2). The shoulders on lines 1a and 5a (bottom) are due to non-averaged hfs anisotropy of the methyl groups and not due to additional couplings. (The deuterated RC's were coagulated resulting in a considerable increased τ_R value and solid state ENDOR line shapes.)

spectrum by using the ENDOR hfc's. The result is shown in Figure 4, in trace *a* a dimer and in trace *b* a monomer was chosen for the simulation. It is obvious that P 870$^+$ in *Rp. sphaeroides* is a dimer of BChl *a* molecules, which is also in accordance with recent EPR results from Wasielewski *et al.*[35]

The reduction factors presented in Table II are in contradiction to earlier solid state ENDOR studies on P 870$^+$, where a halving of all observed hfc's was found.[7-11] Our temperature dependent ENDOR studies showed that this result was due to an incorrect assignment of the observed broad resonances in the frozen state.[30]

TABLE II

Comparison of isotropic hfc's a (MHz) of BChl a_p^+ (255 K) and P 870$^+$ (298 K) from *Rp. sphaeroides* R-26 (ENDOR/TRIPLE data)

Type (Position)	BChl a_p^+	P 870$^+$	RF[a]
CH_3 (1a)	+4.85	+4.00	1.21
CH_3 (5a)	+9.50	+5.60	1.70
β-H	+11.61	+3.30	3.52
β-H (3, 4, 7, 8)	+13.00	+4.45	2.92
β-H	+13.59	+8.60	1.58
β-H	+16.43	+9.50	1.73

[a] RF: reduction factor of hfc's a (BChl a^+)/a (P 870$^+$). The β-proton hfc's are not individually assigned, they are compared according to their ordering in magnitude. The smallest hfc of P 870$^+$ (+1.4 MHz) is not considered since no assignment was made.

The comparison between the hfs data obtained for P 870$^+$ in *Rp. sphaeroides* R-26 and in another bacterium (*R. rubrum* G-9)[36] shows that the couplings are different. This is true not only for the β-proton hfc's which are strongly dependent on the geometry of rings II and IV, but also for the methyl couplings. No such differences of the hfc's of isolated BChl a_p^+ and BChl a_{gg}^+ (different side chains) could be found. These observations point to a possible geometrical distortion of the dimer by the protein environment.

Structural proposal for P 870$^+$

Beyond the dimeric nature of P 870$^+$ the hfs data contain information about its structure. By adopting the ESE results of Bowman and Norris[37] P 870$^+$ can be described by a supermolecule, in which the unpaired electron is delocalized in a super MO extending over both BChl *a* halves. This requires an interplanar distance $\leq$3.5 Å (van der Waals distance of aromatic π-systems). The ENDOR results have shown that the halves are equivalent, i.e., they must be related to each other by a C_2-symmetry axis. A model which fulfills this requirement has already been proposed.[38–40] In order to show if our experimental observations can be explained in the framework of such a model, we have performed a series of simple HMO calculations on a dimer constructed from the π-skeletons of the two BChl *a* molecules, including the keto groups at rings I and III. The best

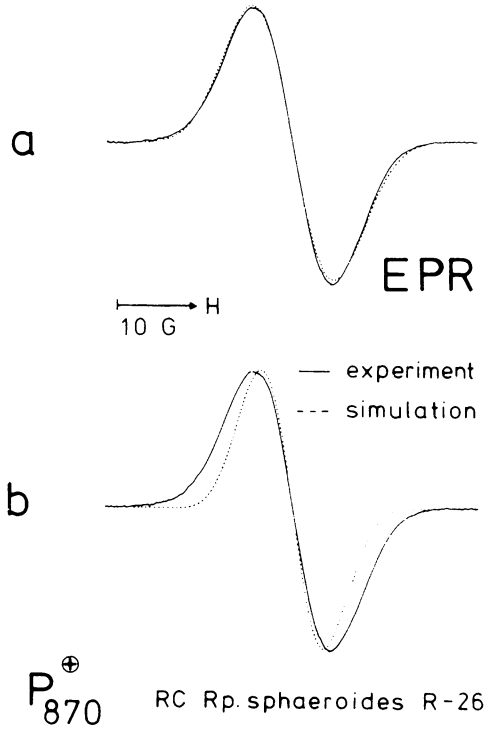

FIGURE 4 Experimental and simulated EPR spectra of P 870$^+$ in solution. For the simulation the ENDOR couplings and assignments from Table II were used; trace a: simulation assuming a dimer (twice the number of protons belonging to each hfc, linewidth 2.3 G obtained from the residual small hfc in P 870$^+$ and the ^{14}N couplings in BChl a^+); trace b: simulation assuming a monomer (linewidth $2^{1/2} \times 2.3 = 3.2$ G).

agreement with the experiments was obtained for a dimer geometry with the following characteristics (Figure 5): (i) interplanar distance 3–3.5 Å (ii) maximum overlap in the region of rings III and V, (iii) two parallel planes (shifted against each other by 7–8 Å), α-face of the first BChl *a* partially overlapping the β-face of the second BChl *a*.

This proposed dimer geometry must, however, be considered as preliminary since the theoretical approach—so far—has been very simple (enforced by computer limitations). More sophisticated calculations (Extended HMO and SCF methods) are already in progress.

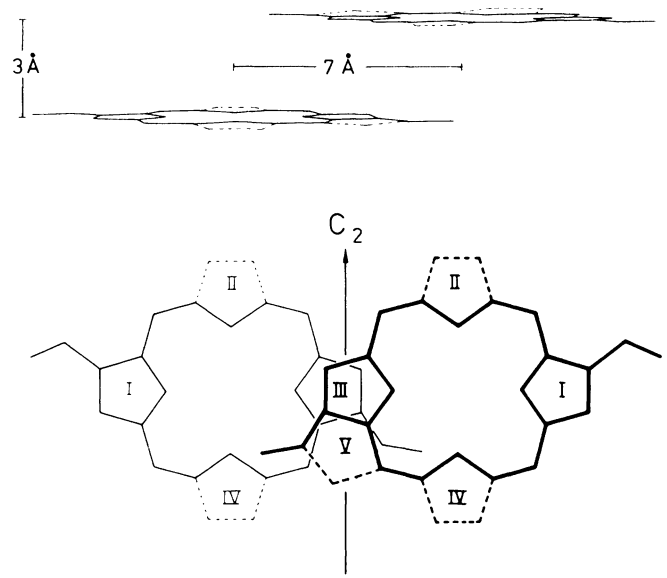

FIGURE 5 Horizontal and perpendicular view of the dimer model. Only the π-skeletons and the hydrated rings (broken lines) of the two monomeric halves are shown. Standard geometries with X-Ray modifications for the keto group at rings III were used for the Hückel MO calculations (CNDO/2 parametrization).

Furthermore we are currently trying to extend our ENDOR/TRIPLE studies to the primary donor and acceptors in other bacteria and in plant systems.

Note added in proof: After completion of this work, a manuscript by O'Malley and Babcock[42] has been brought to our notice in which P 870$^+$ is proposed to consist of a monomeric BChl a^+ with altered spin distribution (see also ref. 36).

Acknowledgements

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