The Electron Transfer Pathway of the Na\(^+\)-pumping NADH:Quinone Oxidoreductase from Vibrio cholerae*

Oscar Juárez, Joel E. Morgan, and Blanca Barquera

From the Department of Biology and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180

The Na\(^+\)-pumping NADH:quinone oxidoreductase (Na\(^+\)-NQR) is the only respiratory enzyme that operates as a Na\(^+\) pump. This redox-driven Na\(^+\) pump is amenable to experimental approaches not available for H\(^+\) pumps, providing an excellent system for mechanistic studies of ion translocation. An understanding of the internal electron transfer steps and their Na\(^+\) dependence is an essential prerequisite for such studies. To this end, we analyzed the reduction kinetics of the wild type Na\(^+\)-NQR, as well as site-directed mutants of the enzyme, which lack specific cofactors. NADH and ubiquinol were used as reductants in separate experiments, and a full spectrum UV-visible stopped flow kinetic method was employed. The results make it possible to define the complete sequence of redox carriers in the electron transfer pathway through the enzyme. Electrons flow from NADH to quinone through the FAD in subunit F, the 2Fe-2S center, the FMN in subunit C, the FMN in subunits B and C (FMNB and FMNC, respectively), and finally riboflavin. The reduction of the FMNC to its anionic flavosemiquinone state is the first Na\(^+\)-dependent process, suggesting that reduction of this site is linked to Na\(^+\) uptake. During the reduction reaction, two FMNs are transformed to their anionic flavosemiquinone in a single kinetic step. Subsequently, FMNC is converted to the flavohydroquinone, accounting for the single anionic flavosemiquinone radical in the fully reduced enzyme. A model of the electron transfer steps in the catalytic cycle of Na\(^+\)-NQR is presented to account for the kinetic and spectroscopic data.

The Na\(^+\)-pumping NADH: ubiquinone oxidoreductase (Na\(^+\)-NQR)\(^2\) is a molecular energy transducer present in the membrane of many marine and pathogenic bacteria, including Vibrio cholerae (1). The enzyme accepts electrons from NADH and donates them to ubiquinone. This redox reaction releases a significant amount of energy, which is used to pump Na\(^+\) across the cell membrane, creating a Na\(^+\) gradient as well as an electrical potential (2, 3). Na\(^+\)-NQR catalyzes the same redox reaction as the H\(^+\)-pumping NADH:quinone oxidoreductase (complex I), but there is nothing to indicate a common mechanism. The two proteins have significantly different cofactor compositions and do not share any homology beyond substrate/cofactor motifs. In fact the pumping stoichiometry of the two enzymes is reportedly different; Na\(^+\)-NQR translocates one Na\(^+\)/electron, whereas as complex I moves at least 2 H\(^+\)/e\(^-\) (4–6).

In Na\(^+\)-NQR electrons are carried through the enzyme by means of at least five redox cofactors: a noncovalently bound FAD and a 2Fe-2S center, in the NqrF subunit, where the binding site for NADH is also located, two covalently attached FMNs in subunits B and C (FMNB and FMNC, respectively), and a noncovalently bound riboflavin that has not been definitively localized but that is believed to reside in the B or C subunit (see Fig. 1) (7–12). Describing the way in which these redox cofactors work together to move electrons from NADH to quinone is a necessary step in understanding how Na\(^+\)-NQR functions as a molecular energy converter. It is important to know the sequence of redox carriers along the path taken by electrons as they flow through the enzyme, as well as the kinetics and energetics of each step and how they are influenced by Na\(^+\) and the electrical potential.

The reaction of the oxidized enzyme with excess NADH, leading to fully reduced state, can be used to investigate the internal electron transfer processes in Na\(^+\)-NQR. This is not the physiological reaction of the enzyme, which probably never becomes fully reduced during steady state turnover with NADH and quinone. Nevertheless, this provides a useful way to apply transient kinetic measurements to the mechanism of the enzyme. Bogachev et al. (13) used this method to study the reduction of the wild type enzyme from Vibrio harveyi. These studies led to a model of the reaction, which takes into account the 2Fe-2S center and three flavin redox cofactors. However, assignment of kinetic processes to specific cofactors is still an open question. The flavins in Na\(^+\)-NQR, now known to be four, can all potentially give rise to the same visible and EPR spectra. Na\(^+\)-NQR can exhibit three different flavosemiquinone radical EPR signals, depending on the overall oxidation state of the enzyme: a neutral flavosemiquinone in the oxidized (as prepared) enzyme, an anionic flavosemiquinone (I) in the fully reduced enzyme, and a second, spectroscopically distinct, anionic flavosemiquinone (II) in the partially reduced enzyme. By studying site-directed mutants that result in the loss of specific cofactors, we have recently assigned anionic flavosemiquinone I to FMNB, anionic flavosemiquinone II to FMNC, and the neutral flavosemiquinone to the riboflavin cofactor (8, 9).
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To define the roles of the different redox cofactors in the kinetics of the reduction of the enzyme, we have used stopped flow methods to study a series of mutants in which cofactors have been removed, individually or pair-wise. The mutants include ones that remove: the 2Fe-2S center (NqrF-C76A), FMN\textsubscript{N} (NqrB-T236Y), FMN\textsubscript{C} (NqrC-T225Y), and a double mutant, which lacks both FMNs (NqrB-T236Y/NqrC-T225Y). The results indicate that electrons follow a linear pathway through the cofactors of the enzyme without significant parallel flow. Each mutation appears to interrupt smooth electron flow through the enzyme at a different point. The results allow each of the redox cofactors of Na\textsuperscript{+}-NQR to be assigned a place in the sequence of electron carriers and to distinguish which processes are controlled by kinetics or thermodynamics. The results also account for the multiple flavosemiquinone radicals observed in different equilibrium redox states of the enzyme, in terms of transitions observed during rapid reduction (9, 14). On this basis, we present a model of electron flow in Na\textsuperscript{+}-NQR and of the steps coupled to Na\textsuperscript{+} translocation in the enzyme.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—V. cholerae expressing the recombinant wild type Na\textsuperscript{+}-NQR and mutants were grown as reported previously (1, 8, 14, 15).

Protein Purification—Wild type and mutant Na\textsuperscript{+}-NQR proteins were purified using affinity chromatography (1). Protein concentration was measured with the Bicinchonic acid assay (Pierce). Total flavin concentration was determined at 450 nm in denatured enzyme preparations by the addition of 6 M guanidinium. The flavin/protein molar ratios for wild type Na\textsuperscript{+}-NQR, NqrB-T236Y, NqrC-T225Y, NqrB-T236Y/NqrC-T225Y, and NqrF-C76A were 3.6 ± 0.2, 2.5 ± 0.3, 2.8 ± 0.4, 1.7 ± 0.3, and 3.7 ± 0.4 (7, 8).

Stopped Flow Experiments—Fast spectrophotometric measurements were performed using an Applied Photophysics SX.18MV-R stopped flow instrument equipped with a PD.1 diode array (diode separation of 2.17 nm) that is capable of acquiring spectra over the range from 185 to 715.5 nm at a rate of 1/1.28 ms. Wild type and mutant enzymes were desalted in buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5% glycerol, 0.05% (w/v) n-dodecyl β-maltoside and were rapidly mixed (1:1) in buffer containing either: 600 μM K\textsubscript{2}-NADH (potassium salt); K\textsubscript{2}-NADH, 200 mM NaCl or 2 mM ubiquinol-1, 200 mM NaCl, 2 mM dithionite. Ubiquinol-1 was prepared from ubiquinone-1 as reported in Ref.16 using sodium dithionite or sodium borohydride as reductants.

Data Analysis—The stopped flow measurements were repeated at least 12 times per experimental condition and tested in three different enzyme preparations. To remove noise caused by the jitter of the xenon lamp and other sources, a temporal base-line correction was carried out. The absorbance data in the range 650–720 nm, where the redox reactions do not make a significant contribution, were averaged and subtracted from the spectrum of each time point. The resulting data matrices were averaged in MATLAB. The averaged matrices were analyzed in Applied Photophysics PC Pro-K software for global analysis of multiwavelength kinetic data. The results were fitted using reaction schemes of 1–4 steps (depending on the mutant tested) with linear or multi-branched pathway models. In all cases the most parsimonious fits were obtained with linear models (judged from the residual plots and variance of the fits).

FIGURE 1. Top panel, scheme representing Na\textsuperscript{+}-NQR. The six subunits (NqrA-F) are depicted together with the redox cofactors and the proposed pathway of electron flow through the enzyme (see “Discussion”). Bottom panel, scheme illustrating the results presented in Figs. 2–6. The scheme depicts the redox states of the cofactors after each kinetic phase in the reaction with NADH (without sodium), in the wild type enzyme, and in the cofactor mutants. State 0 is the initial state. At the top of the scheme, the redox cofactors, FAD, 2Fe-2S center, FMN, FMNC, and riboflavin (RIB) are shown in the order of electron flow through the enzyme. The top box represents the wild type enzyme. The mutants that lack specific cofactors are depicted in the boxes below. For each mutant, the name of the mutant (for example, NqrC-T225Y) is shown in place of the missing cofactor, representing a missing step in the sequence of electron transfer carriers and thus a blockage in the pathway. Each complete circle represents sites for two electrons; single electron reduction is shown using semi-circles. For each step the newly reduced sites are shown with black fill, whereas sites that remain reduced from the previous step are shown with gray fill.
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Figure 2: Time courses at the absorbance maximum (450 nm) for flavins showing the reduction of the wild type enzyme (black line), NqrB-T236Y mutant (red line), NqrC-T225Y (blue line), and the double mutant, NqrB-T236Y/NqrC-T225Y mutant (green line).

RESULTS

The reaction with NADH, which transforms the oxidized enzyme to its fully reduced form, is a useful model reaction that allows rapid kinetic methods to be applied to study electron transfer in Na⁺-NQR. The reduction of wild type Na⁺-NQR by NADH has been studied by stopped flow measurements, and several intermediate steps in the reaction have been resolved. However, it must be borne in mind that this is not the physiological turnover reaction of the enzyme and that Na⁺-NQR probably never becomes fully reduced during steady state turnover. This can be seen in the fact that in the wild type enzyme, the third and fourth kinetic phases of this reaction have rates of 35 and 0.7 s⁻¹, respectively (measured at 4°C; see below), both far lower than the 150 s⁻¹ steady state turnover rate under comparable conditions with NADH and ubiquinone. Nevertheless, the reaction of the fully oxidized enzyme with NADH in the absence of an oxidizing substrate provides a useful way to apply transient kinetic measurements and analysis to the mechanism of this enzyme.

We have applied this method to study a series of mutants in which redox cofactors have been specifically removed. These mutants include ones that lack the following cofactors: 1) FMNₐ (NqrB-T236Y), 2) FMNₐC (NqrC-T225Y), 3) both of these FMN cofactors (double mutant NqrB-T236Y/NqrC-T225Y), and 4) the 2Fe-2S center in NqrF (NqrF-C76A) (Fig. 1).

Fast Kinetics of Reduction of Wild type Na⁺-NQR—The reaction of Na⁺-NQR with excess NADH was carried out using a stopped flow spectrophotometer that allows the full UV-visible spectrum to be recorded at every time point. The time course at 450 nm, the absorbance maximum for oxidized flavins, is shown in Fig. 2. The full spectrum data for the wild type enzyme, in the absence of Na⁺, were best fitted as a four-step process. The spectral components for a linear reaction model (A → B → C → D → E) are shown as difference spectra in the left-hand panels of Fig. 3 (for rate constants see Table 1). It is important to note that a linear reaction model means that the kinetic intermediates are related by a linear scheme (A → B → C → D → E). This is not necessarily equivalent to the idea that the pathway for electrons through the structure of the enzyme never branches.

The results on the wild type enzyme are largely consistent with those from an earlier study on the enzyme from V. harveyi (13). The first component spectrum has a minimum at 460 and 400 nm. This was previously assigned as two-electron reduction of a flavin molecule (F → FH₂) (13, 17). The second component spectrum has a minimum at 525 nm with shoulders at 570 and 625 nm and a maximum at around 430 nm. This was assigned as a one-electron reduction of a neutral flavosemiquinone to the fully reduced (flavohydroquinone) state (FH⁻ → FF⁻) (13). The third component has a minimum at 460 nm, with a small trough at 395 nm and has been assigned as one-electron reduction of flavin to anionic flavosemiquinone (F → FF⁻) (13). The fourth component has a minimum at 480 nm and a maximum at 430 nm. This was originally assigned as reduction of the 2Fe-2S center in NqrF (17).

The extinction coefficients for all one- and two-electron redox transitions of flavins (neutral and anionic) have been determined for glucose oxidase (13, 18). Based on these values and the protein concentration, the first phase (Fig. 3) represents approximately one equivalent of flavin in the F → FH₂ reaction (Δε₄5₀ = 11.2 mM⁻¹ cm⁻¹ compared with 12.3 mM⁻¹ cm⁻¹ for glucose oxidase); the second phase (Fig. 3B) is consistent with one equivalent of flavin undergoing the FH⁻ → FH₂ reaction (Δε₅₇₀ = 4.2 mM⁻¹ cm⁻¹ compared with 3.9 mM⁻¹ cm⁻¹ for glucose oxidase); and the third phase (Fig. 3C) is consistent with two equivalents of flavin in the F → FF⁻ reaction (Δε₄₅₀ = 18.1 mM⁻¹ cm⁻¹ compared with 10.8 mM⁻¹ cm⁻¹ for glucose oxidase). In the V. harveyi study the amplitude of the third phase was significant smaller and was assigned as the transition of one equivalent of flavin (13).

The formation of two equivalents of anionic flavosemiquinone is important in light of the fact that EPR spectra of the fully reduced enzyme show only one equivalent of anionic flavosemiquinone radical (14). The explanation for this may be found in the fourth kinetic phase (Fig. 3D). This was originally assigned as reduction of the 2Fe-2S center (17). However, careful comparison with published reduced-minus-oxidized spectra of iron-sulfur proteins as well as those of flavin redox transitions indicates that this component is more likely to arise from one-electron reduction of an anionic flavosemiquinone to the fully reduced state (FF⁻ → FH₂). In Fig. 3D the spectrum of the fourth phase is shown together with reduced-minus-oxidized spectra of the 2Fe-2S center from Na⁺-NQR of V. cholerae obtained by expression of a fragment of the NqrF subunit (blue line) (19) as well as the spectrum of one-electron reduc-
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FIGURE 3. Reduction of the wild type Na\textsuperscript{+}-NQR from V. cholerae by 300 \textmu M NADH; spectral components from global fit (see "Experimental Procedures") together with reference spectra. Left panels, reactions in the absence of Na\textsuperscript{+}. A, first kinetic phase (black line) compared with the F \to FH\textsubscript{2} transition of the Na\textsuperscript{+}-NQR from V. harveyi (13) (red line); B, second phase (black line) compared with the FH \to FH\textsubscript{2} transition of the Na\textsuperscript{+}-NQR from V. harveyi (13) (red line); C, third phase (black line) compared with an F \to F\textsuperscript{+} transition involving one equivalent of flavin (red line); D, fourth phase (black line), compared with the F\textsuperscript{+} \to FH\textsubscript{2} transition (18) (red line) and the difference spectrum of the reduction of the 2Fe-2S center from the isolated NqrF subunit (19) (blue line). Right panels, reactions in the presence of 100 mM NaCl. E, first phase (black line), compared with the F \to FH\textsubscript{2} transition (red line); F, F\textsuperscript{+} \to FH\textsubscript{2} transition (blue line) and the sum of the spectra for the F \to FH\textsubscript{2} and F\textsuperscript{+} \to FH\textsubscript{2} transitions (red line), respectively. For rate constants see Table 1.

dation of an anionic flavosemiquinone to the fully reduced state (F\textsuperscript{+} \to FH\textsubscript{2}) obtained from glucose oxidase (18). The spectrum of the fourth kinetic phase is consistent with reduction of an anionic flavosemiquinone but clearly different from the 2Fe-2S spectrum. It thus appears that two of the flavin cofactors of the enzyme are converted to anionic flavosemiquinones in the third phase of the reaction but that one of these two is then further reduced to the fully reduced flavohydroquinone in the fourth phase, leaving only the one anionic flavosemiquinone radical observed by EPR in the fully reduced enzyme. With this assignment, the fourth phase would represent one equivalent of flavins in the F\textsuperscript{+} \to FH\textsubscript{2} reaction (\Delta\varepsilon\textsubscript{475} = 5.6 mm\textsuperscript{-1} cm\textsuperscript{-1} compared with 5.9 mm\textsuperscript{-1} cm\textsuperscript{-1} for glucose oxidase (18)). This change in stoichiometry, together with the reassignment of the fourth kinetic phase, leads to considerable clarification of the participation of the four flavins in the overall redox process in the enzyme (see "Discussion").

Na\textsuperscript{+}-NQR is a Na\textsuperscript{+} pump, and the kinetics of the enzyme has been shown to depend strongly on Na\textsuperscript{+} concentrations (13). Therefore, the reduction of the wild type enzyme by NADH was repeated in the presence of 100 mM Na\textsuperscript{+}. The right-hand panel of Fig. 2 shows the time course for this reaction. Compared with the corresponding reaction without Na\textsuperscript{+} (Fig. 2, left-hand panel), the reaction with sodium is visibly simpler. In the global fit, three kinetic phases are now resolved instead of four. The right-hand panels of Fig. 3 show the spectral components for a three-step linear reaction scheme (A \to B \to C \to D). The third phase (Fig. 3G) can be assigned as F\textsuperscript{+} \to FH\textsubscript{2}, and there is a modest increase in the rate constant (from 0.31 to 0.7 s\textsuperscript{-1}). The second phase (Fig. 3F) has essentially the same spectrum as the third phase of the reaction without Na\textsuperscript{+} (Fig. 3C), which was assigned as the formation of two equivalents of anionic flavosemiquinone (see above). The rate constant of this process is significantly faster in the reaction in the presence of Na\textsuperscript{+} (increasing from 4.2 to 35.1 s\textsuperscript{-1}). The first phase (Fig. 3E) appears to be a combination of the first and second phases from the reaction without Na\textsuperscript{+} (Fig. 3A and B), which are now resolved as single phase in the analysis. This correspondence is illustrated in Fig. 3E. The apparent rate constant for this phase is 143.2 s\textsuperscript{-1} as compared with 249.3 and 15.4 s\textsuperscript{-1} for the first and second phases without Na\textsuperscript{+}, respectively. This would be consistent with a significant increase in the rate of reduction of the neutral flavosemiquinone (second phase in the absence of Na\textsuperscript{+}; Fig. 3B), making this process kinetically unsolvable from the initial two-electron reduction of a flavin in the first phase without Na\textsuperscript{+} (Fig. 3A). In this case, the actual rate of the two-electron flavin reduction process could be unchanged, despite the apparent decrease in rate. Thus, the Na\textsuperscript{+} concentration exerts its effects primarily on two phases of the reaction: the reduction of the neutral flavosemiquinone and the formation of the two equivalents of anionic flavosemiquinone. However, it is likely
that the Na\textsuperscript{+} dependence of these phases in the reaction all reflect a single Na\textsuperscript{+}-dependent internal electron step in the enzyme mechanism (see “Discussion”).

These results show that in the wild type enzyme electrons fill the redox acceptors in the following order: 1) F to FH\textsubscript{2}; 2) FH\textsuperscript{+} to FH\textsubscript{2}; 3) 2(F to F\textsuperscript{+}); and 4) F\textsuperscript{+} to FH\textsubscript{2}. However, internal electron flow in the enzyme may be governed by thermodynamics as well as kinetics, so that order does not necessarily correspond to the physical ordering of carriers in the pathway. Furthermore, visible spectroscopy does not distinguish between anionic flavosemiquinones at FMN\textsubscript{B} and FMN\textsubscript{C}. To better define the role of the specific cofactors in the process, the reaction was repeated using a series of mutant enzymes that lack specific cofactors.

**Fast Kinetics of Reduction of NqrB-T236Y Mutant**—In this mutant the threonine 236, where a covalently bound FMN is normally attached, has been replaced by tyrosine, resulting in the loss of the FMN cofactor. In the absence of Na\textsuperscript{+} three phases are resolved in the global fit; the spectral components for a linear reaction scheme (A ↔ B ↔ C ↔ D) are shown in the left-hand panels of Fig. 4. The spectrum and rate constant of the first phase are essentially the same as for the wild type enzyme, consistent with two-electron reduction of flavin (Fig. 4A). The second phase has a spectrum consistent with formation of an anionic flavosemiquinone, together with reduction of an 2Fe-2S center. (Fig. 4B) The spectrum is similar in shape to that of the third phase from the wild type enzyme (Fig. 3C), which has been assigned to formation of anionic flavosemiquinone.

However, the amplitude of this phase is consistent with participation of ~0.3 equivalents of flavin cofactor rather than the two that apparently contribute in the wild type enzyme. Also, the contribution of a 2Fe-2S center can be detected. In the corresponding phase of the wild type enzyme, the contribution of flavins is larger, and it is not possible to determine from the spectrum whether or not there is a contribution from the 2Fe-2S center. However, it is most likely that the two-electron character of the NADH donor leads to the 2Fe-2S center becoming reduced in the same phase as reduction of the neutral flavosemiquinone, in the wild type enzyme reaction (Figs. 1 and 8).

The final phase has a spectrum (Fig. 4C) consistent with reduction of an anionic flavosemiquinone to the fully reduced hydroquinone (Fig. 3D). This agrees with the earlier finding that EPR spectra of the NqrB-T236Y mutant, in the presence of excess reductant, do not show any anionic flavosemiquinone radical signal. The rate of this process (0.22 s\textsuperscript{-1}) is also similar to that of the corresponding phase in the wild type enzyme (0.31 s\textsuperscript{-1}). Interestingly, the second phase in this reaction, in which formation of anionic flavosemiquinone (F → F\textsuperscript{-}) takes place, has a rate of 13.2 s\textsuperscript{-1}, which is higher than the rate of the third phase of the wild type enzyme reaction, in which two anionic flavosemiquinones are formed (4.2 s\textsuperscript{-1}), but quite similar to that of that missing reduction of the neutral flavosemiquinone in the second phase of the wild type enzyme reaction (15.4 s\textsuperscript{-1}).

In the presence of Na\textsuperscript{+}, reduction of the NqrB-T236Y mutant shows only two kinetic phases. The second phase (Fig. 4E) has a spectrum consistent with a F\textsuperscript{-} → FH\textsubscript{2} transition. The rate of this phase (1.3 s\textsuperscript{-1}) is higher than the corresponding phase in this mutant in the absence of sodium (0.22 s\textsuperscript{-1}), but it is similar to the corresponding phase in the wild type enzyme in the presence of Na\textsuperscript{+} (0.7 s\textsuperscript{-1}). The first phase of the reduction of this mutant in the presence of Na\textsuperscript{+} is apparently the sum of the first two phases in the absence of Na\textsuperscript{+} (Fig. 4D). The rate of this phase (140.3 s\textsuperscript{-1}) is essentially the same as that of the first phase from the wild type enzyme without Na\textsuperscript{+} (143.2 s\textsuperscript{-1}), in which reduction of the neutral flavosemiquinone takes place (Fig. 4E). Here again, the 2Fe-2S center may become reduced during the first phase, but if so, its contribution to the spectrum is overshadowed by that of the flavins.

**Fast Kinetics of Reduction of NqrC-T225Y Mutant**—The covalently bound FMN\textsubscript{C} was eliminated by changing threonine 225 to tyrosine. In the absence of Na\textsuperscript{+} two phases are resolved in the global fit, with rate constants of 220 and 0.52 s\textsuperscript{-1}. The spectrum and rate for the first component (Fig. 5A) are essentially the same as those for the first phase of the corresponding reaction in the wild type enzyme (Fig. 3A), which has been assigned as to two-electron reduction of a flavin. The second phase (Fig. 5B) is much slower and has a spectrum consistent with conversion of neutral flavosemiquinone to the fully reduced form (FH\textsuperscript{+} → FH\textsubscript{2}) together with reduction of an 2Fe-2S center. Na\textsuperscript{+} appears to have very little effect on either the rates or the spectra of either phase of this reaction. (Fig. 5, C and D).

### Table 1

| Enzyme | Rate constants (s\textsuperscript{-1}) |
|--------|--------------------------------------|
|        | k\textsubscript{1} | k\textsubscript{2} | k\textsubscript{3} | k\textsubscript{4} |
| Wild type (n = 6) | | | | |
| Na\textsuperscript{−} | 249.3 ± 30.3 | 15.4 ± 4.6 | 4.2 ± 1.7 | 0.31 ± 0.1 |
| + Na\textsuperscript{+} | 143.2 ± 25.2 | 35.1 ± 7.4 | 0.7 ± 0.2 |
| NqrB-T236Y (n = 3) | | | | |
| Na\textsuperscript{−} | 239.0 ± 40.2 | 13.2 ± 4.8 | 0.22 ± 0.1 |
| + Na\textsuperscript{+} | 140.3 ± 36.7 | 1.3 ± 0.6 |
| NqrC-T225Y (n = 4) | | | | |
| Na\textsuperscript{−} | 220.2 ± 31.8 | 0.52 ± 0.2 |
| + Na\textsuperscript{+} | 235.4 ± 25.4 | 1.5 ± 0.4 |
| NqrF-C76A (n = 3) | | | | |
| Na\textsuperscript{−} | 238.3 ± 44.3 | 2.7 ± 0.1 |
| + Na\textsuperscript{+} | 270.1 ± 50.1 |
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**FIGURE 4. Reduction of the NqrB-T236Y mutant by 300 \textmu M NADH; spectral components from global fit (see “Experimental Procedures”) together with reference spectra.** Left panels, reactions in the absence of Na\textsuperscript{+}. A, first kinetic phase (black line) compared with the F \rightarrow FH\textsubscript{2} transition (red line). B, second reduction phase (blue line) compared with an F \rightarrow F\textsuperscript{+} transition involving one equivalent of flavin (red line), reduction of the 2Fe-2S center (green line), and the sum of the 2Fe-2S center and 0.3 equivalents of the F \rightarrow F\textsuperscript{+} transition (black line). C, third phase (black line) compared with the F\textsuperscript{+} \rightarrow FH\textsubscript{2} transition (red line). Right panels, reactions in the presence of 100 mM NaCl. D, first phase (black line), compared with spectra of the F \rightarrow FH\textsubscript{2} (red line), F \rightarrow F\textsuperscript{+} (green line) transition involving one equivalent of each anionic flavosemiquinone, and the sum of the F \rightarrow FH\textsubscript{2} and F \rightarrow F\textsuperscript{+} spectra (blue line). E, second reduction phase (black line) compared with the F\textsuperscript{+} \rightarrow FH\textsubscript{2} transition (red line). For rate constants see Table 1.

**Fast Kinetics of Reduction of NqrB-T236Y/NqrC-T225Y Double Mutant**—This mutant contains only the noncovalently bound FAD in NqrF and the noncovalently bound riboflavin (8). The reaction of the mutant enzyme with NADH consists of a single phase, whether or not Na\textsuperscript{+} is present. The rate constants are almost identical (245 s\textsuperscript{-1} without Na\textsuperscript{+} and 240 s\textsuperscript{-1} with Na\textsuperscript{+}), are as the spectra (Fig. 6, A and C). The spectra appear to show two-electron reduction of a flavin. The spectra and rates are consistent with those of the first phase of the reaction of the wild type enzyme in the absence of Na\textsuperscript{+}. It is worth noting that this kinetic phase is essentially the same in all of the mutants studied here.

**Fast Kinetics of Reduction of NqrF-C76A Mutant**—In this mutant one of the cysteine ligands of the 2Fe-2S center has been changed to alanine, resulting in the complete loss of this cofactor (15). The reduction kinetics in this mutant appear essentially as in the double mutant (above) with only one phase observed with or without Na\textsuperscript{+} (Fig. 6, B and D). When the results of the NqrF-C76A and the NqrB-T236Y/NqrC-T225Y mutants are considered together with results on steady state turnover of the enzyme with NADH and ferricyanide (15), the first phase can be definitively assigned to the two-electron reduction of the FAD in NqrF; the wild type enzyme is capable of transferring electrons from NADH to ferricyanide. This reaction is almost completely abolished in the NqrFS246A mutant, which lacks the FAD. However, in the NqrF-C76A mutant, turnover with ferricyanide is almost the same as in the wild type enzyme. The ferricyanide result allows the FAD to be assigned as the redox carrier between NADH and the 2Fe-2S center. The current stopped flow result on the NqrF-C76A mutant, showing that two-electron reduction of a flavin is the only significant phase remaining from the wild type reaction, allows the redox carrier upstream of the 2Fe-2S center to be assigned to the first kinetic phase of the wild type reaction and hence to the FAD in NqrF.

**Reverse Reaction**—We have recently shown that the riboflavin of Na\textsuperscript{+}-NQR is the site of the neutral flavosemiquinone (8, 9). In all three flavin mutants (NqrB-T236Y, NqrC-T225Y, and the double mutant) as well as the 2Fe-2S center mutant (NqrF-C76A), the reduction by NADH of the neutral flavosemiquinone is dramatically slowed, or abolished, compared with the wild type enzyme. This suggests that the riboflavin is located downstream of both FMN\textsubscript{C} and FMN\textsubscript{B} near the end of the linear electron pathway, where it could be the site from which electrons are donated to quinone. This can be investigated by reacting the oxidized enzyme with ubiquinol, the reduced form of the usual electron acceptor. If this is indeed the final carrier in the pathway, it should be the first to receive electrons from quinol. Moreover, this process should not be blocked in the mutants, which lack any of the FMN cofactors. We have recently shown that reaction of the wild type enzyme with quinol is a single-phase process with a spectrum corresponding to a one-electron reduction of a neutral flavosemiquinone (8). In Fig. 7, the reaction of quinol with the wild type enzyme, the FMN\textsubscript{B} and FMN\textsubscript{C} mutants (NqrB-T236Y and NqrC-T225Y), and the double mutant are com-
pared. In all cases the spectra (Fig. 7A) of the single-phase reaction are similar, reflecting the second-electron reduction of the riboflavin (FH−→FH2). The fact that this is the only phase observed in the wild type enzyme suggests that reduction of sites upstream of the riboflavin is energetically unfavorable. The corresponding time courses are shown in Fig. 7B. In the case of the FMNC mutant, the rate is close to that in the wild type enzyme (Table 2). The rate in the FMNB mutant is somewhat lower than for the wild type enzyme, but it is similar to that in the double mutant. The fact that both mutants that lack FMNB show a similarly slow reaction suggests that it is removal of this flavin that leads to the change in rate.

**DISCUSSION**

These results define, for the first time, the place of each known cofactor of Na+/H+ -NQR in the sequence of electron carriers, including the four flavins and the 2Fe-2S center, and the redox reactions that take place at each site. They also reveal specific reaction steps and cofactors that are associated with Na+ translocation.

The mutants, which lack specific cofactors, are the key to this analysis. In an enzyme with multiple cofactors, electrons may follow a linear pathway, but there are also examples where parallel pathways exist. The distance between redox carriers is a primary factor determining rates of electron transfer (20). Electrons can move rapidly along a series of closely spaced carriers by a sequence of short jumps. Removal of one carrier in a linear pathway can turn two short jumps into one long jump, dramatically reducing the rate of the step. If removal of one cofactor leads to a significant decrease in the rates of reduction of some of the remaining cofactors, this indicates that those cofactors lie downstream of the removed cofactor. However, if removing one of the cofactors makes no change in the rate of reduction of a remaining cofactor, we cannot infer that this cofactor lies upstream of...
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FIGURE 7. Reverse reduction of the wild type Na"+-NQR, NqrB-T236Y, NqrC-T225Y and NqrB-T236Y/NqrC-T225Y mutants by ubiquinol-1 (reverse reduction) in the wild type, NqrB-T236Y, NqrC-T225Y, and NqrB-T236Y/NqrC-T225Y mutants. Each of the cofactor mutants shows an altered reaction; the mutant that lacks FMNC (NqrC-T225Y) is the most severely changed from wild type. These results neatly illustrate that FMNC precedes FMNB in the sequence of electron carriers.

However, a detailed understanding of the results also depends on assignment of spectroscopic signals to the various redox cofactors. In a recent EPR study of these mutants, it was shown that FMNB and FMNC both give rise to anionic semiquinone radicals, and by elimination, that riboflavin gives rise to a neutral semiquinone (9). Because the neutral and anionic radicals also have different UV-visible spectra, these assignments can be used to guide the interpretation of the stopped flow data. Furthermore, as described above, the initial two-electron reduction of a flavin can be assigned to the FAD in the NqrF subunit.

The results allow us to postulate a model of electron flow in Na"+-NQR, presented in Fig. 8. In the model, the reaction of the wild type enzyme, in the absence of Na", proceeds as follows. The process begins with the enzyme in its "as prepared" form (step A), with all the cofactors in their fully oxidized state, except for the riboflavin, which is initially one-electron reduced (1, 8). Donation of the first two electrons from NADH converts the FAD from the flavoquinone to the flavohydroquinone (step B). Donation of the next pair of electrons leads to reduction of the 2Fe-2S center and transient one-electron reduction of one or the other FMN (step C), but in the next stable intermediate (step D) the FAD, 2Fe-2S center and riboflavin, are fully reduced, whereas both FMNs are fully oxidized. Donation of two more electrons leads to one-electron reduction of the two FMNs to anionic flavosemiquinones (step E). Finally, additional reduction results in conversion of both the two anionic FMN semiquinones to its fully reduced state (step F).

It is significant that both FMNs become reduced to the anionic flavosemiquinone in the third kinetic phase, without Na"+ (formation of E), after which, one flavin is reduced from the anionic flavosemiquinone to the hydroquinone (formation of F). This can account for the finding that the EPR spectra of the oxidized form of the enzyme exhibits one equivalent of neutral flavosemiquinone radical, whereas the reduced form of the enzyme exhibits only one equivalent of anionic flavosemiquinone radical, despite the fact that both FMNs are capable of giving rise to anionic flavosemiquinones.

Consistent with the results described above, according to the model (Figs. 1 and 8): 1) Removal of the 2Fe-2S center would not affect reduction of the FAD (intermediate B) but essentially eliminate all steps thereafter. 2) Removal of FMNC would cur-

### Table 2

| Enzyme | Rate constant s⁻¹ |
|--------|-------------------|
| Wild type (n = 5) | 0.031 ± 0.015 |
| NqrB-T236Y (n = 3) | 0.013 ± 0.008 |
| NqrC-T225Y (n = 4) | 0.025 ± 0.007 |
| NqrB-T236Y/NqrC-T225Y (n = 3) | 0.011 ± 0.006 |

the removed cofactor, because there may be a second pathway by which electrons can reach the remaining cofactor. Also this analysis can be misled if removing a cofactor causes significant shifts in the redox properties of another cofactor or long range structural changes. However, if removing a cofactor causes dramatic changes in some steps of a process, but not others, a strong inference can be made that the altered steps are downstream of the removed cofactor. In the case of Na"+-NQR, each of the mutants studied showed a distinct change in the reduction reaction, and analysis of all the results together indicates a linear pathway as described below. The effects of these mutants can be seen, in overview, in the time course of the reaction, followed at absorbance maximum for fully oxidized flavin (450 nm) (Fig. 2). The wild type enzyme reaction shows four clear phases in the absence of Na"+ (left panel) and three in the presence of Na"+ (right panel). Each of the cofactor mutants shows an altered reaction; the mutant that lacks FMNC (NqrC-T225Y) shows the least perturbation, and the mutant that lacks FMNB (NqrB-T236Y) is more significantly perturbed, whereas the double mutant, which lacks both FMNs, is the most severely changed from wild type.
Because Na\textsuperscript{+}-NQR functions as a sodium ion pump, certain steps in the catalytic cycle must be linked to Na\textsuperscript{+} uptake and release. In the wild type enzyme, electron donation from the second NADH leads to reduction of the riboflavin and probably also the 2Fe-2S center (B → D). In contrast, in the mutant that lacks FMN\textsubscript{b}, electron donation from the second NADH leads to the observed reduction of the 2Fe-2S center and the formation of the FMN\textsubscript{c} anionic flavosemiquinone (B → C). In the absence of Na\textsuperscript{+}, where these processes are well resolved, they have almost the same rate constant (15.4 and 13.3 s\textsuperscript{-1}, respectively). Moreover, both processes are strongly accelerated by the presence of Na\textsuperscript{+} (see Table 1). This suggests a common rate-limiting step to be found in the B → C process. Because electron transfer from NADH to FAD is clearly independent of Na\textsuperscript{+} (Refs. 1, 13, 22, and 23 and the current work), this rate-limiting step should be the reduction of either the 2Fe-2S center or the one-electron reduction of FMN\textsubscript{c}. Hayashi and Unemoto (24) have also shown that electron transfer from NADH to menadione, which passes through the 2Fe-2S center, is independent of Na\textsuperscript{+}. This indicates, by elimination, that the formation of the anionic FMN\textsubscript{c} flavosemiquinone is the rate-limiting step and the first Na\textsuperscript{+}-dependent step in the reaction and could thus be linked to Na\textsuperscript{+} uptake.

Our data do not assign Na\textsuperscript{+} release to a specific step of the reaction. However, the fact that reduction of the enzyme by quinol does not proceed beyond reduction of the riboflavin suggests that electron transfer from FMN\textsubscript{b} to riboflavin is a significantly exergonic process. This would be consistent with ejection of Na\textsuperscript{+} from the pump taking place in this step. If true, this would mean that more than one redox cofactor is involved in coupling electron transfer to the translocation of Na\textsuperscript{+}. This would be significantly more complex than a single-site mechanism and requires much additional investigation.

The results presented here describe the complete sequence of redox carriers in Na\textsuperscript{+}-NQR and reveal an important Na\textsuperscript{+}-dependent mechanistic step. These findings provide a foundation for future detailed studies on the mechanism of Na\textsuperscript{+} pumping by the enzyme.

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