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Q-band ENDOR reveals out of plane hydrogen bonds stabilize an anionic ubisemiquinone in cytochrome *bo*₃ from *Escherichia coli*

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Abstract

The respiratory cytochrome *bo*₃ ubiquinol oxidase from *E. coli* has a high affinity ubiquinone binding site that stabilizes the one-electron reduced ubisemiquinone (SQ_H), which is a transient intermediate during the electron mediated reduction of O₂ to water. It is known that SQ_H is stabilized by two strong hydrogen bonds from R71 and D75 to the ubiquinone carbonyl oxygen O1, and weak hydrogen bonds from H98 and Q101 to O4. In the current work, SQ_H was investigated with orientation selective Q-band (~34 GHz) pulsed ¹H ENDOR spectroscopy on fully deuterated cyt *bo*₃ in an H₂O solvent so that only exchangeable protons contribute to the observed ENDOR spectra. Simulations of the experimental ENDOR spectra provided the principal values and directions of the hyperfine (hfi) tensors for the two strongly coupled H-bond protons (H1 and H2). For H1, the largest principal component of the proton anisotropic hfi tensor $T_{Z'} = 11.8$ MHz, whereas for H2 $T_{Z7prime} = 8.6$ MHz. Remarkably, the data show that the direction of the H1 H-bond is nearly perpendicular to the quinone plane (~70° out of plane). The orientation of the second strong hydrogen bond, H2, is out of plane by about 25°. Equilibrium molecular dynamics (MD) simulations on a membrane-embedded model of the cyt *bo*₃ Q_H site show that these H-bond orientations are plausible but do not distinguish which H-bond, from R71 or D75, is

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Notes

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Supporting Information

Relevant methodological details, Figures S1–S8 and Tables S1,S2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

nearly perpendicular to the quinone ring. Density functional theory (DFT) calculations support that the distances and geometries of the H-bonds to the ubiquinone carbonyl oxygens, along with the measured proton anisotropic hfi couplings, are most compatible with an anionic (deprotonated) ubisemiquinone.

Graphical Abstract

Cytochrome *bo*₃ ubiquinol oxidase (cyt *bo*₃) from *Escherichia coli* is a member of the heme-copper oxidoreductase superfamily of enzymes, which includes the mitochondrial cyt *c* oxidase and most prokaryotic respiratory oxidases (oxygen reductases).^{1–4} Cyt *bo*₃ is located in the cytoplasmic membrane where it catalyzes the reduction of molecular oxygen to water using ubiquinol-8 (UQ₈H₂) as the electron donor and also functions as a proton pump, conserving much of the energy available from the redox reaction as the proton motive force.^{5–7} Whereas most of the heme-Cu oxygen reductases utilize cyt *c* as a substrate, cyt *bo*₃ is in a subgroup that utilizes quinol as a substrate.^{2,3} Depending on the detergent utilized, cyt *bo*₃ can be co-purified with or without ubiquinone-8 (UQ₈).⁸ The enzyme purified using the detergent n-dodecyl β-D-maltoside (DDM) contains one equivalent of UQ₈. This “tightly bound” quinone, located at the high-affinity Q_H site, is not displaced from the protein even during catalytic turnover using the soluble substrate ubiquinol-1 (UQ₁H₂).^{9,10} This has provided support for the existence of two separate quinone binding sites in which the high affinity site (Q_H) binds a quinone that acts as a cofactor and the low affinity site (Q_L) binds to the ubiquinol substrate.⁹

The Q_H site stabilizes the one-electron-reduced semiquinone (SQ_H) detected by EPR spectroscopy.^{11,12} This ubisemiquinone can be trapped as a kinetic intermediate during the electron transfer process from the substrate ubiquinol to the low-spin heme *b* component of the enzyme, or can be generated by redox poisoning the enzyme-quinone complex.^{10,13–15} The structure of cyt *bo*₃ published at 3.5 Å resolution does not contain quinone.¹⁶ The quinone binding site in the protein was identified by locating conserved residues as likely candidates within the structure and was then confirmed by site-directed mutagenesis.¹⁶ Residues R71, D75, H98, and Q101 from subunit I were proposed to interact with the bound UQ₈ (Figure S1).^{16,17} Mutations in each of the four proposed Q_H-site residues severely reduce the quinol oxidase activity and eliminate the semiquinone EPR signal, with a notable exception being the D75H mutant.¹⁷ This mutant stabilizes a SQ_H radical with a midpoint potential similar to that of the wildtype (WT) enzyme.¹⁷ Hence, a protein environment stabilizing the SQ radical is necessary but not sufficient for its proper function. A precise spatial arrangement of the SQ and the surrounding residues at the Q_H site is crucial for the efficient electron transfer

process. The interactions of SQ_H with the protein environment were extensively studied using X-band 1D and 2D ESEEM (reviewed in¹⁸).

Auxotroph strains were developed to perform selective ¹⁵N labeling of different nitrogens in Arg, His and Gln residues in the cyt *bo*₃ protein.^{19,20} 2D ESEEM studies have identified N_ε of R71 in cyt *bo*₃ as the H-bond donor carrying the most unpaired spin density transferred from SQ_H.²⁰ In addition, weak hfi couplings with the side-chain and peptide nitrogens from R71, H98, and Q101 were resolved, thus providing a full view of the spin density transfer from SQ_H to the nearest residues.^{19,20} These data are supported by the ~10–11 MHz isotropic coupling with the 5'-methyl substituent protons (about twice the value for the SQ in alcohol solutions),^{21–25} and ¹³C couplings in 5'-methyl²⁶ and carbonyls,²⁴ all which indicate significant asymmetry in the distribution of the unpaired spin density.

Two strongly coupled exchangeable protons with hfi anisotropic components of 6.3 and 4.2 MHz were found near SQ_H using X-band 2D ESEEM in conjunction with deuteration of the solvent.²⁵ The 6.3 MHz anisotropic coupling is equal to the value reported for the monoprotonated benzosemiquinone radical generated in frozen alcohol.²⁷ This coupling, along with the isotropic hfi of the methyl protons, were used to support the conclusion that the SQ in the Q_H site of cyt *bo*₃ is a neutral radical.²⁵ However, the DFT calculations utilizing different simplified models of either the ubiquinone anion-radical or neutral radical H-bonding failed to reproduce all experimentally determined ¹H (H-bonds, 5'-methyl), ¹³C (carbonyls), and ¹⁴N (nitrogen H-bond donors) couplings simultaneously.^{20,28–30} Hence, the SQ_H protonation state, as well as the general hydrogen bond network, remain uncertain. What is required to clarify the situation is a more precise, experimentally characterized model of the SQ_H binding in cyt *bo*₃ including the orientations of the H-bonds and the corresponding donor for each strongly coupled proton. This is the goal of the current work.

In order to overcome the existing uncertainties in the structure of SQ_H we describe a pulsed Q-band ¹H ENDOR study on fully deuterated cyt *bo*₃ in an H₂O solvent to characterize the hfi tensors of the exchangeable protons. The full deuteration of the enzyme and quinone molecule limits the observed spectra to these protons only. Previous X-, Q-, and W-band ¹H ENDOR studies of SQs in proteins and model systems are reviewed in detail.^{18,31–35} Earlier Q-band ENDOR has been successfully applied for the characterization of the hydrogen bond network around SQ_A and SQ_B in deuterated bacterial reaction centers^{36,37} and the anion and neutral radicals of deuterated p-benzoquinone in water or alcohols.^{27,38} As in these studies, our Q-band ENDOR work defines the geometries of the H-bonds around the SQ_H from the analysis of the hfi tensors that is supported by DFT calculations allowing us to directly compare our results with those from the previous studies.

The X-band EPR spectrum of a SQ in frozen solutions is a single line with unresolved g-tensor anisotropy. The spectral width is comparable to the excitation width by microwave pulses, so in the X-band experiment, pulses can be considered as giving a complete excitation of the EPR spectrum. Therefore, at this microwave frequency the ESEEM and ENDOR powder spectra exhibit nuclear frequencies from all orientations of the applied magnetic field relative to the hfi tensor principal axes. On the other hand, at Q-band, with a magnetic field ~1.2 Tesla, the principal components of the SQ_H g-tensor are sufficiently well

resolved, allowing for orientation selective measurements. In these types of experiments, particular sections of the EPR spectrum that correspond to molecules with well-defined orientations with respect to the magnetic field are excited one at a time.^{27,36–38} The principal values of the ^1H hfi tensors and their orientations relative to the SQ_H g-tensor reference frame for two exchangeable protons are obtained by simulations of the orientation selective Q-band ^1H ENDOR spectra recorded at different points on the EPR spectrum. ^1H tensors obtained from these measurements were combined with all other EPR parameters referenced above and used as constraints to model the SQ_H spatial conformation within cyt b_{o3} and its electronic structure using MD and DFT calculations.

EXPERIMENTAL PROCEDURES

Sample Preparation

WT cyt b_{o3} was overexpressed by IPTG induction in *E. coli* strain C43(DE3) transformed with plasmid pETcyo as previously described.³⁹ Full deuteration of this enzyme was achieved by growing the bacteria in deuterated water with uniformly deuterated glucose as the sole carbon source. To ensure proper growth, cells were inoculated stepwise into the minimal medium with 80%, 90%, and finally 100% deuterated water. This complex was purified with a NTA-Ni affinity column after solubilizing the isolated membrane fraction in 1% DDM (dodecyl β -D-maltoside). After eluting the protein from the column, five successive buffer exchanges with a 100-kDa Amicon Ultra-0.5 mL (EMD Millipore) were carried out to change the buffer to 50 mM potassium phosphate pH 8.3, 10 mM EDTA, 5% glycerol and ~0.05% DDM. The final concentration of cyt b_{o3} was approximately 500 μM . Sodium ascorbate (10 mM) was added from a freshly prepared 1 M stock. The sample was transferred to a Q-band EPR tube topped with argon, which was further incubated on ice for 3 hours for the SQ signal to develop.⁹ The tube was then frozen in liquid nitrogen for storage.

ESEEM and Davies ENDOR

The instrumentation and pulse sequences for X-band 1-D 4-pulse ESEEM, HYSCORE, and Q-band pulsed Davies ENDOR have been previously described.^{25,37,40} All EPR measurements were performed at 60 K. Only the SQ_H EPR signal is observed in pulsed EPR experiments at this temperature as demonstrated previously.²⁵ The repetition rate in the pulsed EPR experiments was set to 1,000 Hz to avoid saturation of the echo signals. Orientation-selective Davies ENDOR spectra were acquired in stochastic mode with an average of 200 traces.

Spectral Simulations

Q-band ^1H ENDOR simulations were performed in Matlab R2014b with EasySpin v5.0.9 (<http://www.easyspin.org>). The principal values of each rhombic ^1H hfi tensor [$a - T(1+\delta)$, $a - T(1-\delta)$, $a+2T$] were defined using the isotropic constant a , anisotropic coupling T , and rhombicity parameter δ ($\delta=0$ for axial hfi tensor). The orientation of the hfi tensor with respect to the g-tensor reference frame was described using the Euler angles α , β , and γ . These angles are the series of rotations that bring the g-tensor into the hfi tensor eigenframe: (i) counterclockwise rotation around the g-tensor Z axis by α , (ii) counterclockwise rotation

around its new Y axis by β , (iii) counterclockwise rotation around its new Z axis by γ to arrive at the hfi tensor.

For orientation selective spectra, the effective excitation bandwidth at each field position can be estimated from the EPR broadening and the length of the microwave pulses. The EPR broadening was modeled using the EasySpin H-Strain parameter in simulations of the Q-band EPR spectrum (Figure 1). The pulse excitation bandwidth was approximated by multiplying the inverse of the initial microwave π -pulse by two for Davies ENDOR. Additionally, the characteristic suppression of small couplings in Davies ENDOR was taken into account by multiplying the ENDOR simulations with an upside-down Lorentzian function with a width (full width at half-maximum) of $1/2t_p = 2.1$ MHz, where t_p is the length of the first microwave π -pulse (240 ns).⁴¹ All other parameters were the same as those used in the experiments.

The parameters to be optimized in the simulations included those describing the principal values and directions of the hfi tensors (a , T , δ , α , β , and γ) and the ENDOR linewidth broadening parameter (lwENDOR). Optimizations were carried out by a least-squares minimization using a Nelder-Mead style simplex method.⁴² Only data below 51.1 MHz and above 52.4 MHz were included in the least-squares fitting. The errors were estimated by shifting the parameters away from the optimized solution until the least-squares deviation rose above a set threshold. This threshold was predetermined by visually inspecting at which point the simulations clearly did not reproduce the general features of the experimental data.

Geometrical Constraints

Hyperfine couplings were translated into inter-atom distance constraints based on past DFT calculations, EPR characterization and corresponding crystal structures. Euler angles, on the other hand, were converted into angle/dihedral constraints directly with trigonometry (Table S1).

Molecular Dynamics Simulations

In conjunction with the EPR experiments, MD simulations were conducted on a membrane-embedded model of cyt *bo*₃ with bound UQ₈ to assess how the geometric restraints derived from EPR manifest themselves in binding orientations of the quinone to the oxidase. The starting structure was based on chains A–D from the ubiquinol oxidase structure (PDB code: 1FFT).¹⁶ The missing ubiquinone ligand was placed roughly in the middle of the high affinity binding pocket. After adding missing side chains and protons using the VMD⁴³ plugin PSFGEN, the resulting structure was embedded into a bilayer with mixed lipid composition (2:1:1:1 ratio of PMPE:PYPE:POPE:PMPG; a composition that approximately reflects the dominant lipid species in *E. coli* membranes)⁴⁴ using CHARMM-GUI.^{45,46} The bilayer and inserted protein were solvated using explicit TIP3⁴⁷ water molecules, and ionized to a 150 mM concentration of NaCl using the AUTOIONIZE plugin of VMD.⁴³ The final size of the system was $111 \times 111 \times 129$ Å³. CHARMM36 was used for classical force field parameters for the lipid,⁴⁸ protein,⁴⁹ hemes, and ionic⁵⁰ components of the system. Parameters for ubiquinone were obtained from a previous parameterization study.⁵¹ Due to the functionalization of one of the heme groups and a copper-containing complex, additional

topological components derived by analogy to existing parameters that describe the interactions within the protein were also included (provided in Supporting Information).

In addition to the Hamiltonian formed by the classical force field, additional terms were added such that the sampled configurations are consistent with the EPR experiments, as the crystal structure lacks a quinone ligand. The restraint potentials are composed of half-harmonic potential terms applied to specific bonds and angles within the system (details in Supporting Information). For the strong H-bonds determined between O1 of UQ and N_e of R71 as well as protonated D75 (Figure S1), the distance between the H atom of the donor and the O of the acceptor is harmonically ($k = 1000 \text{ kcal/mol/\AA}^2$) constrained to lie between 1.2 and 2.1 Å. The linearity of the H-bond is maintained by applying a harmonic restraining potential ($k = 1 \text{ kcal/mol/degree}^2$) if the angle between the hydrogen and its heavy atom is more than 30 degrees away from the line between the donor and acceptor heavy atoms. Weak H-bonds between O4 of UQ and H98 and Q101 are restrained only by the distance between the donor and acceptor heavy atoms: 1.5–4.0 Å for H98 (protonated N_e) H-bond, and 2.2–4.0 Å for Q101 H-bond. In addition, the angle formed by the H of the donor with O1 and C1 of UQ is also restrained ($k = 1 \text{ kcal/mol/degree}^2$) to a certain range determined by the EPR simulations.

With this setup, NAMD 2.10⁵² was used to conduct 10 independent simulations. Simulations 6–10 began from the crystallographic state, while simulations 1–5 started after rotating the R71 sidechain to start on the opposite side of the quinone plane. Each copy was equilibrated for 5 ns, during which protein heavy atoms more than 8 Å away from the binding site were harmonically restrained ($k = 1 \text{ kcal/mol/\AA}^2$) to their initial position. From this equilibrated starting point, data were collected over 50 ns of production simulations where the EPR restraints were active, but the protein was released.

All simulations were performed in an NPT ensemble, using a Langevin thermostat with a damping coefficient of 1/ps to maintain the temperature at 310 K and a Nosé-Hoover Langevin piston barostat⁵³ with period and decay times of 200 fs to maintain the pressure at 1 atm. The barostat acted in a semi-isotropic manner, where dimensions of the periodic cell were held in constant ratio in the membrane plane. The particle mesh Ewald (PME) method^{54,55} with 1.2 Å grid spacing was used to account for long-range electrostatic contributions during each of the 2 fs time steps.

DFT Calculations

All density functional calculations, including geometry optimization and hyperfine couplings, were performed using the ORCA package⁵⁶ and the B3LYP functional and the EPR-II basis set.^{57,58} Additionally, the optimizations included the third generation (D3) semi empirical van der Waals corrections with Becke-Johnson damping.^{59,60} The conductor like screening model (COSMO) with a dielectric constant of 8.0 was used for all calculations to replicate the protein environment.⁶¹ Geometry optimizations were performed for the three frames selected from the MD trajectory, with all heavy atom coordinates constrained except those belonging to the quinone, in order to optimize the quinone position relative to the residues' positions in that frame.

RESULTS AND DISCUSSION

Q-band EPR spectra

A multifrequency CW EPR study of SQ_H in cyt *bo*₃ was previously performed at X (~9.5 GHz), Q (~34 GHz) and W (~94 GHz)-bands. High-frequency Q- and W-band spectra resolve the g-tensor anisotropy of SQ_H. Numerical simulations of the W-band spectrum indicate a slightly rhombic **g** tensor with principal values $g_x=2.00593$, $g_y=2.00543$, $g_z=2.00220$, $g_{iso}=2.00452$ (error ± 0.00005).²⁴

Q-band CW EPR and two-pulse field-swept ESE spectra of SQ_H in fully deuterated cyt *bo*₃ in an H₂O solvent are shown in derivative mode in Figure 1. The overall lineshape of these spectra is predominantly axial with well-separated $g_{x,y(\perp)}$ and $g_{z(\parallel)}$ components. In addition, the g_z component of the spectra possesses a well-pronounced splitting produced by an exchangeable proton. Two-pulse ESE relaxation rates were found to change with field position, so the CW spectrum was used for simulations. Least-square fitting yields the following parameters for the **g** and H-Strain values: $g = [2.00593 \ 2.00543 \ 2.00228]$, H-Strain = [11.7 14.8 7.8] (MHz). The simulated g-tensor components obtained here are nearly identical to the values reported previously.²⁴ The splitting at g_z was found to be reproduced when the hfi tensor of the hydrogen bond proton with largest components $A(^1H) = [-6.6 \ -5.3 \ 11.7]$ (MHz) (see Table 1) was included in the simulations. The simulated spectrum with the optimized parameters is shown along with the experimental data in Figure 1.

X-band HYSCORE

The low-frequency region of the HYSCORE spectrum (Figure 2) of SQ_H in deuterated cyt *bo*₃ is dominated by a deuterium matrix peak D_{wc}, which comes from weakly coupled nonexchangeable ²H nuclei of the quinone substituents and amino acid residues. Cross-peaks **1D**, located symmetrically on the anti-diagonal relative to the deuterium matrix line, identify strongly coupled deuterium nuclei interacting with the electron spin of SQ_H. Coordinates of the peak maximum (3.5, 5.0) MHz define a hyperfine coupling ~1.56 MHz for ²H, corresponding to a 10.16 MHz coupling scaled for ¹H. This value is consistent with the coupling ~10–11 MHz assigned previously to the 5'-methyl protons based on the HYSCORE and ENDOR spectra of nonexchangeable protons interacting with the SQ_H spin.^{21–25}

In addition to the deuterium lines, the HYSCORE spectrum contains cross peaks **1N** with coordinates (5.1, 3.3) MHz previously observed for SQ_H in native (not perdeuterated) cyt *bo*₃ and assigned to the N_e of R71.¹⁹ These intensive cross-peaks correlate (double-quantum) nuclear transitions of the highest frequency of ¹⁴N_e from opposite electron spin manifolds. This nitrogen is involved in the H-bond with SQ_H and possesses the largest hfi coupling $A_{14N} \sim 1.8$ MHz from the delocalization of unpaired spin density from SQ_H onto the *s*-orbital of the N_e. On the other hand, the appearance of deep ²H ESEEM in the fully deuterated sample produces intensive peaks in the 2D spectrum that leads to cross-suppression of weaker ¹⁴N cross-features and ¹H cross-ridges of low intensity in the HYSCORE spectra.

Four-pulse ESEEM provides an alternate way to detect the exchangeable protons through observation of sum combination peaks.⁶³ The ^1H sum combination spectrum of SQ_H in deuterated cyt bo_3 in H_2O buffer contains three well-resolved lines in the region of the double Zeeman frequency $2\nu_{1\text{H}}$ as shown in Figure 3. In comparison with the previously reported spectrum of SQ_H in a fully protonated background, the intensity of the proton matrix peak $2\nu_{1\text{H}}$ is greatly diminished (Figure 3) and is now comparable in intensity with two other peaks shifted from $2\nu_{1\text{H}}$ to higher frequencies by ~ 0.7 and ~ 1.4 MHz. The shifts of these lines, assigned to strongly coupled exchangeable protons, are the same as in protonated cyt bo_3 and correspond to anisotropic hyperfine couplings of ~ 6.0 and ~ 4.2 MHz.⁶⁴ Thus, 1D and 2D ESEEM spectra of SQ_H in deuterated cyt bo_3 with deuterated quinone in a protonated buffer indicate that the hydrogen bonding network around SQ_H is not significantly perturbed by the deuteration of the protein and quinone molecule.

Q-band ENDOR

Orientation selective pulsed Davies ^1H ENDOR measurements of SQ_H were performed at thirteen evenly spaced points in steps of 0.2 mT on the field-swept ESE spectrum (Figure 1). Accumulated ENDOR spectra in absorption and derivative presentation are shown in Figures 4 and 5. The sample consists of fully deuterated protein and quinone in an H_2O buffer, so only exchangeable protons contribute to the observed spectra. The experimental ENDOR traces exhibit various features of high and low intensity that are resolved from the central 51.72–51.85 MHz ^1H matrix region (Figure 4).

Previous analysis of the X-band HYSCORE spectra of SQ_H in cyt bo_3 reported three sets of cross-ridges from exchangeable protons **H1–H3** with principal values of the hfi tensors in an axial approximation $A_\perp = a - T$: -7.0 , -5.4 , 6.3 MHz and $A_\parallel = a + 2T$: 11.9 , 7.2 , 1.2 MHz, respectively (signs are relative).²⁵ In agreement with these results, the ENDOR spectra at the g_z edge of the EPR spectrum (traces B10–B13) shows a feature of low intensity with a splitting ~ 11.5 MHz. The splitting and lineshape allows us to assign this feature to the proton **H1** with the largest $A_\parallel$ principal value and conclude that the $A_\parallel$ principal axis for this proton is closely collinear with the g_z principal axis. This $A_\parallel$ feature resembles a “single-crystal-like-shape” in trace B10. There are also two pairs of intensive features with resolved maxima of $A < 6$ MHz at the g_z edge. Those two pairs of lines with the splittings 4.1 and 2.7 MHz possess the best resolution and most symmetrical lineshape in trace B10. The ENDOR spectra recorded at other parts of the EPR spectrum exhibit more complex shape and poorer peak resolution, though up to three maxima are clearly seen in traces B4–B8. The width of this central pattern resulting from the overlap of the most intensive areas around the $A_\perp$ features from different protons is increased up to ~ 9 MHz at the $g_{x,y}$ side of the EPR spectrum, preventing identification of any other remaining $A_\parallel$ lines. The peaks with the splitting 2.3 MHz at the g_z edge are present in all spectra except for at the $g_{x,y}$ edge where it is 1.6 MHz. For the simulation of the orientation selective ENDOR spectra we used the representation in first derivative mode (Figure 5), because it better resolves minor variations of the broad features in the traces B1–B9.

The coupling constants a and T determined for the three exchangeable protons from X-band HYSCORE measurements²⁵ were used as initial parameters in the Q-band ENDOR spectral

simulations. The inclusion of rhombicity ($\delta \neq 0$) into all three hfi tensors was found to be necessary to reproduce the experimental line shapes. For protons **H1** and **H2**, approximately axial tensors characteristic of hydrogen bonds fit the ENDOR traces well. On the other hand, a single **H3** with previously determined parameters was insufficient to reproduce the weakly hfi coupled ENDOR region (49.5 MHz to 54 MHz). We believe that the ridge previously thought to be solely produced by **H3** actually results from contributions from more than one proton, each with T less than 2.9 MHz.²⁵ Indeed, simulations with two protons possessing different anisotropic couplings (**H3** and **H4**) can yield a satisfactory fit to the experimental data in the region around ^1H Zeeman frequency. The final ENDOR simulations overlaying the experimental traces in Figure 5 (derivative mode) are shown in red, with the simulation parameters reported in Table 1. The simulation parameters are also demonstrated to reproduce the absorption mode spectra as shown in Figure S2. The hfi coupling parameters agree well with previous reports. Most importantly, the Euler angles of these protons are also determined, and these are crucial to locate the protons with respect to the SQ_H radical (Figure 6).

Orientation of the H-bonds

With the higher resolution of the hfi tensors of H-bond protons afforded by the full deuteration of the protein and orientation-selective Q-band ENDOR, we are now able to consider their assignments. We will focus on the protons **H1** and **H2**, which possess the largest anisotropic couplings. Results from previous FTIR⁶⁵ and detailed ^{15}N HYSCORE²⁰ studies allow us to suggest that the two strongly coupled protons belong to H-bonds between the carbonyl O1 and donor atoms of D75 and R71 (Figure S1).

The principal values and principal directions of the hfi tensors provide insight into the locations of the hydrogen bonded protons. Specifically, the Euler angles α and β in Table 1 describe the in-plane and out-of-plane orientations of the $T_{Z'(\parallel)}$ axis with respect to the SQ_H g-tensor reference frame (see Figure 6 for a pictorial representation of the Euler angles). In contrast to α and β , the Euler angle γ describes the directions of the perpendicular components of the hfi tensor ($a - T(1 + \delta)$ and $a - T(1 - \delta)$), and because of the very low values of δ found for **H1** and **H2**, is not considered in this discussion.

When $T_{Z'(\parallel)}$ is assumed to lie along the O...H direction, α and β provide a direct means of determining the direction of the H-bond and, therefore, locating the proton with respect to the carbonyl oxygen. This assumption about the ^1H tensor orientation is valid to a good approximation when the dominant contribution to the anisotropic hfi comes from the magnetic dipolar interaction between the unpaired π -electron spin density on the carbonyl oxygen and its H-bonded proton, and is confirmed by our DFT calculations for values up to $T \sim 5$ MHz. DFT provides a more realistic model of the unpaired electron spin density distribution over the quinone molecule, resulting in a more accurate prediction of the anisotropic hfi tensors. Calculations on the optimized structures of SQ_A and SQ_B in bacterial RCs from *Rb. sphaeroides* were found to be in strong agreement with the ENDOR defined Euler angles.³⁷

Table 2 summarizes the principal values and orientations of the principal axes of the hfi tensors determined by the same Q-band ENDOR approach for the protons of the H-bonds

with carbonyl oxygens of the anion-radical of BQ- d_4 in H_2O ,³⁸ the monoprotonated benzoquinone BQH $^{\bullet}$ - d_4 in 2-propanol $(CH_3)_2CHOD$,²⁷ and semiquinones of deuterated UQ $_{10}$ in deuterated RCs from *Rb. sphaeroides* in H_2O .^{36,37} Notably, experiments performed with BQ $^{\bullet}$ - d_4 in H_2O (and alcohols) defining $\beta \sim 90^\circ$ and $\alpha \sim -54^\circ$ allowed the conclusions that the geometry of H-bonds is largely in plane with the quinone ring along the lone pair orbitals of the sp^2 hybridized oxygen.³⁸ The H-bond protons are characterized by almost purely anisotropic hfi couplings, with $T \sim 3$ MHz. These experimentally observed values are well supported by DFT calculations.^{38,66} A hfi coupling of $T \sim 3$ MHz is consistent with a proton participating in a planar hydrogen bond, forming an angle $\pm 60^\circ$ with the C=O bond (Euler angle α , refer to Figure 6) and an O $\cdots$ H bond length ~ 1.8 Å.

A Q-band ENDOR study on the monoprotonated benzoquinone (BQH $^{\bullet}$ - d_4) produced by UV illumination of BQ dissolved in 2-propanol at cryogenic temperatures reported a similar in plane orientation ($\beta=90^\circ$) of the $T_{Z'}$ axis of the anisotropic hfi tensor for the proton in the covalent O-H bond.²⁷ This axis deviated equally ($\alpha = -56^\circ$) from the C=O bond in comparison to the similar axis for the H-bonded proton. DFT calculations performed on different structures of BQH $^{\bullet}$ coordinated by four, three, or zero 2-propanol molecules have also found the O-H proton lying in the quinone plane and approximately along the direction of the lone pair orbital of the oxygen. The O-H bond length was found to be around 1.0 Å. The largest component of the anisotropic hfi tensor is parallel to the O-H direction ($\alpha = -43^\circ$ to -50°) similarly to the H-bonded protons. The DFT results show very good agreement with the experimental anisotropic hfi components but predict a larger absolute value for the negative isotropic coupling (-8.0 MHz vs -6.2 MHz from experiment).²⁷

In contrast to the results from the model systems in organic solvent, the angle β characterizing the orientation of the largest component of the anisotropic hfi tensor for the protons of the H-bonds with SQ $_A$ and SQ $_B$ in RCs from *Rb. sphaeroides* is $50-73^\circ$ (i.e. deviation from the quinone plane is $17-40^\circ$). In proteins, one factor influencing the geometry of hydrogen bonds and proton hfi couplings is the structure of the quinone-binding site, particularly the location of suitable H-bond partners for the SQ oxygens. As a result, the hydrogen bonds are likely forced either above or below the ring plane. In addition, the components of the anisotropic hfi tensor are usually larger than the value of $T \sim 3$ MHz for H-bonded protons with water and alcohol that suggests shorter H-bonds in proteins.¹⁸ The extent of the deviation of the hydrogen bond from the quinone plane can influence the isotropic and anisotropic coupling of the hydrogen-bonded proton and should be verified by DFT calculations.^{30,67}

In light of this discussion, the hfi tensor **H2** in cyt *bo3* possesses characteristics typical for an H-bonded proton between the SQ and protein residues: $T \sim 4.3$ MHz, a small isotropic coupling, and an out of plane deviation of the $T_{Z'}$ principal axis (and O $\cdots$ H direction) of about 25° . The in-plane deviation of the H-bond from the lone pair direction is close to zero (lone pair direction is 60° , while the α for **H2** is 55°) though it is noted that the angle α is determined with significantly lower accuracy than angle β (error margins are 2 to 3 times larger in Table 1). In contrast to **H2**, the components of the anisotropic hfi tensor for **H1** are closer to the tensor of the proton in the covalent O-H bond. Together with the large isotropic coupling for methyl protons these data best fit the description of a monoprotonated, neutral

radical for SQ_H.²⁵ However, there remains a need for additional modeling of SQ_H to support this conclusion, particularly due to the significant difference in the isotropic constant with the value reported for BQH[•], as well as the unusual orientation of the O...H bond in the direction almost normal to the quinone plane ($\beta \sim 20^\circ$). The lack of bound quinone in the existing X-ray structure prevents DFT computations of the SQ_H electronic structure. Therefore, at the first stage of our theoretical analysis we have performed equilibrium MD simulations on a membrane-embedded model of cyt *bo*₃ ubiquinol oxidase with bound ubiquinone.

Molecular Dynamics

The EPR measurements place restraints on allowable binding geometries of SQ_H and the oxidase. MD simulations of the quinone and oxidase together places this geometric information of protons into the context of the protein pocket to obtain structural insights of SQ_H binding. As discussed in Experimental Procedures, MD simulations of the *bo*₃ oxidase with a quinone bound to the “high affinity” pocket are carried out with additional harmonic constraints enforcing structural features derived from pulsed EPR. These biased simulations are designed to mimic the situation of SQ binding, and set up the initial geometries for subsequent DFT calculation. Additionally, the flexibility of working in silico can permit different hypotheses to be tested as to the identity of the **H1** and **H2** protons. It is known that the strongly H-bonded protons **H1** and **H2** are on the O₁ side of the SQ_H, however, their assignment to residues D75 and R71 is ambiguous.^{18,20} Parallel MD runs were set up with proton **H1** being assigned either to the β -carboxyl of D75 or to the N_e of R71 to test both possibilities.

By the end of each 50 ns-long MD trajectory, the protein RMSD is commensurate with the crystal structure resolution in all 10 trajectories (Figure S3). To facilitate the evaluation of the quinone conformation, frames from the trajectories were grouped into 4 clusters based on the structural similarity of the quinone binding site under restraint (Figure S4). The representative structures from each group are shown in Figure S5. It is clear that these structures are quite different from each other, suggesting that accessible quinone conformational space is quite large. These restrained conformations cover a wide distribution of the relevant bond lengths and angles (Figures S6 and S7) defining the geometry of SQ_H within the protein binding pocket. From the MD trajectories, some general structural features of SQ_H binding are observed. For instance, the 2-methoxy group tends to interact with D75, and the two H-bond donors R71 and D75 most likely are located on opposite sides of the SQ_H plane. The MD runs used previously determined parameters for the fully oxidized quinone,⁵¹ which cannot alone correctly represent SQ_H. This prevents us from using frames directly from MD to evaluate the SQ_H binding conformation. We have further performed structural optimization using DFT on selected candidate structures. These structures were chosen since they simultaneously satisfied all bond lengths, bond angle and dihedral angle constraints derived from EPR. Three frames closely satisfying all the geometrical parameters determined by the EPR simulations are shown in Figure 7. Representative distances and angles are listed in Table 3. The side chains of D75 and R71 are always located on opposite sides (above or below) of the quinone plane and the H-bonds from these two residues are quite short (~ 2 Å O...H distance). In contrast, the H-bonds

between H98/Q101 N_ε and the O4 carbonyl are considerably longer and do not exhibit a particular spatial pattern.

DFT calculations

Initial models for DFT calculations were generated using the three frames selected from the MD trajectories with hydrogen bonds from the carboxylic acid OH group of D75 and the N_εH guanidium group of R71 to O1, and with the N_εH of the imidazole group of H98 to O4. By comparing different models, the calculated values can provide valuable insight into understanding how structural variations between the three different frames will affect the resulting EPR parameters. Table 4 shows the calculated principal values for the anisotropic hfi tensor and isotropic coupling for two H-bonded protons from residues R71 and D75, which are the main focus of this study. Comparison of the values for the three frames indicates that calculated hfi tensors for both H-bonded protons are consistent with experimentally determined tensors **H1** and **H2** for MD2 only. The largest calculated principal value 11.3 MHz for the H-bonded proton of D75 and 8.4 MHz for the proton of R71 are in a very good agreement with the experimental values 11.8 MHz (**H1**) and 8.4 MHz (**H2**), respectively. In addition, the calculated tensors possess low hfi tensor rhombicity $\delta=0.06$ (D75) and 0.15 (R71) comparable with that estimated from spectral simulations $\delta=0.1$ for **H1** and **H2**.

In the DFT optimized model the **H2** proton is significantly out of the quinone plane and located practically under the O1 atom, i.e. $\beta = 180^\circ$ (Table 3). This angle is substantially different from the EPR spectral analysis ($\beta \sim 115^\circ$). We have investigated the dependence of the calculated hfi couplings on this angle. As shown in Table S2 rotation of this angle for the optimized DFT model leads to only minor changes in calculated hfi couplings for the hydrogen bonded protons. In contrast, a more planar **H2** proton ($\beta = 170^\circ, 160^\circ, 150^\circ$) possesses progressively smaller ¹H and ¹³C hfi couplings for the 5'-methyl group, yielding a better agreement with experimental values. However, it is impossible to evaluate the conformation dictated by EPR simulation ($\beta \sim 115^\circ$) here because at 115° the R71 sidechain has severe steric clashes with the 2-methoxy group which is pointing down in this DFT model. In summary, judging from the DFT calculations so far, it appears that **H1** belongs to D75.

The small values of anisotropic hfi couplings for protons **H3** and **H4** (~1 MHz) indicate weakly coupled exchangeable protons in the SQ_H environment. The calculations performed in our previous work²⁰ exploring idealized small models of the SQ_H-protein interactions with geometry optimization have shown couplings of this order for the N_η protons of the R71. Segments of O-H directions defined by Euler angle uncertainties for these protons are consistent with the location of N_ηH₂ in the MD2 (Figure 7).

Calculated hfi couplings can also be compared with previous experimental determinations of ¹H (10–11 MHz)^{21–25} and ¹³C (–6.1 MHz)²⁶ isotropic constants for the 5'-methyl group and ¹³C anisotropic hfi tensors and isotropic constants for C₁ (–8.9, –17.3, 26.1) MHz, $a=4.7$ MHz and C₄ (–7.9, –11.3, 19.3) MHz, $a = 0.9$ MHz carbonyl atoms in SQ_H, respectively.²⁴ All these characteristic hfi couplings have been measured in several studies of SQs and indeed are the principal indicator that the spin density distribution in the SQ_H is

highly asymmetric compared with a practically symmetric distribution in the anion radical generated in water or alcohol solutions.¹⁸

DFT calculations for the ^1H and ^{13}C isotropic couplings of the 5'-methyl group of SQ_H , listed in Table 4, are in good agreement with experimentally determined values for MD1, but are elevated ($\sim 2.5\text{--}3.0$ MHz for ^1H and ~ 1.5 MHz for ^{13}C) for MD2 and MD3. In contrast, the fit is poorer for ^{13}C carbonyl tensors. It is noted that the experimental hfi tensors for $^{13}\text{C}_1$ or $^{13}\text{C}_4$ labeled quinone were measured from Q- and W-band EPR spectra in frozen samples, where the hfi splittings are poorly resolved only at the high-field component with $g_z \sim 2.0022$.²⁴ No any other splittings are resolved. The complete tensors were obtained indirectly from simulation of the axial spectral lineshape. Such analysis provides, at best, a reasonable estimate of the largest component of the hfi tensor only. Particularly, it clearly indicates that A_{zz} of C_1 is larger than that of C_4 .

Our ^{13}C , C_1 and C_4 computations for frames MD1–MD3 show greater asymmetry than observed experimentally for SQ_H . For example, if we consider the computed $A_z = a + T_{zz}$ components of the hfi tensors $\sim 39\text{--}58$ MHz for C_1 , and in particular the very low $|A_z| \sim 0.9\text{--}12$ MHz for the C_4 position, the experimentally reported values are ~ 30 MHz for C_1 and ~ 20 MHz for C_4 in SQ_H .²⁴ The better agreement of the DFT calculations with the hydrogen bonded and methyl group protons described above would indicate that the inferior ^{13}C agreement for C_1 and C_4 is probably due to computational overestimation of the CO polarization which historically has been a difficult to predict using DFT calculations. Previous reports^{28,30} indicated relatively better agreement between experiment and computation for $^{13}\text{C}_1$ and $^{13}\text{C}_4$ hfi tensors. These models used, principally, neutral water molecules as H-bond donors to both O_1 and O_4 . This type of neutral and symmetrical H-bond donation to both oxygens generally leads to less polarization of the CO groups compared with our charged one-sided models obtained from the MD simulations.

Results of our calculations may be understood from the Mulliken unpaired spin populations for MD2 (Figure S8), which places relatively large spin density on O_4 (0.30) but very low spin density on C_4 (0.04). Difference between spin populations of O_1 (0.13) and C_1 (0.23) is not so significant. The experimental ^{17}O couplings for the carbonyl oxygens in SQ_H are thus far not available. However, the difference between the calculated A_z of $^{17}\text{O}_1$ (~ -60 MHz) and $^{17}\text{O}_4$ (~ -110 MHz) in MD2 exceeds the largest difference between $^{17}\text{O}_1$ (~ -74 MHz) and $^{17}\text{O}_4$ (~ -105 MHz) calculated previously for the anion-radical SQ_H model with three H-bonds to O_1 (3/0, 1HO-1HO-1HN) among other different models.²⁸ All demonstrate the highly asymmetric nature of the spin density distribution on the SQ_H which was originally described and analyzed in detail in ref. 20.

A neutral versus an anionic semiquinone

Our previous assignment of SQ_H as a neutral radical was based on (i) The high anisotropic coupling 6.3 MHz for one of the exchangeable protons corresponding to a short, in plane $\text{O}\cdots\text{H}$ bond and (ii) An isotropic coupling constant $\sim 10\text{--}11$ MHz for the protons of the 5'-methyl substituent, which is two-fold larger than that of the anion-radical with symmetrical hydrogen bonds.²⁵ In the current work, orientation selective Q-band ENDOR spectra of exchangeable protons near SQ_H allowed us to determine the orientation of the anisotropic

hfi tensor for the **H1** proton in the g-tensor coordinate system. The principal axis of its largest component, coincident with high accuracy with the O...**H1** direction, forms an angle $\beta \sim 20^\circ$ with the normal to the quinone ring plane. The O...**H1** bond direction is nearly perpendicular to the plane of the quinone ring, unlikely to be a covalent bond. The O...**H2** bond direction is also out of plane, but only by about 25° .

From the combined MD and DFT computational efforts, the **H1** proton is assigned to the H-bond between the SQ_H carbonyl O1 and D75 and the **H2** proton is assigned to the H-bond between the same O1 and R71 N_e. The O...H1 and O...H2 distances in the DFT optimized structure of MD2 are equal to 1.61 and 1.74 Å, respectively. These distances are within the typical range of H-bonds to an anionic SQ and significantly exceed the length of the covalent O-H bond.¹⁸ The distances and angles above allow us to conclude that the SQ_H stabilized in cyt *bo3* is the anionic SQ. In addition, the significant asymmetry of the spin density distribution in SQ_H revealed by ¹H and ¹³C 5'-methyl couplings^{21–26} and the ¹³C hfi tensors for C₁ and C₄²⁴ are consistent with this H-bond geometry. The unusual characteristics of **H1** result from the strong $\sim 70^\circ$ out of plane H-bond deviation. The asymmetry of the spin density distribution is likely caused by the additive influence of both H-bonds between carbonyl O1 and D75 and R71 N_e, because DFT calculations give similar values for ¹H and ¹³C 5'-methyl and ¹³C carbonyl couplings for all Frames.

The assignment of SQ_H as an anionic semiquinone also agrees with the conclusion based on the optical absorption spectrum of the formation of SQ_H following pulsed radiolysis of cyt *bo3*.¹⁵ The pH-dependence of the potentiometric titration of SQ_H indicates that the pK_a of the semiquinone Q^{•-}/QH is about pH 7.3,¹² also consistent with the observation of the anionic form of SQ_H in the current work, performed at pH 8.3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

cyt	cytochrome
hfi	hyperfine interaction

2D	two-dimensional
CW	continuous-wave
DFT	density functional theory
B3LYP	Becke3 Lee–Yang–Parr
SQ	semiquinone
EPR	electron paramagnetic resonance
ENDOR	electron-nuclear double resonance
ESEEM	electron spin echo envelope modulation
HYSCORE	hyperfine sublevel correlation
MD	molecular dynamics
RC	reaction center
UQ_n	ubiquinone-n

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Figure 1.

Q-band CW EPR (dashed red) and field-swept two-pulse ESE (blue) spectra of the SQ_H in fully deuterated cyt b_{03} in an H_2O solvent in derivative mode. Experimental traces were smoothed with a 2nd order Savitzky-Golay filter⁶² over an 11 point window (no significant reduction in spectral resolution was observed after applying the filter). The simulated CW EPR spectrum (dashed black) with the optimized parameters (see text) is shown along with the experimental data. The yellow dots represent magnetic field positions selected for Q-band pulsed Davies ENDOR experiments.

Figure 2.

Contour X-band HYSCORE spectrum of deuterated SQ_H in deuterated *bo*₃ oxidase in an H₂O solvent. Experimental parameters: the time τ between the first and second pulses is 360 ns, $\pi/2$ pulse length is 16 ns, magnetic field 343.8 mT, microwave frequency 9.634 GHz, and temperature 60 K.

Figure 3.

Four-pulse X-band ESEEM spectrum of deuterated SQ_H in deuterated bo_3 in an H_2O solvent. Experimental parameters: the time τ between the first and second pulses is 104 ns, $\pi/2$ pulse length is 16 ns, magnetic field 343.8 mT, microwave frequency 9.634 GHz, and temperature 60 K.

Figure 4.

Absorption mode of orientation selective Q-band Davies ^1H ENDOR spectra of SQ_H in fully deuterated $\text{cyt } b_0_3$ in an H_2O solvent. The Davies ENDOR pulse sequence is $(\pi-t-\pi/2-\tau-\pi-\tau-\text{echo})$. Traces were taken at 13 field positions from 1215.0 mT (trace B1, $g_{\text{X/Y}}$) to 1217.4 mT (trace B13, g_{Z}) in steps of 0.2 mT. Experimental parameters: $\pi/2$ -pulse length = 120 ns, time τ between first and second pulses of the two-pulse echo sequence is 600 ns, RF π -pulse length = 16 μs , $t = 17 \mu\text{s}$, microwave frequency 34.090 GHz, and temperature 60 K.

Figure 5.

First derivative of Q-band ^1H Davies ENDOR spectra of SQ_H in fully deuterated $\text{cyt } b\text{o}_3$ in an H_2O solvent. Experimental traces (black) were smoothed with a 2nd order Savitzky-Golay filter⁶² over a 12-point window in the conversion of the absorption mode spectra from Figure 4 to derivative mode. The same filter was applied to the simulated spectra (red) upon conversion to derivative mode to avoid biasing the fit.

Figure 6.

Main conclusion from simulations of orientation-selective ENDOR spectra: both hydrogen bonds to the O1 carbonyl of SQ_H in the *bo*₃ oxidase are considerably out of plane of the quinone. The exact protons locations (above vs below or left vs right) cannot be unequivocally assigned due to the degeneracy in the Euler angles. Principal axis of the largest anisotropic tensor component of the **H1** defining O1 – H1 direction is almost perpendicular to the quinone plane, while the O1 – H2 direction is about 30° off the quinone plane. The axes for the SQ *g*-tensor are shown for clarity. Note, however, that there are no positive or negative directions associated with the *g*-tensor.

Figure 7.

Three frames selected from MD trajectories based on bond lengths, bond angles and dihedral angles between ubiquinone and the key residues (R71, D75, H98 and Q101) in the quinone binding pocket of *bo*₃ oxidase. For simplicity, only the two strongly H-bonded protons are shown in the CPK model.

Table 1

Optimized simulation parameters for Q-band Davies ENDOR spectra.

^1H	T_X^a	T_Y'	T_Z'	a^a	T	δ	Euler Angles ($[\alpha, \beta, \gamma]$)
H1	-6.7	-4.9	11.7	0.0	5.8	0.2	$[0\pm30^\circ, 19\pm7^\circ, 0\pm180^\circ]$
H2	-4.6	-4.0	8.6	-0.4	4.3	0.1	$[55\pm25^\circ, 65\pm10^\circ, 90\pm30^\circ]$
H3	-1.4	-1.0	2.4	1.4	1.2	0.2	$[20^\circ\pm40^\circ, 60^\circ\pm15^\circ, 0^\circ\pm180^\circ]$
H4	-0.4	-1.3	1.6	-0.9	0.8	0.6	$[0^\circ\pm180^\circ, 55^\circ\pm15^\circ, 60^\circ\pm15^\circ]$

^a T_X' , T_Y' , T_Z' , a_{iso} , T are in MHz, α , β , γ are in degrees. The full range of the Euler angles is $[-180, 180]$ degrees for all α , β , γ . However, the symmetrical nature of the $\mathbf{g}$ tensor makes α , β , and γ magnetically equivalent to $(180 - \alpha)$, $(180 - \beta)$, and $(180 - \gamma)$, respectively, reducing the required Euler angle search space to $[-90, 90]$ degrees. Furthermore, the final values are arbitrarily limited to $[0, 90]$ degrees, as the simulations are insensitive to the signs of the angles.

Table 2

Principal values of hyperfine tensors and Euler angles defining the orientation of corresponding principal axes in the g-tensor frame of semiquinones determined by ^1H Q-band ENDOR.

System	T_X^a	T_Y^a	T_Z^a	a	$\alpha^{a,b}$	β	γ	Ref.
$\text{BQ}^{\cdot-}-d_4$ in H_2O	-3.02	-3.0	6.02	0.34	-54	90 ± 5	–	38
$\text{BQH}^{\cdot-}-d_4$ in 2-propanol $(\text{CH}_3)_2\text{CHOD}$	-8.0	-4.7	12.7	-6.2	-56	91	80 ± 4	27
SQ_A (Ala M260)	-4.75	-4.37	9.12	-0.17	16 ± 8	63 ± 2	–	36
SQ_A (His M219)	-5.23	-5.20	10.43	-1.28	-167 ± 2	50 ± 2		
SQ_B (His L190)	-4.95	-4.05	8.4	-0.6	35 ± 14	73 ± 4	72 ± 14	37
SQ_B (Gly L225)	-3.52	-2.88	6.4	-0.2	131 ± 13	57 ± 6	0 ± 22	
SQ_B (Ile L224)	-3.0	-3.0	6.0	-0.2	-146 ± 14	64 ± 7	0 ± 180	

^a T_X^a , T_Y^a , T_Z^a , a are in MHz, α , β , γ are in degrees,

^b Euler angles reported in original publications were converted to the same, most recent EasySpin version 5 standard.

Table 3

Certain distances and angles for the three selected MD frames and DFT optimized MD2.

Frame	D75 R _{OH} , Å ^a	R71 R _{OH} , Å	D75 R _{OO} , Å	R71 R _{NeO} , Å	D75 β (deg)	R71 β (deg)	H98 R _{NeO} , Å	Q101 R _{NeO} , Å
MD1	2.10	2.06	2.98	3.01	103 (below)	25 (above)	3.48	3.85
MD2	1.93	2.01	2.87	2.94	20 (above)	136 (below)	3.66	3.37
MD3	1.83	2.09	2.77	3.00	58 (above)	166 (below)	3.84	3.07
MD2 ^b	1.61	1.74	2.6	2.77	14 (above)	180 (below)	3.76	ND

^aR_{OH} - length of H-bond between O1 and proton of D75 or R71, R_{OO} - distance between O1 and H-bond donor O of D75, R_{NeO} - distance between O1 and H-bond donor N_e of R71, H98 and Q101, β - Euler angle β above or below the quinone plane is defined in the same manner as in Figure 6,

^bDFT optimized.

Table 4

Anisotropic T_I' (X', Y', Z'), and isotropic, a , hfi couplings calculated for H-bonded ^1H and ^{14}N , and ^1H and ^{13}C of SQ_H methyl substituent and carbonyls for the selected MD models. All values are given in MHz.

	MD1		MD2		MD3	
Nucleus (<u>underlined</u>)	$T_{X'} T_{Y'} T_{Z'}$	a	$T_{X'} T_{Y'} T_{Z'}$	a	$T_{X'} T_{Y'} T_{Z'}$	a
R71 – N _e <u>^1H</u>	–2.6 –2.1 4.8	0.2	–4.7 –3.8 8.4	0.2	–3.6 –2.9 6.6	–0.4
R71 – $^{14}\text{N}_e$		0.5		3.6		1.0
H98 – N <u>^1H</u>	–1.4 –1.4 2.8	0.1	–0.6 –0.5 1.1	0.0	–0.4 –0.3 0.7	0.0
D75 COO <u>^1H</u>	–3.3 –3.1 6.4	–0.4	–6.1 –5.2 11.3	–1.8	–4.9 –3.5 8.5	0.0
5' – C <u>$^1\text{H}_3$</u>	–1.5 –1.5 3.0	10.6	–2.1 –1.1 3.2	13.6	–2.1 –1.1 3.2	13.6
5prime; – ^{13}C <u>H_3</u>	–0.5 –0.2 0.7	–6.2	–0.6 –0.1 0.7	–7.7	–0.6 –0.1 0.6	–7.3
<u>C1</u>	–16.9 –14.6 31.6	7.8	–19.4 –17.6 37.0	14.5	–21.5 –19.9 41.3	17.1
<u>C4</u>	–8.8 –4.6 13.4	–12.7	–7.0 –2.6 9.5	–16.5	–5.8 –1.1 6.9	–19.0
<u>O1</u>	27.9 28.8 –56.8	–16.9	24.2 23.5 –47.7	–12.6	23.0 22.1 –45.1	–12.8
<u>O4</u>	44.2 43.7 –87.8	–22.2	45.0 44.7 –89.7	–21.5	47.1 46.7 –93.7	–21.9