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Determination of the Proton Environment of High Stability Menasemiquinone Intermediate in *Escherichia coli* Nitrate Reductase A by Pulsed EPR*

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**Background:** *Escherichia coli* nitrate reductase A (NarGHI) is a membrane-bound enzyme that couples quinol oxidation at a periplasmically oriented Q-site (QD) to proton release into the periplasm during anaerobic respiration. To elucidate the molecular mechanism underlying such a coupling, endogenous menasemiquinone-8 intermediates stabilized at the QD site (MSQD) of NarGHI have been studied by high-resolution pulsed EPR methods in combination with $^1$H$_2$O/$^2$H$_2$O exchange experiments. One of the two non-exchangeable proton hyperfine couplings resolved in hyperfine sublevel correlation (HYSCORE) spectra of the radical displays characteristics typical from quinone methyl protons. However, its unusually small isotropic value reflects a singularly low spin density on the quinone carbon carrying the methyl group, which is ascribed to a strong asymmetry of the MSQD binding mode and consistent with single-sided hydrogen bonding to the quinone oxygen O1. Furthermore, a single exchangeable proton hyperfine coupling is resolved, both by comparing the HYSCORE spectra of the radical in $^2$H$_2$O and $^3$H$_2$O samples and by selective detection of the exchanged deuterons using Q-band $^2$H Mims electron nuclear double resonance (ENDOR) spectroscopy. Spectral analysis reveals its peculiar characteristics, *i.e.* a large anisotropic hyperfine coupling together with an almost zero isotropic contribution. It is assigned to a proton involved in a short ~1.6 Å in-plane hydrogen bond between the quinone O1 oxygen and the $\tilde{\alpha}$ of the His-66 residue, an axial ligand of the distal heme b$_D$.

**Results:** Three proton hyperfine couplings to this radical with atypical characteristics are characterized.

**Conclusion:** Semiquinone binding is strongly asymmetric and occurs via a single short in-plane H-bond.

**Significance:** Learning how the protein environment tunes the semiquinone properties is crucial for understanding the quinol utilization mechanism by energy-transducing enzymes.

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Quinones are small lipophilic organic molecules found in energy-transducing membranes of all living organisms except methanogens (1). Due to their ability to transfer up to two electrons and two protons, they are widely used in photosynthetic and respiratory electron transfer chains. Quinones can freely diffuse in the hydrophobic core of lipid membranes. They can therefore bind into specific quinone-reactive sites (Q-sites) of membrane proteins in which they function as two-electron and proton carriers and are responsible for exchange of reducing equivalents between different electron transport complexes. In this case, the quinones leave the protein after completion of the redox cycle. Typical examples are the Q$_D$ site of bacterial reaction center (RC) or photosystem II and the Q-sites (Q$_L$ and Q$_R$) of bc$_1$ complex. In contrast, non-dissociable quinones can be tightly bound at specific quinone-reactive sites of proteins in which they can be involved in electron transfer processes as prosthetic groups. Well known representatives of this type include quinones in the QA site in RCs of purple bacteria and in photosystem II or the A$_1$ sites in photosystem I (2, 3).

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The different redox states of quinones may also adopt different conformations in the quinone-binding pockets, as evidenced for ubiquinone and ubisemiquinone at the Q_b site of bacterial RC (4). The functional diversity of Q-sites arises from a particular tuning of the protein environment. Despite the fact that high-resolution structural data are available for several Q-sites, how protein–cofactor interactions relate to and control the functional properties of the bound quinone is largely unknown. In particular, understanding the molecular mechanism underlying the coupling between electron transfer and proton translocation that occurs at dissociable Q-sites requires obtaining structural information on all three forms, quinone (Q), semiquinone (SQ), and quinol (QH_2). For this purpose, high-resolution EPR methods such as ENDOR (electron nuclear double resonance) and ESEEM (electron spin echo envelope modulation) spectroscopies were proven to be valuable by giving detailed structural information on protein-bound semiquinone intermediates, provided that this paramagnetic state can be trapped for spectroscopic studies.

Escherichia coli nitrate reductase A (NarGHI) is a membrane-bound heterotrimeric enzyme induced by anaerobiosis and the presence of nitrate. Involved in the nitrate respiratory pathway, a major alternative to the bacterial oxidative phosphorylation, it couples the oxidation of menaquinols or ubiquinols at a periplasmically oriented Q-site (named Q_D) to the cytoplasmic reduction of nitrate. Thus, both substrate turnovers contribute to the generation of a proton motive force across the cytoplasmic membrane. NarGHI contains eight redox-active metal centers (5–9): a molybdenum cofactor and an Fe_4S_4 cluster (F50) in the nitrate-reducing subunit NarG; one Fe_4S_4 cluster (F54) and three Fe_6S_4 clusters (F1–3) in the electron transfer subunit NarH; and two low spin hemes b in the membrane-anchor subunit NarI, termed b_D and b_P to indicate their distal and proximal position to the catalytic site. Importantly, NarI stabilizes an EPR-detectable semiquinone intermediate of both natural substrates at its quinol oxidation site Q_D, close to heme b_D (10–12). Remarkably, the resulting menasemiquinone species herein referred to as MSQ_3, has the largest thermodynamic stability measured so far in respiratory complexes stabilizing semiquinone intermediates. These peculiar properties render NarGHI ideally suited for investigating the molecular factors responsible for the reactivity of respiratory enzymes toward quinols.

Although no high-resolution structural data revealing the binding mode of the natural quinol/quinone substrate are available, we recently utilized high-resolution EPR techniques on endogenous MSQ_D and ubisemiquinone radical (USQ_D) stabilized in NarGHI-enriched inner membrane vesicles (IMVs) of E. coli to explore their environment using the unpaired electron as a probe. The use of ESEEM and HYSCORE (hyperfine sublevel correlation) spectroscopies on either the wild-type enzyme or the enzyme uniformly enriched with _^{15}N_ nuclei provided direct evidence for nitrogen ligation to MSQ_D and USQ_D. On the basis of the direct determination of the quadrupolar parameters of the corresponding interacting _^{14}N_ by S-band (~3 GHz) HYSCORE experiments, we assigned the latter to an _N_8 imidazole nitrogen and proposed it to arise from the heme b_D axial ligand His-66 (13). The non-zero isotropic hyperfine coupling of this nitrogen suggests that the interaction occurs via a hydrogen bond, allowing electron spin density to be transferred from the radical to the interacting nucleus. Interestingly, these experiments did not support a direct H-bond between MSQ_D (or USQ_D) and Lys-86, a residue in the Q_D site that was previously shown to be essential for quinol oxidation and menasemiquinone detection (11, 14). Indeed, no evidence for the transfer of a measurable spin density on any other nuclei than that mentioned above was found. Thus, we tentatively proposed that a water-mediated interaction is formed between MSQ_D (or USQ_D) and Lys-86, consistent with the latter being involved in reactivity toward quinols (13). Moreover, we have recently shown that a cardiolipin molecule specifically bound to the complex is necessary for quinol substrate fixation at the Q_D site, probably through the action of one of its acyl chains located in the vicinity of His-66 (15). Clearly, additional information is required to improve our understanding of the semiquinone binding mode in the Q_D site and of its functional tuning by the protein environment.

In this work, high-resolution EPR techniques have been used to map the environment and the binding mode of MSQ_D via the detection of proton hyperfine couplings to the radical. Using a combination of X-band (~9 GHz) ESEEM/HYSCORE and Q-band (~34 GHz) Mims ENDOR experiments on MSQ_D prepared in either a protonated or a deuteronated solvent, one exchangeable and two non-exchangeable protons magnetically coupled to the radical were detected. Their detailed characterization allows their assignment to specific protons in the vicinity of the radical. Implications of these results for deciphering the semiquinone binding mode and the catalytic mechanism at the Q_D site are discussed.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—NarGHI was expressed in an E. coli nitrate reductase-deficient strain LCB3063 (RK4353, ΔnapA-B, narG:ery, ΔnarZ::OmpR, Spc) (16) using pVA700 plasmid (Amp^R) (6), which encodes for the narGHJI operon under control of the tac promoter. Cells were grown in Terrific Broth under anaerobic conditions at 37 °C as described in Ref. 11 with ampicillin (100 μg ml⁻¹) and spectinomycin (50 μg ml⁻¹) included in the growth medium.

Purified E. coli NarGHI-enriched IMVs were used for this study, allowing us to maintain an unmodified lipid environment and to study the interaction of NarGHI with its endogenous menaquinol substrate. For this purpose, purified E. coli NarGHI-enriched IMVs were isolated by differential centrifugation and sucrose gradient step as described in Ref. 11 using a buffer containing 100 mM MOPS and 5 mM EDTA at pH 7.5. Deuterium-exchanged samples were prepared using the same membrane extraction protocol with a buffer containing D_H2O (99.9% atom D) instead of H2O. The functionality of NarGHI in our samples was confirmed spectrophotometrically by measuring the quinol:nitrate oxidoreductase activity. Stabilization of the semiquinone at the Q_D site was achieved through redox titrations under the same conditions as those used in our previous works (10, 11, 13). Redox potentials are given in the text with respect to the standard hydrogen electrode. The semiquinone concentration in our samples estimated from the double

**Q_D Site Menasemiquinone in Nitrate Reductase A**
QD Site Menasemiquinone in Nitrate Reductase A

integration of their corresponding EPR spectra and by comparison with a standard (1 mM CuSO4) was estimated in the range of 10–12 μM.

Pulsed EPR/ENDOR Experiments—X-band (~9 GHz) and Q-band (~34 GHz) pulsed EPR/ENDOR experiments were performed using a Bruker EleXsys E580-Q spectrometer equipped with an Oxford Instruments CF 935 cryostat. Spectra were measured at 90 K to avoid contamination from fast relaxing metal centers such as FeS centers in NarGHI (13). For the two-pulse experiments (π/2−τ−π), the echo intensity was measured as a function of magnetic field at fixed time interval τ between the two microwave pulses for field sweep ESE or as a function of τ at a fixed magnetic field value for two-pulse ESEEM.

Two-pulse and four-pulse (π/2−τ−π/2−T/2−π−T/2−π/2) ESEEM and HYSCORE (π/2−τ−π/2−τ1−π−τ2−π/2) experiments were performed at a magnetic field corresponding to the maximum intensity of the MSQD two-pulse field sweep ESE spectrum where all orientations of the semiquinone with respect to the external magnetic field contribute, giving rise to powder ESEEM/HYSCORE spectra (see the supplemental material). Spectra were processed using the Bruker Xepr software. Relaxation decays were subtracted (fitting by polynomial functions) followed by zero-filling and tapering with a Hamming window, before Fourier transformation, which finally gives the spectrum in frequency domain. All spectra are shown in absolute value mode. HYSCORE spectra are presented as contour plots.

Q-band pulsed 2H ENDOR spectra were obtained using the Mims (π/2−τ−π/2−τ−π/2−T−echo) sequence (17). A radio frequency τ pulse was applied during the time interval t. The radio frequency power was delivered by a 2-kilowatt Dressler solid state radio frequency amplifier. It was optimized for radio frequency τ pulse lengths of 40 μs for deuterium Mims ENDOR experiments. Pulsed ENDOR spectra were recorded at a magnetic field corresponding to the gj position of the nearly axial Q-band EPR signal of MSQD (10). Pulsed EPR/ENDOR spectra were simulated in the MATLAB environment using the Easyspin software package (release 3.1.0) (18).

Hyperfine and Quadrupole Interactions—A hyperfine coupling between an S = 1/2 radical and a nucleus with nuclear spin value I consists in general of (i) the isotropic contribution Aiso = 2μμIgμμIβμμI|νμμI(0)|2/3h, where |νμμI(0)|2 is the electron spin density at the nucleus, gμμI and gμμI are electron and nuclear g-factors, respectively, βμμI and βμμI are Bohr and nuclear magnetic moments, respectively, h is Planck’s constant, and (ii) the anisotropic contribution described by the traceless dipolar coupling tensor T. In most cases, T can be assumed to be axial, with principal values (−T1, T1, 2T).

The hyperfine couplings of different isotopes of the same element are proportional to a very good approximation to the corresponding gμμI values. In this study, the direct and simultaneous determination of Aiso and T of the protons interacting with MSQD, were derived from the analysis of HYSCORE cross-peak contours as detailed in the supplemental material (19).

A 2H nucleus has a quadrupole moment that interacts with the electric field gradient at the nucleus. The components of the electric field gradient tensor are defined in its principal axis system and ordered according to |qzz| ≥ |qyy| ≥ |qxx|. This traceless tensor can then be fully described by only two parameters: (i) the 2H nuclear quadrupole coupling constant κ = e2qzzQ/h, where e is the charge of electron, Q is the 2H nuclear electric quadrupole moment; and (ii) the asymmetry parameter η = qyy − qxx/qzz. κ is a measure of the strength of the interaction between the nuclear quadrupole moment and the electric field gradient at the 2H nucleus site due to anisotropic charge distribution around the nucleus, whereas η is a measure of the deviation of this distribution from axial symmetry. Thus, the electric field gradient is related to the specific binding geometry. Its components can, therefore, be used to obtain detailed information on hydrogen bonds (20–26). In this study the parameters κ and η of the 2H interacting with MSQD were estimated by simulation of the Q-band 2H Mims ENDOR spectrum.

RESULTS

X-band Pulsed EPR (Field Sweep, Two-pulse ESEEM)—X-band field sweep ESE spectra of NarGHI-enriched IMVs were recorded at 90 K in samples redox-poised at ~−100 mV prepared in either 1H2O or 2H2O. They show a single line from the MSQ stabilized at the QD site of NarGHI with g ~2.0045 and the width ~0.8 mT in 1H2O (13). Replacement of 1H2O by 2H2O decreases the line width by less than 0.1 mT (Fig. 1A). The weakness of this effect is due to the primary contribution to the line shape of the g-tensor anisotropy, which was previously resolved using numerical simulation of the MSQD Q-band EPR spectrum (10). The two-pulse spin echo decay of the radical measured in 1H2O at 90 K is depicted in Fig. 1B. It mainly shows the modulation associated with weakly coupled protons in the immediate environment, with Zeeman frequencies νj(1H)
most extended anisotropic contour, with the largest deviation indicate a significant anisotropic hyperfine component. Considerably from the normal to the diagonal. These two features observed for cross-peaks 1-1 and 4, and 4

proton satisfy the relationships

\[ A_{|1|} = |A_{iso}| + T \]

\[ |A_{|2|} = |A_{iso} + 2T | \]

\[ A_{iso}A_{T} \]

This indicates that the corresponding hyperfine couplings for a given appearance of these correlations in the (1H) magnetically coupled to the radical. The several protons are magnetically coupled to the MSQD. To increase spectral resolution and thus provide more detailed information about the proton environment of MSQD, HYSCORE experiments were carried out and are shown below.

**X-band \( ^{1}H \) HYSCORE**—The low frequency components of the X-band HYSCORE spectra of MSQD were previously shown and analyzed in detail. They revealed cross-peaks arising from a single\( ^{14}N \) hyperfine coupling assigned to the heme bD ligand His-66 residue (12, 13). In addition to these \( ^{14}N \) signals, several cross-features from protons symmetrically positioned with respect to the \( ^{1}H \) Zeeman frequency \( (\nu_z) \approx 14.7 \text{ MHz} \) are clearly resolved in the 10–20-MHz frequency range in the (+, +) quadrant of these spectra (Fig. 2A). This indicates that several protons are magnetically coupled to the radical. The appearance of these correlations in the (+, +) quadrant indicates that the corresponding hyperfine couplings for a given proton satisfy the relationships \( |T + 2A_{iso}| < 4\nu_z(\text{H}) \) (27). To further analyze the spectrum and discriminate between exchangeable and non-exchangeable features, HYSCORE experiments were also performed under the same conditions in the sample prepared in \( ^{2}H_2O \). Fig. 2 shows the proton region of the corresponding HYSCORE spectra recorded with \( \tau = 204 \text{ ns} \) in \( ^{2}H_2O \) (Fig. 2A) or \( ^{2}H_2O \) (Fig. 2B). In addition to the diagonal peak at \( \nu_z(\text{H}) \approx 14.7 \text{ MHz} \), four pairs of cross-features located symmetrically relative to the diagonal are well resolved in the spectrum shown in Fig. 2A. They are designated 1, 1′, 2, 2′, 3, 3′, 4, and 4′. The ridges 2–2′ exhibit the smallest resolved hyperfine splitting, of the order of \( \sim 2 \text{ MHz} \), whereas the largest one is observed for cross-peaks 1–1′. Cross-ridges 3–3′ possess the most extended anisotropic contour, with the largest deviation from the diagonal, whereas cross-peaks 4–4′ deviate significantly from the normal to the diagonal. These two features indicate a significant anisotropic hyperfine component. Contours 1–1′ and 2–2′ are approximately normal to the diagonal, suggesting a smaller anisotropy. Cross-peaks 1 and 4 partially overlap.

Cross-peaks 3–3′ and 4–4′ completely disappear in the proton HYSCORE spectrum measured in \( ^{2}H_2O \), demonstrating that they are produced by at least one exchangeable proton (Fig. 2B). In contrast, cross-peaks 1–1′ and 2–2′ still appear in the spectrum measured in \( ^{2}H_2O \), showing that they arise from non-exchangeable (i.e., covalently bound) protons.

Quantitative analysis of the cross-peak contour line shapes indicates that cross-peaks 3, 3′, 4, and 4′ are produced by a single exchangeable proton (supplemental Fig. S2 and supplemental Table S1). Hence, HYSCORE signals derive from three protons coupled to MSQD, H1 (1-1′), H2 (2-2′), and H3 (3′-3′-4-4′). Among them, H3 is exchangeable. The isotropic \( (A_{iso}) \) and anisotropic \( (T) \) components of the three \( ^{1}H \) hyperfine tensors are given in Table 1. The magnitude of the hyperfine couplings deduced from the analysis of HYSCORE spectra are consistent with our previous preliminary observations of \( ^{1}H \) continuous wave ENDOR resonances with corresponding estimated hyperfine couplings \( A_1 \approx 5.7 \text{ MHz} \) (H3) and \( A_2 \approx 3.3 \text{ MHz} \) (H2) (10, 11).

**X-band \( ^{1}H \) Four-pulse ESEEM**—Additional information about the interacting protons was obtained from one-dimensional four-pulse ESEEM spectra, which are particularly useful for the observation of proton sum combination lines with improved resolution (27, 28). The four-pulse ESEEM spectrum

\[
\text{FIGURE 2. Proton part of HYSCORE spectra of MSQD in } ^{1}H_2O \text{ (A) or in } ^{2}H_2O \text{ (B) with time } \tau = 204 \text{ ns. The microwave frequency was } 9.6944 \text{ GHz (A) and } 9.6934 \text{ GHz (B), and the magnetic field was } 345.2 \text{ mT. For both spectra, the durations of the } \pi/2 \text{ and } \pi \text{ pulses were 12 and 24 ns, respectively, with equal amplitude. 256 points were recorded in each dimension. } t_1 \text{ and } t_2 \text{ were incremented in steps of 16 ns from their initial value.}
\]

\[
\text{TABLE 1 Hyperfine tensors derived from contour line shape analysis of HYSCORE spectra}
\]

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Proton} & (\text{MHz}) & |A_{iso}| & |A_{T}| & |A_{iso} + 2T| & \text{Assignment} \\
\hline
H_{1a} & 7.67 & 8.03 & 28 & 0.55 & \text{methyl protons} \\
H_{1b} & 7.63 & 8.03 & 28 & 0.55 & \text{methyl protons} \\
H_{2a} & 7.24 & 3.22 & 3.22 & 1.3 & \beta\text{-methylene proton} \\
H_{2b} & 7.24 & 3.22 & 3.22 & 1.3 & \beta\text{-methylene proton} \\
H_{3a} & 7.59 & 5.67 & 5.67 & 1.01 & \text{H-bond proton} \\
H_{3b} & 7.59 & 5.67 & 5.67 & 1.01 & \text{H-bond proton} \\
\hline
\end{array}
\]

\[
\text{Q}_D \text{ Site Menasemiquinone in Nitrate Reductase A}
\]
of MSQD in 1H2O buffer contains two well resolved lines in the region of the proton around 2ν(1H) as shown in Fig. 3A. The most intense line appears exactly at the 2ν(1H) frequency and represents the contribution of weakly coupled protons from the protein environment. In addition, the spectrum exhibits a peak of lower intensity shifted from 2ν(1H) to higher frequencies by ~1.2 MHz. This shifted peak completely disappears in the spectra of the sample prepared in 2H2O (Fig. 3B). This indicates that the line shifted from 2ν(1H) arises from an exchangeable proton. The shift observed in the four-pulse ESEEM is well described by

$$\Delta = 9T^2/16ν(1H)$$  \hspace{1cm} (Eq. 1)

from which the anisotropic component T of the hyperfine coupling can be estimated (see supplemental material). The shift of ~1.2 MHz corresponds to T = 5.7 MHz, which is in very good agreement with the corresponding value determined for H3 from the analysis of the HYSCORE spectra. The expected shifts from H1 and H2 (0.06 and 0.05 MHz, respectively) are too small to be resolved in a four-pulse ESEEM spectrum (supplemental Table S2). The proton sum combination peak therefore confirms the assignment made in the HYSCORE spectra for the exchangeable proton and the hyperfine coupling determined from these spectra.

Q-band 2H Mims ENDOR—Further details concerning exchangeable protons coupled to MSQD were obtained through the use of pulsed 2H ENDOR spectroscopy. Fig. 4 shows the Q-band 2H Mims ENDOR spectrum of MSQD in NarGHI-enriched IMVs prepared in 2H2O. It has been recorded at a magnetic field value corresponding to the maximum intensity of the nearly axially symmetric Q-band EPR spectrum of MSQD with g-tensor principal values g⊥ = 2.0061, g∥ = 2.0051, and gao = 2.0023 ± 0.0001 for gao (Fig. 4, top) (10). The ENDOR spectrum exhibits two pairs of well resolved intense lines located symmetrically with respect to the 2H nuclear Larmor frequency (ν(2H)) ~7.84 MHz) and subject to the nuclear quadrupole interaction. The splitting of their center away from the Larmor frequency (~0.8 MHz) is determined by the hyperfine coupling constant, and the splitting within the pair (~0.13 MHz) is given by the quadrupole interaction. The same holds for the two pairs of less intense lines resolved in the spectrum shown in Fig. 4, separated by ~1.5 MHz and split each by ~0.26 MHz. The hyperfine coupling values of ~0.8 and ~1.5 MHz are first estimates of the A⊥ and A∥ components of an almost purely dipolar hyperfine tensor. They are very close to those found for H3 and scaled to the 3H nucleus, i.e. A⊥ = 0.87 MHz and A∥ = 1.77 MHz. Similarly, a nuclear quadrupole coupling constant κ ~ 4/3 × 0.13 ~0.173 MHz can be estimated from the 0.13-MHz splitting of the two most intense doublets. A numerical simulation of the spectrum is shown in Fig. 4. It was obtained using the proton hyperfine coupling values of H3 deduced from the analysis of the HYSCORE spectra and rescaled by the factor gao(1H)/gao(2H) ~6.5. This best simulation was obtained assuming that the g∥, A⊥, and Q-tensors are collinear, with quadrupole parameters κ (~0.176 ± 0.004) MHz and η = 0.20 ± 0.05. Finally, this procedure allowed us to select unambiguously the right set (|AIso|, |T|) for H3 from the two alternatives (Table 1). Overall, the data show that a single exchangeable proton is coupled to the radical, in agreement with the analysis above.

**DISCUSSION**

**Single Exchangeable Proton Coupling with Peculiar Hyperfine Coupling Characteristics**—Our data clearly show the presence of a single exchangeable proton in the vicinity of MSQD, characterized by |AIso| = 0.06 MHz and |T| = 5.73 MHz. Such hyperfine coupling constants are in the range of those measured for
exchangeable protons coupled to protein-bound semiquinones and assigned to protons hydrogen-bonded to the quinone carbonyl oxygens. Typical examples include exchangeable proton couplings to the menasemiquinone stabilized at the QH site of the aad₃ menaquinol oxidase from Bacillus subtilis ([T] = 5.6 MHz and |Aiso| = 5.4 MHz) or to the photoaccumulated phyllosemi酮one A⁻ in Thermococcus elongatus photosystem I ([T] ~3.7 MHz and |Aiso| ~0.1 MHz) (29, 30). Thus, we assign H3 to a proton involved in H-bonding to one of the MSQ₀ carbonyl oxygens. However, the hyperfine coupling tensor to H3 has atypical properties as it combines both an almost zero isotropic hyperfine coupling constant Aiso and a large anisotropic part T. The magnitude of the H-bond tensor is determined by the geometry of the H-bond. A small Aiso is expected when the bond lies in the molecular plane due to the small overlap between the hydrogen 1s orbital and the oxygen 2pz orbital forming part of the semiquinone singly occupied molecular orbital. Almost purely anisotropic hyperfine tensors have thus been observed for in-plane hydrogen-bonded protons to unsubstituted quinones measured in alcoholic solvent, whereas the corresponding |T| values do not exceed 3 MHz (23, 24, 31, 32). The T value measured for H3 is one of the largest measured so far for a proton hydrogen-bonded to a semiquinone. According to density functional theory calculations, the large T value of H3 is predicted to account for a short hydrogen-bond length, typically in the range of 1.3–1.4 Å (22). In this case, Sinnecker et al. (21, 22, 24) have shown that the point dipole model does not work due to the increased covalent character of the H-bond that is not covered in the point dipole approximation. A more reliable alternative approach is to evaluate H-bond distances from the nuclear quadrupole coupling constant of ²H. As empirically proposed by Soda and Chiba (25) and Hunt and Mackay (26), it has been shown that the nuclear quadrupole coupling constant of ²H nuclei H-bonded to semiquinones follows a r⁻³(O–²H) dependence of the form

\[ \kappa = a - \frac{b}{r(O\cdash H)} \text{[kHz]} \]  

(Eq. 2)

where a and b are empirical parameters (22). Using a = 319 kHz and b = 607 kHz Å³ (20) and the value of \( \kappa = 176 \pm 4 \text{ kHz} \) deduced from our work, we obtain from Equation 2 a bond length of r(O–²H) = 1.62 ± 0.02 Å. This value is in the range of short hydrogen bonds for biological systems. For instance, it is similar to that formed from the carbonyl oxygen O4 of the ubisemiquinone Qₐ⁻ in the RC from Rhodobacter sphaeroides R-26 to the imidazole nitrogen N8 of His M219 (r(O–²H) = 1.60 ± 0.04 Å) (20).

Assignment of Non-exchangeable Proton Couplings—In addition to H3, two non-exchangeable proton couplings H1 and H2 are clearly resolved in the HYSCORE spectra of MSQ₀ measured in ²H₂O. For their assignment, we rely on previous experimental and theoretical studies on phylloquinone (also called vitamin K₁) and menaquinone (vitamin K₂) radicals examined in liquid and solid organic solvents or in proteins (23, 29, 30, 33–37). Indeed, these quinones share the same naphthoquinone ring structure methylated at the second position but differ in their aliphatic side chain attached at the 3-position (see supplemental Fig. S1). It has been shown both experimentally and theoretically that the aliphatic side chain properties have only a weak influence on the proton hyperfine coupling tensors measured in organic solvents (23, 33, 34, 38, 39). From these previous studies, it is evident that the non-exchangeable proton couplings from H1 and H2 > 2 MHz originate from the ring methyl protons, from the β-methylene isoprenyl protons, or from α-protons directly attached to the quinone ring. Due to rapid rotation of the methyl group even at low temperature, methyl protons of vitamin K molecules have equal hyperfine tensors and give prominent ENDOR/ESR signals. They are characterized by an almost axial hyperfine tensor, a predominant isotropic hyperfine coupling value in the range of 6.8–12.3 MHz, and a characteristic relative hyperfine anisotropy (\( A_{\perp}/A_{\parallel} \)) in the range of 0.26–0.45 with \( A_{\perp} > A_{\parallel} > 0 \). The latter value increases up to 0.76 for the methyl protons of the asymmetrically bound ubisemiquinone Qₐ⁻ in the RC from R. sphaeroides (2). Based on these results, we assign H1 to the methyl protons of MSQ₀ with \( A_{\iso} = 5.53 \text{ MHz} \) and \( T = +1.25 \text{ MHz} \) leading to \( (A_{\parallel} - A_{\perp})/A_{\iso} \) value of ~0.68 (Fig. 5).

Hyperfine data for β-methylene protons and ring α protons in semiquinones are less abundant. Unlike the methyl protons, the methylene protons are not expected to rotate freely (40). They are also characterized by a nearly axial hyperfine tensor with \( A_{\perp} > A_{\parallel} > 0 \) (40, 41). In contrast, a high degree of anisotropy is expected for a ring α proton hyperfine tensor due to the short distance of the proton and the spin density (30, 40). Because of the axial symmetry of its hyperfine coupling tensor, we tentatively assign H2 to one of the β-methylene isoprenyl protons with \( A_{\iso} = +0.96 \text{ MHz} \) and \( T = +1.18 \text{ MHz} \) (Fig. 5). Overall, the \(^1\text{H} \) hyperfine coupling constants determined in this work are consistent with those estimated from our previous continuous wave X-band \(^1\text{H} \) ENDOR studies of MSQ₀ (10, 11).

**Strongly Asymmetric Spin Distribution in MSQ₀**—The \(^1\text{H} \) hyperfine coupling constants are an excellent probe of the asymmetry of the spin density distribution in the quinone. In particular, the predominant isotropic component, \( A_{\iso} \) of hyperfine coupling to the methyl protons is directly proportional to the unpaired spin density in the π orbital on the adjacent α-carbon (\( \rho_{\alpha} \)), as described by the McConnell relation, \( A_{\iso} = \rho_{\alpha} B_\alpha^2 / 2 \), where \( B_\alpha \) has been taken in the range from 120 to 212 MHz (42). The corresponding value for the methyl protons of MSQ₀ (\( A_{\iso} = 5.53 \text{ MHz} \)) is the smallest one ever reported.
for these protons for vitamin K molecules bound to proteins or in protic solvents (23, 29, 30, 33, 34, 36, 38, 39). In particular, the spin density on the Cα at the 2-position, which is sensed by the methyl protons, is reduced by ~30% in MSQD, as compared with the symmetrically hydrogen bonded MSQ-4 prepared in 2-propanol (39). This decrease can be explained by a strong asymmetry of hydrogen bonding to the carbonyl oxygens of MSQD in NarGHI, which leads to a redistribution of both the spin density and charges within the quinone ring (2, 43, 44). Indeed, a stronger hydrogen bond to the carbonyl oxygen O1, as compared with oxygen O4, is expected to lead to an increase of spin density on carbon 3 but a decrease of the spin density on carbon 2 as observed here for MSQD. A similar but less pronounced spin density shift has been proposed for the menasemiquinone-9 in the Q4 site of the RC from *Rhodopseudomonas viridis* for which the methyl protons isotropic hyperfine coupling constant is about 6.8 MHz (39). In contrast, the large isotropic constants for methyl protons of the MSQ-7 in fine coupling constant is about 6.8 MHz (39). In contrast, the large isotropic constants for methyl protons of the MSQ-7 in *Thermosynechococcus elongatus* (Aiso = 9.8 MHz) (30) suggest that a stronger hydrogen bond to oxygen O4 is formed as compared with oxygen O1, a strongly asymmetric binding mode reverse to that observed in NarGHI.

**Model for MSQD Binding to NarGHI and Mechanistic Implications**—Altogether, the data inferred from the present work allow us to refine the MSQD binding model previously proposed (13). We conclude that the asymmetrical spin density distribution in MSQD, is primarily due to the strong hydrogen bond formed to the O1 oxygen of MSQD involving the exchangeable H3 proton coupling (Fig. 5). Hence, the latter appears mostly responsible for the transfer of spin density from the radical to the interacting nitrogen nucleus that was deduced from the measurement of a small 14N isotropic hyperfine coupling of Aiso −0.8 MHz to MSQD using HYSCORE spectroscopy. Based on the measurement of its nuclear quadrupole parameters by S-band HYSCORE spectroscopy (κ = 0.49, η = 0.50), this nucleus was assigned to the Nα imidazole nitrogen from the heme b2 axial ligand His-66 (13). In addition, this model is consistent with the relatively small η value found for this imidazole nitrogen that is close to that predicted by density functional theory calculations on imidazoles forming a strong in-plane hydrogen bond to one of the benzosemiquinone oxygen atoms (45). Finally, the presence of the positive charges on the nearby heme Fe2+ ion may also contribute to the observed asymmetrical spin distribution in MSQD.

Interestingly, a single strong and highly ordered H-bond to MSQD was detected in the present work by using both 1H HYSCORE and 3H Q-band ENDOR spectroscopies. In the most recent studies of protein-bound semiquinones, the radicals appear to be coupled to at least two exchangeable protons assigned to those involved in H-bonds (20, 29, 46–49). One-sided H-bond was resolved only for the photoaccumulated phyllosemiquinone A1− in photosystem I (30, 50). Based on their experimental and theoretical results, Niklas et al. (30) have shown that the single detected short ~1.6 Å H-bond can fully account for the observed asymmetry in the spin density distribution of the SQ in the A1 site. Remarkably, both the H-bond length and the ~30% variation of the spin density on the Cα at the 2-position of the phyllosemiquinone in the A1 site with respect to the symmetrically hydrogen bonded radical are comparable with the corresponding values measured for MSQD. Thus, our results indicate that MSQD most likely binds to the protein via a one-sided H-bond.

Consequently, our present work indicates that the O4 carboxyl oxygen of MSQD is not protonated in this intermediate state, showing that at least one proton has to be released to the periplasm consecutively to the first electron transfer step to heme b2. This step likely involves Lys-86, a residue that is located at the protein surface, at the entrance of the QD cavity, and that is required for correct binding of quinol analogues and for semiquinone detection at the Q5 site of NarGHI (11, 13, 14). Thus, we speculate that Lys-86 could be a direct hydrogen bond donor to the quinol molecule, facilitating proton abstraction at the quinol O4 oxygen and, concomitantly, the first electron transfer step. This would be accompanied by a movement of Lys-86 away from the substrate, leading to an asymmetric binding mode of the semiquinone intermediate and allowing for proton release toward the periplasm (Fig. 5). Whether quinol deprotonation at the O1 oxygen is coupled to the first or the second electron transfer step remains unclear. Additional studies aimed at measuring the pH dependence of the redox reactions occurring at the QD site will be useful to further understand how quinol deprotonation and electron transfer are synchronized at the QD site. Such studies are currently being performed in our laboratories.

**Role of Protein Environment in Quinol Utilization and Semiquinone Stabilization**—The high stabilization of MSQD in NarGHI is directly related to the redox potentials of the redox transitions MQH2/MSQ (Em,7.5 = −150 mV) and MSQ/MQ (Em,7.5 = −40 mV), which are both thermodynamically favorable for electron transfer to the b2 heme (10). We questioned whether the unusually high stabilization of the semiquinone state at the NarGHI QD site can be related to the strongly asymmetric binding mode of MSQD, mainly due to the short in-plane H-bond formed to the radical. Remarkably, the two other protein-bound semiquinones with redox properties most strongly affected by the protein environment as compared with the corresponding species in alcoholic solvents are the very low potential (Em ~ −750 mV) phyllosemiquinone anion A1− in photosystem I and the high affinity ubisemiquinone at the QH site of cytochrome bo3, which has a high stability, albeit 10 times smaller than that measured for MSQD. In both cases, the semiquinones interact with the protein environment in a very asymmetric manner (46, 51–53). This contrasts to the much lower stability of the more symmetrically bound semiquinones at the QH site of photosynthetic bacterial RC (48, 54) or at the Q4 site in *R. sphaeroides* bc1 complex (47, 55). In addition, the strong H-bond to MSQD is expected to withdraw electron density and stabilize the semiquinone form, thereby raising the redox potential of the second oxidation step from semiquinone to quinone, as experimentally measured. Consequently, we hypothesize that the atypical binding mode of MSQD could strongly contribute to its unusual redox properties. In addition, other effects such as the electrostatic environment of the nearby protein should also be taken into account. Evaluating
their respective contribution to the MSQ$_D$ redox properties requires further work. Finally, the functional implications of this high stabilization remain to be established.

It has been shown both experimentally and theoretically that the presence of bulky substituents on the quinone ring force hydrogen-bond formation out-of-plane, thereby increasing simultaneously the isotropic and anisotropic coupling of the hydrogen-bonded protons (32, 56). Remarkably, measurement of a small A$_{iso}$ and a simultaneous large T value for the proton hydrogen-bonded to MSQ$_D$ indicates that the protein environment around the radical strongly constrains the geometry of the hydrogen bond by maintaining a short in-plane H-bond to the radical, thus leading to the observed peculiar hyperfine coupling characteristics. We have recently shown that an endogenous USQ$_D$ can also be stabilized at the NarGHI Q$_D$ site (12). It binds to the protein via an H-bond to the same nitrogen as mena- and ubisemiquinone does, i.e. most likely His-66 N$_H$. The similar $^{14}$N HYSCORE pattern observed for both radicals suggests that the H-bond involved in binding USQ$_D$ has similar characteristics to that detected in the present study. This provides further support for its involvement in ubisemiquinone stabilization at the Q$_D$ site. Finally, our work indicates that the protein environment modulates and utilizes various substrates at this Q-site and to none stabilization at the QD site. Finally, our work indicates supports for its involvement in ubisemiquinone stabilization.

Concluding Remarks—From the experiments reported in this work, we conclude that MSQ$_D$ is involved in a single strong in-plane and highly ordered H-bond with a solvent exchangeable proton. This strongly asymmetric binding causes a shift of the electron spin density over the quinone ring consistent with the formation of a strong hydrogen bond to the quinone carbonyl oxygen O1. This peculiar binding mode could strongly contribute to the unusual redox properties of MSQ$_D$.

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REFERENCES

1. Nicholls, D. G., and Ferguson, S. J. (2002) Bioenergetics, Third Ed., Academic Press, London
2. Lubitz, W., and Feher, G. (1999) The primary and secondary acceptors in bacterial photosynthesis III. Characterization of the quinone radicals Q$_A^{\cdot}$ and Q$_B^{\cdot}$ by EPR and ENDOR. Appl. Magn. Reson. 17, 1–48
3. Srinivasan, N., and Golbeck, J. H. (2009) Protein-cofactor interactions in bioenergetic complexes: the role of the A$_{10A}$ and A$_{10B}$ phylloquinones in photosystem I. Biochim. Biophys. Acta 1787, 1057–1088
4. Stowell, M. H., McPhillips, T. M., Rees, D. C., Soltis, S. M., Abresch, P., and Feher, G. (1997) Light-induced structural changes in photosynthetic reaction center: implications for mechanism of electron-proton transfer. Science 276, 812–816
5. Lancelli, P., Savoyant, A., Grimaldi, S., Magalon, A., Guigliarelli, B., and Bertrand, P. (2007) New method for the spin quantitation of $^{4}$Fe-$^{4}$S$^+$ clusters with S = $\frac{1}{2}$: application to the F50 center of the NarGHI nitrate reductase from Escherichia coli. J. Phys. Chem. B 111, 13632–13637
6. Guigliarelli, B., Magalon, A., Asso, M., Bertrand, P., Frixon, C., Giordano, G., and Blasco, F. (1996) Complete coordination of the four Fe-$S$ centers of the $\beta$ subunit from Escherichia coli nitrate reductase: physiological, biochemical, and EPR characterization of site-directed mutants lacking the highest or lowest potential $^{4}$Fe-$^{4}$S$^+$ clusters. Biochemistry 35, 4828–4836
7. Blasco, F., Guigliarelli, B., Magalon, A., Asso, M., Giordano, G., and Rothery, R. A. (2001) The coordination and function of the redox centers of the membrane-bound nitrate reductases. Cell Mol. Life Sci. 58, 179–193
8. Rothery, R. A., Blasco, F., Magalon, A., and Weiner, J. H. (2001) The d-heme cytochrome b subunit (NarI) of Escherichia coli nitrate reductase A (NarGHI): structure, function, and interaction with quinols. J. Mol. Microbiol. Biotechnol. 3, 273–283
9. Bertero, M. G., Rothery, R. A., Palak, M., Hou, C., Lim, D., Blasco, F., Weiner, J. H., and Strynadka, N. C. (2003) Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A. Nat. Struct. Biol. 10, 681–687
10. Grimaldi, S., Lancelli, P., Bertrand, P., Blasco, F., and Guigliarelli, B. (2005) Evidence for an EPR-detectable semiquinone intermediate stabilized in the membrane-bound subunit NarI of nitrate reductase A (NarGHI) from Escherichia coli. Biochemistry 44, 1300–1308
11. Lancelli, P., Magalon, A., Bertrand, P., Guigliarelli, B., and Grimaldi, S. (2007) High stability semiquinone intermediate in nitrate reductase A (NarGHI) from Escherichia coli is located in a quinol oxidation site close to heme b$_D$. Biochemistry 46, 5323–5329
12. Arias-Cartín, R., Lyubenova, S., Ceccaldi, P., Prisner, T., Magalon, A., Guigliarelli, B., and Grimaldi, S. (2010) HYSCORE evidence that endogenous mena- and ubisemiquinone bind at the same Q site (Q$_D$) of Escherichia coli nitrate reductase A. J. Am. Chem. Soc. 132, 5942–5943
13. Grimaldi, S., Arias-Cartín, R., Lancelli, P., Lyubenova, S., Endeward, B., Prisner, T. F., Magalon, A., and Guigliarelli, B. (2010) Direct evidence for nitrogen ligation to the high stability semiquinone intermediate in Escherichia coli nitrate reductase A. J. Biol. Chem. 285, 179–187
14. Bertero, M. G., Rothery, R. A., Boroumand, N., Palak, M., Blasco, F., Ginet, N., Weiner, J. H., and Strynadka, N. C. (2005) Structural and biochemical characterization of a quinol-binding site of Escherichia coli nitrate reductase A. J. Biol. Chem. 280, 14836–14843
15. Arias-Cartín, R., Grimaldi, S., Pommier, J., Lancelli, P., Schaefver, C., Arnoux, P., Giordano, G., Guigliarelli, B., and Magalon, A. (2011) Cardiolipin-based respiratory complex activation in bacteria. Proc. Natl. Acad. Sci. U.S.A. 108, 7781–7786
16. Potter, L. C., Millington, P., Griffiths, L., Thomas, G. H., and Cole, J. A. (1999) Competition between Escherichia coli strains expressing either a periplasmic or a membrane-bound nitrate reductase: does Nap confer a selective advantage during nitrate-limited growth? Biochem. J. 344, 77–84
17. Mims, W. B. (1965) Pulsed ENDOR experiments. Proc. R. Soc. Lond. Ser. A 283, 452–457
18. Stoll, S., and Schweiger, A. (2006) EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. J. Magn. Reson. 178, 42–55
19. Dikanov, S. A., and Bowman, M. K. (1995) Cross-peak lineshape of two-dimensional ESEEM spectra in disordered S = $\frac{1}{2}$, I = $\frac{1}{2}$ spin systems. J. Magn. Reson. 116, 125–128
20. Flores, M., Isaacsen, R., Arbes, E., Calvo, R., Lubitz, W., and Feher, G. (2007) Protein-cofactor interactions in bacterial reaction centers from Rhodobacter sphaeroides R-26: II: geometry of the hydrogen bonds to the primary quinone formula by $^{1}$H and $^{13}$C ENDOR spectroscopy. Bioophys. J. 92, 671–682
21. Sinnecker, S., Flores, M., and Lubitz, W. (2006) Protein-cofactor interactions in bacterial reaction centers from Rhodobacter sphaeroides R-26: effect of hydrogen bonding on the electronic and geometric structure of the primary quinone. A density functional theory study. Phys. Chem. Chem. Phys. 8, 5659–5670
22. Sinnecker, S., Reijerse, E., Neeve, F., and Lubitz, W. (2004) Hydrogen bond geometries from electron paramagnetic resonance and electron-nuclear double resonance parameters: density functional study of quinone radical anion solvent interactions. J. Am. Chem. Soc. 126, 3280–3290
23. Epel, B., Niklas, J., Sinnecker, S., Zimmermann, H., and Lubitz, W. (2006) Phylloquinone and related radical anions studied by pulse electron nuclear double resonance spectroscopy at 34 GHz and density functional theory. J. Phys. Chem. B 110, 11549–11560
Q_D Site Menasemiquinone in Nitrate Reductase A

24. Flores, M., Isaacson, R. A., Calvo, R., Feher, G., and Lubitz, W. (2003) Probing hydrogen bonding to quinone anion radicals by 1H and 2H ENDOR Spectroscopy at 35 GHz. Chem. Phys. 294, 401–413

25. Soda, G., and Chiba, T. (1969) Deuterium magnetic resonance study of cupric sulfate pentahydrate. J. Chem. Phys. 50, 439–455

26. Hunt, M. J., and Mackay, A. L. (1974) Deuterium and nitrogen pure quadrupole resonance in deuterated amino acids. J. Magn. Reson. 150, 402–414

27. Schweiger, A., and Jeschke, G. (2001) Principles of Pulse Electron Paramagnetic Resonance, Oxford University Press, New York

28. Reijerse, E. J., and Dikanov, S. A. (1991) Electron spin echo envelope modulation spectroscopy on orientationally disordered systems: Line shape singularities in $S = \frac{1}{2}$, $I = \frac{1}{2}$ spin systems. J. Chem. Phys. 95, 836–845

29. Yi, S. M., Narasimhulu, K. V., Samoilova, R. I., Gennis, R. B., and Dikanov, S. A. (2010) Characterization of the semiquinone radical stabilized by the cytochrome $a_3$-$600$ menaquinol oxidase of Bacillus subtilis. J. Biol. Chem. 285, 18241–18251

30. Niklas, J., Epel, B., Antonkine, M. L., Sinner, S., Pandela, M. E., and Lubitz, W. (2009) Electronic structure of the quinone radical anion $A_{1}^-$ of photosystem I investigated by advanced pulse EPR and ENDOR techniques. J. Phys. Chem. B 113, 10367–10379

31. O’Malley, P. J., and Babcock, G. T. (1986) Powder ENDOR spectra of p-Benzoquinone anion radical: Principal hyperfine tensor components for ring protons and hydrogen-bonded protons. J. Am. Chem. Soc. 108, 3995–4001

32. MacMillan, F., Lendzian, F., and Lubitz, W. (1995) EPR and ENDOR characterization of semiquinone anion radicals related to photosynthesis. Magn. Reson. Chem. 33, 581–593

33. Das, M. R., Connor, H. D., Leniart, D. S., and Freed, J. H. (1970) An electron nuclear double resonance and electron spin resonance study of semiquinones related to vitamins K and E. J. Am. Chem. Soc. 92, 2258–2268

34. Teutloff, C., Bittl, R., and Lubitz, W. (2004) Pulse ENDOR studies on the radical pair $P_{1/2}^{o}A_{1}^-$ and the photoaccumulated quinone acceptor $A_{1}^-$ of photosystem I. Appl. Magn. Reson. 26, 5–21

35. O’Malley, P. J. (1999) Density functional calculated spin densities and hyperfine couplings for hydrogen-bonded 1,4-naphthosemiquinone and phyllosemiquinone anion radicals: a model for the $A_3$ free radical formed in photosystem I. Biochim. Biophys. Acta 1411, 101–113

36. Hastings, S. F., Heathcote, P., Ingledew, W. J., and Rigby, S. E. (2000) ENDOR spectroscopic studies of stable semiquinone radicals bound to the Escherichia coli cytochrome $b_5$ quinol oxidase. Eur. J. Biochem. 267, 5638–5645

37. Rigby, S. E., Evans, M. C., and Heathcote, P. (2001) Electron nuclear double resonance (ENDOR) spectroscopy of radicals in photosystem I and related type 1 photosynthetic reaction centers. Biochim. Biophys. Acta 1507, 247–259

38. Rigby, S. E., Evans, M. C., and Heathcote, P. (1996) ENDOR and special triple resonance spectroscopy of $A_{1}^-$ of photosystem I. Biochemistry 35, 6651–6656

39. Gardiner, A. T., Zech, S. G., MacMillan, F., Käss, H., Bittl, R., Schlodder, E., Lendzian, F., and Lubitz, W. (1999) Electron paramagnetic resonance studies of zinc-substituted reaction centers from Rhodopseudomonas viridis. Biochemistry 38, 11773–11787

40. Zheng, M., and Dismukes, G. C. (1996) The conformation of the isoprenyl chain relative to the semiquinone head in the primary electron acceptor (Q_b) of higher plant PSII (plastoquinone) differs from that in bacterial reaction centers (ubisemiquinone or menasemiquinone) by ~90 degrees. Biochemistry 35, 8955–8963

41. Kevan, L., and Kispert, L. D. (1976) Electron Spin Double Resonance Spectroscopy, John Wiley & Sons, Inc., New York

42. McConnell, H. M. (1956) Indirect hyperfine interactions in the paramagnetic resonance spectra of aromatic free radicals. J. Chem. Phys. 24, 764–766

43. Lubitz, W., Abresch, E. C., Debus, R. J., Isaacson, R. A., Okamura, M. Y., and Feher, G. (1985) Electron nuclear double resonance of semiquinones in reaction centers of Rhodopseudomonas sphaeroides. Biochim. Biophys. Acta 808, 464–469

44. Feher, G., Isaacson, R. A., Okamura, M. Y., and Lubitz, W. (1985) in Antennas and Reaction Centers of Photosynthetic Bacteria (Michel-Beyerle, M. E., ed) Vol. 42, pp. 174–189, Springer-Verlag, Berlin

45. Fritscher, J. (2004) Influence of hydrogen bond geometry on quadrupole coupling parameters: a theoretical study of imidazole–water and imidazole–semiquinone complexes. Phys. Chem. Chem. Phys. 6, 4950–4956

46. Yap, L. I., Samoilova, R. I., Gennis, R. B., and Dikanov, S. A. (2006) Characterization of the exchangeable protons in the immediate vicinity of the semiquinone radical at the $Q_b$ site of the cytochrome $b_5$, from Escherichia coli. J. Biol. Chem. 281, 16879–16887

47. Dikanov, S. A., Samoilova, R. I., Kolling, D. R., Holland, J. T., and Crofts, A. R. (2004) Hydrogen bonds involved in binding the $Q_b$ site semiquinone in the bc$_1$ complex, identified through deuterium exchange using pulsed EPR. J. Biol. Chem. 279, 15814–15823

48. Martin, E., Samoilova, R. I., Narasimhulu, K. V., Lin, T. J., Ol’Malley, P. J., Wright, C. A., and Dikanov, S. A. (2011) Hydrogen bonding and spin density distribution in the $Q_b$ semiquinone of bacterial reaction centers and comparison with the $Q_b$ site. J. Am. Chem. Soc. 133, 5525–5537

49. Chatterjee, R., Milikisivans, S., Coates, C. S., and Lakshmi, K. V. (2011) High-resolution two-dimensional $^1$H and $^{14}$N hyperfine sublevel correlation spectroscopy of the primary quinone of photosystem II. Biochemistry 50, 495–501

50. Srinivasan, N., Chatterjee, R., Milikisivans, S., Golbeck, J. H., and Lakshmi, K. V. (2011) Effect of hydrogen bond strength on the redox properties of phylloquinones: a two-dimensional hyperfine sublevel correlation spectroscopy study of photosystem I. Biochemistry 50, 3495–3501

51. Grimaldi, S., Ostermann, T., Weiden, N., Mogi, T., Miyoshi, H., Ludwig, B., Michel, H., Prisner, T. F., and MacMillan, F. (2003) Asymmetric binding of the high-affinity $Q_{b}$ semiquinone in quinol oxidase (bo$_3$) from Escherichia coli studied by multifrequency electron paramagnetic resonance spectroscopy. Biochemistry 42, 5632–5639

52. Kačprzak, S., Kaupp, M., and MacMillan, F. (2006) Protein-cofactor interactions and EPR parameters for the $Q_{b}$ quinone-binding site of quinol oxidase: a density functional study. J. Am. Chem. Soc. 128, 5659–5671

53. MacMillan, F., Kačprzak, S., Hellwig, P., Grimaldi, S., Michel, H., and Kaupp, M. (2011) Elucidating mechanisms in heme copper oxidases: the high-affinity $Q_{b}$-binding site in quinol oxidase as studied by DONUT-HYSSCORE spectroscopy and density functional theory. Faraday Discuss. 148, 315–344

54. Rutherford, A. W., and Evans, M. C. (1980) Direct measurement of the redox potential of the primary and secondary quinone electron acceptors in Rhodopseudomonas sphaeroides (wild-type) by EPR spectrometry. FEMS Lett. 110, 257–261

55. Robertson, D. E., Prince, R. C., Bowyer, J. R., Matsuura, K., Dutton, P. L., and Ohsnishi, T. (1984) Thermodynamic properties of the semiquinone and its binding site in the ubiquinol-cytochrome c (c$_{1}$) oxidoreductase of respiratory and photosynthetic systems. J. Biol. Chem. 259, 1758–1763

56. Ol’Malley, P. J. (1998) A density functional study of the effect of orientation of hydrogen bond donation on the hyperfine couplings of benzosemiquinones: relevance to semiquinone-protein hydrogen bonding interactions in vivo. Chem. Phys. Lett. 291, 367–374