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Transient and Pulsed EPR Spectroscopy on the Radical Pair State $P_{865}^{+\cdot}Q_A^{-\cdot}$ to Study Light-Induced Changes in Bacterial Reaction Centers

S. G. Zech, R. Bittl, A. T. Gardiner, and W. Lubitz

Max-Volmer-Institut für Biophysikalische Chemie und Biochemie,
Technische Universität Berlin, Berlin, Germany

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Dedicated to Harry Kurreck on the occasion of his 65th birthday

Abstract. The radical pair state $P_{865}^{+\cdot}Q_A^{-\cdot}$ (P_{865}: primary donor, Q_A: quinone acceptor) in Zn-substituted bacterial reaction centers is investigated using transient and pulsed EPR spectroscopy. For photoexcited samples not frozen in the dark but under continuous illumination, a prolonged lifetime of this radical pair state is observed in agreement with previous studies using time resolved optical spectroscopy. The transient EPR spectra revealed neither a different orientation of the quinone acceptor anion nor a change of its *g*-anisotropy in the sample frozen in the charge separated state as compared with that frozen in the dark. The latter finding indicates a similar hydrogen bonding situation for $Q_A^{-\cdot}$ in both samples. Changes observed in the transient EPR spectra are interpreted as result of contributions from spin-polarized $Q_A^{-\cdot}$ which was generated in part of the sample while freezing under illumination. From the out-of-phase echo modulation pattern observed in the pulsed EPR measurements, it follows that the distance between $P_{865}^{+\cdot}$ and $Q_A^{-\cdot}$ is the same in dark frozen samples and in those frozen under continuous illuminaton. This is in contrast to the model suggested by Kleinfeld D., Okamura M.Y., Feher G.: Biochemistry **23**, 5780 (1984), in which an increased distance and a larger distribution of distances was suggested for samples frozen under illumination. The prolonged lifetime of the radical pair $P_{865}^{+\cdot}Q_A^{-\cdot}$ is discussed in terms of differences in the relaxation behavior of the protein.

1. Introduction

In reaction centers of photosynthetic bacteria, the first step of energy conversion is a charge separation across a membrane. The details of this electron transfer (ET) are governed by the type of cofactors, their interaction with each other and with the local protein environment. After light excitation of the primary electron donor (P_{865}), a bacteriochlorophyll dimer, an electron is transferred via a series of acceptors – a bacteriochlorophyll and a bacteriopheophytin molecule (I) – to a ubiquinone-10 (Q_A). Due to its long lifetime of approximately 25 ms at low

temperatures, the radical pair (RP) state $P_{865}^{+\bullet}Q_A^{-\bullet}$ can be investigated using time resolved EPR techniques.

Light-induced structural changes occurring in the ET process in bacterial reaction centers (RCs) were first discussed by McElroy *et al.* [1]. They found that the oxidized primary donor $P_{865}^{+\bullet}$ can be trapped if the RCs were cooled to cryogenic temperatures under illumination. First direct evidence for light-induced functional changes have been reported by Kleinfeld *et al.* [2]. Using Q_B depleted RCs, their experiment showed an approximately five times prolonged lifetime of the secondary RP state $P_{865}^{+\bullet}Q_A^{-\bullet}$ in *Rb. sphaeroides* R-26 when the sample was rapidly frozen to cryogenic temperatures under continuous illumination compared with samples frozen in the dark followed by light excitation. A shift of the quinone by more than 1 Å resulting in a larger distance between $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$ has been suggested to explain this change in lifetime in samples frozen in the charge separated state $P_{865}^{+\bullet}Q_A^{-\bullet}$. Based on the empirical relationship between distance and ET rate [3], an enlarged distance should result into a prolonged lifetime of the RP. To explain the deviation from a monoexponential decay function, a wider distribution of cofactor distances was suggested (for details see [2]).

Recently, changes in the position of Q_B accompanied by a 180° twist of the quinone head group around the phytyl chain have been detected by X-ray crystallography after freezing the crystals under illumination [4]. The movement of the quinone as a necessary prerequisite for ET from $Q_A^{-\bullet}$ to Q_B was suggested. This might explain the ET from $Q_A^{-\bullet}$ to Q_B which, at low temperatures, only occurs in samples frozen under illumination [2]. On the other hand, no significant change was detectable in the Q_A region. However, this is not surprising since in the experiments of Stowell *et al.* [4] the crystals were frozen in the state $P_{865}^{+\bullet}Q_B^{-\bullet}$ rather than in the state $P_{865}^{+\bullet}Q_A^{-\bullet}$ as done by Kleinfeld *et al.* [2].

Transient EPR experiments on the spin-polarized RP state $P_{865}^{+\bullet}Q_A^{-\bullet}$ have been performed previously to compare light and dark frozen samples [5, 6]. The transient EPR spectra are extremely sensitive to the orientation of $Q_A^{-\bullet}$ with respect to the dipolar axis Z_D, i.e., the axis connecting the two spins on $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$, respectively. The observed changes in the transient spectra published so far [5, 6] did not show a significant reorientation of Q_A for samples frozen under illumination. Furthermore, the small spectral changes found in [6] were not correlated with the change in function, i.e., the prolonged lifetime of $P_{865}^{+\bullet}Q_A^{-\bullet}$. Warming the sample frozen under illumination to about 150 K for one hour shifted the recombination kinetics towards those observed for dark frozen samples. This change was *not* accompanied by a change of the transient spectrum of $P_{865}^{+\bullet}Q_A^{-\bullet}$ of the sample frozen under illumination. Therefore it was concluded that the change of the recombination time has a different origin than the change of the transient spectrum [6].

However, for cofactor distances of more than ≈20 Å, the polarization pattern of a transient EPR spectrum of a spin-correlated RP is not sensitive to a change in the distance of about 1 Å [7]. In contrast, pulsed experiments on the RP state

$P_{865}^{+\bullet}Q_A^{-\bullet}$ allow a precise measurement of the distance between the electron spins within the RP.

In an electron spin echo (ESE) experiment, the spin-polarized RP gives rise to a phase shifted echo (out-of-phase echo) [8, 9]. This echo shows a deep envelope modulation (ESEEM) which is mainly determined by the spin-spin coupling, i.e., the dipolar coupling D and the isotropic coupling J, between the electron spins in the RP. Since the dipolar coupling is related to the distance r between the spins by a simple r^{-3} dependence, changes in the cofactor distances as suggested by Kleinfeld *et al.* [2] should be directly observable in a pulsed EPR experiment.

From the Fourier transforms of the echo modulation it is possible to deduce the dipolar and isotropic coupling separately [10–12]. This has been used previously for the determination of the distance between $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$ in RCs in which the high spin non-heme Fe^{2+} was replaced by diamagnetic Zn^{2+}. A distance of (28.4 ± 0.3) Å between the two electrons has been found for two different types of preparations [11, 12], i.e., for RCs in which the Zn^{2+} substitution was achieved either by chemical exchange [13] or by biosynthetical replacement using the mutant *Rb. sphaeroides* HC(M266) [14]. In this mutant the histidine (H) amino acid residue at position 266 in the M subunit ligating the non-heme Fe^{2+} is replaced by cysteine (C). As a consequence, RCs of *Rb. sphaeroides* HC(M266) incorporate Zn^{2+} instead of Fe^{2+} if grown on a Zn^{2+} enriched medium. The distance of (28.4 ± 0.3) Å between the RP spins derived by pulsed EPR experiments is in excellent agreement with the distance of 28.3 Å between the two cofactors derived from X-ray data [15].

In this study we extend our previous investigations of the light induced spectral changes investigated by transient EPR spectroscopy [6] to the measurement of protonated samples at X-band and deuterated samples at Q-band. Furthermore, pulsed EPR experiments on the RP state are presented that allow a precise comparison of the distance between the radical partners in samples frozen under different conditions.

2. Materials and Methods

2.1. Sample Preparation

For this study we used RCs in which the non-heme Fe^{2+} has been replaced by diamagnetic Zn^{2+} to enable the observation of the decoupled $Q_A^{-\bullet}$ by EPR spectroscopy. Zn substitution was achieved for the fully deuterated sample of *Rb. sphaeroides* R-26 as described previously [16]. Here the same preparation as in several other studies [6, 16–18] has been used. To inhibit ET transfer past $Q_A^{-\bullet}$ at room temperature, an approximately 200 fold molar excess of *o*-phenanthroline was added to the sample.

The protonated Q_B-depleted Zn-RCs of *Rb. sphaeroides* mutant HC(M266) were the same as those used previously for pulsed EPR measurements [12]. Cells were

grown semi-anaerobically in YCC+ medium supplemented with the appropriate antibiotics. Cells were harvested after five days of growth. The isolation and preparation of Zn-RCs were done as described previously [19], except that after the initial LDAO (N,N-dimethyldodecylamine-N-oxide) solubilisation 0.1 mM $ZnCl_2$ was added to all the subsequent buffers to prevent dissociation of the zinc ion from the non-heme iron site.

The samples were filled into a quartz capillary with 2 mm inner diameter and were either rapidly frozen in the dark by plunging them into liquid nitrogen or frozen under illumination. The continuous illumination was performed by a 100 W tungsten halogen lamp. Light with wavelengths below 830 nm and above 900 nm has been cut off by filters. The samples were illuminated for a few seconds at room temperature. A transparent dewar filled with liquid nitrogen was then raised over the sample while continuing the illumination. The samples reached 77 K within about 5 s. In Q_B-depleted or *o*-phenanthroline treated RCs freezing under illumination leads to the RP state $P_{865}^{+\bullet}Q_A^{-\bullet}$ [2] in contrast to Q_B containing samples where freezing results in the $P_{865}^{+\bullet}Q_B^{-\bullet}$ state [2, 4].

2.2. EPR Experiments

The experimental setup for the transient EPR measurements [6] and for the pulsed EPR experiments [12] has been described previously. For Q-band experiments we used the Bruker ER 051 QG microwave bridge and a home built Q-band resonator using the coupling concept of the Bruker 5106 QT resonator. The overall time resolution in the transient EPR experiments was about 50 ns at X-band and 100 ns at Q-band. Transient EPR spectra were recorded in direct detection mode with positive signals in enhanced absorption (A) or negative signals in emission (E). The measurements reported here were all performed at 80 K.

For the out-of-phase echo experiments, a two mw-pulse sequence was applied at time t after creating the RP by the laser flash. The delay time t between laser flash and the first mw pulse was set to $t \approx 800$ ns to avoid influences of the zero quantum coherence on the echo shape [20]. The echo was detected at time $T \approx \tau$ as a function of the pulse spacing τ. The mw-pulse length was 8 ns for the first and 16 ns for the second pulse using a mw power of about 1 kW giving the maximal echo intensity. The magnetic field B_0 was also adjusted to the maximum of the out-of-phase echo intensity which corresponds to the center of the transient EPR spectrum measured prior the pulsed EPR experiments.

It has been shown in theoretical studies [21, 22] that a $\pi/2$–π mw pulse sequence is not necessarily the best choice for ESE experiments on spin-correlated RPs. For a $\pi/2$–π pulse sequence as usually used in ESE experiments, the echo intensity is small at time $T \approx \tau$ after the second pulse. The maximum echo intensity is expected using a $\pi/4$–π sequence yielding a higher sensitivity in the experiment [21, 22]. This unusual pulse angle dependence has been recognized

earlier in FT-NMR spectroscopy of non-equilibrium systems with termed *dipolar order* (see, e.g., [23]).

Since analytical expressions for the out-of-phase echo amplitude as a function of the pulse spacing τ have only been derived for a $\pi/2$–π pulse sequence, we used a $\zeta/2$–ζ pulse sequence. For this sequence, we have shown in a previous study that, over a wide range, the observed modulation patterns are independent of the flip angle [12]. Therefore, the flip angle ζ has been adjusted to the maximum of the echo intensity. This angle ζ_{max}, however, is not expected to be $\zeta_{max} = \pi$ [21, 22].

The pulse angle is determined by the strength of the B_1 field, i.e., the mw power, and by the pulse length t_p and is given by $\zeta = \omega_1 t_p \approx 1/2(g_1 + g_2)\beta B_1 t_p$, where g_1 and g_2 are the g-factors of $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$, respectively and β is the Bohr magneton. Using a pulse sequence with equal duration t_p for each mw pulse, the flip angle ζ can be determined experimentally by increasing the B_1 field until the echo intensity vanishes at time $T \approx \tau$ after the second pulse. This B_1 field corresponds to a π–π pulse sequence for which no echo is expected. Then the pulse length for the first pulse is reduced by half to give a $\pi/2$–π sequence and the B_1 field is decreased gradually until the maximum echo intensity is observed. Thereby, $\zeta_{max} = 130° \pm 10°$ was obtained to yield the maximum out-of-phase echo intensity in a $\zeta/2$–ζ pulse sequence in agreement with numerical simulations previously performed [20]. However, the modulation frequency has been found experimentally to be independent of the pulse sequence which is applied, i.e., a $\pi/4$–π sequence gives the same modulation pattern as the 65°–130° sequence used in this study (data not shown).

3. Results and Discussion

3.1. Recombination of the Radical Pair

The change of the lifetime of the RP state $P_{865}^{+\bullet}Q_A^{-\bullet}$ reported earlier [2] has been reproduced in the present study using time resolved EPR spectroscopy with field modulation and lock-in detection. Figure 1 shows the recombination kinetics of $P_{865}^{+\bullet}Q_A^{-\bullet}$ for the deuterated sample measured at X-band. This change of the recombination kinetics were virtually identical for both samples used in this study, i.e., for protonated Zn-RCs of *Rb. sphaeroides* HC(M266) and deuterated Zn-RCs of *Rb. sphaeroides* R-26. The recombination rates have been measured before and after each experiment discussed below to assure that the sample frozen under illumination showed the corresponding slow recombination rate.

The inset in Fig. 1 shows the spectrum of the RP obtained from the recombination kinetics at various field points. The signal has been integrated within a time gate of 5–30 ms after the laser pulse. At this time, the spin polarization has decayed due to spin-lattice relaxation and the influence of the mw irradiation. Thus, the spectrum accumulated at late times can be described as the superposition of

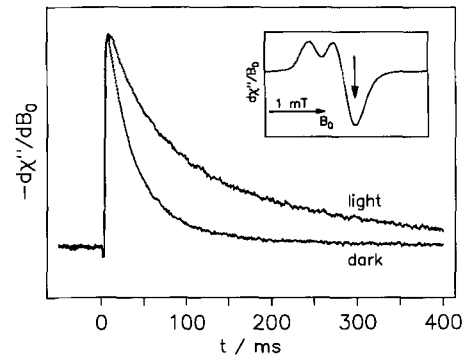

Fig. 1. Recombination kinetics of $P_{865}^{+\bullet}Q_A^{-\bullet}$ in deuterated Zn-RCs of *Rb. sphaeroides* R-26 for samples frozen in the dark and samples frozen under illumination (as indicated). The kinetic traces have been recorded at X-band by field modulation EPR using lock-in detection. Both traces have been scaled to identical maximum amplitude. The inset shows the time-resolved EPR spectrum of $P_{865}^{+\bullet}Q_A^{-\bullet}$ in thermal equilibrium for the dark frozen sample. The arrow indicates the field position (B_0 = 349.9 mT) at which the kinetics have been accumulated. Experimental conditions: modulation frequency is 100 kHz, modulation amplitude is 0.1 mT, time constant is 1 ms, microwave power is 224 μW, microwave frequency is 9.805 GHz, T = 80 K. 4096 events have been averaged for each kinetic trace at 1.3 Hz laser repetition rate.

the $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$ signals in thermal equilibrium, neglecting the small dipolar spin-spin coupling. The arrow indicates the field position at which the recombination kinetics has been measured. At this position, both radicals contribute to the observed kinetics. However, within the signal to noise (S/N) ratio the kinetics were independent of the field position, indicating that the rate of reoxidation of $Q_A^{-\bullet}$ equals the reduction of $P_{865}^{+\bullet}$.

Consistent with the rates measured by optical spectroscopy [2], the decay of the signal is nearly mono-exponential with a time constant of $\tau^{\text{dark}} \approx 25$ ms (97%) for the sample frozen in the dark. In contrast, the sample frozen in the charge separated state $P_{865}^{+\bullet}Q_A^{-\bullet}$, i.e., under continuous illumination, shows a prolonged lifetime with a non-exponential decay behavior and time constants of $\tau_1^{\text{light}} = 46$ ms (49%) and $\tau_2^{\text{light}} = 204$ ms in a bi-exponential least squares fit. The time $\tau_{1/e}$ for a signal decay to reach exp(−1) of its initial amplitude, is $\tau_{1/e}^{\text{dark}} \approx 27$ ms for the dark frozen sample and $\tau_{1/e}^{\text{light}} \approx 101$ ms for the sample frozen under illumination, respectively, in good agreement with the results obtained from optical spectroscopy that gave $\tau_{1/e}^{\text{dark}} \approx 25$ ms and $\tau_{1/e}^{\text{light}} \approx 120$ ms [2].

3.2. Transient EPR Spectra

Transient EPR spectra have been extracted from the 2D (time/field) data set using a digital boxcar integration [17] and are shown in Fig. 2 for samples frozen in the dark (solid lines) and those frozen under illumination (dashed lines). Due to

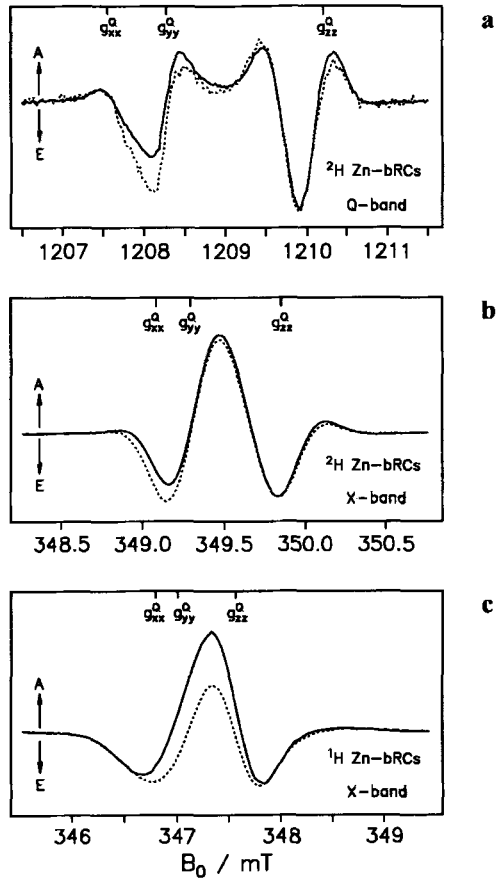

Fig. 2. Transient EPR spectra of the radical pair $P_{865}^{+\bullet}Q_A^{-\bullet}$ in deuterated Zn-RCs of *Rb. sphaeroides* R-26 measured at Q-band (**a**) or at X-band (**b**), and for protonated Zn-RCs from *Rb. sphaeroides* mutant HC(M266) measured at X-band (**c**). The solid lines indicate the spectra for samples frozen in the dark before light excitation, dotted lines show the spectra for samples frozen under illumination. All spectra have been extracted from the 2D-data sets using a digital boxcar integration between 0.5 and 6.0 μs after the laser flash. In the spectra of the samples frozen in the light, a triplet background signal has been removed by a linear baseline correction. The principal values of the $Q_A^{-\bullet}$ *g*-tensor (g_{xx} = 2.0066, g_{yy} = 2.0054, g_{zz} = 2.0022) [18] are indicated. Experimental conditions: microwave power is 224 μW (X-band) and 270 μW (Q-band), microwave frequencies are 33.914 GHz (**a**), 9.804 GHz (**b**) and 9.740 GHz (**c**). T = 80 K. Laser repetition rate is 10 Hz for the dark, approximately 2 Hz for the light frozen samples, respectively.

the reduced hyperfine couplings in the deuterated sample, the same polarization pattern (A/E/A/A/E/A) is observed here at Q-band (Fig. 2a) as previously reported for protonated samples measured at W-band [18]. In the Q-band spectrum the g_{xx} and g_{yy} components of the $Q_A^{-\bullet}$ *g*-tensor are resolved while only g_{zz} still overlaps with $g(P_{865}^{+\bullet})$. Therefore it is possible to obtain the principal values of the quinone *g*-tensor from the transient EPR spectrum [24].

The anisotropy of the *g*-tensor of the quinone is particularly sensitive to the presence and orientation of hydrogen bonds to the carbonyl oxygens [24–26]. Both carbonyl oxygens of $Q_A^{-\bullet}$ are hydrogen bonded asymmetrically to the protein to either His(M219) or Ala(M260) (backbone NH) [27, 28]. Therefore, a shift of the quinone as a cause of freezing the sample under illumination, as suggested by Kleinfeld *et al.* [2], should change the hydrogen bonding situation of $Q_A^{-\bullet}$ and hence its *g*-anisotropy. Comparison of the samples frozen in the dark and those frozen in the light (Fig. 2), shows that all spectra have identical spectral width, independent of the freezing procedure applied. Thus, from the similar *g*-tensor of $Q_A^{-\bullet}$ present in samples frozen under different conditions, we can conclude directly that no change in the hydrogen bonding of $Q_A^{-\bullet}$ occurs while freezing under illumination. This obviously restricts possible changes in the position of the quinone.

Transient EPR spectra are also particularly sensitive to the orientation of the quinone with respect to the dipolar axis Z_D [7, 26, 29]. Since a shift of the quinone without an accompanying reorientation seems to be unlikely, a change in the quinone position should also affect the polarization pattern of the transient EPR spectra. Inspection of the Q-band spectra (Fig. 2a) shows that both spectra start with enhanced absorption. This absorption in the low field part is only observed if the dipolar axis Z_D and the g_{xx} axis of the quinone (running through the two carbonyl oxygens) includes an angle of more than 54.7° (magic angle) [7, 29]. The X-ray data give an angle of about 69° [15] consistent with the transient EPR spectra [18]. Since for both spectra a similar polarization pattern is observed at the low field edge, we can further conclude that no major reorientation of $Q_A^{-\bullet}$ occurs while freezing under illumination. These observations for $Q_A^{-\bullet}$ are in contrast to the large change in position and orientation observed by X-ray crystallography for $Q_B^{-\bullet}$ [4].

However, inspection of Fig. 2 reveals differences between the spectra for samples frozen under illumination compared with frozen in the dark. All spectra of the light frozen samples show an enhanced emission in the low field part compared with dark frozen samples. This is most prominent for the protonated sample measured at X-band (Fig. 2c) in agreement with the behavior found previously [5]. In this reference the assignment of the spectra frozen in the dark and frozen under illumination has to be interchanged (A. J. Hoff, private communication).

In contrast to the dark frozen sample, the integral over the spectra for the samples frozen under illumination does not vanish. This is not consistent with the concept of correlated coupled RPs (CCRP) (see, e.g., [7, 29]). This model predicts a vanishing integral over the whole spectrum. Since the primary donor is excited to a singlet state and this spin state is conserved during the fast ET from P_{865}^{*} to Q_A, no net spin polarization for $P_{865}^{+\bullet}Q_A^{-\bullet}$ is expected. This is indeed the case for the dark frozen samples but not for the samples frozen under illumination (see Fig. 2). Therefore, the spectral changes cannot be interpreted within the CCRP model.

Emissively polarized signals of $Q_A^{-\bullet}$ have been observed previously in chemically prereduced Zn-RCs investigated at low temperatures under illumination

[30–32]. This spin polarization of $Q_A^{-\bullet}$ arises from a transfer of polarization from the RP $P_{865}^{+\bullet}I^{-\bullet}$ to $Q_A^{-\bullet}$ via the exchange coupling J_{IQ} [30, 33]. At Q-band, an anisotropic spin-polarization of $Q_A^{-\bullet}$ has been observed [31, 32]. For Zn-RCs of *Rb. sphaeroides* R-26 the emissive polarization is most easily achieved in the low field region of the spectrum [31], consistent with our results discussed above (see Fig. 2). Considering this, we attribute the change in the spectra to a transfer of spin polarization from the intermediary RP state $P_{865}^{+\bullet}I^{-\bullet}$ to pre-reduced $Q_A^{-\bullet}$, which has been generated in part of the sample during the freezing under illumination. This is corroborated by different observations: (i) The cw-EPR spectra show a large signal for the light frozen sample and almost no signal in the dark frozen sample (data not shown). (ii) The signal to noise ratio for the kinetics measured in the sample frozen under illumination (see Fig. 1) are worse than for the dark frozen sample [6]. (iii) A larger triplet background signal of $^3P_{865}$ and (iv) a reduced intensity for the signal of $P_{865}^{+\bullet}Q_A^{-\bullet}$ are observed for the light frozen sample, indicating a blocked forward ET due to the presence of stable $Q_A^{-\bullet}$ in part of the sample.

According to the reduced signal intensity of $P_{865}^{+\bullet}Q_A^{-\bullet}$ for the light frozen sample, these spectra have been scaled to obtain largest similarity between dark and light frozen samples in the high-field part. This results into a pure emissively polarized signal of $Q_A^{-\bullet}$ in the light minus dark spectrum for protonated samples at X-band as previously reported [30]. The contribution of emissively polarized $Q_A^{-\bullet}$ to the observed spectrum of the deuterated RCs (see Fig. 2b) is slightly larger than that observed in our previous study [6], probably caused by a larger amount of accumulated $Q_A^{-\bullet}$ due to longer illumination of the sample at room temperature. At a temperature of 77 K the accumulated $Q_A^{-\bullet}$ is stable over a time period of several days, i.e., storing the sample for 4 days in liquid nitrogen and re-measuring the transient EPR spectra at 80 K results into spectra indistinguishable from those shown in Fig. 2 (dotted lines).

The superposition of the spectra of $P_{865}^{+\bullet}Q_A^{-\bullet}$ and polarized $Q_A^{-\bullet}$ reflects a heterogeneity of the sample. A quantitative discussion of the changes in the transient spectra is, therefore, difficult. For a simulation of these superimposed spin-polarized spectra of $P_{865}^{+\bullet}Q_A^{-\bullet}$ and $Q_A^{-\bullet}$, knowledge of the interaction between $P_{865}^{+\bullet}$ and $I^{-\bullet}$, $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$, $I^{-\bullet}$ and $Q_A^{-\bullet}$ [33] as well as between $^3P_{865}$ and $Q_A^{-\bullet}$ [34] is required. Because of the very large number of parameters involved, a quantitative analysis including spectral simulations is not performed here.

Furthermore, simulations of the spectra may be complicated due to a change in the ET rate from $I^{-\bullet}$ to Q_A which may accompany the smaller recombination rates. Spectral simulations accounting for different lifetimes of the intermediary RP state $P_{865}^{+\bullet}I^{-\bullet}$ [35–38] showed that the lifetime of $P_{865}^{+\bullet}I^{-\bullet}$ affects mainly the *relative* intensities of emissive and absorptive contributions to the spectrum but left the polarization pattern virtually unchanged within a wide range of transfer times. Thus, a qualitative discussion of the polarization pattern as performed above is indeed feasible without exact knowledge of the $P_{865}^{+\bullet}I^{-\bullet}$ lifetime.

3.3. Measurement of the Distance Between $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$

Additional information on the radical pair state can be obtained from the distance between $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$. This cofactor distance provides a direct test of the above mentioned hypothesis of Kleinfeld *et al.* [2]. These authors proposed a change in the distance within the RP of more than 1 Å accompanied by a wider distribution of cofactor distances.

The cofactor distance between $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$ can be measured precisely by a pulsed EPR experiment performed on the RP state $P_{865}^{+\bullet}Q_A^{-\bullet}$ (see above). If the cofactor distance is affected by the illumination while freezing, the modulation of the out-of-phase echo should change. An increase of the cofactor distance would result into a smaller modulation frequency, i.e., a slower echo modulation as a result of the smaller dipolar coupling. Consequently, a decrease in distance leads to a

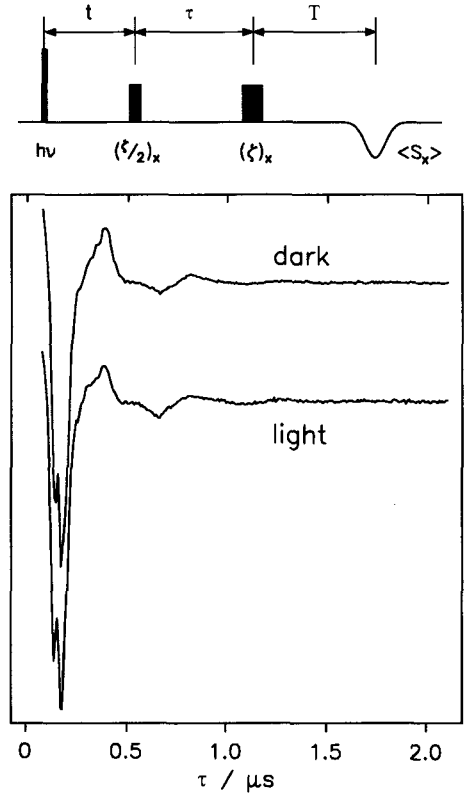

Fig. 3. Out-of-phase echo modulation pattern for the protonated Zn-RCs *Rb. sphaeroides* mutant HC(M266) frozen in the dark (upper trace) or under illumination (lower trace) measured at X-band and a temperature of 80 K. The first 88 ns of the echo modulation are obscured by the spectrometer deadtime. Laser repetition rate is 10 Hz for the dark frozen sample, 1.5 Hz for the sample frozen under illumination; field position B_0 = 347.8 mT, microwave frequency is 9.756 GHz. Other experimental conditions as given in Section 2. Top: pulse scheme used for this experiment.

faster echo modulation. If the distribution of cofactor distances is changed by the freezing procedure, the damping of the out-of-phase echo modulation should be affected because a distribution of cofactor distances leads to a stronger superposition of different modulation frequencies and thus to a faster decay of the echo modulation [39].

In Fig. 3 (upper trace) the out-of-phase modulation measured at 80 K for the sample frozen in the dark is shown. This echo modulation shows a modulation frequency identical to that previously obtained for the same sample at a higher temperature (150 K). In agreement with the studies of Dzuba *et al.* [10, 39], the damping of the modulation is slightly larger at 80 K than at 150 K. The lower trace in Fig. 3 shows the echo modulation of the light frozen sample. Within experimental accuracy, no change in the modulation pattern can be seen. The damping of the echo modulation is also found to be virtually unaffected by freezing the sample under illumination. Therefore one can conclude that a change in the cofactor distances cannot be larger than the experimental accuracy of our experiment (0.3 Å). The distance of (28.4 ± 0.3) Å [11, 12] between $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$ is hence the same in samples frozen under illumination and samples frozen in the dark. In addition, a larger distribution of cofactor distances leading to a faster damping of the echo modulation for samples frozen in the charge separated state is not observed in our experiments.

4. Conclusions

Our experiments show that the light induced changes observed for the lifetime of the RP state $P_{865}^{+\bullet}Q_A^{-\bullet}$ are accompanied by changes in the transient EPR spectra of this state (see Fig. 2). These changes, however, cannot be explained in terms of a reorientation of the quinone. Contributions from emissively polarized $Q_A^{-\bullet}$ are observed which are the result of the inhibition of part of the sample due to the accumulation of stable $Q_A^{-\bullet}$ while freezing the sample under illumination. Changes in the orientation of Q_A, if present, are too small to be detectable by transient EPR spectroscopy. From the similar *g*-anisotropy for $Q_A^{-\bullet}$ present in samples frozen under illumination compared with those frozen in the dark, a similar hydrogen bonding situation for $Q_A^{-\bullet}$ is concluded for both cases.

Pulsed EPR measurements on the RP state $P_{865}^{+\bullet}Q_A^{-\bullet}$ unambiguously show that the distance between $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$ is the same in the sample frozen in the charge separated state and frozen in the dark. Therefore, the change in the recombination rates cannot be explained by a shift of the quinone position resulting in a larger distance between $P_{865}^{+\bullet}$ and $Q_A^{-\bullet}$ as suggested by Kleinfeld *et al.* [2].

The prolonged lifetime of the RP state $P_{865}^{+\bullet}Q_A^{-\bullet}$ for samples frozen under illumination, can be explained by a model recently presented by Brettel [40]. In this model it is assumed that the protein relaxation contributes a substantial part to the overall reorganization energy of the ET process. A suppression of protein relaxation modes by freezing should then result in a change of the driving force

for the ET. In the case of the recombination of the RP state $P_{865}^{+\bullet}Q_A^{-\bullet}$, freezing under illumination could result in a smaller driving force compared to dark frozen samples. Therefore, a slower recombination in the sample frozen under illumination would be expected. This could also explain the observed similarity of the RP lifetime at room temperature with that of samples frozen under illumination [40].

The assignment of changes in the RP lifetime for $P_{865}^{+\bullet}Q_A^{-\bullet}$ to differences in the relaxation behavior of the protein-pigment complex rather than to a change in the cofactor distances or their relative orientation is consistent with our measurements.

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Author's address: Dr. Robert Bittl, Max-Volmer-Institut für Biophysikalische Chemie und Biochemie, Technische Universität Berlin, Straße des 17. Juni 135, D-10623 Berlin, Germany