Characterization of the Exchangeable Protons in the Immediate Vicinity of the Semiquinone Radical at the Q_H Site of the Cytochrome bo_3 from Escherichia coli*

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The cytochrome bo_3 ubiquinol oxidase from Escherichia coli resides in the bacterial cytoplasmic membrane and catalyzes the two-electron oxidation of ubiquinol-8 and four-electron reduction of O_2 to water. The one-electron reduced semiquinone forms transiently during the reaction, and the enzyme has been demonstrated to stabilize the semiquinone. Two-dimensional electron spin echo envelope modulation has been applied to explore the exchangeable protons involved in hydrogen bonding to the semiquinone by substitution of H_2O by D_2O. Three exchangeable protons possessing different isotropic and anisotropic hyperfine couplings were identified. The strength of the hyperfine interaction with one proton suggests a significant covalent O–H binding of carbonyl oxygen O1 that is a characteristic of a neutral radical, an assignment that is also supported by the unusually large hyperfine coupling to the methyl protons. The second proton with a large anisotropic coupling also forms a strong hydrogen bond with a carbonyl oxygen. This second hydrogen bond, which has a significant out-of-plane character, is from an NH or NH nitrogen, probably from an arginine (Arg-71) known to be in the quinone binding site. Assignment of the third exchangeable proton with smaller anisotropic coupling is more ambiguous, but it is clearly not involved in a direct hydrogen bond with either of the carbonyl oxygens. The results support a model that the semiquinone is bound to the protein in a very asymmetric manner by two strong hydrogen bonds from Asp-75 and Arg-71 to the O1 carbonyl, while the O4 carbonyl is not hydrogen-bonded to the protein.

Cytochrome bo_3 (cyt bo_3)3 is a terminal oxidase in the aerobic respiratory chain of Escherichia coli. It catalyzes the two-electron oxidation of ubiquinol-8 with a semiquinone (SQ) intermediate in an overall reaction that releases two protons to solution. The available evidence suggests that the cyt bo_3 ubiquinol oxidase has two Q binding sites (1–3): a low affinity site (Q_h) where the substrate quinol is oxidized and the product is released, and a high affinity site where the bound quinone species acts as a conduit for electrons, similar to the role of the Q_L site in the bacterial reaction center (3–7, 9). The substrate (QH_L2) site, referred to as the low affinity site (Q_h), is equilibrated with the quinone pool in the membrane. The high affinity quinone-binding site (Q_LH2), from which Q is not readily removed, stabilizes the SQ. The quinone bound at the Q_L site functions as a tightly bound cofactor. Pulse radiolysis studies (10) have shown that the tightly bound quinone can be rapidly reduced to the semiquinone species and that the tightly bound quinone is essential for rapid electron transfer to heme b. The first order rate constant for the reduction of heme b is 1.5 × 10^3 s^-1, which is approximately the turnover rate of the enzyme. It is reasonably assumed that there is a rapid (>10^4 s^-1) two-electron reduction of Q_L by the bound substrate Q_LH2, followed by two one-electron intramolecular transfers from the Q_LH2 to heme b. Hence, the suggested electron transfer sequence is as in Reaction 1.

Q_LH2 ⇌ Q_L ⇌ heme b ⇌ heme o_3-CuB → O_2

REACTION 1

The function of the quinone cofactor bound at the Q_L site as a two-electron-one-electron transformer is supported by the electrochemical studies, which show that the Q_L site stabilizes the SQ form of the quinone bound at this site. The midpoint potential for the two-electron reduction of Q_LH2 is ~–130 mV at pH 7.5, and the pH dependence (up to pH 9) of the midpoint potential shows that two protons are taken up by the protein upon reduction, probably forming the protonated dihydroubiquinol: 2 e^- + 2H^- + Q_LH2 → Q_LH2.

The midpoint potential for the one-electron oxidation of Q_LH2 to the SQ species is ~–13 mV, and the pH dependence of the oxidation of the SQ to the fully oxidized quinone indicates oxidation to form the SQ is coupled to the loss of a proton from a group with a pK_a of ~7.5. This has been interpreted as the pK_a of the SQ, predicting that the SQ is an anion (Q') above pH 7.5 but is a neutral species (Q_LH) below pH 7.5 (11).

The x-ray structure of cyt bo_3 (12) does not contain any bound quinone, but site-directed mutagenesis studies (12–15) have identified residues that influence the SQ, resulting in a model for the Q_L binding site (12). This model (12) proposes hydrogen bonds from Asp-75 and Arg-71 to one of the carbonyl oxygens and hydrogen bonds from Glu-101 and His-98 to the second carbonyl oxygen.

Information about the electronic structure of the semiquinone radical in cyt bo_3 and its interaction with the protein environment is available from high resolution EPR studies. The hydrogen-bonded protons around the SQ in cyt bo_3 were previously examined using electron-nuclear double resonance (ENDOR) techniques in conjunction with
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deuterium (D$_2$O) exchange. The Q-band ENDOR spectrum showed only one splitting from exchangeable deuterium consistent with hydrogen bonding to the quinone oxygen(s) (16). Two pairs of exchange-sensitive features were observed in the X-band ENDOR spectra of cyt bo$_3$ bound to either native quinone or to exogenously added quinones (17, 18). The data were interpreted by different laboratories as indicating either one H-bonded proton (18) or two equivalent H-bonded protons (17).

A multifrequency (9, 34, and 94 GHz) EPR study was performed with cyt bo$_3$ bound to ubiquinone-2 selectively labeled with $^{13}$C at either the 1- or the 4-carbonyl carbons (19). The EPR spectra revealed significant differences in the hyperfine tensors of these carbons indicating that the radical interacts with the protein in a highly asymmetric manner (19), consistent with a strong hydrogen bond to the O1 carbonyl oxygen. In addition, one- and two-dimensional electron spin echo envelope modulation (1D and 2D ESEEM) studies have shown that the SQ in cyt bo$_3$ forms an H-bond with a $^{14}$N atom from the protein environment (20), tentatively assigned either as coming from the polypeptide backbone (20) or from an arginine side chain (19).

Thus, previous studies have established the presence of at least one H-bond between the SQ in cyt bo$_3$ and a nitrogen that is part of the protein environment. These results do not exclude additional hydrogen bonds between the quinone oxygens and other protein H-bonding partners, including S and O atoms.

The current work provides additional information about the exchangeable protons in hydrogen bonds to the SQ species in cyt bo$_3$ by using a combination of 1D and 2D ESEEM, and pulsed ENDOR. The major new observation is that there are at least three exchangeable protons with distinct hyperfine couplings in the immediate environment of the SQ. The data support a model in which the semiquinone is stabilized by strong hydrogen bonds from both Asp-75 and Arg-71 to oxygen O1 and with no hydrogen bonds to carbonyl O4, resulting in a highly asymmetric spin distribution.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The pJRHisA plasmid encoding wild-type cyt bo$_3$ was transformed into the C43(DE3) E. coli strain (Avidis, France). Cells were grown in LB medium containing 100 µg/ml ampicillin, 0.3% lactic acid, and 500 µM CuSO$_4$ at 37 °C and harvested at the mid-logarithm phase. Harvested cells were resuspended in 50 mM K$_2$HPO$_4$, 0.1% MgSO$_4$, pH 8.3, and broken by passing through a microfluidizer (Microfluidics Corp., Worcester, MA) at 10,000 p.s.i. (three times), followed by centrifugation at 16,000 × g for 30 min to remove cell debris. Membranes were then isolated from the supernatant by centrifugation at 180,000 × g for at least 5 h. The isolated membranes were resuspended in 50 mM K$_2$HPO$_4$, pH 8.3, and solubilized with 1% n-dodecyl β-D-maltoside (Anatrace, OH) by stirring at 4 °C for 2 h. The unsolubilized material was removed by centrifugation at 15,000 × g for 1 h. The solubilized cyt bo$_3$ was loaded onto a nickel-nitriolatric acid column and purified as described previously (21). The purified protein was then dialyzed overnight in 50 mM K$_2$HPO$_4$, 0.1% n-dodecyl β-D-maltoside, pH 8.3, and concentrated to ∼400 µM. For the deuterated sample, the dialyzed protein was concentrated, exchanged with deuterated 50 mM K$_2$HPO$_4$, 0.1% n-dodecyl β-D-maltoside, pH 8.3, and further concentrated to ∼400 µM. The enzyme was anaerobically reduced under an argon atmosphere with 500-times excess sodium ascorbate, and the reduced sample was then transferred to an argon-flushed EPR tube, followed by rapid freezing in liquid nitrogen.

**EPR Measurements**—The continuous wave and pulsed EPR experiments were carried out using X-band Bruker ELEXSYS E580 spectrometers equipped with Oxford CF 935 cryostats. Unless otherwise indicated, all measurements were made at 50 K. Several types of EPR experiments with different pulse sequences were employed with appropriate phase-cycling schemes to eliminate unwanted features from experimental echo envelopes. Among them are two-pulse and four-pulse sequences. In the two-pulse experiment ($\pi/2$–$\pi$–$\pi$–echo) the intensity of the echo signal is measured as a function of the time interval $\tau$ between two microwave pulses with turning angles $\pi/2$ and $\pi$ to generate an echo envelope that maps the time course of relaxation of the spin system (in ESEEM), or as a function of magnetic field at fixed $\tau$ (in field-sweep ESE). In the 2D four-pulse ESEEM experiment ($\pi/2$–$\pi$–$\pi/2$–$\tau$–$\pi/2$–$\pi$–$\pi$–echo), also called HYSCORE (22), the intensity of the stimulated echo after the fourth pulse is measured with $t_2$ and $t_1$ varied, and $\tau$ constant. Such a 2D set of echo envelopes gives, after complex Fourier transformation, a 2D spectrum with equal resolution in each direction. Spectral processing of ESEEM patterns was performed using Bruker WIN-EPR software.

Pulsed ENDOR spectra of the radical in cyt bo$_3$ were obtained using Davies ($\pi$–$\pi$–$\pi/2$–$\pi$–$\pi$–echo) and Mims ($\pi/2$–$\pi$–$\pi/2$–$\pi$–$\pi$–echo) sequences with different pulse lengths. In addition, radio frequency $\pi$ pulse is applied during the time interval $t$ in both sequences. The specifics of these experiments are described in detail elsewhere (23).

**Characteristics of HYSCORE Spectra from I = 1/2 Nuclei**—The most informative experimental data regarding the ligand environment of the semiquinone were obtained from the 2D ESEEM (HYSCORE) experiment (22). The basic advantage of the HYSCORE technique is the creation of 2D spectra with off-diagonal cross-peaks ($r_{\alpha\beta}$, $r_{\alpha\gamma}$) and ($\nu_{\alpha\beta}$, $\nu_{\alpha\gamma}$), whose coordinates are nuclear frequencies from opposite electron spin manifolds. The cross-peaks simplify significantly the analysis of congested spectra by correlating and spreading out the nuclear frequencies. In addition, the HYSCORE experiment separates overlapping peaks along a second dimension and enhances the signal-to-noise ratio through a second Fourier transform. HYSCORE is also valuable for the detection of extended anisotropic peaks of low intensity, which are not seen in 1D ESEEM spectra.

Orientationally disordered (i.e. powder) spectra of $I = 1/2$ nuclei also reveal, in the form of cross-peak contour projections, the interdependence between $\nu_{\alpha}$ and $\nu_{\beta}$ values in the same orientation. Analysis of the contours allows for direct, simultaneous determination of
RESULTS

EPR and ESEEM Spectra of the Semiquinone—The field-sweep ESE spectrum of cyt bo$_3$ recorded at 50 K (Fig. 1) shows only one line from the SQ with $g \approx 2.0047$ and the width $\approx 1.2$ millitesla at the half-height. Some measurements were also performed at 90 K, and the results were similar to those obtained at 50 K. The 1D and 2D ESEEM spectra of the SQ at frequencies $\approx 10$ MHz, appropriate for $^{14}$N nuclei, are identical to those reported previously by Grimaldi et al. (20). These spectra were used to determine the quadrupole coupling constant $K = e^2\eta Q/4\hbar = 0.93$ MHz, and the asymmetry parameter $\eta = 0.51$, which are consistent with assigning this to a $^{14}$N nitrogen from the NH or NH$_2$ groups forming an H-bond with the SQ (see “Discussion”).

Proton HYSCORE—Fig. 2 (A–C) shows the proton part of the HYSCORE spectrum ($\tau = 136$ ns) of the SQ radical in the cyt bo$_3$ sample prepared in $^1$H$_2$O buffer. In addition to a diagonal peak with extended shoulders at the proton Zeeman frequency ($v_H \approx 14.7$ MHz), the spectra in Fig. 2 contain up to six pairs of resolved cross-peaks located symmetrically relative to the diagonal. They are designated 1, 2, 2’, 3, 3’, and 4. The cross-peaks labeled 1 demonstrate the largest hyperfine splitting, of the order $\approx 10$ MHz. The cross-peaks 2 possess the most extended anisotropic contour in the area further up the diagonal. The peaks 2’, 3, 3’, and 4 are located in a similar area of the plot close to each other and partially overlap at the low intensity levels. The contours of these peaks could be separated at the higher levels of the intensity, as shown in Fig. 2C, and used for quantitative analysis. The 2, 2’, 3, and 3’ contours deviate from the normal to the diagonal, indicating a significant anisotropic component. In contrast, contours 1 and 4 are approximately normal to the diagonal, suggesting smaller anisotropy. Cross-peaks 2–4 (Fig. 2D) completely disappeared in the HYSCORE spectra obtained under the same conditions using the sample with $^2$H$_2$O, showing that these are produced by exchangeable protons. However, cross-peaks 1 and the diagonal peak, with its shoulders, still appear in the spectra.

Quantitative analysis of the cross-peak contour lineshapes (see “Appendix”) finds that cross-peaks 2 and 2’ are produced by the same proton (H2) and that cross-peaks 3 and 3’ are also from the same proton (H3). Hence, these data derive from four protons coupled to the SQ: H1, H2, H3, and H4. Among them, H2, H3, and H4 are exchangeable pro-
Pulsed ENDOR—Fig. 3 shows Davies pulsed ENDOR spectra for the radical in QH site of cyt bo3 prepared in 1H2O (a and c) and 2H2O (b and d). Length of the first inverting microwave \( \pi \) pulse is 240 ns for a and b, and 64 ns for c and d. Inset, Mims ENDOR spectrum of the SQ in 1H2O (f) and 2H2O (g).

Additional factors that may influence the width of these lines in the spectra due to overlap with some spectral features from exchangeable protons around the proton Zeeman frequency is suppressed in the spectra recorded were obtained with two different lengths t_p of the inverting \( \pi \) pulse. The spectrum recorded with t_p = 240 ns shows complex overlap of the lines in the middle of the spectrum around the proton Zeeman frequency (vH). It is seen that the shoulders of this complex line decrease after 1H/2H exchange. The intensity from the weakly coupled protons around the proton Zeeman frequency is suppressed in the spectra recorded with shorter duration t_p = 64 ns. The spectrum in 1H2O contains two pairs of peaks located symmetrically relative to the vH with splittings of ~11 MHz and ~5 MHz. The pair of peaks with the smaller splitting is absent in the spectrum of the sample prepared in 2H2O, thus indicating their assignment to the exchangeable proton(s). In contrast, a new doublet with splitting ~0.73 MHz, centered at the deuterium Zeeman frequency (vH) and corresponding to the proton splitting ~5 MHz, appears in these spectra (Fig. 3). One can also note that the shape of the peaks with ~11 MHz splitting also changes in this spectrum due to overlap with some spectral features from exchangeable protons. The shape of these peaks after deuterium substitution becomes more asymmetric, allowing us to specify parallel and perpendicular canonical frequencies with the splittings |A_|| \sim 10 MHz and |A_\perp| \sim 13 MHz. These features correspond to the cross-peaks from H1 in the HYSSCORE spectra, and they are assigned to the methyl protons with the hyperfine couplings shown in Table 1 (see also "Appendix").

The current data can be compared with the results from previous studies. Hastings et al. (17) reported two splittings |A_\|= 10.8 MHz and |A_\|= 14.4 MHz (corresponding to a = 12 MHz and T = 1.2 MHz) that were not sensitive to deuterium exchange in X-band ENDOR spectra of decyl-ubiquinone in the QH site of cyt bo3. These peaks were assigned to the protons of the methyl substituent. MacMillan et al. (18) found |A_\|= 9.3 MHz and |A_\|= 13.0 MHz (a = 10.5 MHz and T = 1.2 MHz) for these protons by pulsed X-band ENDOR of native ubiquinone-8 in cyt bo3. The most significant loss of intensity after 1H/2H exchange was reported by these authors at frequencies corresponding to the couplings |A_\|= 5.1 MHz and |A_\|= 11.7 MHz (17) and |A_\|= 4.5 MHz and |A_\|= 9.1 MHz (18). In both of these studies, the features were assigned to the same proton(s) and used for the calculation of the anisotropic component of the hyperfine tensor and H-bond length.

Veselov et al. (16) performed orientation-selected Q-band ENDOR experiments with the native SQ in the QH site of cyt bo3. They reported the isotropic coupling of the methyl protons to be ~11 MHz and a loss of intensity in the 4- to 5-MHz region around the proton Zeeman frequency after 1H/2H exchange. Accordingly, the deuterium ENDOR spectrum recorded at \( g_\perp \) exhibits only one resolved splitting ~0.8 MHz, corresponding to a proton coupling of 5.2 MHz.

In summary, the current work, as well as the previous experiments, have all found a remarkably large anisotropic coupling constant ~10–11 MHz from the methyl protons. In addition, these studies also show similar exchangeable proton and deuterium ENDOR features. Proton ENDOR spectra show a loss of intensity after 1H/2H exchange, which is most significant at frequencies corresponding to the couplings |A_\|= 4.5–5.1 MHz and |A_\|= 9.1–11.7 MHz.

The powder ENDOR spectrum simulated with the contribution of all three exchangeable protons H2–H4 using the hyperfine tensors given in Table 1 reproduces intense features with a splitting of ~5 MHz resulting from the overlap of lines from all three protons and |A_\| peaks with the splitting ~12 MHz from H2 (Fig. 4, "Appendix"). The shape of these features depends on the linewidth of individual ENDOR transitions. Additional factors that may influence the width of these lines in the spectra are the non-axiality of the hyperfine tensors and "strain" in the values of the hyperfine couplings. Thus, the 2D ESEEM data for protons H2, H3, and H4 can explain the exchangeable features in the ENDOR spectra described above, and those data previously assigned...
by other authors (17, 18) to one or two equivalent H-bonded protons (see “Discussion”).

**DISCUSSION**

_A Neutral versus an Anionic Semiquinone_—The hyperfine couplings for both the non-exchangeable methyl protons as well as for the exchangeable protons obtained from HYSCORE and ENDOR experiments provide several important clues about the nature of the SQ species in cyt bo_3. Previous studies (16–18), along with the current work, have found \( a = 10–11 \text{ MHz} \) for the methyl protons. This is the largest isotropic constant reported for these protons for ubiquinones bound to proteins or in solution. Hyperfine coupling of 5.5–6.5 MHz has been reported for ubiquinone anion-radicals in different solvents (25–27). The value changes only slightly in the SQ_H and SQ_Qb sites of the photosynthetic reaction center (28). The proton isotropic constant in the rotating methyl group is directly proportional to the \( \pi \)-spin density on the attached carbon atom and described by the McConnel relation \( a = 81 \mu \text{D} \) (29). Thus, the unpaired \( \pi \)-spin density on the carbon attached to the methyl group of the SQ in cyt bo_3 is \( \sim 2\text{-fold larger} \) (\( \sim 7\% \) to \( \sim 13\% \)) compared with what is observed with anionic SQ radicals in solution.

In the benzoquinone anion radical, all of the \( \alpha \)-protons have an isotropic coupling constant of 6.2 MHz. In contrast, the neutral benzoquinone radical shows two types of \( \alpha \)-proton couplings of 13.5 and 1.4 MHz. Similar couplings have been observed for the methyl protons of duroquinone, which is 5.33 MHz in the anionic radical but 13.4 MHz and \( \sim 1 \text{ MHz} \) in the neutral radical. The isotropic coupling constant of the OH proton in these neutral radicals has a value of \( \sim 1.5–3 \text{ MHz} \) (30). Analogously, the methyl protons of an anion radical of ubiquinone-0 possess an isotropic constant 6.5 MHz and the corresponding value in the neutral radical with protonated O1 is equal to 12.8 MHz (27). Thus, the data are consistent with the assignment of the SQ bound to the Q_H site being a neutral species (Q-H).

The differences in hyperfine couplings in comparing anionic and neutral SQ radicals result from the protonation of one of the carbonyl oxygens of the semiquinone in the neutral radical, which leads to a shift of spin density and charges within the quinone ring. The bound oxygen possesses a larger negative charge to stabilize the interaction with the proton and, thus, the spin density is partly shifted within the semiquinone. When O1 is protonated, the increase in spin density at carbons C1, C3, C5, and O4 is expected. On the other hand, for O4 protonation, the increase will be seen on atoms C2, C4, C6, and O1 (28). The unpaired \( \pi \)-spin density on the carbon attached to the methyl group of the SQ in cyt bo_3 is entirely consistent with a neutral radical with a protonated O1 oxygen.

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The current data also demonstrate three exchangeable protons with significant differences in hyperfine couplings. Two of these protons possess large anisotropic hyperfine couplings with \( |T| \sim 4.2 \) and 6.3 MHz, which exceed the value \( |T| \sim 3 \text{ MHz} \) observed for in-plane hydrogen-bonded protons in alcoholic solutions (25, 31, 32). Recent DFT calculations (33) have shown that the hydrogen-bonded proton with \( |T| \sim 3 \text{ MHz} \) corresponds to an O . . . H distance of \( \sim 1.8 \text{ Å} \), whereas \( |T| = 5.2 \text{ MHz} \) is consistent with 1.4 Å. Hence, the measured coupling of \( |T| = 6.3 \text{ MHz} \) requires an even shorter O . . . H bond distance, of the order 1.2 Å, implying substantial covalent character. This result provides additional support for the interpretation that the SQ in cyt bo_3 can be considered not as a hydrogen-bonded anion but as a protonated neutral radical.

Prominent peaks from the \( \beta \)-protons of the isoprenyl tail were not observed in ENDOR and 2D ESEEM. The lack of these features indicates that these protons have small hyperfine couplings and contribute only to the spectral area close to the proton Zeeman frequency. Veselov et al. (16) studied these protons in greater detail by using orientation-selected Q-band ENDOR and found that the hyperfine couplings with these protons in the Q_H site (\( \sim 2–4 \text{ MHz} \)) are smaller than for the SQ in solution. This is also consistent with the redistribution of the spin density of the SQ in the Q_H site resulting from the protonation of O1.

Previous studies have shown that the one-electron reduction of the fully oxidized quinone at the Q_H site (Q/Q^-) is coupled to the protonation of a group with a \( pK_a \) of \( \sim 7.5 \). If it is the SQ itself that is protonated, this would predict a neutral semiquinone dominating below pH 7.5 (Q/Q^-H) and an anionic species above pH 7.5 (Q^-Q^-). However, the EPR spectra from previous publications (11, 17, 20) show that the large hyperfine coupling of the methyl protons is present from pH 6 to 9. No transition from a neutral radical (large hyperfine coupling to the methyl hydrogens) to a species with characteristics of the anionic radical (smaller hyperfine coupling to the methyl hydrogens) is observed as the pH is increased above pH 7.5. This eliminates the SQ itself as being the protonatable group with a \( pK_a \) of 7.5 and indicates that the pH dependence of the \( E_m \) of the (Q/SQ) couple is more complicated than previously thought. Pulsed radioisotope experiments have monitored the transient formation of the SQ radical in the Q_H site at pH 7 by ultraviolet absorption (10). The data were interpreted to show an anionic radical being formed based on the absence of a resolved absorption maximum at 420 nm, which is characteristic of a neutral radical. However, the pulse radioisotope data are not compelling and are complicated by the spectrum of the N-methyl nicotinamide radical generated as the reductant in these experiments. As already pointed out, the EPR spectra in the current work are not compatible with an anionic SQ species, although the transient species may not be identical to the stabilized form of the SQ.

**Comparison with Recent DFT Calculations**—DFT calculations can be used to provide insight into the detailed relations between the structure, environment, and EPR characteristics of the ubisemiquinone radical. A recent study (34) modeled the SQ bound to the Q_H site of cyt bo_3, calculating the g-tensor and the \( ^{13}C, ^1H, \) and \( ^{17}O \) hyperfine tensors with the assumption that the SQ is an anionic species and testing a variety of H-bonding schemes.

The calculation performed optimized the SQ-protein complex considered as an anion-radical involved in the hydrogen bonds with oxygen and nitrogen atoms. The O . . . H bond lengths in the optimized structures were varied between 1.75 and 2.12 Å for the protons H-bonded to O1 and O4 for the different models of the complex. It was concluded that a single-sided binding model cannot account for the experimentally observed g-tensor nor for the asymmetry of the \( ^{13}C \) carbonyl hyperfine tensors. A model with two hydrogen bonds to O1 and one hydrogen...
bond to O4 (2-1 model) was suggested for the Q₆ site, but a model with one more hydrogen bond on each side (3-2 model) could not be excluded.

The model proposed is in agreement with our observation of three exchangeable protons around the SQ. However, the calculated hyperfine couplings with H-bonded protons (34) are inconsistent with the experimental values determined in the current work. The components of the calculated hyperfine tensors with 2.5 MHz < |T| < 4 MHz are systematically lower than those found for H2 and H3. The low values of the calculated hyperfine tensors result from the H-bond lengths considered in the model. The experimental coupling of the third H-bond in the current work (H4) is smaller than the calculated values, which leaves doubt as to whether this H-bond is to either of the carboxyl oxygens.

An important discrepancy between the calculations of the favored 2-1 model (34) and the experimental data is that the calculations predict the isotropic coupling with the methyl protons to be only 6–7 MHz, compared with the measured values near 10–11 MHz. Better agreement is found with the 2-0 model, which predicts coupling of 0.51 MHz.

The g-tensor calculations systematically yield higher gₓ value for all models of coordination than experimentally observed. The authors note that the “artificial” shortening of hydrogen bonds to 1.6 Å in the 2-1 model reduce the value of gₓ, but this case is excluded as “very unlikely,” although the model with one shortened H-bond to oxygen O1 provides good agreement with the ¹³C experimental values.

The experimental data presented in the current work, however, strongly support a model with a short H-bond to oxygen O1, i.e. an O–H bond with significant covalent character. It is likely that a more satisfactory description of the EPR parameters could be obtained by considering the SQ to be a neutral radical.

Non-planarity and Exchangeable Protons H2 and H3—In proteins, one factor influencing the geometry of hydrogen bonds and proton hyperfine couplings is the structure of the quinone-processing site, particularly the location of suitable hydrogen-bond partners for the semiquinone oxygens. As a result, the hydrogen bonds are likely forced either above or below the ring plane. The DFT calculations show that deviation of the hydrogen bond from the quinone plane leads to a simultaneous increase of the isotropic and anisotropic coupling of the hydrogen-bonded proton (35). This demonstrates the importance of the H-bond geometry in determining the magnitude of the H-bond tensor. This effect is not accounted for in the simple point-dipole model used for estimation of H-bond length. This geometry factor can be considered as an alternative explanation of the large hyperfine values found for the two exchangeable protons and reinforces their assignment to the protons of hydrogen bonds with the carbonyl oxygens. It is important, however, that H-bond deviation does not influence the hyperfine couplings with the ring protons (35) and thus cannot explain the increase of the couplings with methyl protons. The calculations also predict a significant increase of the isotropic hyperfine coupling for the out-of-plane protons that is contradicted by the hyperfine coupling ~0.7 MHz selected for H2. Therefore, the out-of-plane deviation cannot explain simultaneously the hyperfine coupling for H2 and large methyl proton couplings as can be done by invoking a covalent O–H bond to the O1 oxygen in the SQ. However, the characteristics of the exchangeable proton H3 with |a| = 1.2, and |T| = 4.2 MHz can be explained by the formation of the out-of-plane H-bond. In DFT calculations, similar characteristics were obtained for the out-of-plane deviation ~45° of the H-bond with typical in-plane characteristics, i.e. a ~ 0 and |T| = 3 MHz (35).

The Nature of H-bonded Nitrogen—Based on the crystal structure, it was proposed (12) that the carbonyl oxygens of the bound ubiquinol can form up to four hydrogen bonds with the protein, to Asp-75, Arg-71, His-98, and Gln-101. Site-directed mutagenesis studies (12, 13) have confirmed that these four residues are functionally important and that mutants at each position alter or eliminate the SQ that is stabilized at the Q₆ site (13). Hence, there are three potential hydrogen bonds in the proposed model with nitrogen atoms: Arg-71, His-98, and Gln-101.

Grimaldi et al. (20) initially assigned the quadrupole parameters K = 0.93 MHz and η = 0.51 for the H-bonded ¹⁴N observed in the ESEEM spectra to an amide (peptide) backbone nitrogen –NH–C=O. The following publication from the same laboratory (19) noted that the nitrogen of an arginine residue cannot be excluded as a possible ligand, as suggested by the proposed structure of the Q₆ site in cyt bo₃. However, the arguments for such assignment were not provided.

The amide nitrogen in free peptides, such as in metal complexes of diglycine and H-bonded to sulfur atoms of iron-sulfur clusters (36–40), has a narrow range of quadrupole coupling constants, K = 0.75–0.85 MHz, determined by the electronic structure and the geometry of the planar peptide group; this coupling constant is only slightly perturbed by hydrogen bonding. This has been confirmed by the calculations of the quadrupole coupling tensor (39, 41, 42). The quadrupole constant K for nitrogen in cyt bo₃ slightly exceeds the K values reported for peptide nitrogens in different compounds. The assignment by Grimaldi et al. (see Fig. 5 in Ref. 20) is based on the comparison with quadrupole coupling constants of peptide nitrogens in model compounds and nitrogens at the QA site of the reaction centers (43, 44) and the QA site in photosystem II (45) involved in H-bonding with quinones and assigned to peptide nitrogens. Importantly, however, the K values in these cases are all in the range 0.75–0.85 MHz, i.e. they are systematically lower than the K value in cyt bo₃.

Higher values of the K ~ 0.9–1.0 MHz have been reported for the NH₂ group in primary aliphatic and aromatic amines and amides (46), and for the substituent in nitrooxide radicals in frozen alcohol solutions (8). Available experimental data also do not allow the NH group to be excluded as a possible donor of the H-bond (46). Hence, the ¹⁴N detected in the ESEEM spectra of the quinone radical in the Q₆ site could, indeed, be from an NH₂ or NH group of the Arg-71 side chain.

The initial model of the Q₆ binding site (12) also predicts hydrogen bonds to the nitrogen atoms from His-98 and Gln-101. The quadrupole characteristics of the ¹⁴N detected in ESEEM spectra could not belong to any imidazolate nitrogen of a histidine residue, which exhibit significantly smaller K values. A hydrogen bond to His-98 can be rigorously excluded based on these data. The remaining possibility, which is consistent with the structural model (12), is a hydrogen bond between the -NH₂ group of Gln-101 and the O4 carbonyl atom.

Model of the SQ Environment—The current data report three differently coupled exchangeable protons around the SQ in cyt bo₃. One of the protons requires a very short O . . . H, strongly indicating that the SQ species is a neutral radical. Of particular interest is the previously reported D75H mutant that, though enzymatically inactive, stabilizes the SQ with the same midpoint potential as does the wild-type enzyme (13). However, the EPR spectrum of the D75H mutant does not possess the hyperfine structure resulting from the large hyperfine coupling to the methyl protons (13). This suggests that the D75H mutant stabilizes the anionic form of the SQ. Hence, it is proposed that H2 is a hydrogen bond from Asp-75 to oxygen O1, which has covalent character, resulting in the neutral radical at the Q₆ site.

The excess of negative spin density on O1 resulting from the strong (covalent) H-bond with Asp-75 facilitates the formation of the second hydrogen bond by proton H3 (|a| = 1.2 MHz, |T| = 4.2 MHz) between...
O1 and a nitrogen donor from Arg-71 observed in ESEEM spectra. These two protons, H2 and H3, stabilize the strong asymmetry in the distribution of the unpaired spin density that is observed.

Currently, one can propose several structural possibilities for H4. It can be the second proton of the -NH2 group not participating in H-bond with the SQ but involved in magnetic interaction with the unpaired electron spin. Unpaired spin density transfers on this proton via the bridge O...H-N-H. Also H4 could come from the formation of a hydrogen bond to the oxygen of a methoxy group, which would be expected to possess a significantly smaller unpaired spin density than found on the carbonyl oxygen. Alternatively, H4 could be a proton at a second shell distance.

Clearly, further work is needed to clarify the situation. However, the data presented in the current work are by far the most definitive by directly addressing the characteristics of hydrogen bonds for the SQ stabilized at the QH site.

CONCLUSION

The present study reports for the first time the finding of three exchangeable protons with different hyperfine couplings in the environment of the SQ species in QH site of cyt bo3 quinol oxidase. The analysis of the hyperfine couplings with methyl protons of the SQ and exchangeable protons allows several conclusions about the nature of the SQ and the structure of its environment as follows. 1) The SQ stabilized by cyt bo3 at the QH site possesses the characteristics of a neutral radical. The data suggest significant covalent character of the O-H bond of carbonyl oxygen O1. This is supported by the unusually large hyperfine coupling to the methyl protons and one exchangeable proton. The exchangeable proton is shared between Asp-75 and O1 oxygen of the SQ. 2) A second exchangeable proton is shared between carboxyl oxygen O1 and a nitrogen-containing group of Arg-71. 3) A third exchangeable proton is weakly coupled to the SQ electron spin and is not involved to the direct binding with carboxyl oxygens. 4) It is proposed that there is one-sided binding of the semiquinone to the protein via O1, supporting the previous conclusion by Grimaldi et al. (19), based on the analysis of the 13C couplings of carbonyl carbons. Hence, there is a highly asymmetric binding of the SQ in the QH site of cyt bo3, ruling out the hypothesis by Hastings et al. (17) that postulates a binding site with two equivalent hydrogen bonds.

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APPENDIX

Analysis of HYSCORE Spectra—The contour lineshape in the powder 2D spectrum from the 1H nuclei (nuclear spin I = 1/2, Zeeman frequency νz) for axial hyperfine interactions is described (27) by Equation 1.

\[ ν_a = (Q_a ν_β^2 + G_a)^{1/2} \]  (Eq. 1)

where

\[ Q_a = \frac{T + 2a - 4ν_β}{T + 2a + 4ν_β} \]  (Eq. 2)

and

\[ G_a = 2ν \left( \frac{4ν_β^2 - a^2 + 2T^2 - aT}{T + 2a + 4ν_β} \right) \]  (Eq. 3)

For each cross-peak contour, the frequency values along the ridge can be plotted as \( ν_a^2 \) versus \( ν_β^2 \), transforming the contour lineshape into a straight line segment whose slope and intercept are proportional to \( Q_a \) and \( G_a \), respectively. These values can then be used to obtain two possible solutions of isotropic (a) and anisotropic (T) couplings with the same value of \( |2a + T| \) and interchanged \( A_+ = |a - T| \) and \( A_1 = |a + 2T| \) (24).

The coordinates \( ν_a \) and \( ν_β \) of arbitrary points along the ridge formed by the uppermost contour for each proton cross-peak 1–4 were measured from HYSCORE spectra recorded with different τ values at a constant magnetic field value and plotted as sets of values for \( ν_a^2 \) versus \( ν_β^2 \). Initially, the larger frequency of each point was arbitrarily selected as \( ν_a \), and the smaller as \( ν_β \) for all cross-peaks. The points have been fitted by linear regression to give the slopes and intercepts. In this presentation we have found that the slopes of the linear regression for the 2 and 2’ lines have an inverse relationship, i.e., \( Q_{ν_a} (2) = 1/Q_{ν_β} (2') \). This means that the 2’ peak is produced by the same proton H2 but belongs to the cross-feature with opposite assignment of the nuclear frequencies, i.e., \( ν_β > ν_a \) instead of \( ν_α > ν_β \) (24). In other words, 2 and 2’ are parts of the same cross-feature located on different sides relative to the diagonal of (+ +)-quadrant (see also Fig. 6). A similar relation between slopes \( Q_{ν_a} \) (3) = 1/Q_{ν_β} (3’) was observed for cross-peaks 3 and 3’, thus indicating that they are produced by the same proton H3.

Fig. 5 shows the plot where the smaller coordinates for cross-peaks 2’ and 3’ were assigned to \( ν_β \) and larger ones to \( ν_a \) in contrast to peaks 1,2,3,4. In such a presentation, the points from 2 and 2’ fit the linear regression well, thus confirming their assignment to the proton, H2. The same is true for peaks 3 and 3’ produced by proton H3. Peaks 1 and 4 result from proton(s) H1 and H4. The slopes and intercepts for the linear regressions shown in these figures are presented in Table 2, together with two possible sets of \( (a, T) \) satisfying Equation 1 for protons H1–H4, and \( A_+ \) and \( A_1 \) values.

In addition to the experimental points, the curve \( |ν_a + ν_β| = 2ν_1 \)
respectively. This presentation is similar for both sets of ridges. The wider and more extended ridges possess greater intensity.

MHz; $T_1$.

them. This was the situation for the simulated spectra from H1 and H4.

towards the proton Zeeman frequency in the field 345 milliteslas) is plotted in Fig. 5 to explain the nature of the two solutions determined by Equation 1. The points at which the curve crosses each extrapolated straight line correspond to the nuclear frequencies $v_\alpha$ and $v_\beta$ at canonical orientations. For an axial hyperfine tensor, there are two possible assignments of the parallel or perpendicular orientations and consequently, two sets of hyperfine tensors, one for each assignment. This approach gives hyperfine couplings identical to those determined from the slope and intercept.

The Hyperfine Couplings of Protons—Selection of the right ($\alpha$ and $T$) for protons 1–4 from the two alternatives (Table 2) is based on the simulation of the HYSCORE spectra and/or comparison with the ENDOR data. The cross-peaks in HYSCORE spectra possess a maximum at some intermediate frequencies with zero intensity in the canonical orientations of the hyperfine tensor, and the spectra simulated for two possible sets do not always provide convincing support for any of them. This was the situation for the simulated spectra from H1 and H4.

Selection of the preferred set ($\alpha$ and $T$) for H1 was made on the basis of the ENDOR data. Of the two sets found for H1 from the HYSCORE spectra, the set with $\alpha = \pm 10.0$ MHz and $T = \pm 1.7$ MHz gives $|A_\alpha| = 8.5$ MHz and $|A_\beta| = 14.0$ MHz, which are reasonably close to the couplings $|A_\alpha| = 9.3$–10.5 MHz and $|A_\beta| = 13$–14.0 MHz observed in ENDOR spectra in this and previous reports (17, 18). This set marked by bold font in Table 2 is selected as a correct combination. Thus, we assign cross-peaks 1 to the protons H1 of the methyl group with the couplings $\alpha = 10$ MHz and $T = 1.8$ MHz. The choice of absolute signs follows from the *triple* ENDOR experiments (25, 26).

The preferred set for protons H2 and H3 are found from the simulation of HYSCORE spectra. Fig. 6 shows the ideal contour presentation of the cross-peaks (Fig. 6, A2 and A3) and the calculated HYSCORE spectra with $\tau = 136$ ns for two sets of ($\alpha$ and $T$) from Table 2 (Fig. 6, B2 and B3, and C2 and C3). The contour presentation of the spectrum for each proton is identical for both sets. This shows that cross-peaks of these protons are extended ridges located at both sides relative to the

### TABLE 2

Parameters derived from contour lineshape analysis of HYSCORE spectra

| Proton | $Q_\alpha$ | $G_\alpha$ | $(\alpha$ and $T)^a$ | $A_\alpha = \alpha - T$ | $A_\beta = \alpha + 2T$ |
|--------|----------|----------|---------------------|------------------------|------------------------|
| H1     | -2.17 (0.03) | 595.98 (2.77) | $\pm 11.7, \pm 1.7$ | $\pm 13.4$ | $\pm 8.3$ |
| Peaks 1 | -1.18 (0.01) | 516.78 (0.88) | $\pm 5.6, \pm 6.3$ | $\pm 11.9$ | $\pm 7.0$ |
| H2     | -0.94 (0.03) | 437.89 (4.47) | $\pm 0.7, \pm 6.3$ | $\pm 7.0$ | $\pm 11.9$ |
| H3     | -1.29 (0.02) | 489.89 (2.79) | $\pm 1.2, \pm 4.2$ | $\pm 5.4$ | $\pm 7.2$ |
| Peaks 3 | -1.29 (0.02) | 489.89 (2.79) | $\pm 4.6, \pm 1.7$ | $\pm 6.3$ | $\pm 1.2$ |
| H4     | -1.29 (0.02) | 489.89 (2.79) | $\pm 2.9, \pm 1.7$ | $\pm 12$ | $\pm 6.3$ |

$^a$ The analysis described in the text provides only relative signs of $\alpha$ and $T$ values. For both $\alpha$ and $T$, the signs given in this table can be exchanged so as to correspond to the opposite assignments (larger frequency of the proton cross-peak to $v_\alpha$ and smaller one to $v_\beta$). Because of this uncertainty, this table shows all possible signs for each set ($\alpha$ and $T$), and corresponding $A_\alpha = \alpha - T$, and $A_\beta = \alpha + 2T$.

![Figure 6. The calculated HYSCORE spectra for protons H2 (A2–C2) and H3 (A3–C3). A2 and A3 represent an ideal contour presentation for the cross-peaks from H2 and H3, respectively.](image-url)
The ENDOR spectra show a loss of intensity after $^1$H/$^2$H exchange, which is most significant at frequencies corresponding to the couplings $|A_4| = 4.5$–5.1 MHz and $|A_5| = 9.1$–11.7 MHz. The HYSCORE analysis has not identified the exchangeable proton with $|A_4|$ and $|A_5|$ simultaneously matching these two features. On the other hand, among selected sets, only $|A_4| = 1.9$ MHz of H2 corresponds to the $|A_5| = 9.1$–11.7 MHz. The HYSCORE data show that the signs of $A_4$ and $A_5$ for H2 and H3 are opposite. This means that the spectral lines corresponding to the two electron spin manifolds for each of these protons overlap in the frequency interval from $-|A_4|/2$ to $+|A_4|/2$ around the proton Zeeman frequency, producing the enhanced intensity in the center of spectrum between the intensity maxima at $\pm |A_4|/2$ (Fig. 4). The $|A_4|/2$ for H2 and H3 equal to 3.5 and 2.7 MHz, respectively, and the intervals of enhanced intensity almost coincide.

The simulations of HYSCORE spectra for H4 do not lead to a convincing identification of the preferred set. The ENDOR spectra suggest the appearance of the $|A_4|/2 = 3.1$ MHz peaks for $a = \pm 4.6$ MHz and $T = \pm 1.7$ MHz, i.e., located between the $|A_4|/2$ lines from H2 and H3, that would probably produce the single maximum from the overlap of $|A_4|/2$ features from three different protons. Indeed, the simulated ENDOR spectrum with the contribution of all three protons demonstrates intense features with a splitting of $\sim 5$ MHz resulting from the overlap of lines from all three protons and $|A_4|/2$ features with the splitting $\sim 12$ MHz from H2 (Fig. 4). The shape of these features depends on the linewidth of individual ENDOR transitions. The second possibility for H4 predicts the appearance of $|A_3|$ peaks with the splitting $\sim 1$ MHz. However, we, as well as others (17, 18), do not find convincing evidence for such splitting when comparing ENDOR spectra of the samples prepared in $^2$H$_2$O and $^3$H$_2$O.

These considerations show that by using HYSCORE data for the three exchangeable protons, one can explain features in the ENDOR spectra previously assigned by other authors (17, 18) to one or two equivalent H-bonded protons.

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