Published in final edited form as:

Biochemistry. 2012 November 13; 51(45): 9086–9093. doi:10.1021/bi300834w.

Hydrogen bonding between the Q_B site ubisemiquinone and Ser-L223 in the bacterial reaction centre – a combined spectroscopic and computational perspective[^]

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Abstract

In the Q_B site of the *Rba. sphaeroides* photosynthetic reaction centre the donation of a hydrogen bond from the hydroxyl group of Ser-L223 to the ubisemiquinone formed after the first flash is debatable. In this study we use a combination of spectroscopy and quantum mechanics/molecular mechanics (QM/MM) calculations to comprehensively explore this topic. We show that ENDOR, ESEEM and HYSCORE spectroscopic differences between the mutant L223SA and the wild type sample (WT) are negligible, indicating only minor perturbations in the ubisemiquinone spin density for the mutant sample. Qualitatively this suggests that a strong hydrogen bond does not exist in the WT between the Ser-L223 hydroxyl group and the semiquinone O₁ atom, as removal of this hydrogen bond in the mutant should cause a significant redistribution of spin density in the semiquinone. We show quantitatively, using QM/MM calculations, that a WT model in which the Ser-L223 hydroxyl group is rotated to prevent hydrogen bond formation with the O₁ atom of the semiquinone predicts negligible change for the L223SA mutant. This, together with the better agreement between key QM/MM calculated and experimental hyperfine couplings for the non-hydrogen bonded model, leads us to conclude that no strong hydrogen bond is formed between the Ser-L223 hydroxyl group and the semiquinone O₁ atom after the first flash. The implications of this finding for quinone reduction in photosynthetic reaction centres are discussed.

[^]This investigation was supported by the NIH GM062954 Grant and DE-FG02-08ER15960 Grant from Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Sciences, US DOE (S.A.D.), NSF Grant MCB-0818121 (C.A.W.) and NCRR/NIH Grant S10-RR15878 and S10-RR025438 for pulsed EPR instrumentation. PJOM acknowledges the use of computer resources granted by the EPSRC UK national service for computational chemistry software (NSCCS).

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Supporting Information Available. 1-D four-pulse ESEEM, ²H HYSCORE, ¹⁴N HYSCORE, ¹⁵N HYSCORE and Field Swept ESE spectra for WT and SL223A samples are compared. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The primary event in photosynthesis is light-driven charge separation, catalyzed by the reaction center (RC) protein-pigment complex. Light activation results in electron transfer from the primary donor, P, a dimer of (bacterio)chlorophyll, through a series of cofactors of low potential. On time scales longer than a nanosecond, the charge separation in RCs from purple bacteria resides on the primary donor and on the acceptor quinones. The primary quinone, Q_A , is tightly bound and functions as a one-electron redox species, transferring electrons sequentially to the secondary quinone, Q_B . This secondary quinone, Q_B , is reversibly bound and can be doubly reduced via the semiquinone (SQ) form of Q_A (SQ_A or Q_A^- , depending on context) with the uptake of two protons. The fully reduced, protonated quinol is released and replaced by another quinone (reviewed in (1, 2)). The purple bacterial RC therefore uses light energy to produce reduced ubiquinol, which is then used as a substrate by the cytochrome bc_1 complex. This creates the proton electrochemical gradient needed for ATP production from ADP. In the Q_B binding site the two neutral forms, quinone and quinol, are weakly bound, but the negatively charged semiquinone free radical intermediate, SQ_B or Q_B^- , is tightly bound and stabilized. Hydrogen-bonding by the SQ oxygen atoms to nearby amino acid donor groups can be expected to contribute significantly to its stability. Hydrogen bonded residues are also likely to be the immediate source of protons for reduction of the quinone to the quinol form. Ubiquinol is also the substrate for cytochrome bc_1 in the respiratory electron transfer chain where it is generated by reduction in Complexes I and II.(3) Photosystem II in cyanobacteria, algae and higher plants uses a similar mechanism to the purple bacterial reaction centre to reduce plastoquinone to plastoquinol, which is the substrate for the cytochrome b_6f complex.

Quinone substrate binding sites such as Q_B are often difficult to characterize experimentally as such sites will have low occupancy due to the binding and unbinding of the substrate and product. Of the quinone reduction sites, the Q_B site in the bacterium *Rhodobacter (Rba.) sphaeroides* is the best characterized by experimental methods. High resolution X-ray crystal structures of the site are available (4–6) and a wide range of spectroscopic methods such as EPR (7) and FTIR (8) have been used to examine the quinone and semiquinone forms. Details of putative hydrogen bond donors have been elucidated from the structural data and mechanisms of protonation to the ubiquinol have been proposed.(9) Crystal structures show Q_B can occupy at least two different configurations, a tightly bound proximal position and a distal position more distant from the $Fe^{II}-(His)_4$ complex.(6, 10) Q_B is always seen to occupy the proximal location in preparations where the RC was frozen under illumination – indicating that it is this conformation that traps the semiquinone (SQ_B) state. Structures with Q_B in the proximal position show HN_δ of His-L190 (an Fe-ligand) as a potential hydrogen bond donor to the carbonyl oxygen O_4 , and backbone -NH groups from Ile-L224 and/or Gly-L225 plus the hydroxyl group of Ser-L223 as potential H-bond donors to the O_1 carbonyl oxygen (Figure 1). The donation of a hydrogen bond from the hydroxyl group of Ser-L223 is debatable, with reports appearing for and against the presence of such a hydrogen bond to the O_1 of the quinone or the semiquinone. Early reports, allied to the initial crystal structures, were supportive of such a H-bond (6), and electrostatic calculations suggested that the Ser-L223 hydroxyl group would be hydrogen bonded to the ionized side-chain of Asp-L213 in the ground state (oxidized Q_B) but switch to the anionic semiquinone, Q_B^- .(11) However, subsequent FTIR studies (12) argued against significant H-bond formation to either the quinone (Q_AQ_B) or semiquinone ($Q_AQ_B^-$) state. Nevertheless, this interaction is believed to be a key factor for eventual protonation of the semiquinone leading to formation of the fully reduced quinol and recent ENDOR studies (13) have appeared to show Ser-L223 hydrogen bonding to the Q_B semiquinone in the $Q_AQ_B^-$ state. This would favour the model where the Ser-L223 hydroxyl group acts initially as a H-bond donor to Asp-L213 when the quinone form is present in the site but, upon reduction to the SQ state after a single flash, rotation of the hydroxyl group is proposed to lead to formation of a hydrogen bond to the O_1 atom of the SQ.

In this report we examine the effects of replacing the Ser-L223 residue by Ala, generating the L223SA mutant. Alanine will not hydrogen bond with the quinone or semiquinone O₁ atom and therefore changes to the EPR spectroscopic properties would be expected in the mutant if the Ser-L223 residue did form a strong hydrogen bonding interaction with the O₁ atom in the wild type (WT). We use ENDOR and ESEEM spectroscopies to probe any differences that exist between the WT and L223SA mutant. We also model the mutation using QM/MM calculations enabling us to quantitatively calculate any changes that should occur for comparison with the experimental determinations.

Experimental and Methods Section

Reaction center mutation and expression

The Serine-L223 to Alanine (L223SA) mutation was introduced by standard procedures for site directed mutagenesis, based on Stratagene's QuikChange method (Stratagene, La Jolla, CA), as previously described.(14, 15) The mutant was constructed in the complementation vector, pLMX415His6, which contains an engineered reaction center operon (*puf*) lacking *pufA* and *pufB* of the light harvesting complex 1 (LH1), and with a hexahistidine tag at the C-terminus of the M subunit. pLMX415His6 is derived from the broad host-range plasmid pRK415 and was transferred from *E. coli* strain S17-1 into *Rba. sphaeroides* by conjugation. The expression strain, GaBM, was derived from the green Ga parent and lacked RCs and both light harvesting pigment complexes (LH1 and LH2).(15) Cells were grown heterotrophically on Sistrom's minimal medium with malate, in the dark, and with 2 µg/mL tetracycline. Pigmentation was induced by transition to semiaerobic conditions. For reaction center isolation, cell membranes were solubilized in 1% lauryldimethylamine-N-oxide (LDAO), and purified using nickel affinity chromatography, essentially as described.(16)

Sample preparation

In order to isolate SQ EPR signals, the native, high spin Fe²⁺ must be replaced by diamagnetic Zn²⁺. Procedures for biochemical metal exchange, along with the methods of bacterial cell growth and RC isolation, were as previously described.(17) ¹⁵N enrichment of RCs was accomplished during cell growth by using ¹⁵N-labeled ammonium sulfate (Cambridge Isotopes) in the minimal growth medium. Prior to EPR sample generation, the detergent, LDAO, used in RC isolation, was exchanged for Triton X-100 by diluting approximately 100-fold in 10 mM Tris, pH(pD) 7.9, 20 µM EDTA, 0.03% Triton X-100 and reconcentrating. The same method was used for deuterium-exchanged samples, by diluting in D₂O buffer and incubating for 24 hours before reconcentrating. Samples with Q_A⁻ were made by chemical reduction with 8 mM Na-dithionite. For samples with Q_B⁻ the RCs were combined with a 3-fold excess of both ubiquinone-50 (Q-10) and cytochrome *c*, reduced in 10 mM ascorbate. To trap the Q_B SQ, the sample was illuminated by a single laser flash at 532 nm (Spectra Physics Quanta-Ray GCR-11 Nd-YAG) and immediately frozen in liquid nitrogen. Routine optical assay of Fe/Zn-exchanged RC preparations showed a minimum of 80% reconstitution of Q_B activity. However, pulse EPR measurements showed no sign of Q_A SQ signals in Q_B SQ samples in either ¹H-HYSCORE or in ¹⁴N- or ¹⁵N-HYSCORE, indicating that the functional reconstitution was complete, probably because of the much higher concentrations involved.

EPR, ENDOR and ESEEM experiments

The instrumentation for X-band and Q-band CW EPR measurements was as previously described.(18) The instrumentation, pulse sequences and spectral processing for X-band 1D 4-pulse ESEEM ($\pi/2-\tau-\pi/2-t-\pi-t-\pi/2$ -echo) and 2D 4-pulse ESEEM (HYSCORE) ($\pi/2-\tau-\pi/2-t_1-\pi-t_2-\pi/2$ -echo) were also as described.(18) Pulsed ENDOR spectra of the semiquinones were obtained using Davies ($\pi-t-\pi/2-\tau-\pi-\tau$ -echo) and Mims ($\pi/2-\tau-\pi/2-t-\pi-$

2- τ) sequences with different pulse lengths. In addition, a radio frequency π pulse is applied during the time interval t in both sequences. The specifics of these experiments are described in detail elsewhere.(19)

Computational studies

Starting with the *Rba. sphaeroides* structure of Axelrod *et al* (20) (PDB file 1dv3), we created a model of the Q_B site. This model consisted of L subunit residues 177–242, and M subunit residues 218–220, 233–235 and 265–267 plus the non-heme Fe²⁺. Hydrogens were added and the native Fe²⁺ ion was replaced by Zn²⁺. The ubiquinone isoprene chain was reduced to CH₂CHCH₂. For the optimisation studies, two-layer ONIOM calculations ONIOM(B3LYP/6-31G(d):UFF) were performed. The QM layer contained the ubisemiquinone Q_B, His-L190, Gly-L225, Ile-L224, Ser-L223, Zn and its other ligands. The remaining atoms formed the MM layer. Linking between the QM and MM layers was achieved using hydrogen link atoms. Keeping all heavy atoms except the semiquinone fixed, we optimised the semiquinone geometry within the site. All hydrogen atom positions were optimised. In one model, WT-HB, the hydroxyl group of the Ser-L223 residue was initially positioned within hydrogen bonding distance of the quinone O₁ atom. In a second model, WT-NHB, it was rotated away from hydrogen bonding range. To model the L223SA mutant, the CH₂OH group of Ser-L223 in the WT models was replaced with a methyl group. Charges for the MM layer were generated using the qEq method and electrostatic embedding, i.e., ONIOM-EE was employed.(21) This geometry was then used in a further single point ONIOM (B3LYP/EPR-II:UFF) calculation to obtain spin densities and hyperfine coupling constants (hfc). For the Zn atom the 6–31G(d) basis set was used. All calculations were performed using Gaussian 09 software.(22)

Results and Discussion

¹H ENDOR studies of semiquinones have proven very successful in measuring hyperfine couplings to rotating methyl groups and hydrogen bonded protons.(7) Strong ENDOR responses are seen for both types of protons. For the ubisemiquinone free radical the 5' methyl group ¹H hfc is directly proportional to the unpaired spin density at the C₅ position and is an excellent probe of the spin density distribution around the ring. In quinone binding sites or in protic solvents changes in the spin density distribution of the ring are caused by hydrogen bond donation to the O₁ and O₄ atoms. Using density functional theory calculations it was shown that hydrogen bond formation to the oxygen atoms of the semiquinone leads primarily to a redistribution of spin density from the oxygens to the C_{ipso} carbon atom.(23, 24) The increased spin density at the C_{ipso} atom can then lead via spin polarization to a decreased spin density at the neighbouring ortho carbon atoms. This will in turn, via a secondary spin polarization mechanism, lead to an increased π spin density at the meta positions. The extent of this redistribution of spin density is directly proportional to both the strength and number of hydrogen bonds formed. For a symmetrical hydrogen bonding situation with equal hydrogen bonding to both O₁ and O₄ one observes decreased O spin density and increased C_{ipso} spin density, and this is the situation that usually exists in protic solvents. In protein binding sites however different strengths and/or quantities of hydrogen bonds can occur for the O₁ and O₄ atoms. If hydrogen bond formation occurs to only one oxygen atom, this is manifested in an asymmetric spin density for the SQ, as demonstrated by the phyllosemiquinone present in Photosystem I.(25, 26) For example, if hydrogen bond donation occurs only at the O₄ atom this will lead to enhanced spin density at the C₄, C₆, C₂ and O₁ atoms with corresponding decreases at O₄, C₃, C₅ and C₁. This would be reversed for hydrogen bond formation only to O₁. Usually, rather than exclusive hydrogen bonding to only one side, there is a difference in the extent and strength of hydrogen bonds formed to the two oxygens leading to a situation intermediate to the extremes described above. For the Q_B site in *Rba. sphaeroides*, X-ray crystal structure data

(5, 10) have shown that the O₄ atom is a potential hydrogen bond acceptor to the N_δH of His-L190. The O₁ atom has potential hydrogen bond acceptance with the peptide NH of Gly-L225 and Ile-L224 and the Ser-L223 OH group. Due to its rotational freedom the Ser-L223 hydroxyl group can be rotated away from hydrogen bond donation to O₁ and, indeed, in some models it has been proposed to hydrogen bond to Asp-L213 instead.(11, 12) Based on the previous report of a hydrogen bond to Ser-L223 by Q-band ENDOR (13) we assumed in a previous publication (18) that the hydroxyl group of this residue is rotated such that a hydrogen bond donation occurs to the SQ_B O₁ atom. This hydrogen-bonded model is denoted WT-HB. In this study we additionally look at an alternative model (WT-NHB) where the Ser-L223 hydroxyl group is rotated away from the SQ_B atom, thereby preventing hydrogen bond donation to the SQ_B O₁ atom. The calculated values for WT-NHB are given in Table 2 and compared with those obtained with the hydrogen bonded model, WT-HB. The changes in calculated hyperfine couplings are as expected from our qualitative discussion above. Removal of the hydrogen bond from Ser-L223 hydroxyl leads to an increase in the magnitudes of the O₁, C₂, C₆ and C₄ anisotropic hfc's, with a corresponding reduction in the O₄, C₅, C₃ and C₁ values. As the anisotropic hyperfine coupling magnitudes are a direct measure of the π spin density at these atom positions this reflects the spin density changes as well.

In a previous publication (18) we pointed out the poor agreement between the ¹H isotropic hfc of the 5'-methyl group for the WT-HB model (7.3 MHz) and the experimental value (5.4 MHz). This was surprising as this hfc is one of the most accurately calculated for the Q_A site and other semiquinone sites. We now believe that this discrepancy was due to the use of the WT-HB model for this calculation. The extra hydrogen bond from the hydroxyl of Ser-L223 to the SQ_B O₁ leads, as explained above, to an elevated spin density value at the C₅ position. This leads in turn to a larger ¹H hfc for the 5'-methyl group. The absence of the Ser-L223 hydrogen bond in the WT-NHB model leads to a smaller C₅ spin density value with a resultant smaller ¹H isotropic hfc of 5.8 MHz for the methyl group, which is in better quantitative agreement with the experimental value of 5.4 MHz. It is also of particular note from Table 2, that the calculated ¹³C A_{zz} (a + T₃₃) value for C₄ of the WT-NHB model (32.8 MHz) is of larger magnitude than the 23.5 MHz calculated for the corresponding A_{zz} of C₁. For the WT-HB model the calculations show the opposite, with a larger magnitude A_{zz} value for C₁ of 33.1 MHz compared with a C₄ value of 27.3 MHz. Experimental EPR spectra clearly show (7) the larger A_{zz} magnitude of the C₄ (32.2 MHz) hyperfine coupling versus C₁ (27.7MHz). The extra hydrogen bond to O₁ introduced from Ser-L223 in the WT-HB model is calculated to flip the spin density polarization over to a situation where the C₁ has the larger magnitude in clear disagreement with the experimental observations.

The calculated hfc's for the L223SA mutant model are also given in Tables 2 and 3. These are significantly different from the WT-HB model but are practically unchanged from those calculated for the WT-NHB model. The QM/MM calculations therefore predict an identical spin density distribution for the WT-NHB and L223SA models. For the mutant, the calculated ¹H isotropic hfc value for the 5'-methyl group of 5.6 MHz is very close to the WT-NHB model value of 5.8 MHz. The experimental pulsed ¹H ENDOR spectra, in both protonated and deuterated solutions, for mutant and WT models are overlaid in Figure 2. The spectra are essentially identical. The D₂O solvent preparations show intense bands with well-pronounced features defining parallel (**1'**) and perpendicular (**1**) principal values of 7.8 MHz (**1'**) and 4.4 MHz (**1**) for the axial hyperfine tensor of the 5'-methyl protons. These principal values determine the isotropic hyperfine constant ~5.5 MHz. Identical values are observed for the mutant sample showing that, experimentally, no change in the value of this coupling is observed. If a hydrogen bond were present from the Ser-L223 hydroxyl group to the O₁ of SQ_B in the WT, the QM/MM calculated values discussed above indicate that the 5'-methyl hfc should change significantly and be clearly evident in the spectra of Figure 2a.

The unchanged position of these bands in the L223SA spectrum signifies that Ser-L223 is not hydrogen bonded to the O₁ atom of SQ_B in the WT.

In addition it is instructive to look for direct evidence of hydrogen bonding via the presence of ENDOR bands due to the hydrogen bonded protons. As mentioned above, these usually give rise to strong ¹H ENDOR signals and if such a hydrogen bond exists from the Ser-L223 hydroxyl group in the WT, it will be absent in the L223SA spectrum. The QM/MM calculations for the hydrogen bonded WT-HB model (Table 3) indicate that large hyperfine coupling values for the parallel component of 6.8 MHz and the perpendicular component of 4.5–4.7 MHz are expected for the Ser-L223 hydrogen bond interaction. The absence of this interaction in the mutant should be clearly visible by comparing the protonated solvent WT and mutant spectra, Figure 2b. Overlap of the WT and L223SA spectra in Figure 2b show no major differences. Some minor intensity changes are noted, especially the features (2) corresponding to a splitting of ~3 MHz, which we ascribe to small rearrangements of the Q_B binding site after the mutation. In supplementary information, four-pulse ESEEM, showing sum-combination peaks, and ²H HYSCORE spectra are shown to be similar for both WT and L223SA samples as well further supporting the absence of Ser-L223 hydrogen bonding in WT. The four-pulse X-band ¹H spectra are mostly sensitive to the anisotropic hyperfine couplings. Similarity of the spectra in WT and mutant samples indicate that anisotropic couplings are not responsible for the features (2) in ENDOR spectra. Therefore, one can suggest that the observed difference results from small variations in the isotropic hyperfine coupling of one or more of hydrogen bond protons. The QM/MM calculations (Table 3) do show that the Gly-L225 ¹H values are slightly lower for the mutant compared with the WT-NHB model. This is presumably linked to the slightly longer Gly-L225 H-bond distance calculated for the mutant model and shown in Table 1.

In addition to the ENDOR studies described above, we have also conducted ¹⁴N and ¹⁵N HYSCORE studies on both WT and L223SA mutant samples. Figures 3 and 4 show comparison of the ¹⁴N and ¹⁵N HYSCORE spectra in WT and mutant samples. The ¹⁴N spectra consist of two pairs of cross-peaks (1) and (2) produced by HN_δ of His-L190 and peptide NH of Gly-L225, respectively.(17, 18) There is essentially no visible difference between ¹⁴N spectra. The ¹⁵N spectra also show cross-peaks (1) and (2) from the same nitrogens and an additional narrow peak (3) at the diagonal point (¹⁵v_N, ¹⁵v_N) from other weakly coupled nitrogens.(17, 18) Some differences between WT and mutant are seen in the ¹⁵N HYSCORE spectra, notably the larger width of the cross-peaks (1) from the His nitrogen and larger intensity of the diagonal peak (3). The change in lineshape (1) may be due to a slight change of the coupling or rhombicity of the anisotropic hyperfine tensor. The change in the diagonal peak (3) intensity may indicate reorganization of the protein environment induced by the mutation, effectively decreasing the average distance from SQ_B to the nearest nitrogens. The lack of any significant difference in ¹⁴N and ¹⁵N hyperfine couplings between the WT and L223SA is in agreement with the QM/MM calculations of these parameters in Table 2, which shows essentially no change in the calculated values of these couplings for all three models.

The results of this work, showing no strong hydrogen bonding between Ser-L223 and Q_B⁻ in the Q_AQ_B⁻ state, are in apparent contrast with the CW-ENDOR study by Paddock *et al.* (27) The Paddock *et al.* conclusions were based purely on the observation of ¹H ENDOR intensity changes, on mutation, which were attributed directly to hydrogen bonded proton hyperfine couplings from the L223-Ser hydroxyl group in the WT sample. As mentioned above, we do observe some intensity changes in this hydrogen bonding region on mutation but not as extensively as reported in the other study. They did not report any ¹H hyperfine coupling values for the 5' methyl group, which is a clearer indicator of the hydrogen bonding status of the SQ. In addition the main results in their study were obtained with an

extensively mutated reaction center, in which Q_A is missing due to the positioning of a mutant tryptophan residue in the quinone binding site. In the case of RCs singly mutated in this manner (Ala-M260→Trp), the X-ray crystal structure shows that a chloride ion is bound in the empty Q_A site.(28) It is easy to imagine that the presence of this anion could mimic the state with Q_A^- present, thereby elevating the pK_a of Q_B^- .

Implications for the role of Ser-L223 in Q_B protonation

The absence of a hydrogen bond from Ser-L223 to Q_B^- , as demonstrated here, is in agreement with previous conclusions drawn from FTIR studies.(12) It is also in accord with the finding that the rate of the *first* electron transfer, from Q_A^- to Q_B is not dependent on the identity of the residue at L223.(29)

The conclusion is that Ser-L223 is not a hydrogen bond donor to the semiquinone Q_B^- species generated after the first flash. However, the residue is known to significantly influence the rate of reaction and proton transfer associated with the *second* electron transfer step, i.e., from Q_A^- to Q_B^- .(28)

The Ser-L223→Ala mutant is fully inhibited in the proton-activated transfer of the second electron Q_B^- (29), indicative of complete fracture of the proton delivery pathway to O_1 of the semiquinone. It is believed, therefore, that Ser-L223 functions as the proton donor to the O_1 atom of Q_B^- , leading to formation of a transient neutral semiquinone, $Q_B H$, prior to acceptance of a second electron from Q_A^- to form $Q_B H^-$.(1, 2, 29) However, in the $Q_A Q_B^-$ state observed in this work, no hydrogen bond is formed between Ser-L223 and Q_B^- and, instead, Ser-L223 is likely to be hydrogen bonded to the carboxylate group of Asp-L213 after the first flash. Following the second flash, formation of the $Q_A^- Q_B^-$ state likely promotes formation of a hydrogen bond from Ser-L223 to Q_B^- facilitating proton donation. The pK_a of Q^-/QH in vitro has been estimated (30, 31) to be in the range 4–6 but the doubly negative charged state, $Q_A^- Q_B^-$, can be expected to have an increased pK_a value for both Q_A^- and Q_B^- . Elevation of this value for Q_B^- may favour cleavage of the Ser-L223 hydrogen bond to Asp-L213 and rotation of the hydroxyl group to hydrogen bond to Q_B^- . This sets the stage for formation of $Q_B H$, accompanied by proton influx from the bulk phase (the periplasm, *in vivo*) via a chain of proton acceptors leading to Ser-L223 via the Asp-L213 residue, and restoration of the original hydrogen bonding interaction between the serine and aspartate. Subsequent electron transfer from Q_A^- to $Q_B H$ and uptake of another proton at the O_4 atom leads to formation of $Q_B H_2$. The quinol can then diffuse out of the site and be replaced by another quinone molecule from the pool.

Conclusions

ENDOR and ESEEM data show that the spectroscopic differences between the mutant L223SA and WT samples are negligible indicating minor perturbations in the SQ_B spin density for the mutant sample. Qualitatively this suggests that, after the first flash, a strong hydrogen bond does not exist in the WT between the Ser-L223 hydroxyl group and the SQ_B O_1 atom, as removal of this hydrogen bond in the mutant should cause a significant

redistribution of spin density distribution of the semiquinone reflected in altered hyperfine couplings. We show quantitatively, using QM/MM calculations, that a WT model in which the Ser-L223 hydroxyl group is rotated to prevent hydrogen bond formation with the SQ_B O₁ atom predicts negligible change for the L223SA mutant. This, together with the better agreement between key QM/MM calculated and experimental hfc's for the non-hydrogen bonded model, leads us to conclude that no strong hydrogen bond exists between the Ser-L223 hydroxyl group and the SQ_B O₁ atom in the WT after the first flash.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

DFT	density functional theory
B3LYP	Becke3 Lee Yang Parr
QM	quantum mechanics
MM	molecular mechanics
SQ	semiquinone
RC	reaction center
ONIOM	Our Own N-layered Integrated Molecular Orbital and Molecular Mechanics
EPR	electron paramagnetic resonance
ENDOR	electron nuclear double resonance
ESEEM	electron spin echo envelope modulation
HYSCORE	hyperfine sublevel correlation
hfc	hyperfine coupling constant

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Figure 1. Optimised Q_B site model for (a) WT-HB and (b) WT-NHB. In (a) hydrogen bond donation to the O₁ atom occurs from the peptide NH groups of Gly-L225, Ile-224 and the hydroxyl group of Ser-L223. In (b) the Ser-OH group does not donate a hydrogen bond to the O₁ atom. Arrow points to the position of the Ser-L223 hydroxyl group hydrogen atom.

Figure 2.

The pulsed ^1H ENDOR spectra of the SQ_B in WT (black) and L223SA (blue) in $^2\text{H}_2\text{O}$ and of the SQ_B in WT (red) and L223SA (green) RCs in $^1\text{H}_2\text{O}$. The spectra were measured using the Davies pulse sequence (π -T- $\pi/2$ - τ - π -echo with an RF π pulse applied during the time interval T), the length of microwave pulses $t_{\text{MW}} = 88, 44, 88$ ns, respectively, $\tau = 380$ ns, length of RF pulse = $16 \mu\text{s}$, MW Frequency = 9.690 GHz, temperature 70 K.

Figure 3. Comparison of ^{14}N HYSCORE spectra: WT protein (red) and L223SA mutant (blue) in H_2O (magnetic field 345.6 mT, $\tau=136$ ns, 9.690 GHz).

Figure 4. Comparison of ^{15}N HYSCORE spectra: ^{15}N uniformly labeled WT protein (red) and L223SA mutant (blue) in H_2O (magnetic field 345.0 mT, $\tau=200$ ns, 9.6871 GHz).

Table 1

SQ_B optimised carbonyl covalent bond and hydrogen bonding distances. The O1 to Ser O distance is given in brackets. All distances in ångstroms

Bond	WT-HB	WT-NHB	L223SA
C4-O4	1.28	1.28	1.28
C1-O1	1.28	1.27	1.28
O4 - HN _δ His-L190	1.58	1.55	1.54
O1 - HN Gly-L225	2.00	1.83	1.91
O1 - HO Ser-L223	1.81(2.80)	3.10(2.85)	-
O1 - HN Ile-L224	2.30	2.10	2.10

Table 2

Calculated ^{13}C , ^{17}O and ^1H hyperfine couplings (MHz) for Q_B site hydrogen bonding interactions in WT and the L223SA mutant model.

Position	WT-HB			L223SA			WT-NHB		
	Anisotropic T_{33}, T_{22}, T_{11}	Isotropic A	Anisotropic T_{33}, T_{22}, T_{11}	Isotropic A	Anisotropic T_{33}, T_{22}, T_{11}	Isotropic A	Anisotropic T_{33}, T_{22}, T_{11}	Isotropic A	Isotropic A
$^{13}\text{C}(1)$	30.6	3.3	25.2	-1.5	25.2	-1.7	25.2	-1.7	-1.7
	-16.6		-14.1		-14.0		-14.0		
	-14.0		-11.1		-11.2		-11.2		
$^{13}\text{C}(4)$	26.3	1.0	28.3	2.3	29.2	3.6	29.2	3.6	3.6
	-14.4		-15.4		-15.4		-15.4		
	-11.9		-12.9		-13.0		-13.0		
$^{17}\text{O}(1)$	-66.9	-18.3	-71.6	-18.4	-72.1	-18.3	-72.1	-18.3	-18.3
	-33.6		38.0		35.6		35.6		
	-33.3		37.1		36.5		36.5		
$^{17}\text{O}(4)$	-62.6	-18.2	-61.6	-17.8	-60.5	-17.9	-60.5	-17.9	-17.9
	31.4		30.3		29.9		29.9		
	31.2		29.7		30.5		30.5		
$^1\text{H CH}_3(5')$	2.6	7.6	2.6	5.6	2.5	5.8	2.5	5.8	5.8
	-1.7		-1.6		-1.6		-1.6		
	-1.0		-1.0		0.9		0.9		

Table 3

Calculated ^1H and ^{14}N hyperfine couplings (MHz) for Q_B site hydrogen bonding interactions in WT and L223SA mutant.

Position	WT-HB			L223SA			WT-NHB		
	Anisotropic T_{33}, T_{22}, T_{11}	Isotropic A	Anisotropic T_{33}, T_{22}, T_{11}	Isotropic A	Anisotropic T_{33}, T_{22}, T_{11}	Isotropic A	Anisotropic T_{33}, T_{22}, T_{11}	Isotropic A	Isotropic A
^1HN His-L190	10.2 -5.3 -4.9	-0.4	10.7 -5.6 -5.1	-0.2	10.3 -5.4 -4.9	-0.1			
^1HN Gly-L225	6.6 -3.7 -2.9	-0.6	7.3 -4.0 -3.3	-0.7	8.4 -4.5 -3.8	-0.8			
^1HN Ile-L224	3.9 -2.1 -1.8	-0.1	5.0 -2.6 -2.3	-0.2	4.9 -2.6 -2.3	-0.1			
^1HO Ser-L223	7.6 -4.0 -3.7	-0.8			1.8 -1.0 -0.8	0.0			
$^{14}\text{N}_\text{d}$ His-L190	0.4 -0.2 -0.2	1.5	0.4 -0.2 -0.2	1.4	0.4 -0.2 -0.2	1.4			
^{14}NH Gly-L225	0.3 -0.2 -0.1	0.6	0.3 -0.2 -0.1	0.6	0.3 -0.2 -0.1	0.9			
^{14}NH Ile-L224	0.2 -0.1 -0.1	0.1	0.2 -0.1 -0.1	0.2	0.2 -0.1 -0.1	0.1			