Electron Paramagnetic Resonance Studies of Zinc-Substituted Reaction Centers from *Rhodopseudomonas viridis* 1


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**Abstract:** The primary quinone acceptor radical anion QA•− (a menaquinone-9) is studied in reaction centers (RCs) of *Rhodopseudomonas viridis* in which the high-spin non-heme Fe2+ is replaced by diamagnetic Zn2+. The procedure for the iron substitution, which follows the work of Debus et al. (Debus, R. J., Feher, G., and Okamura, M. Y. (1986) Biochemistry 25, 2276–2287), is described. In *Rps. viridis* an exchange rate of the iron of ~50% ± 10% is achieved. Time-resolved optical spectroscopy shows that the ZnRCs are fully competent in charge separation and that the charge recombination times are similar to those of native RCs. The g tensor of QA−• in the ZnRCs is determined by a simulation of the EPR at 34 GHz yielding g1 = 2.00597 (5), g2 = 2.00492 (5), and g3 = 2.00216 (5). Comparison with a menaquinone anion radical (MQ4−•) dissolved in 2-propanol identifies QA−• as a naphthoquinone and shows that only one tensor component (g3) is predominantly changed in the RC. This is attributed to interaction with the protein environment. Electron−nuclear double resonance (ENDOR) experiments at 9 GHz reveal a shift of the spin density distribution of QA−• in the RC as compared with MQ4−• in alcoholic solution. This is ascribed to an asymmetry of the QA binding site. Furthermore, a hyperfine coupling constant from an exchangeable proton is deduced and assigned to a proton in a hydrogen bond between the quinone oxygen and surrounding amino acid residues. By electron spin−echo envelope modulation (ESEEM) techniques performed on QA−• in the ZnRCs, two 14N nuclear quadrupole tensors are determined that arise from the surrounding amino acids. One nitrogen coupling is assigned to a NO−(1)−H of a histidine and the other to a polypeptide backbone N−H by comparison with the nuclear quadrupole couplings of respective model systems. Inspection of the X-ray structure of *Rps. viridis* RCs shows that His(M217) and Ala(M258) are likely candidates for the respective amino acids. The quinone should therefore be bound by two H bonds to the protein that could, however, be of different strength. An asymmetric H-bond situation has also been found for QA−• in the RC of *Rhodobacter sphaeroides*. Time-resolved electron paramagnetic resonance (EPR) experiments are performed on the radical pair state P960−•QA−• in ZnRCs of *Rps. viridis* that were treated with o-phenanthroline to block electron transfer to QB. The orientations of the two radicals in the radical pair obtained from transient EPR and their distance deduced from pulsed EPR (out-of-phase ESEEM) are very similar to the geometry observed for the ground state P960QA in the X-ray structure [Lancaster, R., Michel, H. (1997) Structure 5, 1339].

In bacterial reaction centers (RCs) 1 the light-induced charge separation is achieved with the donation of an electron from the lowest excited singlet state of the primary donor P•+; a bacteriochlorophyll (BChl) dimer, to an electron transport (ET) chain comprising monomeric BChl, bacteriopheophytin (BPh), and two quinones (1, 2). The quinones act in sequence in the ET process in the RC. They have different redox potentials. The primary quinone, QA, accepts only one electron and is not protonated, whereas QB exhibits the typical two-electron two-proton chemistry (3). The quite different behavior of QA and QB is traced back to the impact of the protein surrounding. The interactions with specific amino acids, e.g., via hydrogen bonds, are thought to fine-tune the structural and electronic pro-

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1 Abbreviations: AAS, atomic absorption spectroscopy; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; CCRP, correlated coupled radical pair; ENDOR, electron−nuclear double resonance; EPR, electron paramagnetic resonance; ESE, electron spin echo; ESEEM, electron spin−echo envelope modulation; ET, electron transfer; FeCy, ferricyanide; hfc, hyperfine coupling constant; LDAO, lauryldimethylamine N-oxide; MQn, menaquinone-n; mw, microwave; NQR, nuclear quadrupole resonance; QA, primary quinone electron acceptor; QB, secondary quinone electron acceptor; RC, reaction center; RF, radical pair; RT, room temperature; UQn, ubiquinone-n; ZnRC, zinc-substituted reaction center.

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6 Abbreviations: AAS, atomic absorption spectroscopy; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; CCRP, correlated coupled radical pair; ENDOR, electron−nuclear double resonance; EPR, electron paramagnetic resonance; ESE, electron spin echo; ESEEM, electron spin−echo envelope modulation; ET, electron transfer; FeCy, ferricyanide; hfc, hyperfine coupling constant; LDAO, lauryldimethylamine N-oxide; MQn, menaquinone-n; mw, microwave; NQR, nuclear quadrupole resonance; QA, primary quinone electron acceptor; QB, secondary quinone electron acceptor; RC, reaction center; RF, radical pair; RT, room temperature; UQn, ubiquinone-n; ZnRC, zinc-substituted reaction center.
properties of QA and QB for optimum function in the RC (4).

Information on the geometrical structure of the two quinones and their binding sites can be derived from X-ray crystallography performed on RC single crystals (5, 6). The method of choice to study details of the electronic structure is EPR spectroscopy performed on the radical anions QA− and QB− formed in the ET process (4). In native, iron-containing RCs the two quinone radicals are, however, strongly magnetically coupled to the non-heme, high-spin Fe2+ and only broad spectra can be obtained at cryogenic temperatures (7–10). Information about the electronic structure of the semiquinone states by EPR techniques as described above for Rh. sphaeroides. This is important not only for a more thorough spectroscopic characterization of the RC of R. viridis but also for better understanding Nature’s basic concept to form the specific quinone binding sites, i.e., binding sites for cofactors with unusual properties in a membrane protein. Recently, we have reported on the biosynthetic enrichment of Zn in the RC of R. viridis by growing bacteria on low-iron/high-zinc medium (23). This method gave only fairly low yields of Zn incorporation (∼5%). In this paper we report on the replacement of the Fe2+ by Zn2+ in the RC of R. viridis by the chemical method introduced by Debus et al. (11). The obtained ZnRCs are characterized and the primary quinone radical anion QA− and the radical pair P960+ QA− are investigated by using EPR, ENDOR, ESEEM, and transient/pulsed EPR techniques, respectively.

MATERIALS AND METHODS

Preparation of FeRCs of R. viridis. Wild-type Fe-containing Rps. viridis RCs were prepared by a protocol developed from ref 24. Approximately 20 g (wet weight cell paste) of R. viridis cells were ultrasonicated and the membrane fragments were adjusted to OD1cm,1012 = 100 with 20 mM Tris·HCl, pH 8.0. Lauryltrimethylammonium N-oxide (LDAO) was added dropwise to 1% and stirred for 10 min at room temperature (RT) in the dark. Following ultracentrifugation (50 000 rpm, Beckman 60Ti rotor, 90 min), the supernatant was decanted and retained, whereas the pellet could be discarded as it contained no RC/core conjugates, whereas the remaining supernatants were pooled, concentrated, and loaded onto a DEAE column equilibrated with 10 mM Tris·HCl, 0.1% LDAO, and 0.1 mM EDTA, pH 8.0 (TLE buffer).
The brown RCs were washed with sufficient TLE until the eluate contained no pigment/denatured protein (gauged by absorption at 280 nm) and then eluted with a salt gradient and dialyzed into the appropriate detergent prior to use. A typical yield from a preparation was around 50 mg of RC from 20 g of cell paste with an optical purity ratio of A305/A300 = 2.4. For further preparation the detergent LDAO was exchanged with sodium cholate (NaChol) following (11).

Preparation of ZnRCs of *Rps. viridis*. *Rps. viridis* cultured on high-Zn²⁺/low-Fe²⁺ medium incorporates only approximately 5% Zn²⁺ into the RC (23). To obtain a higher zinc enrichment it was necessary to adapt the biochemical procedure used to reversibly dissociate the H-subunit and exchange the non-heme iron developed by Debus et al. (11) for *Rb. sphaeroides*. This method has the potential to produce RCs with ≥90% Zn²⁺. *Rps. viridis* FeRC (OD\textsubscript{832} = 30) in NaChol were diluted to OD\textsubscript{832} = 10 so that the final buffer solution was 50 mM Tris-HCl and 0.033% NaChol, pH 8.0. The RCs were slowly stirred at 4 °C, o-phenanthroline was added (4 mM), and the solution was allowed to equilibrate for 2 min, after which 1.5 M potassium thiocyanate (KSCN) was slowly added. This was incubated at 4 °C for precisely 60 min, after which time the solution was placed into dialysis (four changes over 24 h) against 10 mM Tris-HCl, 0.025% NaChol, and 0.1 mM EDTA, pH 8.0, to remove any unspecifically bound zinc ions. This procedure results in a fraction of RCs that do not reassociate correctly; i.e., the sample contains a mixture of nonreassociated H and LM subunits in addition to correctly reassembled RCs. A fresh 0.5 M dithionite solution was added, mixed, and shock-frozen by immersion in liquid nitrogen. The samples were stored at 77 K. Samples were added, mixed, and shock-frozen by immersion in liquid nitrogen. The samples were stored at 77 K. Samples were then stored at 77 K. Samples were then concentrated (Centricon) and the procedure was repeated three times. The D₂O-exchanged ZnRCs were reduced as described above with D₂O-based solutions.

Generation of Q₅⁻ by Dithionite Reduction. *Rps. viridis* ZnRCs (OD\textsubscript{832} = 100) were dialyzed overnight in anaerobic TCE buffer. A fresh 0.5 M dithionite solution was prepared in 1 M Tris-HCl, 0.025% NaChol, and 0.1 mM EDTA, pH 8.0. The RC solution was transferred under argon to an EPR tube that was being continuously flushed with argon. Whereupon 1/10 volume of dithionite solution was added, mixed, and shock-frozen by immersion in liquid nitrogen. The samples were stored at 77 K. Samples were also prepared by 10-fold dilution of ZnRCs (OD\textsubscript{832} = 100) into D₂O buffer at room temperature for approximately 3 h. The sample was then concentrated (Centricon) and the procedure was repeated three times. The D₂O-exchanged ZnRCs were reduced as described above with D₂O-based solutions.

Generation of MQ₄ Radical Anion in Solution. The menaquinone-4 anion radical (MQ₄⁻) was used as model system for Q₅⁻ in the RC. It was generated by dissolving the quinone (approximately 1 mM) in slightly basic (potassium tert-butylicate, 10-fold molar excess) anaerobic solutions of either protonated or fully deuterated 2-propanol. The solution was further deoxygenated in the EPR sample capillary by bubbling with purified oxygen-free argon for 2–3 min and then shock-frozen by immersion in liquid nitrogen.

**Q-Band EPR Measurements.** Q-Band cw EPR (34 GHz) spectra have been recorded on a Bruker ER 200D spectrometer equipped with a Bruker Q-band microwave bridge, Bruker ER5106 QT resonator, and an Oxford CF 935 cryostat. The Q-band EPR powder spectra have been analyzed with a self-written simulation and fit program that is based on the work of Rieger (25) using a modified Levenberg–Marquardt nonlinear least-squares model (26). This simulation routine includes second-order effects and can deal with an arbitrary number of nuclei with noncollinear g and hf tensors.
**Time-Resolved EPR Measurements.** All time-resolved EPR experiments were performed on a Bruker ESP 380 E spectrometer as previously described for transient EPR (27) and pulsed EPR experiments (28). 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 time resolution of the setup in transient EPR mode was about 50 ns at X-band and 100 ns at Q-band. The transient EPR spectra were extracted from the two-dimensional (time/field) data set as the difference between two time gates, one after and one before the laser flash as described in ref 29. This removes all contributions from stable radicals in the sample, i.e., signals not initiated by the laser flash. Light excitation was achieved by a Q-switched and frequency-doubled Nd:YAG laser (Spectra Physics GCR 130, \(\lambda = 532\) nm, 8 ns pulse width, \(\leq 5\) mJ/pulse incident to the sample).

About 40 \(\mu\)L of the sample (OD\(_{322} = 90\)) was filled into a quartz tube with 2 mm inner diameter and 3 mm outer diameter and frozen in the dark in liquid nitrogen. For detection of the transient Q-band spectrum of \(P_{960}^+\) \(Q_{\lambda}'^+\), an oxidation of the cytochrome hemes was achieved by addition of a 80-fold excess of a 1:9 (v/v) solution of \(K_4\text{Fe(CN)}_6/\text{K}_3[\text{Fe(CN)}_6] \) (FeCy) before the sample was frozen (30) (see below for further details).

**ESEEM and ENDOR Spectroscopy.** The stimulated echo (three-pulse) ESEEM experiments on \(Q_{\lambda}'^-\) were also obtained on the Bruker ESP 380 E spectrometer. Two-dimensional experiments were performed in order to get the complete spectral information. The subtraction of the relaxation decay and the evaluation of the time domain spectra were done with the ESP 380 software as described in detail in ref 31.

The out-of-phase ESEEM measurements of the RP state \(P_{960}^-\) \(Q_{\lambda}'^-\) were carried out as described earlier (28). Here we used a \(\zeta/2-\zeta\) pulse sequence with \(\zeta \approx 130^\circ\) (21). The magnetic field was set to the center of the transient EPR spectrum.

\(^1\text{H}-\text{ENDOR}\) spectra of \(Q_{\lambda}'^-\) and \(\text{MQ}_{4}'^-\) were recorded on a Bruker ESP 300 E spectrometer with a self-built ENDOR/TRIPLE extension (32, 33) and a TM\(_{10}\) ENDOR cavity of local design (34). The temperature was controlled between two time gates, one after and one before the laser flash as described in ref 29. This removes all contributions from stable radicals in the sample, i.e., signals not initiated by the laser flash. Light excitation was achieved by a Q-switched and frequency-doubled Nd:YAG laser (Spectra Physics GCR 130, \(\lambda = 532\) nm, 8 ns pulse width, \(\leq 5\) mJ/pulse incident to the sample).

**RESULTS AND DISCUSSION**

**Characterization of the ZnRCs**

**Preparation of ZnRC of \(Rps.\ viridis\).** To obtain chemically exchanged \(Rps.\ viridis\) ZnRCs, FeRCs were prepared and treated with KSCN to dissociate the H subunit and with \(o\)-phenanthroline to chemically exchange the non-heme iron with Zn\(^{2+}\). Native (panel A) and Coomassie-stained SDS–PAGE gels (panel B) of this procedure are presented in Figure 2. In panel A, lane 1, the Fe-containing RCs that undergo the procedure are shown. Lane 2 illustrates the effect of the KSCN treatment in that the nonpigmented H subunit is dissociated to form the LM subunit, which migrates more slowly than the native RC. Lanes 3 and 4 show the preparation after Affi-Gel chromatography: lane 3 is the column eluate containing the LM complex, whereas lane 4 is the putative Zn-containing RC, which is eluted from the column with the cysteine solution. It can be seen clearly that the H subunit has reassocciated correctly to form the intact RC complex. This has been confirmed in panel B, where the same samples were loaded onto a SDS–PAGE gel and stained with Coomassie blue. Lane 1 is FeRC and all four subunits of the complex are visible. In lane 2 the H-subunit has been dissociated from LM but is still present in the sample; however, after Affi-Gel treatment, the column eluate in lane 3 clearly contains only LM with the H subunit no longer present. Lane 4 is the Zn-containing RC sample, which was used for the subsequent experiments and clearly consists of intact RCs with all four subunits. We have also tried to prepare iron-free RCs by a similar procedure as described above, without success. Presumably, the preparation is not very stable.

The typical absorption spectrum of intact ZnRCs obtained from our procedure was identical to that of wild-type FeRCs. However, the overall yield obtained (given as the amount of ZnRCs recovered from the Affi-Gel column against the amount of FeRCs at the beginning of the procedure) is approximately 10%; this compares with approximately 80% reported for \(Rb.\ sphaeroides\) (11). Furthermore, the amount of Zn\(^{2+}\) incorporated is also substantially less: 50% \(\pm 10\%\) as against 90% for \(Rb.\ sphaeroides\) (see below). A possible reason is that the reaction conditions used are not ideal for \(Rps.\ viridis\). A systematic optimization of the various parameters might lead to a better preparation with enhanced Zn\(^{2+}\) content and yield.

**Determination of Zn\(^{2+}\) Content in ZnRCs.** The amount of Zn\(^{2+}\) substitution can be checked, in principle, by atomic absorption spectroscopy (AAS), mass spectrometry, or standard (cw) EPR methods. These methods are, however, not able to determine directly the Zn\(^{2+}\) replacing the non-heme iron. EPR spectroscopy determines the amount of \(Q_{\lambda}'^-\) decoupled from the Fe\(^{2+}\). The detection by AAS is difficult and additionally complicated for \(Rps.\ viridis\) RCs because of the bound cytochrome, which contains four iron ions.

Figure 3 shows the time-resolved Q-band EPR-spectra of \(P_{960}^-\) and \(Q_{\lambda}'^-\) recorded at low microwave powers, i.e.,
under nonsaturating conditions for both radicals. According to the late integration window (5–10 ms after the laser flash), the spectra show a superposition of the signals of P_{960}\textsuperscript{+}Q_{\Lambda}^{-} and Q_{\Lambda}^{-} in thermal equilibrium (see Figure 3B). As can be seen, the \(g\) and \(g\) components of the \(g\) tensor of Q_{\Lambda}^{-} (not resolved at Q-band) are separated from the P_{960}\textsuperscript{+} signal at \(g = 2.0026\) (1), while the \(g\) component overlaps with the EPR line of P_{960}\textsuperscript{+} (see below and Table 2 for details).

The superposition of the steady-state cw spectra from P_{960}\textsuperscript{+} and Q_{\Lambda}^{-} obtained in separate samples from either FeCy-treated (P_{960}\textsuperscript{+}) or Na2SO4-treated (Q_{\Lambda}^{-}) ZnRCs of Rps. viridis (Figure 3B) closely resemble the experimental spectrum of the radical pair shown in Figure 3A. From this superposition the amount of decoupled Q_{\Lambda}^{-} can be calculated by double-integration of both cospectra and by comparing the relative intensities of the Q_{\Lambda}^{-} and the P_{960}\textsuperscript{+} signals. From this calculation we conclude that the amount of decoupled Q_{\Lambda}^{-} and thus the degree of Zn\textsuperscript{2+} substitution is about 50\% ± 10\%.

The AAS measurements suggested that the degree of Zn\textsuperscript{2+} is of the order of 20\%. However, due to the fact that AAS measurements are inherently more difficult with Rps. viridis RCs than Rb. sphaeroides (see above), we feel that the higher figure derived from the EPR is more accurate. In principle, some of the RCs could contain no metal. However, optical recombination kinetics experiments indicate that essentially no metal-free RCs are present in our samples (see below).

**Charge Recombination Kinetics in Rps. viridis ZnRCs.**

The kinetics of charge recombination between P_{960}\textsuperscript{+} and Q_{\Lambda}^{-} or Q_{B}^{-} were measured to establish whether the reversible dissociation and reassociation of the H subunit by chaotropic agents resulted in a functional RC. Figure 4 compares the charge recombination of P_{960}\textsuperscript{+}Q_{\Lambda}^{-} (left) and of P_{960}\textsuperscript{+}Q_{B}^{-} (right) in native FeRCs and ZnRCs. The formation and decay of the charge-separated states were monitored by measuring the flash-induced absorption changes at 980 nm. The decay kinetics of P_{960}\textsuperscript{+}Q_{\Lambda}^{-}, measured in the presence of 4 mM o-phenanthroline, and of P_{960}\textsuperscript{+}Q_{B}^{-}, measured after addition of UQ_{8}, could be fitted well with the sum of two exponentials. The time constants and relative amplitudes are summarized in Table 1. Our native FeRCs solubilized in LDAO exhibit biphasic recombination kinetics of P_{960}\textsuperscript{+}Q_{\Lambda}^{-} and of P_{960}\textsuperscript{+}Q_{B}^{-} that are almost identical to those previously published (35, 36). In ZnRCs containing approximately 50\% Zn\textsuperscript{2+} similar recombination kinetics were found. The recombination of P_{960}\textsuperscript{+}Q_{\Lambda}^{-} was slightly slower (by about a factor of 1.6) compared to FeRCs (see Figure 4 and Table 1), whereas the recombination kinetics of P_{960}\textsuperscript{+}Q_{B}^{-} were essentially the same in FeRCs and ZnRCs. Little or no effect on the recombination reactions were observed when the RCs were solubilized in NaChol instead of LDAO (see Table 1). This is important since Q_{\Lambda} cannot be reduced by dithionite in LDAO-solubilized RCs to form Q_{\Lambda}^{-}.

It has already been established that Zn-reconstituted RCs from Rb. sphaeroides, containing approximately 90\% Zn\textsuperscript{2+}, exhibit electron transfer kinetics that are essentially the same as those of native RCs (11, 37, 38). The small changes in the charge recombination rates observed in our work between ZnRCs and FeRCs from Rps. viridis are in agreement with these results. From the work with RCs from Rb. sphaeroides it is also known that the removal of Fe\textsuperscript{2+} and/or the H subunit strongly affects the charge recombination rates (11, 39). Especially the recombination of P_{960}\textsuperscript{+}Q_{B}^{-} is significantly slower. If one assumes that these findings also hold for RCs from Rps. viridis, our observation that the recombination kinetics of P_{960}\textsuperscript{+}Q_{B}^{-} were essentially the same in our ZnRCs and native FeRCs indicate that the ZnRCs show very little contamination with RCs containing no divalent metal ion or with particles lacking the H subunit.

**Experiments on the Anion Radical Q_{\Lambda}^{-} in ZnRCs**

**Determination of the g-Tensor Principal Values of Q_{\Lambda}^{-} in ZnRCs of Rps. viridis.**

The Q-band EPR spectra of Q_{\Lambda}^{-} in Rps. viridis ZnRCs and the respective model system MQ_{\Lambda}^{-} in frozen 2-propanol are presented in Figure 5. The principal g-tensor values were obtained from simulations and
The model system in alcoholic solution, one can conclude that H bonds to the CO groups of the quinones are present in both environments (4, 12). The observed slight increase of the \( g_v \) value of \( \text{QA}^- \) could be explained as a result of a difference in the polarity of the surrounding of the quinones in solution and in the hydrophobic protein pocket (5). Furthermore, it could be due to an asymmetry of the H bonds to the carbonyl oxygens in the protein as suggested by various spectroscopic studies on isotopically labeled \( \text{QA}^- \) in \( \text{Rh. sphaeroides} \) (15, 16, 42–44). This view is supported by the recent X-ray structure analysis of \( \text{Rps. viridis} \) RC single crystals (5). In the original structure (Brookhaven Protein Data Bank entry 1PRC) (45) the distances from the proximal carbonyl oxygen (\( O_1 \), closest to the Fe\( ^{2+} \)) to the nitrogen \( N^{(1)} \) of His M217 and the distal carbonyl oxygen (\( O_1 \)) to the backbone nitrogen of Ala M258 were given as equidistant (3.1 \( \text{Å} \)). In the improved RC structure (Brookhaven Protein Data Bank entry 2PRC) (5) these distances are different, i.e., 2.9 and 3.1 \( \text{Å} \), respectively (see Figure 6).

Electron Spin Echo Envelope Modulation of \( \text{QA}^- \) in ZnRCs of \( \text{Rps. viridis} \). ESEEM spectroscopy is used to probe the surroundings of \( \text{QA}^- \) in the \( \text{Rps. viridis} \) ZnRC with respect to nitrogen in the vicinity of the menaquinone. For \( ^{14} \text{N} \) nuclei \( (I = 1) \), sharp lines from the three zero-field nuclear quadrupole resonance (NQR) transitions can be observed in ESEEM spectra (48, 49), when the so-called cancellation condition is fulfilled; i.e., \( |\nu_n - |A/2| < 2K/3 \) (50). Then, the hfc and nuclear Zeeman term cancel each other in one \( m_i \) state and the three NQR transitions \( \nu_{-j} \), \( \nu_{-\bar{j}} \), and \( \nu_0 \) are observed that are related to the quadrupole parameters by \( K = (\nu_{-j} - \nu_{-\bar{j}})/6 \), and \( \eta = 3\nu_0(\nu_{-j} + \nu_{-\bar{j}}) \) (48, 49). Here \( K = c^2qQ/4h \) and \( \eta = (q_{-j} - q_{-\bar{j}})q_0 \), in which \( Q \) is the nuclear quadrupole moment and \( q \) is the electric field gradient with principal components \( q_{i} \) (\( i = x, y, z \)). In the other \( m_s \) state a double quantum transition, \( \nu_{dQ} \), is observed from which the effective hfc can be calculated (48, 49).

In Figure 7 the ESEEM spectrum of \( \text{QA}^- \) is shown. Two sets of corresponding NQR transitions—marked \( \times \) and \( \Diamond \) —could be assigned in the spectrum to two different \(^{14}\text{N} \) nuclei. The resulting NQR coupling constants, \( K \), and asymmetry parameters, \( \eta \), are collected in Table 3.

From the measured NQR parameters of an \(^{14}\text{N} \) nucleus, an identification of amino acid residues binding to \( \text{QA}^- \) is possible (17). On the basis of the X-ray structure analysis, likely candidates are His(M217) and Ala(M258); see Figure 6.
Table 2: Comparison of the Principal Values of the Electronic g Tensors of Q_{\alpha}^{-} \cdot in ZnRCs of Rps. viridis and Rb. sphaeroides and the Respective Model Compounds

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<th>quinone</th>
<th>g_{x}</th>
<th>g_{y}</th>
<th>g_{z}</th>
<th>g_{iso}</th>
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<td>Q_{\alpha}^{-}, Rps. viridis \textsuperscript{b}</td>
<td>2.00597 (5)</td>
<td>2.00492 (5)</td>
<td>2.00216 (5)</td>
<td>2.00435 (5)</td>
<td>this work</td>
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<td>MQ_{c}^{-} in 2-propanol-d_{4}</td>
<td>2.00579 (5)</td>
<td>2.00498 (5)</td>
<td>2.00218 (5)</td>
<td>2.00432 (5)</td>
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<td>2.00215 (5)</td>
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\textsuperscript{b} All values from simulations of Q-band EPR spectra; numbers in parentheses are the errors in the last digit.

Figure 6: Binding sites of the non-heme iron and Q_{A} in the RC of Rps. viridis. Carbon atoms are shown in black, nitrogen atoms in dark gray, oxygen atoms in light gray, and the iron in white. Iron–protein interactions and hydrogen bonds are shown as dashed lines. The distances between the donor and acceptor of the respective hydrogen bonds are indicated. For histidine M217 the ring nitrogen \epsilon is ligated to the iron and the nitrogen \delta(1) forms a H-bond to oxygen O_{6} of the quinone. This figure was drawn with the program BobScript (46), an extension of MolScript (47), using the Brookhaven Protein Data Bank coordinate file 2PRC (5).

Figure 7: Three-pulse (stimulated echo) ESEEM spectra (projections of full 2D data set after Fourier transformation onto the T axis) of Q_{\alpha}^{-} \cdot in Rps. viridis ZnRCs. Corresponding sets of zero-field NQR transitions are indicated by symbols (x, \diamond). Experimental conditions: temperature 15 K, T \times t = 512 \times 128 points. Start values: T_{0} = 200 ns, t_{0} = 120 ns. Increments: \Delta T = 24 ns, \Delta t = 8 ns, \pi/2 pulse = 16 ns. A four-phase cycle was employed. Total accumulation time was approximately 3 h. Top: Three-pulse sequence used in the experiment.

6. Alanine contains one amino nitrogen in the peptide bond, whereas in histidine there are additional nitrogen atoms present in the imidazole ring. Published \( K \) and \( \eta \) values of the imidazole nitrogens in histidine and of typical peptide-bond nitrogens in triglycine and polyglycine are given in Table 3. A comparison with the NQR data of the \( 14 \text{N} \) nuclei determined in this work shows that the evaluated \( K \) values of 0.80 and 0.41 and \( \eta \) values of 0.52 and 0.69 can be assigned to a peptide nitrogen and to a N(\text{N}) from a histidine ligand, respectively. A quite similar result was deduced from ESEEM investigations of the ubiquinone radical anion Q_{\alpha}^{-} \cdot in Rb. sphaeroides R-26 (17–19) and of the plastoquinone radical anion, Q_{\alpha}^{-} \cdot, in plant photosystem II (51, 55–57); see Table 3.\textsuperscript{3}

It can be concluded that one of the carbonyl oxygens of Q_{\alpha}^{-} \cdot in Rps. viridis is coupled to His(M217). It is reasonable to assign the detected peptide nitrogen to Ala(M258), which most likely takes part in a second H bond to the other carbonyl oxygen of Q_{\alpha}^{-} \cdot. This is in agreement with structural data from the recent X-ray crystallographic study of Rps. viridis RCs (Figure 6). Analysis of the double quantum transitions (Figure 7) provides a rough estimate of the hyperfine couplings of the nitrogen nuclei. This yields a value on the order of 2\nu(14\text{N}) \approx 2 MHz that is much larger than the dipolar coupling of about 0.2 MHz that can be estimated for point dipoles separated by a distance of \approx 3 \text{Å}. Thus, a significant delocalization of the unpaired electron on the quinone to both amino acid residues must take place. Such a transfer of spin density probably involves the hydrogen bonds and could either be by direct overlap or by spin polarization as postulated earlier by Lendzian et al. (18) and by Deligiannakis et al. (55). Density functional theory (DFT) calculations of hydrogen-bonded quinone radical anions show that such transfer of spin density to the H-bond donor indeed takes place (59).

\textsuperscript{3} Pelouquin et al. (58) have recently assigned NQR parameters measured for Q_{\alpha}^{-} \cdot in photosystem II to a backbone NH (probably of alanine) and to another unassigned peptide nitrogen.
ENDOR of Menaquinone Anion Radical. For an interpretation of the ENDOR spectrum of $Q_{A^-}$ in *Rps. viridis* ZnRCs, an understanding of the electronic structure of a menaquinone model anion radical is indispensable. Since it is known that the length of the isoprenoid chain does not influence the spin density distribution, we have chosen to investigate MQ$_4^{−}$ dissolved in 2-propanol as a model system.

For the quinone radical anion in solution, ENDOR lines are expected to occur in pairs symmetrically displayed about the Larmor frequency, $v_n$, of the respective nucleus (for $|A| < 2v_n$) (60):

$$v_{ENDOR}^+ = |v_n ± A/2|$$

From the line splitting the hfc $A$ can directly be obtained. In frozen solution the ENDOR signals from each proton—or group of equivalent protons—are smeared out over the whole range of the anisotropic hf tensor, and tensor principal components can be obtained from the spectral features in the powder-type ENDOR spectrum (for details see refs 60 and 61). Axially symmetric hyperfine coupling tensors of freely rotating methyl groups exhibiting a small hf anisotropy can, for example, easily be analyzed yielding the two principal values, $A_{II}$ and $A_1$. Furthermore, exchangeable protons coupled to the radical can be discriminated by investigation of the quinone radical in deuterated solvents. Due to the smaller Zeeman frequency of $^2$H, ENDOR lines of deuterons usually do not contribute to the $^1$H ENDOR range.

Panels A and B of Figure 8 show the $^1$H ENDOR spectra of MQ$_4^{−}$ at 123 K in protonated and deuterated 2-propanol. We observe several line pairs (labeled a,a′, b,b′, etc.), whose respective hyperfine splittings are summarized in Table 4. In the spectrum obtained in deuterated 2-propanol only $^1$H ENDOR lines from nonexchangeable protons of the MQ$_4^{−}$ molecule remain; other lines, e.g., from protons in hydrogen bonds, which are replaced by deuterons, are missing.

The spectral shape of pattern i,i′ and h,h′ resembles that of an axially symmetric hyperfine tensor, as expected for protons of a freely rotating methyl group. We therefore assign these line pairs in Figure 8B to the $A_{II}$ and $A_1$ component of the CH$_3$ protons at position 3 (10.1 and 6.6 MHz, respectively). The resulting isotropic value of 7.8 MHz is in fair agreement with the value for vitamin $K_1^{−}$ in liquid solution (7.3 MHz) that has a similar structure (62, 63). The observed anisotropy ($A_{II} − A_1$)/$A_{iso}$ is rather large for protons of a rotating methyl group. However, for anion radicals of other methyl-substituted quinone radical anions, similar values were found (4, 64). The magnitude of the anisotropy can be explained by the additional effect of the large spin density of the adjacent carbonyl group on the dipolar hyperfine coupling of the methyl protons. Couplings c,c′ and e,e′ (1.5 and 3.0 MHz, respectively) in Figure 8B are assigned to the two methylene protons of the side chain at position 2 (Figure 1). The reduction of these coupling constants as compared to those of vitamin $K_1^{−}$ in liquid solution (3.7 MHz) (62, 63) indicates a preferred out-of-plane conformation of the side chain in frozen solution. This leads to a larger dihedral angle for the CH$_2$ protons ($θ = 60^°$) as compared with $θ_{CH} = 45^°$ for a rotating CH$_3$ group. The $β$-proton hfcs are proportional to the $π$-spin density at the neighboring carbon atom, $ρ_c^π$, and to $cos^2 θ$ according to (65)

$$A_{iso}(H_β) = ρ_c^π (B_0 + B_2 cos^2 θ)$$

in which $B_0$ is usually negligibly small. Therefore, the $π$ spin densities at carbon positions 2 and 3 could be of comparable magnitude, even though the hfcs of their $β$-protons are different. A preferred out-of-plane conformation of the side chain has recently been reported to be present in several methyl-substituted quinones in frozen solution (66). The $α$-protons at positions 5–8 of the quinone (Figure 1) are expected to be small and are therefore assigned to couplings a,a′ and b,b′ and may contribute to some extent also to coupling c,c′.

![Figure 8: Powder-type cw ENDOR spectra (X-band) for MQ$_4^{−}$ in 2-propanol (A) and in fully deuterated 2-propanol-d$_6$ (B). In panel C the difference spectrum (A – B) is shown. The line pairs from which the hf splittings are obtained are marked (a,a′, b,b′, etc.); see Table 4. Note that in spectrum C only exchangeable protons from the surrounding medium contribute to the spectrum, e.g., protons involved in H bonds to the quinone oxygens. Experimental conditions: mw power 4 mW, accumulation time 80 min, rf power 150 W, 12.5 kHz FM, deviation ±25 kHz, and field setting, center of EPR line.](image)

![Table 4: Hyperfine Couplings for MQ$_4^{−}$ in 2-Propanol from ENDOR Spectroscopy](table)
In Figure 8C the difference between the spectra obtained in protonated and deuterated solvent is shown. Here, one observes only hf interactions resulting from exchangeable protons from the surrounding solvent. Similar spectra have been obtained for quinones with other substituents, e.g., plastoquinone-9, ubiquinone-10, and duroquinone anion radicals (62, 64, 67, 68). The hfcs assigned to exchangeable protons (a,a’, d,d’, f,f’, and g,g’) are also given in Table 4. Their assignment is not immediately obvious. The two broad features giving rise to hf splittings f,f’ and g,g’ are assigned to A⊥ components of two large hf tensors belonging to protons that are probably involved in hydrogen bonds to oxygens 1 and 4. The respective A∥ components arise either both from lines d,d’ or one from d,d’ and the other from a,a’, whereby the latter assignment is unlikely, since it leads to a rather large positive isotropic hf coupling of one H-bond proton.

On the basis of a comparison with literature values for other semiquinones (64), we assign couplings d,d’ (−2.2 MHz) and f,f’ (+4.6 MHz) to an exchangeable proton with a purely dipolar tensor (A∥ = +4.6, A⊥ = −2.3, Aiso ≈ 0.1 MHz). This proton most probably lies in the quinone plane. An A⊥ component belonging to A∥ = +5.5 MHz cannot be determined with confidence. It is probably overlapping with d,d’. We therefore assume that this second tensor is somewhat larger than the first one. We assign the two detected exchangeable protons to be hydrogen bonded to each of the carbonyl oxygens at position 1 and 4. We are unable to show, however, which is bound to which oxygen. The length of the first H bond estimated from the point-dipole approximation:

\[ A'(\theta) = \frac{c}{r^3} (3 \cos^2 \theta - 1) \]

with \( c = 79 \text{ MHz \ Å}^3 \) and a spin density on the oxygen estimated from \(^1\text{O} \) hf data (64, 69) of \( \rho = 0.20 \) gives \( r_{OH} = 1.9 \text{ Å} \). The second bond is expected to be somewhat shorter due to the larger A∥ value. The line pair a,a’ (Figure 8C) belonging to the hf of an exchangeable proton is assigned to a hydrogen in a second solvation shell of the quinone.

The point-dipole approximation is not very reliable for short distances and distributed spin densities. More reliable results are obtained from DPT calculations performed on H-bonded quinone anion radicals (59, 70, 71), which yield both dipolar and isotropic hfcs for all nuclei for a certain geometry. For the naphthoquinone radical H-bonded in plane by methanol molecules (\( r_{OH} \approx 1.8 \text{ Å} \)), dipolar tensor components of +5.8, −2.8, and −3.1 MHz (Aiso = +0.2 MHz) are calculated (59). This tensor is somewhat larger than the one obtained for MQ2− in our experiments, indicating that for MQ2− the H-bonds are somewhat longer. Increased values are calculated for out-of-plane H-bonding (59). Due to the substitution pattern of MQ2−, such a situation is likely to occur for this radical and the second larger H-bond tensor found in our experiments could be assigned to an out-of-plane H-bond.

ENDOR of QA− in ZnRCs of Rps. viridis. The ENDOR spectra obtained for QA− in ZnRCs from Rps. viridis are shown in Figure 9. The values for the hyperfine couplings are summarized in Table 5. In the spectrum recorded in deuterated buffer (Figure 9B) only the hf splittings from the nonexchangeable protons attached to the quinone and the protein matrix are detected. Since it is difficult to distinguish between ENDOR lines from methyl and side-chain methylene protons at positions 3 and 2, respectively, we base our assignment on the comparison with MQ2− in frozen alcoholic solution (Figure 8). The largest splitting i,i’ (8.5 MHz) is assigned to the A∥ component of the hyperfine tensor of methyl protons at position 3. Since for this position a similar hyperfine anisotropy can be expected as for MQ2− in vitro, we assign the corresponding A⊥ component to splitting h,h’ (6.0 MHz). The resulting isotropic part Aiso is 6.8 MHz.

Splits g,g’ and e,e’ (5.0 and 3.5 MHz, respectively) are then assigned to the two methylene β-protons of the side chain. From the X-ray structure, dihedral angles \( \theta \approx -60^\circ \) and \( +57^\circ \) are obtained for the two methylene β-protons for the neutral QA molecule. This shows that the side chain is oriented perpendicular to the quinone plane and the two methylene protons should have similar hfcs. Line pair g,g’ is assigned to the A∥ and e,e’ to the A⊥ component of their

### Table 5: Hyperfine Couplings for QA− in ZnRCs of Rps. viridis from ENDOR Spectroscopy

<table>
<thead>
<tr>
<th>Transition</th>
<th>HFC (MHz)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>a,a’</td>
<td>0.3</td>
<td>α-protons or matrix</td>
</tr>
<tr>
<td>b,b’</td>
<td>0.9</td>
<td>α-protons or matrix</td>
</tr>
<tr>
<td>c,c’</td>
<td>1.3</td>
<td>α-protons or matrix</td>
</tr>
<tr>
<td>d,d’</td>
<td>2.0</td>
<td>α-protons or matrix</td>
</tr>
<tr>
<td>e,e’</td>
<td>3.5</td>
<td>2-CH3</td>
</tr>
<tr>
<td>f,f’</td>
<td>4.6</td>
<td>H-bond (A∥)</td>
</tr>
<tr>
<td>g,g’</td>
<td>5.0</td>
<td>2-CH2</td>
</tr>
<tr>
<td>h,h’</td>
<td>6.0</td>
<td>3-CH3 (A∥)</td>
</tr>
<tr>
<td>i,i’</td>
<td>8.5</td>
<td>3-CH3 (A⊥)</td>
</tr>
</tbody>
</table>

* For labeling of transitions, see spectra in Figure 9. Error ±0.2 MHz. See Figure 1 for molecular structures and numbering of menaquinone.
Comparison of the hyperfine values of QA−* in the RC and MQ−* in alcoholic solution (Tables 4 and 5) shows that the methyl proton hfc (Aiso) is significantly smaller in the RC, whereas the methylene proton hyperfine splittings are increased. The spin density at position 3, which is sensed by the methyl proton coupling, is reduced by 13% in QA−* as compared with MQ−* in vitro. Assuming a similar out-of-plane orientation of the methylene side chain for MQ−* in vitro (66) as for QA−* in Rps. viridis, it can be deduced from eq 2 that the spin density of position 2 is significantly increased in QA−*. Since in eq 2 cos2 θ = 0.5 for a rotating methyl group, and cos2 θ = 0.25 for θ = 60°, assumed for the CH2 group, the data indicate that in QA−* the spin density at carbon 2 is larger than at carbon 3. This finding is very similar to the situation found for UQ10−* in vitro and QA−* in the RC of Rb. sphaeroides (4).

The observed shift of spin density can be understood by postulating a different environment of the two carbonyl groups. One intriguing possibility is the formation of a proton in a hydrogen bond between the protein and one carbonyl oxygen of QA−* in the RC as in the corresponding spectrum of MQ−* in RCs of Rb. sphaeroides; the other one must be very small (4).

Comparison with Crystallographic Data. In Figure 6 the structure of the QA site in Rps. viridis is shown. In the ground-state X-ray crystallographic study two H bonds between the protein and QA are indicated (5). In this recent structural refinement a shorter distance has been found between O4 of QA and the Nα(1) of His(M217) (2.9 Å) than between O4 of QA and the backbone nitrogen of Ala(M258) (3.1 Å). This is in qualitative agreement with our spectroscopic data. It has been shown by X-ray crystallography of RC single crystals of Rb. sphaeroides in the ground state and the charge-separated state PΨQA−* (73) that no significant change in structure and position of QA−* as compared with QA occurs.

Experiments on the Radical Pair State P960+QA−* in ZnRCs

Transient EPR Experiments of P960+QA−*. As shown in a number of transient EPR studies, information about the relative orientation of the two cofactors involved in a radical pair (RP) can be deduced from simulations of the transient EPR spectra, based on the CCRP (correlated coupled radical pair) model as reviewed in refs 20, 74 and 75. To get a reliable set of orientational parameters, measurements of the RP spectra in various frequency bands are required.

A characteristic X-band electron spin polarization (ESP) pattern with E/A/E (E = emission, A = enhanced absorption) is well-established for ZnRCs of Rb. sphaeroides (76) and can be attributed to the RP state PΨQA−*. Similar experiments with Rps. viridis are more complicated due to the bound cytochrome c subunit. The redox potentials of the four hemes have been measured (77, 78) and have the following sequence: P960, heme 3, heme 4, heme 2, heme 1. Without addition of oxidants or reductants, hemes 4, 2, and 1 are oxidized but heme 3 is only partially (~50%) oxidized (G. Fritzsch, personal communication). Upon laser excitation, in a fraction of the RCs, the primary donor P960+ will be reduced by the nonoxidized heme 3 fraction within 250 ns, which is much faster than the recombination of the RP state PΨQA−* (approximately 1.5 ms). As a result, after reduction of PΨQA−* this fraction of the sample contains prereduced QA−*, which blocks ET past the bacteriopheophytin (BPh). Further light excitation of this RC fraction results in the formation of the intermediary RP state PΨQA−*BPh−, followed by charge recombination to the triplet state of the primary donor, 1P960 (79). The observation of the RP signal of PΨQA−* alone is therefore not possible in Rps. viridis since the heme 3 cannot be fully oxidized without oxidizing part of P960.
different polarization pattern has been reported for native is due to centers in which heme 3 reduced P 960 in the literature for P 960 (see ref obtained, which are in good agreement with values reported in Figure 10B. The difference spectrum is depicted in Figure 10A is expected to be very small at a temperature of 100 K. For native RCs of Rb. sphaeroides R-26, a strong increase of the relaxation of the spin polarization of P 865 [Fe 2+Q 960]− is observed at increasing temperature, followed by a drastic decrease of its signal intensity (86). Although the signal of P 865 [Fe 2+Q 960]− is visible up to ~200 K without a change in polarization at early times, the intensity reduces by about a factor of 5 from 6 to 100 K. Thus, the intensity of P 865 [Fe 2+Q 960]− is at least one order of magnitude smaller than for P 865 [Fe 2+Q 960]−. In analogy to these results, the contribution of P 960 [Fe 2+Q 960]− to the transient spectra discussed here has been neglected. This is in accordance with the spectrum of the native sample (Figure 10B) where no additional signal in the g = 2 region, which could be ascribed to P 960 [Fe 2+Q 960]−, is observed.

For a detailed discussion of the geometrical arrangement of the two cofactors, a measurement of the RP signal at various frequency bands is necessary, since the relative influence of the hyperfine coupling on the spectra decreases with increasing microwave (mw) frequency. Furthermore, at higher mw frequencies the spectrum is better resolved as the g anisotropy becomes dominant. On the other hand, since the spectrum is spread out over a wider range, the absolute intensity per Gauss tends to decrease at higher mw frequencies. Therefore, for a measurement of the RP spectrum at Q-band, the triplet background has to be suppressed as much as possible. For this purpose, we added an 80-fold excess of FeCy to the sample (see the Materials and Methods section). This led to a decrease of the triplet background by a factor of 5 but decreased the intensity of the RP signal only slightly (≤10%). Further addition of FeCy resulted in oxidation of P 960, monitored by the optical absorption band at 960 nm, and a drastic decrease of the RP signal intensity (data not shown).

Figure 10A shows the transient X-band EPR spectrum of Rps. viridis recorded at 100 K. It can be attributed to a superposition of a triplet signal and the RP signal of [Fe 2+Q 960]−. From the triplet spectrum, the D and E values of |D| = 162.0 ± 1.5 cm−1 and |E| = 41.0 ± 1.5 cm−1 are obtained, which are in good agreement with values reported in the literature for P 960 (ref 80 and references therein). The observed polarization pattern (A/E/A/E/A/E) can be explained as a result of selective population of the [T 70] sublevel due to the recombination of the intermediary radical pair P 960+·BPh−· (79). Oxidation of heme 3 by FeCy results in a drastic decrease of the P 960 intensity (see below). Therefore, we can conclude that the P 960 state observed here is due to centers in which heme 3 reduced P 960+· in the charge-separated state P 960+·[Fe 2+Q 960]−, i.e., in centers containing Q 960. It should be noted that at a temperature of 100 K, the A/E/A/E/A/E polarization pattern for P 960+· is also observed for Fe-containing (native) RCs (Figure 10B). This is in contrast to previous work (30, 81, 82) in which a different polarization pattern has been reported for native Q 960-containing RCs of Rps. viridis. The reason for the observed differences remains to be clarified.

For an analysis of the RP spectrum, a subtraction of the triplet spectrum is necessary. The spectrum of P 960+·[Q 960]− shows a smaller spectral width than that of P 865+·[Q 960]−, consistent with the smaller g anisotropy of Q 960− in Rp. viridis as compared with Q 960− in Rb. sphaeroides. The relative intensities of the two emissive peaks are changed in comparison with Rb. sphaeroides R-26 (83).

Due to the low amount of Zn substitution (~50%, see above), transient EPR signals, arising from the RP P 960+·[Fe 2+Q 960]− in RCs that still contain the non-heme iron, can disturb the observed spectrum for P 960+·[Q 960]− in the ZnRCs. The spin-polarized EPR spectrum of P 960+·[Fe 2+Q 960]− shows an A/E/A polarization pattern (30) similar to the one observed in native RCs of Rb. sphaeroides R-26 (84, 85). However, the contribution of this signal to the observed spectrum shown in Figure 10A is expected to be very small at a temperature of 100 K. For native RCs of Rb. sphaeroides R-26, a strong increase of the relaxation of the spin polarization of P 960+·[Fe 2+Q 960]− is observed at increasing temperature, followed by a drastic decrease of its signal intensity (86). Although the signal of P 960+·[Fe 2+Q 960]− is visible up to ~200 K without a change in polarization at early times, the intensity reduces by about a factor of 5 from 6 to 100 K. Thus, the intensity of P 960+·[Fe 2+Q 960]− is at least one order of magnitude smaller than for P 960+·[Fe 2+Q 960]−. In analogy to these results, the contribution of P 960+·[Fe 2+Q 960]− to the transient spectra discussed here has been neglected. This is in accordance with the spectrum of the native sample (Figure 10B) where no additional signal in the g = 2 region, which could be ascribed to P 960+·[Fe 2+Q 960]−, is observed.

For a detailed discussion of the geometrical arrangement of the two cofactors, a measurement of the RP signal at various frequency bands is necessary, since the relative influence of the hyperfine coupling on the spectra decreases with increasing microwave (mw) frequency. Furthermore, at higher mw frequencies the spectrum is better resolved as the g anisotropy becomes dominant. On the other hand, since the spectrum is spread out over a wider range, the absolute intensity per Gauss tends to decrease at higher mw frequencies. Therefore, for a measurement of the RP spectrum at Q-band, the triplet background has to be suppressed as much as possible. For this purpose, we added an 80-fold excess of FeCy to the sample (see the Materials and Methods section). This led to a decrease of the triplet background by a factor of 5 but decreased the intensity of the RP signal only slightly (≤10%). Further addition of FeCy resulted in oxidation of P 960, monitored by the optical absorption band at 960 nm, and a drastic decrease of the RP signal intensity (data not shown).

Figure 11A shows the transient Q-band EPR spectra of P 960+·[Q 960]− in the FeCy-treated sample after subtraction of the small remaining triplet background. For comparison, the RP spectra of P 960+·[Q 960]− of Rb. sphaeroides R-26 is depicted in Figure 11B. Both spectra show a similar polarization pattern (A/E/A/E/A/E). The low-field A/E/A part of the P 960+·[Q 960]− spectrum is shifted to slightly higher fields compared with P 965+·[Q 960]−, corroborating the smaller spectral width already found at X-band. From the similar polarization pattern in the low-field part of the spectra a similar orientation of the quinones in both RCs is deduced. The Q-band spectra can be simulated by using the orientational parameter obtained from the X-ray structures of Rps. viridis (5) and Rb. sphaeroides R-26 (6, 87), respectively. The simulations are shown in Figure 11 by dotted lines. The simulation is satisfactory for the quinone part of the spectrum (low-field), while in the P part (high-field) some differences between experiment and simulation are evident. Mainly two
It should be noted that the ESEEM experiment on a spin-correlated light-induced RP is substantially different from the similar experiment on a stabilized single radical. ESEEM experiments on a single radical are able to yield small electron–nuclear hyperfine couplings and quadrupole interactions as described above. The echo modulation frequency in an ESEEM experiment on the RP state is the same in protonated and deuterated $^{28}$S as well as in $^{15}$N- or $^{15}$N-enriched samples ($^{31}P$). For this reason, contributions from hyperfine and quadrupole interactions can be neglected in these experiments and the spectra are only dominated by the electron–electron interaction.

Figure 12A shows the out-of-phase echo modulation of $P_{960}^{+}\cdot Q_{A}^{-}$ in ZnRCs of $R. sphaeroides$ measured at 150 K. The ESEEM is similar to that of $P_{665}^{+}\cdot Q_{A}^{-}$ in ZnRCs of $R. sphaeroides$ R-26 (28). In particular, the modulation frequency deviates only slightly, indicating that the distances between the spins in both RCs are very similar. Detailed information on the cofactor distance can be achieved, e.g., by a comparison between the Fourier transform of the experimental ESEEM after reconstruction of the signal within the spectrometer deadtime and its numerical simulation according to the CCRP model mentioned above. In Figure 12B, the sine Fourier transform (SFT) of the experimental out-of-phase echo modulation is shown together with the numerical simulation. For the upper limit of isotropic coupling $J$, a value of $J \approx 1.0 \mu T$ consistent with the simulation of the transient EPR spectra (92), has been derived for both RCs. From the numerical simulation we obtained a dipolar coupling of $D = -116 \pm 5 \mu T$ for $R. viridis$. From this value, a distance of 28.8 \pm 0.3 \AA between the centers of the spin densities on $P_{960}^{+}$ and $Q_{A}^{-}$ in $R. viridis$ is calculated. The distance obtained for ZnRCs of

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**Figure 11:** Transient EPR spectra of $R. viridis$ (A) and $R. sphaeroides$ R-26 (B) measured at Q-band at $T = 100$ K. Integration window 1.5–5.0 $\mu$s after the laser flash; mw power 133 $\mu$W. Simulations with the cofactor arrangement obtained from X-ray structures (5, 87) are shown as dotted lines. Simulation parameters ($R. viridis$): $g(P^{+}) = 2.00309$, $g(A) = 2.00251$, $g(Q_{A}) = 2.00205$; $g(Q_{A}) = 2.00251$, $g(Q_{A}) = 2.00205$; $g(Q_{A}) = 2.00251$, $g(Q_{A}) = 2.00205$; $g(Q_{A}) = 2.00251$, $g(Q_{A}) = 2.00205$; $g(Q_{A}) = 2.00251$, $g(Q_{A}) = 2.00205$; $g(Q_{A}) = 2.00251$, $g(Q_{A}) = 2.00205$; $g(Q_{A}) = 2.00251$, $g(Q_{A}) = 2.00205$. From these experiments and the spectra are only dominated by the electron–electron interaction.

**Figure 12:** (A) Two-pulse ESEEM time traces for the RP $P_{960}^{+}\cdot Q_{A}^{-}$ in $R. viridis$ ZnRCs at $T = 150$ K. (B) Sine Fourier transforms of the experimental ESEEM traces after dead-time reconstruction (--) and numerical simulations (---). The data evaluation and parameters obtained from the simulations are discussed in the text. Top: pulse sequence used for the experiment.
**Zn-Substituted RCs from Rps. viridis**

*Rhs. sphaeroides* in a similar experiment was 28.4 ± 0.3 Å (28). From the X-ray structure of *Rps. viridis* (5) a cofactor distance³ of 28.3 ± 0.4 Å between P₉₀⁺ and Qₐ⁻ is derived, assuming that the spin on P₉₀⁺ is located mainly on the L half of the dimer and the spin on Qₐ⁻ is located in the center between the carbonyl oxygens. This distance is in reasonable agreement with the distance between the RP spins measured in our pulsed EPR experiment.

**SUMMARY AND CONCLUSION**

In reaction centers of *Rps. viridis* the non-heme high-spin Fe²⁺ has been partially replaced by Zn²⁺. The resulting RCs have been purified and characterized by biochemical and spectroscopic methods. This includes time-resolved optical measurements to determine the electron-transfer kinetics as well as cw, transient and pulsed EPR techniques to obtain the g tensor and hyperfine couplings of the primary quinone acceptor radical anion, Qₐ⁻, as well as structural parameters from the radical pair P₉₀⁺Qₐ⁻. The data have been compared with those obtained earlier from ZnRCs of *Rhs. sphaeroides*.

The analysis of the transient EPR spectra of the radical pair states P⁺Qₐ⁻ in both *Rps. viridis* and *Rhs. sphaeroides* showed that the relative orientation of these radicals is very similar. It also agrees with the available X-ray crystallographic structure (5, 6, 87) in the ground state. This indicates that no major reorientation of the cofactors takes place during charge separation (cf. also ref 73). The distance between the spins in the radical pairs was determined from out-of-phase ESEEM experiments. It is the same within experimental error for both bacterial RCs, in agreement with the X-ray crystallographic structures.

The g-tensor of Qₐ⁻ in *Rps. viridis* RCs measured by Q-band EPR characterizes this species as a naphthoquinone-type radical anion. The shifts of the g tensor components—in particular of gₓ—with respect to a model compound in alcoholic solution is similar to that observed for ubiquinone in the A-site of *Rhs. sphaeroides* ZnRCs (40). It seems to be characteristic for the specific binding sites of Qₐ in the protein.

The proton hyperfine coupling constants of Qₐ⁻ determined by cw ENDOR spectroscopy clearly show a shift of spin density in the quinone ring as compared with the menaquinone radical anion in alcoholic solution. This effect is qualitatively identical to that found for Qₐ⁻ in *Rhs. sphaeroides* (4, 14). Furthermore, D₂O exchange experiments identified at least one exchangeable proton in Qₐ⁻ that could be assigned to a hydrogen bond between a carbonyl group of Qₐ⁻ and the protein. The magnitude of the observed coupling indicates a fairly strong H-bond. A second H-bond could not be clearly resolved in the spectra. The shift of spin density in the quinone ring is attributed mainly to asymmetric H-bonding of the two C–O groups of Qₐ⁻. On the basis of a valence bond model (4), the stronger bond should be to the oxygen O₄ adjacent to the CH₃ group of the menaquinone; i.e., to the less sterically hindered carbonyl group. This finding is in agreement with data obtained for Qₐ⁻ in *Rhs. sphaeroides* from EPR/ENDOR on selectively isotope-labeled systems (15, 16) and FTIR (43, 44) techniques. A similar situation was also found for menaquinone in *Rps. viridis* RCs by FTIR (93, 94).

Support for a second H-bond comes from our ESEEM data that show interaction of the semiquinone anion radical Qₐ⁻ with two nitrogens from the surrounding protein. A comparison of the respective ¹⁴N nuclear quadrupole coupling constants with those of various amino acid residues indicates that one nitrogen most probably belongs to a N¹H—H of histidine and one to an N–H of the protein backbone. Two different H-bonds to the C–O groups of Qₐ⁻ in *Rhs. sphaeroides* have been found experimentally by following the intensity change of the respective ENDOR signals during D₂O exchange (4, 95). A recent X-ray structure analysis of *Rps. viridis* RCs (5) shows that the carbonyl oxygens of Qₐ are within H-bond distance to His(M217) and Ala(M258).

A very similar situation is found for the Qₐ site in *Rhs. sphaeroides* RCs (6, 87).

Obviously, Nature has used the same basic concept to form the peculiar Qₐ binding site in both bacterial species to achieve the specific function of this electron carrier. It is postulated that the binding site described here will also be found in other photosynthetic bacteria and in photosystem II of plant photosynthesis that perform similar functions.

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**REFERENCES**


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³ Considering the coordinate error of the X-ray structural data for each atom (0.3 Å) (5), the error for the P₉₀⁺Qₐ⁻ distance can be estimated to be at least 0.4 Å.

² It is assumed that the spin is predominantly on the BCHl b dimer half bound to the L protein subunit in analogy to the dimer in RCs of *Rhs. sphaeroides*. For a localization of the spin on the M half, a distance of 28.0 ± 0.4 Å is obtained; for a symmetric spin distribution in the dimer, a distance of 27.9 ± 0.4 Å is found (center between the two dimer halves).