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THE REACTION CENTER TRIPLET STATE OF THE PHOTOSYNTHETIC BACTERIUM *RHODOBACTER SPHAEROIDES* R26: ELECTRON SPIN ECHO SPECTROSCOPY OF A SINGLE CRYSTAL

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Electron spin echo detected EPR spectra have been obtained of the triplet state of a single crystal of the reaction center of *Rhodobacter sphaeroides* R26. The crystal is found to be monoclinic and to contain four magnetically inequivalent sites. The magnetic relaxation behaviour of this triplet has been investigated and the lifetimes of the triplet sublevels have been determined.

1. Introduction

Recently, the first successful crystallization of the reaction center (RC) proteins of the photosynthetic bacterium *Rhodospseudomonas (Rps) viridis* and *Rhodobacter (Rb) sphaeroides* R26 has been reported [1,2]. Subsequent X-ray diffraction for *Rps. viridis* revealed the atomic structure of the crystal with a resolution of 2.9 Å [3], while X-ray diffraction data on *Rb. sphaeroides* R26 showed a striking structural homology with *Rps. viridis* [4,5]. The mutual orientation of the RC pigments being known, spectroscopic measurements may contribute a great deal to the knowledge of the electronic properties of the excited states of the RC and of the electrostatic interaction between the pigments.

In photosynthesis light energy is converted into chemical energy, the primary steps being

in which P is the primary donor, I an early transient electron acceptor and X the first stable acceptor. The lifetime of the radical pair $[\text{P}^+\text{I}^-]$ is about 200 ps. This lifetime can be lengthened by prereducing the second acceptor X. Illumination then results in the formation of the triplet of the primary donor, ^3P . The triplet yield becomes almost unity at low temperatures in accordance with the radical-pair mechanism [6],

In the past application of cw and pulsed EPR techniques has proved to be very useful in the study of the triplet state of randomly oriented RCs [7,8]. However, the full potential of magnetic resonance techniques can only be exploited when such experiments are performed on oriented single crystals. The crystallization of the photosynthetic RC therefore allows for exciting, hitherto impossible, EPR investigations. Gast et al. recently performed cw EPR studies on single crystals of the RC of *Rps. viridis* and *Rb. sphaeroides* R26 [9,10]. They obtained EPR spectra as a function of the direction of magnetic field with respect to the crystal axes in the crystal planes. From simulations of these spectra they derived the number of magnetically inequivalent sites and the directions of the principal axes of the zero-field splitting tensor with respect to the crystal axes.

A further elucidation of the magnetic properties, and in particular of the spin density distribution in the triplet state, may be achieved by electron nuclear double resonance (ENDOR) spectroscopy. So far ENDOR studies, performed either directly or indirectly via electron spin echo envelope modulation spectroscopy, have only been reported for randomly oriented triplets [11,12]. The advantage of perform-

ing an ENDOR study of the triplet state in a single crystal is that the ENDOR frequencies can be measured for well-defined orientations of the RC with respect to the direction of the magnetic field. The orientational dependence of those frequencies, in principle, yields directly the hyperfine tensors of the protons and nitrogens present in the RC complex, thus offering information concerning the spin density distribution. In our opinion the application of pulsed ENDOR spectroscopy, i.e. the combination of electron spin echo (ESE) and ENDOR techniques, seems the most promising way of performing an ENDOR study for the single crystal.

As the initial step on this route, the present paper reports the results of the first ESE study of a single crystal of the RC of *Rb. sphaeroides* R26. From the ESE-detected EPR spectra we conclude that there are four magnetically inequivalent positions in the unit cell of our crystal. The symmetry relations found between the fine structure axes of these sites show that the crystal of the RC of *Rb. sphaeroides* R26 studied by us is monoclinic.

The lifetimes of the individual triplet sublevels have been determined. Additionally, the decay of the Hahn echo and of the stimulated echo for orientations of the magnetic field along the fine structure axes will be described.

2. Materials and methods

RCs were prepared from *Rb. sphaeroides* R26 as described by Feher and Okamura [13]. They were crystallized using a slightly modified version of the method of Allen and Feher [2]. Crystals were grown by vapour diffusion in an aqueous solution of 7.5 mM Tris-HCl, 0.45 mM EDTA, 0.075% w/v (weight/volume) NaN_3 , 3% w/v 1,2,3-heptane triol, 0.3% w/v LDAO and 1.5 M $(\text{NH}_4)_2\text{SO}_4$, and conserved in an aqueous solution containing 10 mM Tris-HCl, 0.7 mM EDTA, 1% w/v 1,2,3-heptane triol, 0.1% w/v LDAO and 2.7 M $(\text{NH}_4)_2\text{SO}_4$.

The ESE experiments were performed with a homodyne X-band ESE spectrometer similar to the one described in ref. [14]. The cavity was a stripline resonator comparable to the one described by Mims [15] with a loaded Q of about 75 at a resonance frequency of 9.2 GHz. On the body of the cavity a gear

arrangement was constructed to allow for rotation of the sample tube. This rotation in combination with the rotation of the magnet makes it possible to orient a crystal in every direction with respect to the magnetic field. The microwave excitation pulses were amplified to typically 100 W by a Space microwave pulse amplifier 6001 modulator which produce $\pi/2$ pulses of 15 ns. In the detection circuit a limiter (alpha MT-3283C) and a pin switch (HP33144A) were used before the GaAs-FET preamplifier (W&J 5306-513) to protect the preamplifier and shorten its recovery time. With this set up we reduced our spectrometer "dead-time" under superfluid helium conditions to 160 ns.

The optical excitation was provided by the output of a pulsed tunable dye laser (Quanta Ray PDL-1) pumped by a frequency-doubled Nd:YAG laser (Quanta Ray DCR-2). Using a solution of the dye Kiton Red in methanol we obtained 40 mJ pulses at 590 nm. The laser operated at a repetition rate of 10 Hz and served as a trigger for the ESE spectrometer.

For the experiments to be described below a crystal with dimensions of $2 \times 2 \times 1.5$ mm was used. In order to reduce the second acceptor, this crystal was soaked for 30 min at room temperature in the storage solution to which sodium ascorbate (1 M) and 40% glycerol by volume were added, then transferred into the sample tube with a droplet of this solution and illuminated at 170 K by the laser. All ESE experiments were performed at 1.2 K.

3. Results and discussion

3.1. Field swept ESE

ESE-detected EPR spectra have been obtained for the RC triplet state in a single crystal of the RC of *Rb. sphaeroides* R26. The complete study of these spectra as a function of the direction of the magnetic field with respect to the crystal revealed four magnetically inequivalent sites. Single-sweep spectra are depicted in fig. 1 for orientations of the magnetic field along the principal directions x , y and z of the fine structure tensor of one of the sites. The signals corresponding to the other sites hardly show up in these spectra because they are broadened away. Fig. 1 also shows the ESE-detected EPR spectrum for a frozen

Fig. 1. The ESE-detected EPR spectra of the triplet of the RC of *Rb. sphaeroides* R26 in a single crystal with (a) $B \parallel y$, (b) $B \parallel x$ and (c) $B \parallel z$. In (d) the ESE-detected EPR spectrum of a frozen solution of RCs of *Rb. sphaeroides* R26 is depicted. The delay δ_2 between the laser flash and the $\pi/2$ pulse is 2 μ s. The interval τ between the $\pi/2$ and π pulse is (a) 540 ns and (b), (c), (d) 270 ns. The resonance frequency is 9180 MHz. The temperature is 1.2 K.

solution of RCs of *Rb. sphaeroides* R26. The signals in the EPR spectra derive from the RC triplet state, apart from the intensity present in the central $g=2$ part of the spectrum. The latter echo, also present in the absence of laser excitation, is a residue of a background signal that originates from the sodium ascorbate and the cavity itself. This signal has been suppressed almost completely by applying a $\pi/2$ microwave pulse just before the laser flash.

The polarization pattern of the triplet EPR signals shows the familiar AEEAAE intensity distribution (A, enhanced absorption; E, emission) that results

from the dominant population of the $m_s=0$ triplet sublevel as described by the radical pair mechanism [6]. The width of the EPR lines for the magnetic field along the principal directions is about 20 G, the signals for $B \parallel z$ being somewhat narrower than for the other two stationary directions. However, severe line broadening already occurs when the magnetic field is turned only 10° away from the principal directions.

From the values of the magnetic fields corresponding to the resonances in figs. 1a to 1c the following zero-field parameters have been calculated: $X/h=283 \pm 2$ MHz, $Y/h=90 \pm 2$ MHz and $Z/h=-375 \pm 2$ MHz ($D/h=561$ MHz ($D=0.0187$ cm $^{-1}$); $E/h=97$ MHz ($E=0.0032$ cm $^{-1}$)). These zero-field splittings are within the experimental error identical to those obtained from ODMR studies on RCs of *Rb. sphaeroides* R26 in a frozen solution [16].

As mentioned above spectra like those in fig. 1 were observed for four magnetically inequivalent sites. Labeling the principal z direction of the fine structure tensor of the triplet at site i ($i=1$ to 4) by z_i , the mutual orientation of the directions z_i showed that the angle between z_1 and z_2 equals that between z_3 and z_4 , the angle between z_1 and z_3 equals that between z_2 and z_4 , while the angle between z_1 and z_4 does not equal that between z_2 and z_3 . Similar relations have been found between the principal x directions, albeit that we were not able to determine two of these directions (x_2 and x_4) accurately. The symmetry relation between the four sites is only compatible with a monoclinic crystal structure. From the direction of z_1 and z_4 (or, equivalently, z_2 and z_3) the orientation of the unique axis of this monoclinic crystal follows because this axis must bisect the angle between z_1 and z_4 (and between z_2 and z_3). The monoclinic symmetry then shows up in fig. 2, where the observed principal x and z directions are drawn in a stereographic Wulff projection with the unique axis perpendicular to the plane of the paper.

In their study of the crystallization of RCs of *Rb. sphaeroides* R26 Allen and Feher observed a variety of crystal forms [2]. X-ray diffraction data for two of those forms showed that one crystal was orthorhombic (spacegroup C222) and one monoclinic (space group P2). The latter one contained two RCs per unit cell. On the other hand Gast and Norris performed a cw EPR study on a single crystal of the RC of *Rb. sphaeroides* R26 [10]. They concluded that

Fig. 2. Stereographic projection indicating the directions of the principal x and z axes of the fine structure tensor of the four sites. The closed and open circles refer to the upper and lower half of the sphere. The direction perpendicular to the plane of the paper corresponds to the unique axis of the monoclinic crystal.

their crystal was orthorhombic, which was confirmed by subsequent X-ray analysis (space group $P4_12_12_1$) [4]. Apparently the crystals studied by those two groups belong to another space group than the crystal for which we observed our EPR spectra. Although our crystal is monoclinic as one of the crystals described in ref. [2], the observation of four magnetically inequivalent sites points to at least four RCs per unit cell in our case.

3.2. Time-domain experiments

In utilizing the ESE technique we are working in the time domain, which allows the study of dynamical properties of the RC triplet state. The phase memory time as well as the spin-lattice relaxation time and the triplet sublevel lifetimes have been studied.

In fig. 3a the decay of the two-pulse Hahn echo for $B \parallel z$ has been depicted. The intensity of the Hahn echo reduces to almost zero for τ values larger than $2 \mu\text{s}$. Moreover, the decay is strongly modulated, which most probably results from the ^{14}N nuclear quadrupole interaction [12]. Similar observations

Fig. 3. Time-domain spectra of the high-field transition for $B \parallel z$. (a) Hahn echo decay. $\delta_1 = 200 \text{ ns}$; $\delta_2 = 2 \mu\text{s}$. (b) Stimulated echo decay. $\delta_1 = 200 \text{ ns}$; $\delta_2 = 2 \mu\text{s}$; $\tau = 300 \text{ ns}$. (c) Hahn echo intensity as a function of the delay δ_2 after the laser flash. $\tau = 270 \text{ ns}$.

were made for the decay along the x and y fine structure axes. This relaxation behaviour agrees with observations made for a frozen solution of RCs of *Rb. sphaeroides* R26 [17].

The spin-lattice relaxation time T_1 has been investigated by applying a π pulse and measuring the echo intensity after a variable time interval using a standard $\pi/2$ - π pulse sequence. Within the triplet lifetime there is no effect of spin-lattice relaxation on the echo intensity, i.e. T_1 must be long as compared to the triplet lifetime.

The decay of the stimulated echo has been measured for orientations of the magnetic field along the principal directions x , y and z . A typical example of such a decay is shown in fig. 3b for $B \parallel z$. Similar behaviour has been observed for randomly oriented samples [12]. Our observation of a long T_1 indicates that the fast decay of the stimulated echo does not

reflect spin-lattice relaxation but must be attributed to spin diffusion processes.

The lifetimes of the three triplet sublevels have been investigated by measuring the echo intensity as a function of the delay δ_2 between the laser flash and the two-pulse echo sequence for both the high- and the low-field transition. The decrease of the echo intensity for $B\parallel x$ and $B\parallel y$ can be described by a single exponential function with a characteristic time of $60 \pm 6 \mu\text{s}$. In contrast, the decay curve obtained with $B\parallel z$, shown in fig. 3c, could not be described by a single-exponential function. The superposition of two exponential functions ($t_1 = 65 \pm 6 \mu\text{s}$, $t_2 = 400 \pm 50 \mu\text{s}$) with about equal pre-exponential factors provides a sufficient description. Because a fast component in the echo decay for $B\parallel z$ had never been reported for random samples, we performed the same experiment along the $B\parallel z$ "turning point" for a frozen solution of RCs. Here too, a double-exponential decay was observed with lifetimes of 67 ± 7 and $600 \pm 70 \mu\text{s}$ and pre-exponential factors of 0.15 and 0.85 respectively.

Assuming only the $m_s=0$ triplet sublevel to be populated (S-T₀ mixing), the observed decay times for $B\parallel x$, y and z can be taken to represent the triplet sublevel lifetimes. The lifetimes as measured for $B\parallel x$ and $B\parallel y$ are close to those reported for whole cells [18]. This also holds for the slow component of the z level decay but the fast component has not been observed before. The decay of the fast component for $B\parallel z$ equals the decay time found for $B\parallel x$ and $B\parallel y$. The 15% fast component in the z level decay for randomly oriented triplets could then in principle be due to a slight admixture of the x and y triplet sublevels to the z "turning point". For our well-oriented single crystal, however, this contribution should be negligible and certainly not considerably larger than for a random solution of RCs. The single-crystal fast component for $B\parallel z$ also cannot be attributed to radical pair S-T _{± 1} mixing, since in that case the echo intensity (which is proportional to the population difference between the T₀ and T _{± 1} levels) should initially increase, contrary to our observations. An explanation based on time-dependent contributions of the background signal also can be ruled out, because the lifetimes derived from the decay curves of the low-field transitions (which contain contributions of the background signal) and the high-field

ones (which are free of background signal) are found to be equal to within 10%. We have to conclude that the origin of the fast-component present in the z level decay is as yet not understood. One might think that it derives from RCs with a slightly different configuration of the constituent molecules [19].

Summarizing, we have proved the feasibility of a study based on ESE spectroscopy in combination with pulsed laser excitation of the triplet state of the RC of *Rb. sphaeroides* R26 in a single crystal. The ESE-detected EPR spectra showed the present crystal to be monoclinic with four magnetically inequivalent sites. The relaxation behaviour of the triplet in the single crystal does not differ significantly from that previously observed for randomly oriented systems. However, the double-exponential decay of the triplet z sublevel population found in both the single crystal and the random system is a new phenomenon. Now that this ESE study has been successful, a pulsed ENDOR study has come within reach and is presently being performed.

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