A NEW FLAVIN RADICAL SIGNAL IN THE Na\(^{+}\)-PUMPING NADH:QUINONE OXIDOREDUCTASE FROM *VIBRIO CHOLERAE*: AN EPR/ENDOR INVESTIGATION OF THE ROLE OF THE COVALENTLY BOUND FLAVINS IN SUBUNITS B AND C

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SUMMARY

The Na⁺-pumping NADH-ubiquinone oxidoreductase has six polypeptide subunits (NqrA-F) and a number of redox cofactors including: a non-covalently bound FAD and a 2Fe-2S center in subunit F, covalently bound FMN’s in subunits B and C, and a non-covalently bound riboflavin in an undisclosed location. The FMN cofactors in subunits B and C are bound to threonine residues by phosphoester linkages. A neutral flavin-semiquinone radical is observed in the oxidized enzyme, while an anionic flavin-semiquinone has been reported in the reduced enzyme. For this work, we have altered the binding ligands of the FMN’s in subunits B and C by replacing the threonine ligands with other amino acids, and studied the resulting mutants by EPR and ENDOR spectroscopy. We conclude that Na⁺-NQR forms three spectroscopically distinct flavin radicals: 1) a neutral radical in the oxidized enzyme, which is observed in all of the mutants, and most likely arises from the riboflavin, 2) an anionic radical observed in the fully-reduced enzyme, which is present in wild type, and the NqrC-T225Y mutant, but not the NqrB-T236Y mutant, 3) a second anionic radical, seen primarily under weakly reducing conditions, which is present in wild type, and the NqrC-T225Y mutant, but not the NqrB-T236Y mutant. Thus, we can tentatively assign the first anionic radical to the FMN in subunit B and the second to the FMN in subunit C. The second anionic radical has not been previously reported. In ENDOR spectra, it exhibits a larger linewidth and larger 8α-methyl proton splittings, compared to the first anionic radical.

INTRODUCTION

The Na⁺-translocating NADH:quinone oxidoreductase (Na⁺-NQR) is a primary sodium pump present in the inner membrane of many marine and pathogenic bacteria including Vibrio alginolyticus, Vibrio harveyi, Haemophilus influenzae and Vibrio cholerae. This enzyme oxidizes NADH and reduces ubiquinone as the first step in the aerobic respiratory chain. The free energy from the electron transfer reaction is harnessed to the translocation of sodium ions across the membrane, creating a sodium motive force, which is used by the cell for metabolic work (1-8).

Na⁺-NQR is made of six subunits that accommodate several cofactors: One non-covalently bound FAD and a 2Fe-2S center in subunit F, two covalently bound FMN’s in subunits B and C, and riboflavin in an unknown location (1,9-13). The covalently bound FMN’s in Na⁺-NQR are bound to the protein by phosphoester bonds between the phosphate of the FMN and the -OH group of a threonine residue, (T236 in NqrB and T225 in NqrC). In both cases the threonine is part of a highly conserved sequence of amino acids, SGAT (11,12,14). The role of these FMN’s in electron transfer and in Na⁺-translocation has not been elucidated.

An unusual feature of Na⁺-NQR is the occurrence of more than one flavin radical. In a previous EPR spectroscopy study, we showed that the air-oxidized enzyme (resting state) exhibits a neutral flavin semiquinone, while in the reduced enzyme, an anionic flavin semiquinone, deprotonated at the N(5) position of the isalloxazine ring (15,16). Neither of these signals has been assigned to any of the flavins in Na⁺-NQR. However, both signals were observed in mutants, such as NqrF-S246A, where the FAD cofactor has been knocked out, indicating that the radical signals must arise from other flavins of the enzyme. In the present paper, we present new mutations, which eliminate two of the remaining three flavins: the covalently bound FMN’s in subunits B and C. Using these mutants, together with the wild-type enzyme, we have now made the first detailed ENDOR study of the flavin radicals of Na⁺-NQR.
The results reveal a new anionic flavin radical in the partially reduced enzyme which has a larger linewidth and larger \( \delta \)-methyl proton splittings than the anionic flavin radical in the fully reduced enzyme. Our findings allow us to tentatively assign the neutral radical to the riboflavin cofactor, the anionic radical, from the partially reduced enzyme, to the FMN in NqrB, and the anionic radical, from the fully reduced enzyme, to the FMN in NqrC.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and growth conditions**

*Vibrio cholerae* O395N1ΔnqrA-F which lacks the genomic copy of the *nqr* operon was used as host for expression of the wild-type and mutant Na\(^{+}\)-NQR’s. Cells were grown in LB medium at 37 \( ^\circ \)C. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) competent cells were used for mutagenesis.

**Site directed mutagenesis**

The plasmid pBAD-*nqr* was used as template for mutagenesis and also for expression of the recombinant wild-type Na\(^{+}\)-NQR and mutants. Mutants in the NqrB-T236 and NqrC-T225 residues were constructed using the primers listed in Table 1. Site-directed mutagenesis was carried out using “Quikchange” kit (Stratagene, La Jolla, CA). Mutations were checked by restriction digestions, using restriction sites introduced by means of silent mutations in the mutagenesis primers. The mutations were subsequently confirmed by direct DNA sequencing from the mutated plasmids. Mutated plasmids were introduced into *Vibrio cholerae* O395N1ΔnqrA-F by electroporation as reported previously (1). This was followed by gel filtration on D-Salt Polyacrylamide 6000 (Pierce, Rockford, IL) using a buffer (“final buffer”) containing 50 mM sodium phosphate, pH 8.0, 100 mM NaCl, 5% glycerol, 10 mM EDTA and 0.05% dodecyl maltoside (Anatrace, Maumee, OH). The resulting preparations were frozen in aliquots and stored at liquid nitrogen temperature. Protein concentrations were determined using the BCA Protein Assay Kit from Pierce, and SDS-PAGE containing 6M urea (4%-16% acrylamide) was used to check the purity of the proteins.

Steady state turnover of Na\(^{+}\)-NQR was measured as either (1) oxidation of NADH and reduction of Q-1 (ubiquinone analog) or (2) oxidation of NADH and reduction of the artificial electron acceptor ferri cyanide. These reactions were followed spectrophotometrically, using a full-spectrum method as reported before (1).

**UV-visible spectroscopy**

UV-visible spectra were recorded on an Agilent 8453 spectrophotometer.

**Flavin analysis by denaturation, visible and fluorescence spectroscopy**

Wild-type and mutant enzymes were denatured by addition of 6 M guanidine chloride. The denatured protein was passed through a Centricon filter (molecular weight cut-off 3 kDa) to remove the protein and the covalently bound flavins. The amount of flavin in the sample, before and after filtration, was measured by absorbance (UV-visible) and by fluorescence spectroscopy.

**HPLC analysis of non-covalently bound flavins**

The filtrate obtained as described above was also analyzed by HPLC as described previously (1).

**EPR and ENDOR experiments**

X-band EPR spectra were collected on a Varian E-122 spectrometer. The samples
were run as frozen glasses at ~ 60 K using an Air Products Helitran cryostat with liquid helium. Pulsed ENDOR spectra were collected on a Bruker E580-10 Elexsys spectrometer. Samples were maintained at 85K using liquid helium in an Oxford CF935 Cryostat controlled by an Oxford ITC-4 temperature controller. Pulsed ENDOR experiments were carried out using a Davies three-pulse scheme (\(\pi - T - \pi/2 - \tau - \pi - \tau -\) echo, with the RF pulse applied during time T). The magnetic fields were calibrated with a Varian NMR Gauss meter. All instruments were maintained by the IERC, University of Illinois at Urbana-Champaign. EPR spectra were simulated with the “SIMPOW6” program developed at the University of Illinois, which is based upon “QPOW” (17). ENDOR spectra were simulated using “SIMEND”, which is a version of “SIMPIP” (http://ierc.med.uiuc.edu/~nilges/software.html) that calculates a frequency-swept spectrum and includes an intensity weighting function for the Boltzman population and for angle-selection. The angle selection is determined primarily by the anisotropic N(5) and N(10) hyperfine splittings, as well as the H(5) proton hyperfine splitting in the case of the neutral radical:

\[
I_{\alpha} = \sum_{n,m} \sum_{m'} \sum_{m''} \left[ \sum_{n} TM_{mn} (\beta_{n} - \beta_{m}) F(H_{\alpha} - H_{\alpha}(m, n, m', \theta, \phi), \Delta H(\theta, \phi)) \right]
\]

where \(n\) and \(m\) run over all spin levels, \(TM\) is the transitions moment, \(\beta\) is the Boltzmann population; \(H_{\alpha}\) and \(H_{\alpha}(m, n, m', \theta, \phi)\) are calculated using first-order perturbation theory, and the anisotropic Gaussian linewidth \(\Delta H(\theta, \phi)\) is obtained by fitting the residual linewidth due to the remaining protons other than H(5). The contributions from the individual protons are calculated separately and then summed together.

Because the sensitivity decreases with frequency due to instrumentation, an empirical weighting or roll-off function was added:

\[
G(\nu) = \frac{1}{1 + (\nu/\nu_{0})^{n}}
\]

where \(n\) is found to be around 2.6 to 2.8 and \(\nu_{0}\) is approximately 4 MHz. This correction is an empirical one that also includes corrections for other sources of frequency-dependent sensitivity such as relaxation.

**RESULTS**

**Mutations of the threonine ligands of the covalently bound FMNs**

As part of our ongoing project to study the spectroscopy and redox chemistry of the flavins in Na\(^{+}\)-NQR we have used site-directed mutagenesis to change NqrB-T236 and NqrC-T225 to different amino acids. The objective was to either eliminate or perturb each FMN cofactor individually in order to study the effect on enzyme activity and on the flavin radicals.

The FMN cofactors in Na\(^{+}\)-NQR subunits B and C are both covalently attached by phosphoester bonds to threonine residues: T236 in NqrB and T225 in NqrC. In both cases the threonine is part of a highly conserved sequence of amino acids (SGAT) (11,12). The same sequence has recently been identified as part of an FMN binding site in the NosR flavoprotein of *Pseudomonas stutzeri* (18).
to serine, alanine, cysteine, leucine, and tyrosine. The serine mutant enzyme was very much like the wild-type. The enzyme was active, and all cofactors were incorporated, including the covalently bound flavin in subunit B. This is consistent with the fact that serine has an −OH group and can replace threonine in forming a phosphoester bond. The alanine, cysteine and leucine mutations resulted in enzymes that were too unstable to be studied.

The tyrosine mutant (NqrB-T236Y) assembled, but did not incorporate the covalently bound FMN in NqrB. Wild-type Na⁺-NQR shows two fluorescent bands in PAGE, corresponding to subunits NqrB and NqrC, whereas the NqrB tyrosine mutant showed only one fluorescent band, corresponding to the NqrC subunit (Figure 1), although all six subunits could be observed in Coomassie-stained gels (not shown). Analysis of the total flavin content by UV/vis and fluorescence (see Materials and Methods) showed that the mutant enzyme contained approximately 3.3 flavins, and that the ratio of covalently- to noncovalently-bound flavins was about 1:2.3. HPLC analysis of the soluble flavins showed that the non-covalently bound FAD and riboflavin cofactors were present in the mutant, though the riboflavin was apparently present in a stoichiometry of only 30% consistent with the smaller amount of flavin detected by the UV/vis and fluorescence assays (Figure 2). EPR spectra of the reduced enzyme showed that the 2Fe-2S center was also assembled (Figure 4). The enzyme was not able to carry out its physiological reaction, measured as transport electrons from NADH to Q1, but was active with NADH and the artificial substrate ferricyanide, which is believed to accept electrons from the FAD cofactor in NqrF (rate: ~300 μmoles reduced ferricyanide min⁻¹ mg⁻¹) (1). Thus, the NqrB-T236Y enzyme assembles with all cofactors except the covalently bound FMN in NqrB. This made it an ideal vehicle to study the role of this flavin in the formation of the radical signals of Na⁺-NQR.

The NqrC subunit is relatively hydrophilic and is predicted to have one or two transmembrane helices. The conserved motif (SGAT) that includes Threonine-225 is predicted to be in a large hydrophilic loop. NqrC-T225 was mutated to leucine (not shown) and to tyrosine. In both cases the Na⁺-NQR complex was expressed and assembled, but without incorporation of the covalently bound flavin in NqrC. An SDS-PAGE gel (Figure 1) shows that the NqrCT225Y mutant exhibits only one fluorescent band, corresponding to NqrB. Although the NqrC protein is seen under subsequent Coomassie blue staining, only the NqrB band is fluorescent indicative of a covalently bound flavin. The NqrC mutants were more stable (i.e., easier to obtain in good yield) than any of the NqrB mutants, probably indicating that FMN in NqrB plays a role in the assembly of the Na⁺-NQR complex. The NqrC-T225Y preparation was found to contain the same amount of FAD and riboflavin, per unit protein, as the wild-type enzyme (not shown). The NqrC mutants were also inactive in NADH:quinone oxidoreductase activity but, like the NqrB mutants, were active when ferricyanide was used as an electron acceptor (rate: ~300 μmoles reduced ferricyanide min⁻¹ mg⁻¹).

CW EPR Results
Since Na⁺-NQR exhibits flavin radical EPR signals; we used EPR spectroscopy to study the NqrB-T236Y and NqrC-T225Y mutants in more depth. The EPR spectra of the wild-type Na⁺-NQR and the NqrC-T225Y mutant are compared in Figure 3. For the air-oxidized enzyme, the EPR spectra of the wild-type and NqrC-T225Y are identical and have the same 20.0 G linewidth, which is typical for a neutral flavin radical (19,20). For the enzyme reduced with NADH or sodium dithionite, the spectra are very similar, but some minor differences are seen in linewidth and intensity. In the case of dithionite reduction the linewidth of the radical is slightly smaller (13.6 G) for NqrC-T225Y than for the wild type (14.6 G),
though both values are typical for anionic flavin radicals. (21) With NADH reduction the observed linewidths are slightly larger than those with dithionite reduction (15). For NqrC-T225Y the value is 14.7 G while for the wild-type it is 15.3 G. Thus, the EPR of the NqrC-T225Y and the wild type show only small differences, primarily slightly narrower lines for the NqrC-T225Y under reducing conditions.

The flavin radical EPR signals of the NqrC-T225Y mutant are similar to those of the wild-type enzyme, as are those of the FAD and 2Fe-2S mutants (10,22). In contrast, the EPR spectra of the NqrB-T236Y mutant show a number of significant differences compared to the wild-type enzyme. Most important is that reduction of the enzyme by dithionite can result in complete abolition of the free radical signal. As shown in Figure 4, as the concentration of dithionite is increased, the amplitude of the free radical signal decreases until it is essentially gone at concentrations above 10 mM. In the case of NADH the effects are not as great, but the flavin radical signal is still found to decrease as the concentration of NADH is increased (Figure S1, supplementary material). At face value, these results suggest that the FMN in NqrB is the parent of the anionic flavin radical.

Air oxidized samples of NqrB-T236Y give rise to an EPR signal with a linewidth of 20 G which is characteristic of a neutral radical. However, the spin concentration is less than a third of that seen in wild-type samples (radical to 2Fe-2S ratio of 0.28:1). The highest concentration of flavin radical is seen when the concentration of reductant is around 0.3 mM (Figure 4 and S1) where the ratio of radical concentration to 2Fe-2S concentration is found to be around 0.45 to 1. The linewidth of the flavin radical signal decreases upon reduction; this line narrowing is significant even at low concentrations of reductant. For reduction with dithionite the line narrows to 15.6 Gauss while for reduction with NADH it narrows to 16.6 Gauss. This narrowing suggests that a significant amount of anionic flavin radical is still formed upon reduction of this mutant, even though the radical disappears under strongly reducing conditions.

If mutation of NqrB-T236 to tyrosine, prevents the incorporation of FMN into this subunit, as indicated by our gel results, and if the FMN in NqrB is the source of the anionic radical as suggested by the disappearance of the radical signal under strongly reducing conditions, then one would expect that this enzyme would exhibit only the EPR signal with a linewidth of 20 Gauss corresponding to the neutral radical. The significant narrowing of the radical signal, especially in the initial stages of reduction, would appear to be inconsistent with the assignment of the anionic flavin radical to the FMN of NqrB.

ENDOR spectra of wild-type Na⁺-NQR under oxidizing and fully reducing conditions

As noted in our earlier work (1,15), ENDOR spectroscopy is much more definitive in distinguishing between neutral and anionic flavin radicals. In particular, the splittings from the C(8α)-methyl protons are quite distinct for neutral and anion flavin radicals; couplings are typically <10 MHz for neutral radicals and >10 MHz for anion radicals. To understand why an anionic flavin radical is still present in the NqrB mutant under certain conditions and to confirm that radicals observed in the NqrB mutant are indeed the same neutral and anionic radicals observed in wild-type samples, we have performed pulsed ENDOR experiments on the NqrB and NqrC mutants as well as wild-type Na⁺-NQR. The ENDOR spectrum of the enzyme under weakly reducing conditions is expected to include contributions from both neutral and anionic flavin radicals and the contributing spectra are expected to overlap to some extent. To better aid the analysis of such mixed spectra we have used spectral simulation. As a prerequisite, it was first necessary to be able
to correctly simulate the complete spectra of both the pure neutral and pure anionic radicals.

To simulate the spectrum of the neutral or anionic flavin radical it was necessary to include not only the splittings from the C(8α), the C(6), the C(1′)H, C(9), and C(7α) protons which were reported earlier (15), but also the second, C(1′)H2, proton on the ribityl side chain and the N(3) amide proton. Because peaks with small hyperfine splittings can be suppressed when the length of the τe pulse is small, τe was set to 320 ns so as to minimize distortion of the central part of the ENDOR spectrum around 14.5 MHz, the proton Zeeman frequency. This central region includes splittings from the C(9), C(7α), and N(3) protons. For a value of τe of 320 ns, most, but not all of the matrix peak, which is primarily due to remote protons such as those at more distant positions on the ribityl side chain, is suppressed. To compensate for this, a generalized matrix peak was added in the simulations.

Because the proton ENDOR spectra consist of large number overlapping peaks, simulation is not straightforward. Several strategies were employed to make this problem tractable.

In order to provide a more complete basis for the simulations, spectra were acquired with the magnetic field set near the EPR absorption maximum (g = 2.0026) and also with the field set 25 Gauss (g = 1.9899) above the absorption maximum. Unlike the ENDOR spectrum obtained with the field set to the absorption maximum. Unlike the ENDOR spectrum obtained with the field set to the absorption maximum, ENDOR data obtained with the field set off-center show significant angle selection due to the highly anisotropic N(5) and N(10) couplings. This results in a “single-crystal-like” z-axis spectrum.

In addition, the absorption and first derivative spectra were both used to judge the goodness of the simulation. Only a simulation that consistently fit both the 0-

and 1-derivative spectra could be chosen as “best fit.” Also, the choice of starting values used in simulations was guided by DFT calculations of the FAD flavin radical in DNA photolysase (23) as well as our own DFT calculations of the neutral and anionic flavin radicals using the structure of the FMN radical in flavodoxin from *Clostridium beijerinckii* (24); see Supplementary Material.

Figures 5 and 6 show the simulations for the neutral and anionic flavin radicals, respectively. As can be seen the simulations reproduce the shape of the ENDOR spectra very well. These simulations do not include contributions from the N(5) and N(10) nitrogens or the contribution of the N(5) proton, in the case of the neutral radical. The N(5) and N(10) nitrogens and the N(5) proton have highly anisotropic hyperfine tensors and simulations predict that these nuclei should give rise to weak broad peaks around 1 to 3 MHz and around 27 and 33 MHz. It is important to note that the linewidths for the C(8α)methyl protons are significantly smaller for the anionic flavin radical than for the neutral flavin radical.

In order to fit our spectra we needed to include splittings from both of the C(1′) methylene protons. In contrast to DNA photolysase where the two methylene proton splittings are significantly different (20), in Na+-NQR the difference between the two methylene splittings is much smaller for both the neutral and the anionic radicals. For DNA photolysase the Ax component of the C(1′) proton with the larger coupling is large enough (11.3 MHz) so as to give a well defined peak outside the C(8α)-methyl proton peaks. For both the neutral and anionic flavin radicals in Na+-NQR, no separate peaks are seen outside the C(8α)-methyl proton peaks and simulation shows that the C(1′) proton peaks overlap with the C(8α)-methyl proton peaks. The hyperfine coupling for the two β-methylene C(1′) protons is due to hyperconjugation, which gives rise to a coupling which is dependant
on the dihedral angle between the πσ orbital on the N(10) and the Cβ-Hβ bond.

\[ A^{H1} = \rho_s [B \cos^2 \theta + C] \quad A^{H2} = \rho_s [B \cos^2 (\theta - 120) + C] \] (1)

where \( B \sim 100 \) MHz and \( \rho_s \) is the πσ spin density. The C term is usually small and can be neglected. From our data on the neutral and anionic radicals we find that \( \theta \) is equal to 57.7° and 56.7°, respectively, whereas for DNA photolyase an angle of 50.4° was determined. Examination of the structure of FAD in DNA photolyase and that of FMN the flavodoxin in Clostridium beijerinckii reveals a number of differences in the structure of the ribityl side chain. First, the side chain of FAD in DNA photolyase is oriented so that a hydrogen bond is formed between the OH group at the C(3') position and the N(1) nitrogen, while for the FMN in Clostridium beijerinckii, the shorter side chain is rotated about the C(2')-C(3') bond to give a hydrogen bond between the OH at the C(2') position and the N(1) nitrogen. This difference in hydrogen bonding results in a dihedral angle between the C(1')-C(2') bond and the πσ orbital of N(4) that is half as large (4.3°). Thus, the C(1') proton splittings of both the neutral and anionic radicals in Na⁺-NQR are much more consistent with the structure of the FMN in Clostridium beijerinckii than that of the FAD in DNA photolyase (20,24).

**ENDOR Spectra of the Mutants under Weakly Reducing Conditions**

Figure 7 shows ENDOR spectra for the NqrB-T236Y mutant. For the air-oxidized sample the spectrum is identical to that of wild-type Na⁺-NQR, confirming that the neutral radical is unchanged in this mutant. For reduced samples, the appearance of methyl proton peaks with splittings of 10 – 12 MHz clearly indicates the formation of an anionic radical. However, the spectrum for 1 mM dithionite is clearly different from that of the anionic flavin radical seen in the fully reduced wild-type Na⁺-NQR (Figure 6a). Simulation of the 1 mM dithionite spectrum using a mix of neutral and anionic spectra is not able to account for the shape of the spectrum. The most noticeable difference between the NqrB-T236Y mutant spectrum and that for the fully reduced wild-type spectrum is a broadening of the C(8α)-methyl proton splittings. Fitting of the NqrB-T236Y mutant spectrum could be greatly improved by increasing the linewidths corresponding to \( A_{||} \) and \( A_{\perp} \) from 0.10 and 0.10 MHz to 0.46 and 0.56, respectively. It is also noticeable in Figure 7 that the spacing between the methyl proton peaks is slightly larger than that expected for fully reduced wild-type Na⁺-NQR. Simulation gave values of \( A_{||} \) and \( A_{\perp} \) of 12.3 ± 0.2 and 10.2 ± 0.1 MHz, respectively compared to the expected values of 11.82 and 9.84 MHz. Additionally, the fit was improved if the values of \( A_x \) and \( A_y \) for the C(6) proton were decreased slightly to −8.5 ± 0.3 and −7.9 ± 0.5, respectively. Differences in other hyperfine splittings are not measurable, in part due to the low signal-to-noise that results from the low occupancy of the radical in the NqrB mutant. The simulations shown in Figure 7 for 0.3 and 1 mM dithionite also include a contribution from the ordinary neutral radical with a neutral to an anion ratio of 50:50 and 23:77, respectively.

Because the presence of this new second or intermediate radical, which we will call anionic flavin radical (II), are most noticeable when the enzyme is only partially reduced, ENDOR spectra of wild-type Na⁺-NQR were also acquired at reductant concentrations similar to those used to observe this signal in the NqrB-T236Y mutant. Figure 8 shows ENDOR spectra of
wild-type Na\textsuperscript{+}-NQR at dithionite concentrations of 0.1, 0.2, and 0.3 mM. While the anionic radical is seen to grow as evidenced by the presence of the C(8α)-methyl proton peaks at 10 to 12 MHz, the peaks are not as sharp as those seen in the fully reduced spectrum shown in Figure 6a. These spectra could not be accounted for by a simulation of mixture of the neutral radical and the anionic flavin radical (I), which is observed under fully reduced conditions. The spectra could be accounted for well by a simulation (Figure 8) that included a mixture of three different flavin radicals: the neutral, anion (I), which is observed in the wild type under fully reduced conditions, and anion (II), which is observed in the NqrB-T236Y mutant, with neutral to anionic (II) to anionic (I) ratios of 90:8:1, 36:36:23, and 15:35:50 for 0.1, 0.2, and 0.3 mM dithionite, respectively. Because all three radicals contribute to the wild-type spectra, the anionic flavin radical (II) is not as easily discerned as it is in the NqrB T236Y mutant spectra. However, the long broad wings just beyond the C(8α)-methyl proton peaks in both the experimental and simulated spectra (shown with arrows in figure) indicate the presence of anion flavin radical (II). Reduction with 0.3 mM NADH gave results identical to those for 0.3 mM dithionite.

ENDOR spectra were also recorded for the NqrC-T225Y mutant. Just as with the CW-EPR spectra, no differences were seen between the ENDOR spectra of the NqrC-T225Y mutant and wild-type Na\textsuperscript{+}-NQR for samples that were either air-oxidized or reduced with 10 mM NADH or dithionite. However, significant differences were seen for samples that were weakly reduced. Figure 9 shows ENDOR spectra for the NqrC-T225Y mutant under reducing conditions when the formation of an anion flavin radical is first seen. Unlike the wild-type spectra, the expected sharp C(8α)-methyl proton peaks of anion flavin radical (I) are seen even at the lowest of concentration of reductant. Simulations showed that the spectra could be readily fitted using only two radicals, the neutral and anion radical (I), with ratios of 81:19, 31:69, and 20:80 for 0.1, 0.2, and 0.3 mM NADH, respectively.

Thus, in the NqrB-T236Y mutant, only the intermediate anion flavin radical (II), with its broad C(8α)-methyl proton peaks, is formed upon reduction, while in the NqrC-T225Y mutant, only anionic flavin radical (I), with its sharp C(8α)-methyl proton peaks is formed upon reduction.

**DISCUSSION**

This completes a set of mutants that knock out each known, localized cofactor of Na\textsuperscript{+}-NQR. The enzyme contains five known cofactors: Covalently-bound FMN’s in subunits B and C, non-covalently bound FAD and a 2Fe-2S center in subunit F, and riboflavin, non-covalently bound in an unknown location; there may also be a tightly bound quinone. Here, we have presented two new mutants: NqrB-T236Y and NqrC-225Y, which knock out the covalently bound FMN’s in NqrB and NqrC respectively. Previously, we reported mutants that knocked out the FAD and 2Fe-2S (10). Perturbation of the riboflavin and the possible ubiquinone will have to await their localization. In both its reduced and oxidized forms, Na\textsuperscript{+}-NQR exhibits flavin radical EPR spectra. The oxidized enzyme has a neutral flavin radical, while the reduced enzyme has an anionic radical. In the NqrF-S246A mutant, which knocks out the FAD, neutral and anionic radicals are still observed.

Here, we have studied the NqrB-T236Y and NqrC-T225Y mutants, as well as the wild-type enzyme, using EPR and ENDOR spectroscopy. The ENDOR study shows that what was previously believed to be a single type of anionic radical in Na\textsuperscript{+}-NQR actually has two distinct forms: one with rather sharp C(8α)-methyl proton peaks (anionic radical I), and one with broader C(8α)-methyl proton peaks (anionic radical II).
In the wild-type enzyme, under oxidizing conditions, a neutral flavin radical is observed. When the enzyme becomes partially reduced, a mixture of anionic radicals I and II is observed, while in the fully-reduced enzyme, only anion radical I is observed. All of the mutants studied thus far exhibit the neutral radical under oxidizing conditions; however, deviations from the wild-type behavior are observed upon reduction of some of the mutants. In the NqrB T236Y mutant, partial reduction of the enzyme leads to anionic radical II alone, but complete reduction abolishes all radical signals. In the NqrC T225Y mutant, as the enzyme becomes reduced, anionic radical I is observed, without anionic radical II. This radical signal persists in the fully-reduced enzyme. As we reported previously, under oxidizing conditions, mutants that lack the FAD in subunit F, exhibit the neutral radical in their EPR spectra.

These results argue against either of the two FMN’s or the FAD as the source of the neutral flavin radical signal and indicate instead that this radical is located on the riboflavin cofactor. The observation that the concentration of neutral radical and that of riboflavin are both substantially diminished in the NqrB-T236Y mutant supports this conclusion.

The simplest explanation of these results is that the three different radical signals observed in Na+-NQR arise from three different flavins: the neutral radical from the riboflavin, anionic radical I from the FMN in NqrB, and anionic radical II, from the FMN in NqrC. Reduction of the FAD in NqF appears to take place by a two electron step, which does not populate any EPR-visible states. Reduction of the enzyme would then involve at least four one-electron flavin redox transitions and one two-electron flavin transition: 1) the one-electron reduction of the neutral riboflavin semiquinone to the corresponding hydroquinone, and 2) the one-electron reduction of the FMN in NqrB to form the anionic semiquinone (anionic radical I), 3) the two-electron reduction of FAD in NqrF. The reaction also includes reduction of the 2Fe-2S center, and possibly a tightly-bound quinone cofactor.

This conclusion differs from that of Bogachev et al. (25) who studied the reduction of Na+-NQR from Vibrio harveyi by spectro-electrochemistry using full-spectrum absorbance detection. In these measurements they observed only three redox transitions involving flavins: 1) two-electron reduction of a flavoquinone, 2) one-electron reduction of a flavoquinone to form an anionic flavo-semiquinone and 3) one-electron reduction of a neutral flavo-semiquinone. Our discovery of the second anionic semiquinone suggests a more complicated mechanism.

One possible way that these results could be reconciled would be if one anionic radical disappears as the other is formed, that is, if anionic radical II becomes reduced to the flavohydroquinol, at the same redox potential where anionic radical I is formed from the flavoquinone. In this way, the total amount of anionic radical would never be larger than 1. The difference between the two anionic radicals is subtle, and in visible absorbations spectra, the replacement of one anionic flavin radical by another could go undetected. This explanation would require the lower redox potential (F $\rightarrow$ F$^2^-$) of the NqrC-FMN to be very close to the higher redox potential (F $\rightarrow$ F$^-$) of the NqrB-FMN. This model makes very clear predictions about the results of redox titrations of Na+-NQR monitored by EPR/ENDOR, since the transition of anionic radical I to radical II should be clearly resolved.

Our data do not rule out the possibility that the two anionic semiquinone radicals arise from the same flavin moiety, and that the differences between them reflect changes in
the environment of a single center. In this case, in the wild-type enzyme, the transition from anionic radical II to radical I would have to be driven by the reduction of a different redox center, mediated though an electrostatic or conformational interaction. In this scheme, neither of the FMN cofactors could give rise to the anionic radicals, since both NqrB-T236Y and NqrC-T225Y each exhibit one of the anionic radical signals. Thus, these mutations would have to exert their effects remotely, by locking the anionic flavin radical center into one of two alternate conformations. However, this scheme would not reconcile the optical stopped-flow data with the ENDOR results, since it would require the riboflavin to give rise to all three types of radical signal in Na⁺-NQR, whereas the results of Bogachev et al. (25) require that the neutral and at least one of the anionic flavin radicals arise from distinct centers.

Assuming that our assignments are correct, it appears that the FMN cofactors form structurally similar anion flavin radicals. Both FMN’s are bound to threonine residues that lie in the same conserved motif (SGAT). What allow us to differentiate these anionic radicals are differences in the environment around the radicals. The line broadening of the C(8a)-methyl proton peaks in radical anion (II) can be attributed to one of two possible environmental sources. One possibility is restricted rotation of the methyl groups due to steric hindrance from the surrounding protein. The more freely the methyl group rotates, the more equivalent the three methyl protons will be and the narrower the linewidth. The second possibility is random dislocations of the radical by its environs (in particular those induced by hydrogen bonding), which could result in differing distributions of spin densities on the isoalloxazine ring and a distribution of hyperfine splittings (an effect known as hyperfine strain).

In addition to the differences in linewidths of the C(8α)-methyl proton peaks, the two anions differ in their apparent redox potentials. While anionic flavin radical (I), the NqrB FMN radical anion is readily observed at near stoichiometric levels (~90%) under strongly reducing conditions, anion radical (II), the NqrC FMN radical, has a maximum occupancy below 50% and is much more readily reduced than anion radical (I), the NqrB FMN radical. This difference is most likely due to changes in hydrogen bonding that could stabilize the two-electron reduced FMN hydroquinone in NqrC but not the corresponding FMN hydroquinone in NqrB. This difference in redox potentials could help to reconcile the current EPR/ENDOR data with the stopped-flow kinetic data, as described above.

In future studies, redox titrations and time-resolved measurements on the reduction of Na⁺-NQR, monitored by EPR and ENDOR should be able to shed further light on these questions, as should stopped-flow studies on the kinetics of reduction of the flavin mutants.
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FOOTNOTES

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The abbreviations used are: Na⁺-NQR, sodium-translocating NADH:quinone oxidoreductase; IERC, Illinois EPR Research Center; Ni-NTA, nickel-nitrilotriacetic acid.

The values for the C(8α), the C(6), the C(1')H, the C(9), and C(7α) protons obtained from these spectral simulation do not differ significantly from the assignments given in our earlier ENDOR studies. (15) Because the simulation of the CW spectra used to obtain the N(5) and N(4) nitrogen splittings as well as the N(5) proton splitting for the neutral radical included C(8α), C(6), and C(1')H proton splittings, we re-simulated the CW spectra using our ENDOR-refined values. The simulated spectra were essentially the same and except for some changes in residual linewidths, the simulation parameters changed little.

FIGURE LEGENDS

Figure 1: SDS-PAGE of purified Na⁺-NQR samples under UV-light illumination. A. Wild-type and NqrB-T236S and NqrC-T225Y mutant proteins are shown. B. Wild-type and NqrB-T225Y mutant proteins are shown. Approximately 30 μg of protein was used in each case.

Figure 2: HPLC elution profile of the soluble flavins in Na⁺-NQR after denaturation by 6M guanidine-HCl. The elution profile of the soluble flavins was monitored by the absorbance at 450 nm. Wild-type (WT) and the NqrB-T236Y mutant proteins are shown.

Figure 3: EPR spectra of wild-type Na⁺-NQR and NqrC-T225Y mutant. A) air-oxidized; B) NADH reduction (10mM); C) sodium dithionite reduction (10mM NqrC-T225Y and 60mM wild-type). The protein concentration for all samples was approximately 300 μM. The reduced samples were prepared under anaerobic conditions.

Figure 4: EPR spectra of NqrB-T236Y mutant at different levels of reduction with sodium dithionite. Protein concentration of the samples was approximately 100 μM. Samples were prepared under anaerobic conditions.

Figure 5: ENDOR spectra of wild-type Na⁺-NQR under air-oxidizing conditions. The protein concentration for all samples was approximately 300 μM. Simulated spectra are in black. A) Spectrum taken with the magnetic field on the center of the CW-EPR resonance; B) First
derivative spectrum of A); C) Spectrum taken with the magnetic field off center of the CW-EPR resonance. D) First derivative spectrum of C).

**Figure 6:** ENDOR spectra of wild-type Na⁺-NQR at 60 mM sodium dithionite. The protein concentration for all samples was approximately 200 μM Simulated spectra are in black. A) Spectrum taken with the magnetic field on the center of the CW EPR resonance; B) First derivative spectrum of A); C) Spectrum taken with the magnetic field off center of the CW EPR resonance. D) First derivative spectrum of C).

**Figure 7:** ENDOR spectra of NqrB-T236Y mutant at different concentrations of sodium dithionite. The protein concentration for all samples was approximately 100 μM. Simulated spectra are in red.

**Figure 8:** ENDOR spectra of wild-type Na⁺-NQR at different concentrations of sodium dithionite. The protein concentration for all samples was approximately 200 μM for all samples. Simulated spectra are in black. The arrows point to the broad wings which are indicative of anion flavin radical (II).

**Figure 9:** ENDOR spectra of NqrC-T225Y mutant at different concentrations of NADH. The protein concentration for all samples was approximately 100 μM. Simulated spectra are in black.
TABLES

Table 1. Mutagenesis primers sequence

| MUTAN | SEQUENCE OF FORWARD PRIMER |
|-------|-----------------------------|
| **NqrB:** |                            |
| T236A  | 5’GGCTACTCAGGCGGCGCGGCGCTGAG |
| T236C  | 5’GGCTACTCAGGCGAGCATGCGCGCTGAG |
| T236L  | 5’GGCTACTCAGGGGCCCTTCGCGCTGAG |
| T236S  | 5’GGCTACTCAGGGCCTGACTCAGCGCGCTGAG |
| T236Y  | 5’GGCTACTCAGGGCGCTACGCGCTGAG |
| **NqrC:** |                            |
| T225L  | 5’GTAGACGGTCTGTCTGGCGCATTTTGTTAACCAGTAATGGCGTACAA |
| T225Y  | 5’GTAGACGGTCTGTCTGGCGCATATTGACCAGTAATGGCGTACAA |

The sequences in bold indicate the mutation sites and the underlined sequences indicate the restriction sites introduced.
Table 2. Flavin content in wild-type and mutants of the Na⁺-NQR complex

| STRAIN        | FLAVIN CONTENT(1) | SOLUBLE/TOTAL (2) |
|---------------|-------------------|-------------------|
| Wild-type     | 3.9               | 0.5               |
| NqrB:         |                   |                   |
| T236S         | 3.7               | 0.5               |
| T236Y         | 2.35              | 0.55              |
| NqrC:         |                   |                   |
| T225L         | 2.7               | 0.655             |
| T225Y         | 2.8               | 0.67              |

(1) mg flavin after guanidine denaturation/mg protein. An extinction coefficient of 12 mM⁻¹cm⁻¹ was used. Average value of three measurements.

(2) Total amount of flavin after 6M guanidine treatment. “SOLUBLE” indicates the amount of flavins found in the supernatant fraction after filtering the guanidine denatured protein. “TOTAL” indicates the amount of flavin in the denatured sample prior to filtration. A filter with molecular weight cut of 3 k Da was used.
Table 3. Spin Hamiltonian parameters for the neutral semiquinone obtained from simulation of ENDOR spectra. Errors are given in parenthesis.

|        | $A_x$  | $A_y$  | $A_z$  | $A_{iso}$ | $W^b$  | $\text{Int}^c$ |
|--------|--------|--------|--------|-----------|--------|---------------|
| C(8α)H| 9.92 (0.06) | 8.19 (0.03) | 8.19 (0.03) | 8.77 | 0.18, 0.32 | 3.00 |
| C(6)H | -2.58 (0.24) | -6.25 (0.26) | -6.12 (0.26) | -4.98 | 0.17 | 1.00 |
| C(1')H$^1$ | 10.48 (0.59) | 6.50 (0.47) | 6.50 (0.55) | 7.83 | 0.17 | 1.00 |
| C(1')H$^2$ | 7.65 (0.25) | 5.34 (0.22) | 4.76 (0.29) | 5.92 | 0.17 | 1.00 |
| C(9)H | 2.41 (0.10) | 1.33 (0.10) | 2.28 (0.15) | 2.01 | 0.16 | 1.03 |
| C(7α)H | -0.13 (0.09) | -1.40 (0.04) | -1.40 (0.04) | -0.98 | 0.10, 0.40 | 2.19 |
| N(3)H | 1.75 (0.41) | -1.50 (0.35) | -3.52 (0.46) | -1.09 | 0.23 | 0.99 |
| Hmat$^d$ | 0.35 | 0.18 | -0.09 | 0.15 | 0.43 | 1.19 |

a. Isotropic and principal hyperfine values in MHz
b. Linewidth in MHz (Gaussian peak-to-peak). For C(8α)H and C(7α)H the two values correspond to $A_||$ and $A_\perp$, respectively.
c. Relative weighting intensities for $t_e$ equal to 320 ns.
d. Matrix peak. Assignment of $A_x$ versus $A_y$ is arbitrary.
Table 4. Spin Hamiltonian parameters for the semiquinone anion I obtained from simulation of ENDOR spectra. Errors are given in parenthesis.

|         | $A_x$    | $A_y$    | $A_z$    | $A_{iso}$ | $W^b$ | Int$^c$ |
|---------|----------|----------|----------|-----------|-------|---------|
| C(8α)H | 11.82(0.06) | 9.84 (0.02) | 9.84 (0.02) | 10.50 | 0.10 | 3.00 |
| C(6)H  | -4.16 (0.52) | -9.45 (0.20) | -8.95 (0.24) | -7.56 | 0.08 | 1.01 |
| C(1')H$^1$ | 11.42(1.80) | 3.86 (0.50) | 2.64 (0.82) | 5.99 | 0.21 | 1.00 |
| C(1')H$^2$ | 7.00 (0.44) | 3.12 (0.30) | 2.03 (0.62) | 4.01 | 0.21 | 1.00 |
| C(9)H  | 3.68 (0.24) | 1.17 (0.18) | 0.41 (0.46) | 1.78 | 0.18 | 1.08 |
| C(7α)H | -0.51 (0.10) | -1.96 (0.04) | -1.96 (0.04) | -1.45 | 0.15 | 1.68 |
| N(3)H  | 1.26 (0.48) | -1.42 (0.40) | -3.57 (1.50) | -1.24 | 0.28 | 0.78 |
| Hmat$^d$ | -0.05 | 0.05 | 0.12 | 0.04 | 0.49 | 1.25 |

a. Isotropic and principal hyperfine values in MHz
b. Linewidth in MHz (Gaussian peak-to-peak).
c. Relative weighting intensities for $t_e$ equal to 320 ns.
d. Matrix peak. Assignment of $A_x$ versus $A_y$ is arbitrary.
FIGURE 1

A

WT B- C-
T236S T225Y

NqrC
NqrB

B

WT B-T236Y

NqrC
NqrB
FIGURE 2

[Graph showing the peaks of FAD and RIBOFLAVIN for B-T236Y and WT samples.]
FIGURE 3

A) OXIDIZED

B) NADH

C) DITHIONITE

MAGNETIC FIELD (GAUSS)
FIGURE 7

OXIDIZED

0.3 mM

1.0 mM

RF (MHz)
A new flavin radical signal in the Na+-pumping NADH: Quinone oxidoreductase from Vibrio cholerae: An EPR/ENDOR investigation of the role of the covalently bound flavins in subunits B and C

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