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# OPTICALLY EXCITED TRIPLET STATES IN THE BACTERIA *RHODOPSEUDOMONAS SPHAEROIDES* 'WILD-TYPE' DETECTED BY MAGNETIC RESONANCE IN ZERO-FIELD

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## Summary

Optically-detected magnetic resonance (ODMR) experiments in zero-field on the photosynthetic bacteria *Rhodopseudomonas sphaeroides* revealed triplet states belonging to molecules which have a prompt emission in the optical region from 590 to 700 nm and a delayed emission between 700 and 830 nm. The zero-field parameters of these triplet states are $29 < |D| < 34 \cdot 10^{-3}$ cm$^{-1}$ and $4 < |E| < 8 \cdot 10^{-3}$ cm$^{-1}$, the decay rates of the $|D| + |E|$ transitions being in the order of 60—340 ms. The correlation between optical emission and radio-frequency was used to separate the total optical emission from 590—700 nm into individual emissions, belonging to molecules whose triplet states were studied by ODMR in this region.

Comparing the fluorescence microwave double resonance (FMDR) spectra with the results of excitation spectroscopy, as well as comparing the zero-field parameters and the decay rates with that of Mg-porphyrins in matrices given in the literature, allowed the identification of the emitting molecules as Mg-porphyrins which are produced by the biosynthesis of bacteriochlorophyll in the cells.

## Introduction

ODMR experiments in zero-field have shown to be a very sensitive tool to investigate triplet states of organic molecules. The high sensitivity and the

Abbreviations: EEDOR, electron-electron double resonance; FMDR, fluorescence microwave double resonance; ODMR, optically-detected magnetic resonance; FDMR, fluorescence-detected microwave resonance; DEDMR, delayed emission-detected microwave resonance; BChl, bacteriochlorophyll.

independence of crystalline orientation of the molecules are due to the correlation of magnetic and optical spectroscopy which are demonstrated by measurements of randomly oriented molecules, especially porphyrins in matrices [5].

This technique, zero-field optically-detected magnetic resonance (ODMR), has become very attractive for the study of biological molecules such as proteins [7] and porphyrins in photosynthesis [1,2,8]. Particularly, in photosynthetic bacteria, the triplet state of the reaction center can be monitored via the fluorescence emission of the antenna molecules from 900 to 950 nm [1,2]. But the emission of the antenna from 900 to 950 nm represents only a very restricted region of the total emission of *Rhodopseudomonas sphaeroides* as demonstrated in Ref. 3.

This emission is heavily structured and only partially identified. Therefore, we attempted ODMR experiments in the whole region of the prompt and delayed emission of the complicated system of *Rps. sphaeroides* with the purpose of finding further triplet states to determine the origins of at least some bands in the total emission. Our original hope was that significant help in the identification of the emitting molecules would be afforded by the results of excitation spectroscopy which was carried out in a parallel study. Further clarification was expected from the comparison of the zero-field parameters and the decay kinetics of the radio-frequency transitions with corresponding results on similar molecules measured in matrices as given in the literature.

## Materials and Methods

*Rps. sphaeroides* 'wild type' was grown anaerobically in the medium described by Cohen-Bazire et al. [9]. The cells were centrifuged and redissolved in phosphate buffer twice under a nitrogen atmosphere. Subsequently, they were centrifuged again and redissolved in phosphate buffer with 10 mM morpholinopropanesulfonic acid (pH 8) and reduced with excess dithionite. After 2-fold dilution with extremely pure glycerol, the samples were diluted to an absorbance of 1 at 590 nm and quickly frozen to liquid helium temperature.

Zero-field resonance was detected by fluorescence-detected microwave resonance (FDMR) [6] and by delayed emission-detected microwave resonance technique (DEDMR) [10]. FDMR as well as DEDMR means that the microwave frequency is swept at fixed optical wavelengths. In contrast to these experiments, fluorescence microwave double resonance (FMDR) means that the optical wavelength is scanned over the total fluorescence spectrum at fixed microwave frequency, the power of which is amplitude-modulated.

Broadband excitation (400—450 nm) was performed using a XBO 450 light source followed by $H_2O$, $CuSO_4$, KV399 (Schott), DT450 and DT470 (Balzers) filters. The emission was monitored with a 0.6 m Jobin Yvon model HRS 1 grating monochromator fitted with a cooled photomultiplier (extended S 20). The microwave sources were HP models 8690B and 8620A followed by Hughes model 1177 TWT power amplifiers. A digital multipulse time base synchronized the repetitive sweeps with the sweep of the computer of average transients. The FMDR spectra were recorded by amplitude modulation of the microwave power and a lock-in analyser (Ithaco dynatrac 3).

## Results

*FDMR experiments*

As a check on the quality of the samples, first the measurements by Clarke [1] and Hoff [2] were reproduced qualitatively. These experiments revealed the emission of the reaction center of *Rps. sphaeroides* in the wavelength range between 900 and 950 nm. Further, FDMR experiments which have been performed in successive regions of the whole emission spectrum from 590 to 950 nm showed, thus far, additional FDMR signals between 590 and 700 nm. Within this spectral region, amplitude and transition frequencies of the FDMR signals depend on the wavelength selected by the monochromator. In general, the FDMR signals occur between 200 and 1400 MHz. The width of the magnetic resonance signals is in the order of 50—100 MHz and is nearly independent of the width of the optical window which is 1.8 nm. The wavelength dependence of the radio-frequency transitions is demonstrated in Fig. 1 for the

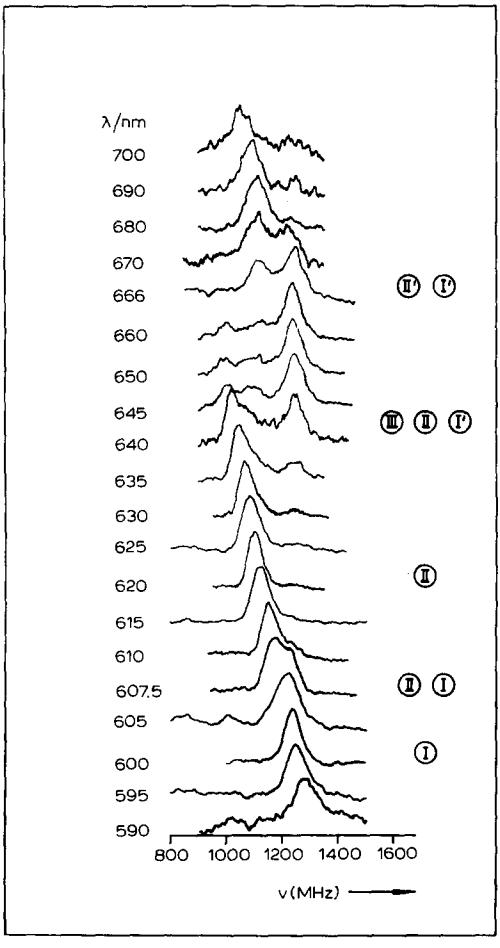



**Fig. 1. FDMR signals of the $|D| + |E|$ transition, dependent on the wavelength of the prompt emission of *Rps. sphaeroides*.**

frequency range between 1000 and 1400 MHz, the highest frequencies found. Similar signals could also be found in the frequency ranges 300—500 and 600—800 MHz. It should be mentioned, that the lowest radio-frequency transitions could only be detected with electron-electron double resonance techniques (EEDOR).

Starting with the lowest row in Fig. 1, just one radio-frequency transition (1290 MHz) appears monitoring the fluorescence at 590 nm. The FDMR signal is shifted slightly in frequency from 1290 to 1220 MHz upon changing the wavelength of the optical detection to 605 nm. This transition is assigned to a triplet state I. At and above 607.5 nm, a further resonance at 1177 MHz appears besides the signal of triplet I. This radio-frequency transition is assigned to a triplet state II. On increasing the wavelength, this transition shifts to 1089 MHz at 625 nm. Ambiguities can be avoided by comparing the absolute signal intensities. This information is not contained in the normalized plot of Fig. 1.

The existence of a third triplet state is manifested by the resolution of three radio-frequency transitions at 640 and 645 nm and in the strong asymmetry of the signals at 630 and 635 nm. The latter effect points out the superposition of two radio-frequency transitions. This triplet state, labeled triplet III, occurs in the fluorescence from 630—666 nm.

Transition frequencies comparable to that of triplet I appear again at 635 nm and are detectable upto 670 nm. This group of signals is labeled triplet I'. Similar considerations can be made in the wavelength region from 666 to 700 nm with regard to triplet II. In analogy, these signals are called triplet II'.

The results detailed above are summarized in Table I. The fluorescence wavelength of the maximum radio-frequency-induced FDMR signals, the transition frequencies belonging to these wavelengths, the FDMR linewidth, and the resulting zero-field parameters $|D|$ and $|E|$ are given. Additionally, the recovery times $\tau(\nu_1)$ ($1/e$ time) of the fast transients detected at the radio-frequency transitions $\nu_1$ are listed. According to Ref. 6, this means that $\tau(\nu_1)$ is the longer decay time of the two levels combined by the radio-frequency. Because of the

TABLE I

TRANSITION RADIO FREQUENCIES, LINEWIDTHS, ZERO-FIELD PARAMETERS OF THE FDMR SIGNALS AND THE RECOVERY TIME $\tau(\nu_1)$ OF THE FAST TRANSIENT SIGNAL $\nu_1$

| | $\nu_1$ (MHz) | $\nu_2$ (MHz) | $\nu_3$ (MHz) | $\Delta\nu_1$ (MHz) | $\Delta\nu_2$ (MHz) | $\tau(\nu_1)$ (ms) | $\lvert D\rvert$ ($10^{-4}$ cm$^{-1}$) | $\lvert E\rvert$ ($10^{-4}$ cm$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Triplet I (600 nm) | 1237 | 786 | 455 | 66 | 100 | 344 | 334 | 74 |
| Triplet I' (660 nm) | 1238 | 788 | 450 | 68 | 99 | 343 | 334 | 74 |
| Triplet II (620 nm) | 1100 | 697 | 403 | 78 | 70 | 138 | 297 | 67 |
| Triplet II' (685 nm) | 1107 | 694 | 413 | 96 | 72 | 131 | 297 | 68 |
| Triplet III (635 nm) | 1045 | 687 | 358 | 50 | 58 | 64 | 286 | 59 |

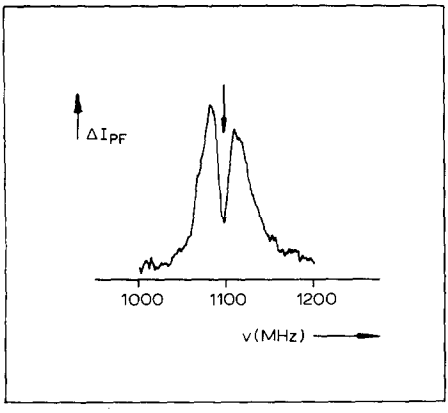
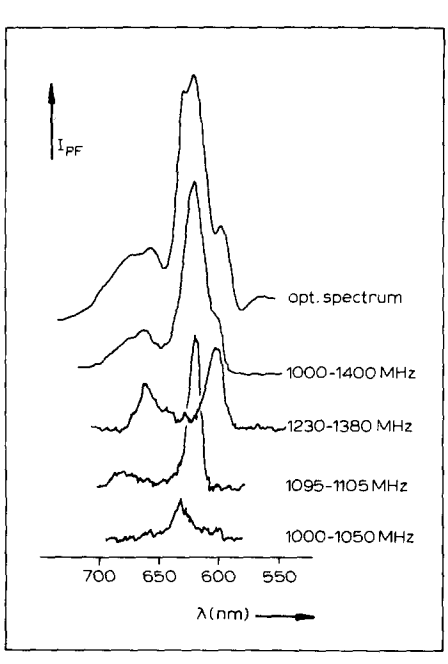



Fig. 2. Hole-burning with a stationary microwave irradiation of 100 mW at 1103.8 MHz (arrow) performed at the ODMR signal of triplet II at 620 nm. The half-width of the hole is about 11.9 MHz.

low signal-to-noise ratio, it was not possible so far to lower the excitation intensity and to extrapolate $\tau(\nu_1)$ to the excitation rate zero. $\tau(\nu_1)$ can only be regarded as a rough approximation to the real kinetics of the triplet sub-levels.

The broad FDMR signals were further analyzed by hole-burning experiments [11]. Fig. 2 shows the $|D| + |E|$ transition of triplet II as an example of all signals investigated. At the frequency of the stationary microwave irradiation, a hole



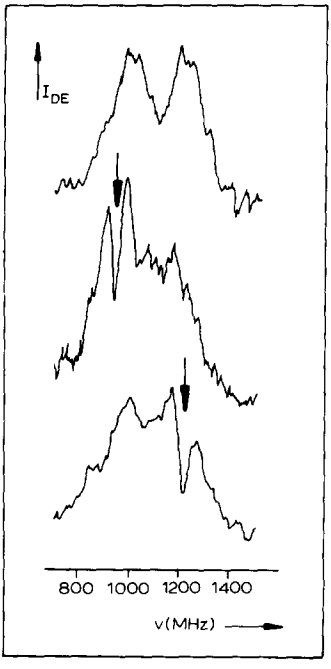




Fig. 3. Comparison of the FMDR spectra of triplet I. . . .III, the experimental superposition of the three FMDR spectra and the spectrum of the prompt emission.

Fig. 4. DEDMR transitions at 1198 and 970 MHz detected at 710 nm. The lower two signals show hole-burning experiments with 200 mW at 938—971 MHz and 1200—1239 MHz (arrow).

appears with a linewidth of about 10 MHz. Table I demonstrates that all three transition frequencies, as well as the decay kinetics, are identical for triplet I and I′ and triplet II and II′, respectively. Therefore, it can be concluded that triplet I and I′, as well as II and II′, are identical, which is confirmed by the FMDR spectra described in the following.

*FMDR experiments*

In contrast to the FDMR experiments, in which the microwave frequency is scanned continuously at fixed optical wavelengths, in FMDR experiments the microwave frequency is fixed (but amplitude-modulated) and the optical wavelength is scanned continuously. Only those components of the optical emission are detected via the lock-in tuned to the amplitude modulation frequency which originate from molecules the stationary triplet population of which is changed by the amplitude modulation of the radio-frequency.

In Fig. 3, the optical spectrum is compared with the FMDR spectra obtained by radio-frequency irradiation at the transition frequencies of the triplet states I, II and III separately, as well as all frequencies together. The almost quantitative agreement of the optical spectrum with the FMDR spectrum of all three triplets demonstrates the quality and the validity of the separation of the total emission into the emission of the individual molecules.

*DEDMR experiments*

Monitoring the delayed emission of *Rps. sphaeroides* in preliminary experiments, ODMR (DEDMR) signals could be observed between 700 and 830 nm. In these preliminary experiments, only the triplet state belonging to 710 nm (maximum delayed emission) was examined in detail. The transition frequencies found were: 1198, 970 and 230 MHz. The transition lowest in frequency could only be detected with the double-resonance technique as in the case of the FDMR experiments. Fig. 4 shows the signals at 1198 and 970 MHz in the upper row. The inhomogeneity of the signals is demonstrated by hole-burning experiments (lower row).

## Discussion

*FDMR, FMDR*

First, focusing on Figs. 1 and 3, the results of the FDMR and the FMDR experiments allow the quantitative separation of the prompt emission of *Rps. sphaeroides* from 590 to 700 nm into the emission of three species of molecules whose triplet states have been detected by our ODMR experiments. Not only the 0.0 transition of the fluorescence could be found but at least one vibronic band could be associated with them (i.e., 600—660 nm, 623—685 nm). Because of the excitation spectra which show only the absorption bands of the emitting species, it can be excluded that the microwave transitions detected on the different fluorescence bands belong to triplet states of other molecules from which the energy is transmitted to the fluorescing ones.

For the purpose of identifying these molecules which belong to a manifold of molecules in this complex biological system, the results of emission and excitation spectroscopy on *Rps. sphaeroides* [3] are considered and listed in

TABLE II

COMPARISON OF THE RESULTS OF THE EXCITATION SPECTROSCOPY [3], FDMR AND FMDR

| | FDMR, FDMR | | | | Excitation spectroscopy | |
|---|---|---|---|---|---|---|
| | $\|D\|$ ($10^{-4}$ $cm^{-1}$) | $\|E\|$ ($10^{-4}$ $cm^{-1}$) | $\tau(\nu_1)$ (ms) | Max. (FMDR) (nm) | Max. (PF) (nm) | Molecule |
| Triplet I | 334 | 74 | 344 | 600, 660 | 597, 656 | Mg-protoporphyrin-IX monomethyl ester |
| Triplet II | 297 | 67 | 138 | 623, 685 | 626, 688 | Mg-2,4-divinylphaeoporphyrin-$a_5$ monomethyl ester |
| Triplet III | 286 | 59 | 64 | 635 | | |

Table II with the ODMR data. The first striking feature is the nearly quantitative coincidence between the fluorescence maxima assigned by excitation spectroscopy and the assignment of FMDR. Therefore, there is no doubt that the molecules with triplet states I and II which are found in the present work are associated with two of the molecules identified by excitation spectroscopy. Triplet III emitting at 635 nm has not been observed and identified up to now. A rough identification will be tried via the values of the zero-field parameters and the kinetics of decay as demonstrated now.

Unfortunately, no ODMR data for the molecules listed in Table II are known, neither in vivo nor in matrices. Therefore, our results will be compared with those obtained with similar molecules. In this connection, 'similar' means Mg-porphins differing only in the side groups. These porphins are porphyrins with four non-reduced pyrrole rings [12,13]. Only for Mg-porphins are the triplet decay times found to be as long as those found in the experiments presented here [14].

An additional indication of the nature of a triplet state are the zero-field parameters $|D|$ and $|E|$, in which D represents the delocalization of the triplet spin on the molecular skeleton [14]. We compare our experimental results (triplet I, II and III) with the data on Mg-protoporphyrin molecules in solution. These have $|D| = 33.1 \cdot 10^{-3}$ $cm^{-1}$ [5], which corresponds within experimental error quantitatively to that of triplet I (Mg-protoporphyrin-IX monomethyl ester).

Triplet II, Mg-2,4-divinylphaeoporphyrin-$a_5$ monomethyl ester, has long decay times of the triplet state also. The $|D|$ value of $29.7 \cdot 10^{-3}$ $cm^{-1}$ is about 20% lower than that of triplet I and Mg-protoporphyrin, respectively. This can be understood by a further delocalization of the electron in which it extends further onto the isocyclic pentanon ring of this molecule.

Triplet III could not be identified by excitation spectroscopy, but the decay kinetics and zero-field parameters (Table II) indicate that the molecule has to be similar to a Mg-porphin.

*DEDMR*

Prompt and delayed emission ($t = 0.3. . . .10$ ms) differ strongly in the spectral range 700—850 nm, a strong indication that the delayed emission originates from triplet states as phosphorescence. The excitation spectrum of the delayed

emission bears a likeness to that of a molecule similar to Mg-protoporphyrin. The zero-field parameters support this fact and indicate that the molecule responsible for the phosphorescence is not identical with the molecules responsible for triplets I, II and III.

*Linewidth and wavelength dependence of the ODMR transitions*

Broad ODMR signals, which increase in breadth when monitored at the periphery of the optical band (compare Figs. 1 and 3) and which show a correlation between zero-field parameters and the wavelength monitored, are already known for biological molecules in frozen glasses, e.g. tryptophan in ethyleneglycol-$H_2O$ [16]. The broad lines are due to a multiplicity of sites with the same optical origin but varying zero-field splittings. The 'homogenous' linewidth of the individual site could be estimated by hole-burning experiments to be 10 MHz, comparable to that of biological molecules in Schpolski matrices [17].

Although there is a close analogy to the data known in the literature, there exists one important difference: the data presented here are found monitoring the fluorescence, not the phosphorescence.

A change in the optical wavelength of about 15—20 nm results in a shift of 50—70 MHz, or, in other words, the frequency of the $|D| + |E|$ transition increases with increasing singlet energy by 0.1 MHz $\cdot$ $cm^{-1}$ in a similar manner for triplets I, II and III, a further evidence for comparable molecules I. . . .III.

These results may be of relevance for models recently given in the literature [18] in which selective spin-orbit interaction is responsible for the shifts. In these considerations the energy difference $(\Delta E^S - \Delta E^T)$ between the singlet and triplet energy of the individual site is responsible for the correlation of optical energies and triplet splittings, as it was originally suggested by Francis and Harris [19] for $k$-dependant triplet energies.

Considering that the inhomogeneous distribution of singlet energies (500 $cm^{-1}$, this work) is comparable to that of triplet energies (300 $cm^{-1}$ [16]), the results of the FDMR are an excellent confirmation of the ideas of Lemaistre and Zewail [18]. A further quantitative evaluation of our data is in progress and will be presented elsewhere (Beck, J. and von Schütz, J.U., unpublished results).

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