Riboflavin Is an Active Redox Cofactor in the Na⁺-pumping NADH:Quinone Oxidoreductase (Na⁺-NQR) from Vibrio cholerae*

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Here we present new evidence that riboflavin is present as one of four flavins in Na⁺-NQR. In particular, we present conclusive evidence that the source of the neutral radical is not one of the FMNs and that riboflavin is the center that gives rise to the neutral flavosemiquinone. The riboflavin is a bona fide redox cofactor and is likely to be the last redox carrier of the enzyme, from which electrons are donated to quinone. We have constructed a double mutant that lacks both covalently bound FMN cofactors (NqrB-T236Y/NqrC-T225Y) and have studied this mutant together with the two single mutants (NqrB-T236Y and NqrC-T225Y) and a mutant that lacks the noncovalently bound FAD in NqrF (NqrF-S246A). The double mutant contains riboflavin and FAD in a 0.6:1 ratio, as the only flavins in the enzyme; noncovalently bound flavins were detected. In the oxidized form, the double mutant exhibits an EPR signal consistent with a neutral flavosemiquinone radical, which is abolished on reduction of the enzyme. The same radical can be observed in the FAD deletion mutant. Furthermore, when the oxidized enzyme reacts with ubiquinol (the reduced form of the usual electron acceptor) in a process that reverses the physiological direction of the electron flow, a single kinetic phase is observed. The kinetic difference spectrum of this process is consistent with one-electron reduction of a neutral flavosemiquinone. The presence of riboflavin in the role of a redox cofactor is thus far unique to Na⁺-NQR.

The Na⁺-translocating NADH:quinone oxidoreductase (Na⁺-NQR)² is a primary sodium pump, found only in prokaryotes, that couples electron transfer from NADH to ubiquinone to the transport of sodium ions across the membrane (1, 2). The enzyme is the entry point for electrons into the respiratory chain of many marine and bacterial pathogens, including Vibrio cholerae (3–6). The Na⁺-NQR complex is made up of six subunits and includes a total of 24 transmembrane helices (7). Several important functional sites have been localized in the sequence of the enzyme. The electron donor, NADH, binds at conserved motif in the cytosolic domain of the NqrF subunit (8). This subunit also includes attachment sites for a noncovalently bound FAD cofactor and a 2Fe-2S center (8–10). The NqrB and C subunits both include attachment sites for covalently bound FMN cofactors. In each case the binding site is a conserved S(T)GAT motif, where FMN is attached to the final threonine by a phosphoester bond (11–13). The enzyme must also include a site where the electron acceptor quinone binds, but its location has not been defined.

We have previously reported that in addition to these cofactors, Na⁺-NQR consistently contained approximately one molecule of riboflavin, which was not a degradation product of FAD or FMN. This was the first time that riboflavin was implicated as an enzyme cofactor. This finding has received some experimental corroboration. Recently, Tao et al. (17) have reported finding riboflavin in the intact wild type enzyme and also in a subcomplex containing only the B and C subunits of Na⁺-NQR. However, this paper did not address the questions of the assignment of any of the spectroscopic signals observed or the catalytic role of riboflavin in Na⁺-NQR.

The conclusion that riboflavin is a bona fide component of Na⁺-NQR has not been universally accepted, and as recently as 2008, Na⁺-NQR was described as containing only three flavin molecules (18).

To understand the role played in Na⁺-NQR by flavins in general and riboflavin specifically, we began a study whose goal was to determine which cofactor gives rise to each of the well defined spectroscopic signals in Na⁺-NQR. The enzyme exhibits a number of different flavin EPR signals, depending on the overall redox state of the enzyme (17, 19). A neutral flavin radical is present in the oxidized (as isolated) form of the enzyme. Two different anionic flavosemiquinone radical signals are detected at different levels of reduction. Using a combination of site-directed mutagenesis and EPR spectroscopy, we have attempted to determine which flavin or flavins in the enzyme give rise to each of the three observed radical signals. According to our analysis, the anionic radicals are formed by the FMN molecules attached to subunits B and C. The neutral radical was observed in the oxidized form of the wild type enzyme, as well as in mutants where the FAD and each of the FMN cofactors

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2 The abbreviations used are: Na⁺-NQR, sodium-pumping NADH:quinone oxidoreductase; HPLC, high pressure liquid chromatography.
were individually deleted. This indicated, by elimination, that the neutral flavosemiquinone radical arises from the riboflavin cofactor in Na\(^+\)-NQR (20). This contradicts earlier work suggesting that the source of the neutral radical was one of the two FMN molecules (21).

However, the results from single flavin deletion mutants are not completely conclusive. For example it is possible that one or both of the FMN cofactors could be a neutral flavosemiquinone in the oxidized form of the enzyme and then deprotonate to become an anionic flavosemiquinone when the remaining redox centers of the enzyme become reduced. It is also possible that deletion of one FMN cofactor alters the redox or spectroscopic properties of the other FMN.

To eliminate these possibilities, we have now constructed a double mutant, NqrB-T236Y/NqrC-T225Y, in which both of the covalently bound FMNs have been removed. Here, we show that the double mutant contains riboflavin. This is further evidence that riboflavin is a *bona fide* cofactor of Na\(^+\)-NQR and not a product of degradation of other cofactors. The double mutant exhibits the neutral flavosemiquinone EPR signal, but neither of the anionic radicals is observed. The presence of only the neutral flavosemiquinone EPR signal indicates that this radical is not derived from either FMN cofactor. We also studied the NqrF-S246A mutant, which lacks the FAD cofactor. This mutant also exhibited a neutral flavosemiquinone EPR signal that is spectroscopically identical to the ones obtained from the double mutant and the wild type enzyme.

Given this assignment of the neutral radical to the riboflavin cofactor, we have begun to define the role of riboflavin in the redox reactions of the enzyme. It has previously been shown that the neutral flavosemiquinone participates in the electron transfer pathway of Na\(^+\)-NQR from *Vibrio harveyi* (21). Here, we have characterized a reaction in which the oxidized enzyme is reduced by ubiquinol, the reduced form of the usual electron acceptor. In this “reverse reduction” reaction the disappearance of the neutral flavosemiquinone can be observed as the only significant kinetic process. This demonstrates that the riboflavin is a true redox cofactor of the enzyme and suggests that it may be located close to the electron output site in the sequence of redox carriers in Na\(^+\)-NQR.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—*V. cholerae* cells, expressing the recombinant Na\(^+\)-NQR wild type or mutants, were grown at 37 °C in LB medium in the presence of 100 μg/ml ampicillin and 50 μg/ml streptomycin at 37 °C with constant agitation of 200 rpm. Expression of the *nqr* operon was achieved by the addition of arabinose to the culture medium during the log phase of growth. The cells were grown in two 30-liter (working volume) fermenters (BioFlo-5000; New Brunswick Scientific) with a constant agitation of 300 rpm. The cells were harvested by continuous flow centrifugation (Cepa Z41; New Brunswick Scientific), washed with 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 200 mM NaPi, pH 8, 300 mM NaCl, 5 mM imidazole, 5% (v/v) glycerol and frozen in aliquots at −80 °C until needed.

**Site-directed Mutagenesis**—The single mutants NqrB-T236Y, NqrC-T225Y, and NqrF-S246A were constructed previously, as described in Ref. 20. The double mutant, NqrB-T236Y/NqrC-T225Y was constructed using a QuickChange mutagenesis kit (Stratagene). Plasmid DNA from the existing pBAD-nqr-B-T236Y, which includes the entire *nqr* operon, was used as a template for the NqrC-T225Y mutagenesis reaction to create the double mutant. The primers used for this reaction were the ones used previously to construct the NqrC-T225Y single mutant (20). The double mutant was checked by restriction digestion analysis and DNA sequencing. The plasmid containing the double mutant was transformed in a strain of *V. cholerae* that lacks of the entire *nqr* operon (7). The expression of the double mutant NqrB-T236Y/NqrC-T225Y was tested by Western blotting using anti-His, tag antibodies (Invitrogen).

**Protein Purification and Other Analytical Methods**—Na\(^+\)-NQR wild type and mutants were purified using the protocol reported previously (3). After the affinity chromatography step, the double mutant protein was further purified by gel filtration chromatography (HiLoad 16/60 Superdex 200 column) using “Tris buffer” (50 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.1% (w/v) *n*-dodecyl β-maltoside, 5% (v/v) glycerol). The aliquots were frozen in liquid nitrogen until needed. Protein concentration was determined using the bichoninic acid method (Pierce).

Protein was denatured by the addition of 6 mM guanidine-hydrochloride, pH 7, or 2% SDS (w/v), 6 mM urea. The denatured samples were analyzed by visible and fluorescence spectroscopy. UV-visible spectra were recorded in a diode-array spectrophotometer (Agilent). Fluorescence spectra of the denatured protein were recorded in Horiba-Jobin-Yvon Fluolog Tau3 fluorimeter. After denaturation, the mixture of protein and soluble cofactors was separated using centrifugal Amicon filters (3-kDa cut-off), (Millipore), and the supernatant was analyzed by visible and fluorescence spectroscopy. The flavins present in this supernatant were further analyzed by HPLC (Shimadzu) following the method reported in Ref. 14, using a C18 5X column.

**Preparation of Ubiquinol**—Ubiquinol-1 was prepared from ubiquinone-1 by the method of Trumpower and Edwards (22) using dithionite and sodium borohydride as reductants.

**Stopped Flow Measurements**—Reduction of the oxidized enzyme by ubiquinol was carried out using an Applied Photo-physics SX.18MV-R stopped flow spectrophotometer equipped with a PD.1 diode array capable of acquiring from 185 to 715 nm (diode separation of 2.17 nm) at a rate of up to one full spectrum every 1.28 ms. The enzyme was first transferred to low sodium Tris buffer (50 mM Tris-HCl, pH 8, 0.05% (w/v) *n*-dodecyl β-maltoside, 1 mM EDTA, 5% (v/v) glycerol) using centrifugal concentrators and then incubated with *Escherichia coli* phospholipids (*E. coli* total lipid extract; Avanti Lipids) (0.25 mg/mg of protein) on ice for 10 min. The ubiquinol-1 solution was prepared in Tris buffer with 200 mM NaCl and 4 mM dithionite to prevent air oxidation of ubiquinol-1. The enzyme and reductant were each loaded into one of the two syringes of the stopped flow system and allowed to equilibrate to the working temperature (4 °C) prior to mixing. The over-sampling function of the instrument was used to reduce noise.
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To obtain a good signal to noise ratio, each measurement was repeated 12–15 times, and the experiment was repeated with three different batches of enzyme.

Data Analysis—Data matrices from replicate acquisitions were averaged using MATLAB (The Mathworks). The averaged matrices were analyzed using Applied Photophysics PC Pro-K software for global analysis of multiwavelength kinetic data.

EPR Experiments—Reduced samples for EPR analysis were prepared using a vacuum line. The samples were reduced with NADH or dithionite in anaerobic conditions. 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.

RESULTS

Effect of the Double Mutation NqrB-T236Y/NqrC-T225Y on the Cofactor Composition of Na\textsuperscript{+}-NQR—These two mutations eliminate the ligands for the FMN cofactors in subunits NqrB and NqrC. The resulting protein contains only soluble flavins and the 2Fe-2S center. In the case of NqrB, the change from threonine to tyrosine was the only viable mutation found; other amino acids yielded a poorly assembled protein. Bacteria carrying the double mutant were able to grow and express the full Na\textsuperscript{+}-NQR complex, but the yield of pure protein was only ~10–20% of the yield for the recombinant wild type.

Attempts to Delete Three of the Four Flavins in Na\textsuperscript{+}-NQR—We tried to obtain an enzyme with riboflavin as the only flavin cofactor by constructing a triple mutant, NqrB-T236Y/NqrC-T225Y/NqrF-S246A. Unfortunately, it proved to be impossible to obtain useful amounts of the mutant protein. The cells carrying the mutant plasmid grew extremely poorly, and the quantities of enzyme in the cell were very low, making it difficult to obtain a pure preparation.

Fluorescence Spectra of the Denatured NqrB-T236Y/NqrC-T225Y Double Mutant—To confirm that the presence of riboflavin in Na\textsuperscript{+}-NQR is not the result of the degradation of the other flavins in the enzyme, (i.e. degradation of the covalently bound flavins in NqrB or in NqrC or the noncovalently bound FAD in NqrF), we analyzed the flavin content of a double mutant, which lacks the two covalently bound flavins. This mutant should only have the two noncovalently bound flavins: FAD and riboflavin. To this end, the protein was denatured using guanidine or SDS/urea, and the soluble components were separated using a centrifugal concentrator with a molecular mass cut-off of 3 kDa. The denatured protein and the supernatant were analyzed by visible and fluorescence spectroscopy. Fig. 1 shows two fluorescence emission spectra: one corresponding to FAD and the second corresponding to the total content of flavins in the denatured sample and the other corresponding to the spectrum of the supernatant after filtration, which corresponds to the soluble flavins in the enzyme. The two spectra are close to identical, indicating that this mutant contains only soluble flavins. The protein/flavin ratio is 1:1.6, suggesting that there are two flavin molecules in the protein.

HPLC Identification of the Soluble Flavins in the Double Mutant NqrB-T236Y/NqrC-T225Y and in NqrF-S246A

Mutant—To identify the nature of the soluble flavins in the denatured double mutant protein, the supernatant obtained after denaturation was injected in a C-18 column and separated by HPLC. Flavin standards were run to localize the retention time for FAD, FMN, and riboflavin as reported before (14). As in the wild type enzyme, the HPLC profile presents two peaks, the first corresponding to FAD and the second corresponding to riboflavin. The ratio between FAD:riboflavin in the double mutant is 1:0.8. Similar ratios were obtained for samples denatured with either guanidine or urea/SDS. These results clearly show that riboflavin is still present in the enzyme even in the absence of the two covalently bound flavins. The lower FAD:riboflavin ratio of in the double mutant, is probably due to the loss of the smaller riboflavin cofactor during enzyme isolation and sug-
suggests that the mutant may structurally less stable than the wild type enzyme. We also used HPLC to analyze the flavin content of the NqrF-S246A mutant, which lacks the noncovalently bound FAD in subunit F. Unlike the double mutant, this enzyme contains the two covalently bound FMNs in NqrB and NqrC, but these will be removed from the sample by the filtration step, leaving only the soluble flavins. The HPLC profile of this mutant shows only one peak with a retention time corresponding to riboflavin (Fig. 2).

**EPR Characterization of the Double Mutant NqrB-T236Y/NqrC-T225Y and the NqrF-S246A Mutant**—Previously, we used EPR to study mutants that eliminate each of the three flavins of known location in Na⁺-NQR: the two covalently bound FMNs in NqrB and NqrC and the noncovalently bound FAD in NqrF (9, 20). The wild type enzyme, in its oxidized form (as prepared), exhibits a neutral flavosemiquinone radical. Upon reduction, two different anionic flavosemiquinone radicals can be observed depending on the level of reduction. By studying these mutants, the two anionic radicals were assigned to the FMN in NqrB and NqrC. The mutant that lacks the FAD showed all of the EPR signals observed in the wild type enzyme. Furthermore, the neutral radical was observed in the oxidized form of all of the mutants, indicating that this signal does not arise from the FAD or either of the FMNs. Based on these results, we suggested that riboflavin is the site of the neutral radical in Na⁺-NQR (20).

To confirm this result, we have now analyzed the double mutant, NqrB-T236Y/NqrC-T225Y by EPR in its oxidized, partially reduced, and fully reduced forms. If our previous conclusion is correct, the only flavin radical observed in this mutant should be a neutral radical in the oxidized form of the enzyme, and this signal should disappear upon reduction, with no appearance of the anionic radicals. Fig. 3A shows the EPR spectra of the double mutant in the oxidized, partially reduced and fully reduced forms.

The oxidized spectrum shows a signal from a flavosemiquinone with a line width of 20 G (2 millitesla), typical of a neutral flavosemiquinone radical (19, 23). This signal gains intensity upon partial reduction, but the line width does not change, indicating that in the mutant the neutral radical is partially oxidized, and...
after the addition of relative small amount of NADH, this form goes back to the one-electron reduced form. This is consistent with the behavior of the NqrB-T236Y single mutant. After the addition of an excess of reductant (dithionite), the neutral flavosemiquinone radical signal disappears completely, and the only signal observed is that of the 2Fe-2S center. Fig. 3B shows the double derivative spectra of oxidized forms of the double mutant and the wild type enzyme, showing that the neutral flavosemiquinone radicals in these two enzymes are essentially identical. As a further test, we carried out a more detailed analysis by EPR of the NqrF-S246A mutant, in which the binding site for FAD has been eliminated. EPR spectra of the oxidized form of this mutant enzyme show the neutral radical signal (20-G, 2-milliesla line width) (Fig. 3C). Upon addition of excess reductant, this signal is completely abolished, and an anionic semiquinone radical appears (15-G, 1.5-milliesla line width). These data confirm that riboflavin is the only possible candidate for the neutral radical in the enzyme.

It is interesting to note that like our results for the NqrB-T236Y single mutant, the amount of the neutral radical is less than stoichiometric. This contrasts with our previous observation that the neutral radical was in near stoichiometric amount in the wild type enzyme. This may be the result, in part, of the lower concentration of riboflavin in these mutants but also a change in the redox equilibrium of the neutral radical as indicated by the observation that the amplitude of the neutral radical increased in the double mutant upon partial reduction (Fig. 3A). This could result from a change in the redox potential of the neutral radical itself or due to removal of the FMN in NqrB. This would suggest a strong coupling between this FMN and the neutral radical.

**Reduction of the Riboflavin Cofactor in Na⁺-NQR by Ubiquinol—**So far, we have shown that riboflavin is present in Na⁺-NQR and is the flavin responsible for forming the neutral radical. With this assignment, we can begin to investigate the role of riboflavin in the mechanism of the enzyme. Bogachev et al. (21, 24, 25) observed the disappearance of a neutral flavosemiquinone species during the reduction of the enzyme by NADH, in optically monitored stopped flow measurements. This indicates that riboflavin plays a role in the redox mechanism of Na⁺-NQR. To investigate this further, we studied reduction of the enzyme from the opposite direction, reacting the oxidized enzyme with ubiquinol, an analog of the usual electron acceptor, in its reduced form. This reaction should reduce the enzyme by injecting electrons into to the normal electron exit site.

The reaction was carried out by mixing the fully oxidized enzyme with ubiquinol in a stopped flow system. In the wild type enzyme one major kinetic phase is observed. The difference spectrum of this process was obtained from a single-exponential global fit to the data, as shown in Fig. 4A. This spectrum is consistent with one-electron reduction of a neutral flavosemiquinone to the fully reduced form. The time course of the reaction at 575 nm is shown in Fig. 4B. This confirms the participation of riboflavin as a *bona fide* redox cofactor in Na⁺-NQR. It also suggests that riboflavin is the final redox carrier in Na⁺-NQR before electrons are donated to ubiquinone but that in the reverse reaction, reduction of the enzyme by ubiquinol provides only sufficient driving force to reduce this one redox carrier. However, this does not definitively establish riboflavin as the final carrier. The end point of the reaction is governed by equilibria rather than kinetics, and these results do not rule out the possibility of an intervening low potential carrier. The sequence of electron carriers in Na⁺-NQR will be the subject of a separate publication.3

**DISCUSSION**

The presence of riboflavin as a component of Na⁺-NQR was first reported by Barquera et al. (14). This study used biochemical methods to show that the enzyme contains riboflavin in addition to one FAD and two FMNs for a total of four flavins. However, this initial work did not assign any specific spectroscopic signals to the riboflavin or defined its role in the redox reactions of the enzyme. The finding of riboflavin has not been universally accepted (25) but has recently been confirmed in biochemical studies by Tao et al. (17).

Having shown that riboflavin is present in Na⁺-NQR, we set out to define its spectroscopic and functional role. Because the enzyme exhibits at least three identifiable flavosemiquinone radical EPR signals under different conditions (19, 20), we initiated a project to study the correspondence between structurally and chemically identifiable flavin cofactors and spectroscopic signatures (EPR and UV-visible). Three of the four flavins are known to be attached to identifiable motifs in the sequence. We constructed a series of mutants in which each of the three flavins was individually deleted: NqrF-S246A, which lacks the noncovalently bound FAD; NqrB-T236Y, which lacks one of the covalently bound FMNs; and NqrC-T225Y, which lacks the other covalently

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bound FMN. The two FMNs deletion mutants showed changes in the anionic flavosemiquinone EPR signals, but all three mutants showed essentially the same flavosemiquinone radical EPR signal as observed in the wild type enzyme. On this basis, by elimination, we assigned the neutral flavosemiquinone to the riboflavin. These results with mutants, and the resulting assignment, have yet to be confirmed by other workers.

However, it remained possible that the assignments based on the single mutants were not conclusive. It could be imagined that one or both FMN cofactors could be a neutral semiquinone in the oxidized form of the enzyme changing to an anionic semiquinone when the enzyme became reduced or that deletion of one flavin cofactor could change the redox chemistry and spectroscopy of other flavins. To rule out these possibilities we constructed a double mutant in which both of the covalently bound FMNs are absent. This double mutant exhibits essentially the same neutral flavosemiquinone EPR signal as observed in the wild type enzyme. This rules out the possibility that either FMN could be the neutral flavosemiquinone radical. On the basis of these data we cannot completely exclude the possibility that in the double mutant, the neutral flavosemiquinone radical originates from the FAD, whereas in the NqrF-S246A mutant, it arises from one or both FMNs. For this to be true, it would require a remarkably complex combination of events. Also, the FAD is unlikely to have the single mutants were not conclusive. It could be imagined that one or both FMN cofactors could be a neutral semiquinone with mutants, and the resulting assignment, have yet to be confirmed by other workers.

The location of riboflavin in the structure of the enzyme remains unknown. As described above, functionally, it seems to be located near the output site of the enzyme, where electrons are donated to quinone. This would be consistent with the work of Tao et al. (17), who reported biochemical evidence that locates riboflavin in NqrB or in NqrC subunits and tends to argue against a location in the NqrF subunit, which contains the electron-input site where NADH binds.

The results in this paper conclusively establish that riboflavin is an active redox cofactor of Na\(^{+}\)-NQR, giving rise to the neutral flavosemiquinone and that it is likely to be the final electron carrier in the enzyme before the quinone. At this point, the presence of riboflavin as a bona fide redox cofactor is a property unique to Na\(^{+}\)-NQR.

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